Modifikation von Zellen mit synthetischer mRNA für Anwendungen im Bereich der Regenerativen Medizin

Modification of cells with synthetic mRNA for applications in regenerative medicine

DISSERTATION

der Mathematisch-Naturwissenschaftlichen Fakultät
der EBERHARD KARLS UNIVERSITÄT TÜBINGEN

zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Heidrun Steinle
aus Überlingen

August 2020

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Prüfung: 14. Januar 2021

Stellvertretender Dekan: Prof. Dr. József Fortágh

1. Berichterstatter: Prof. Dr. Meltem Avci-Adali

2. Berichterstatter: Prof. Dr. Stefan Stevanovic

„Lerne von Gestern, lebe heute, vertraue auf morgen.
Das wichtigste ist, nicht aufhören zu fragen.“

Albert Einstein (1879-1955)

INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

Ein wichtiger Bestandteil der regenerativen Medizin sind Zelltherapien. Die transplantierten Zellen können sich in das verletze Gewebe integrieren und dort körpereigene Reparatur- und Regenerationsmechanismen anregen. Somit kann es zur Heilung des geschädigten Gewebes und Organen kommen. Limitierungen, wie der Mangel an patientenspezifischem Zellmaterial, die Überlebens- und Integrationsfähigkeit nach Transplantation, können durch Modifikation der Zellen überwunden werden. Das Proteinexpressionsprofil der Zellen wird dabei verändert, um ihre natürliche Aktivität zu verbessern oder ihre zelluläre Entwicklung zu verändern. Die RNA-basierte Modifikation von Zellen stellt eine vielversprechende Methode für therapeutische Anwendungen dar, da die Expression von gewünschten Proteinen Integrations- und Rückstands-frei erfolgt.

Die Bildung neuer Blutgefäße in ischämischem Gewebe, z. B. nach einem Myokardinfarkt, kann die Regeneration und Funktion der Organe verbessern. Daher wurde im Rahmen dieser Arbeit synthetisch modifizierte mRNAs, die für VEGF-A, ANG-1 und SDF-1α kodieren, in murine endotheliale Progenitorzellen (EPCs) transfiziert, um das natürliche angiogenetische Potenzial der EPCs zu fördern. Dabei zeigte die Analyse typischer Parameter zur Gefäßbildung, wie die chemotaktische Migration, die Wundheilungskapazität, die in vitro und in vivo Gefäßneubildung, ein verbessertes angiogenetisches Potenzial der mRNA-modifizierten EPCs im Vergleich zu unbehandelten EPCs. Insbesondere die EPCs, die mit ANG-1 mRNA oder gleichzeitig mit allen 3 mRNAs (VEGF-A, ANG-1 und SDF-1α) transfiziert wurden zeigten signifikant verstärkte proangiogene Eigenschaften. Auf diese Weise veränderte Zellen könnten somit nach einer Transplantation zur Verbesserung der Blutversorgung in geschädigtem oder ischämischem Gewebe führen.

Induziert pluripotente Stammzellen (iPSCs) können als unendliche patientenspezifische Quelle für verschiedene Zellarten dienen. Hierfür können iPSCs durch Differenzierung in gewünschte Zelltypen für zukünftige therapeutische Applikationen generiert werden. Für die nichtintegrative Reprogrammierung von neonatalen Fibroblasten in iPSCs wurde ein mRNA-basierter Ansatz mit einem selbst-replizierenden RNA (srRNA)-basierten Ansatz verglichen. Die generierten iPSCs wiesen typische Charakteristika der pluripotenten Stammzellen auf und waren in der Lage in alle 3 embryonalen Keimblätter (Mesoderm, Endoderm und Ektoderm) zu differenzieren. In den iPSCs wurden keine srRNA-Rückstände oder chromosomale Abnormalitäten festgestellt. Daher sind die so generierten Zellen besonders gut für den therapeutischen Einsatz geeignet. Die Reprogrammierung der Zellen konnte durch eine einmalige srRNA Transfektion durchgeführt werden. Weiterhin stellte sich heraus, dass die Reprogrammierung mittels srRNA effizienter, kostengünstiger und mit weniger Arbeitsaufwand verbunden war als die tägliche Transfektion der Zellen mit mehreren mRNAs. Nach der erfolgreichen Etablierung des srRNA-basierten Protokolls zur Herstellung von iPSCs wurden auch adulte Zellen reprogrammiert. Dazu wurden einerseits renale Epithelzellen (RECs) nicht-invasiv aus Urin gewonnen und andererseits Zellen aus Kieferperiost (JPCs) xeno-frei isoliert. Nach Bestätigung der Pluripotenz, der Differenzierungsfähigkeit in die 3 Keimblätter in vitro und in vivo und der Eliminierung der srRNA, wurden die iPSCs zu gewebespezifischen Zellen differenziert und erfolgreich auf ihre spezifische Markerexpression untersucht. Die REC-iPSCs wurden innerhalb von 10 Tagen zu schlagenden Kardiomyozyten differenziert und anhand einer Video-basierten Analyse von Ca^{2+} Intermediaten wurde die Kontraktilität der generierten

Zellen bestätigt. Die Behandlung der generierten Kardiomyozyten mit dem Aktivator Isoproterenol führte zur Erhöhung der Schlagrate und die Inhibitor-Behandlung mit Nifidipin bewirkte die Hemmung der Kontraktionen in den Zellen. Die JPC-iPSCs wurden zuerst in induziert mesenchymale Stamm/ Stroma-ähnliche Zellen (iMSCs) und dann weiter zu osteogenen Zellen differenziert, die eine starke Mineralisierung aufwiesen.

Darüber hinaus wurden in dieser Arbeit verschiedene Hydrogele bezüglich der mRNA Freisetzung über einen längeren Zeitraum hinweg untersucht, um die transiente mRNA vermittelte Produktion von Proteinen zu verlängern. Chitosan-Alginat-Hydrogele zeigten eine verzögerte Freisetzung von Cyanin 3-markierter Luciferase (Cy3-hGluc) mRNA über 3 Wochen.

Die Bioaktivität der freigesetzten hGLuc mRNAs und die anhaltende Proteinexpression konnten durch die Produktion der Luziferase, vermittelt durch die in die mRNA Hydrogele integrierten HEK293 Zellen und die Messung der Luziferase-Aktivität über 21 Tage bestätigt werden. Rheologische Messungen zeigten stabile gelartige Eigenschaften der mit mRNA beladenen Hydrogele über den Zeitraum von 3 Wochen. Somit konnte durch die verzögerte Freisetzung der mRNAs aus dem Hydrogel, die Proteinexpression in den Zellen verlängert werden ohne die mRNA mehrfach applizieren zu müssen.

Der Einsatz von nicht Genom-integrierenden RNAs zur gezielten Überexpression spezifischer Proteine in patientenspezifischen Zellen eröffnet enorme Möglichkeiten im Bereich der regenerativen Medizin und des Tissue Engineerings. So können mittels mRNA oder srRNA modifizierte autologe Zellen zur Zelltherapie oder Generierung 3-dimensionaler Gewebekonstrukte eingesetzt werden und so eine personalisierte Therapie und Regeneration von Geweben ermöglichen.

ABSTRACT

Cell-based therapies are an important tool for regenerative therapies. The cells integrate into the injured tissue and stimulate the body's own repair and regeneration mechanisms. Thus, damaged tissues and organs can be treated and even healed. To overcome limitations, such as the lack of autologous cell material, survival and integration capacity after transplantation, cells can be modified by altering their protein expression profile to either enhance their natural activity or to alter their cellular fate. The RNA-based modification of cells provides a versatile method for therapeutic applications, as the modifications by the mRNA-mediated expression of desired proteins is integration- and vector-free.

The formation of new blood vessels in ischemic tissue, e.g. B. after a myocardial infarction, can improve the regeneration and function of organs. Therefore, in this work synthetic modified mRNAs encoding VEGF-A, ANG-1 and SDF-1α were transfected into murine endothelial progenitor cells (EPCs) in order to promote the natural angiogenic potential of the EPCs. The analysis of typical parameters for vascularization, such as chemotactic migration, wound healing capacity and in vitro and in vivo angiogenesis/ tube formation, demonstrated an improved angiogenic potential of the mRNA-modified EPCs compared to untreated EPCs. In particular, the cells transfected with ANG-1 mRNA or those transfected simultaneously with all 3 mRNAs (VEGF-A, ANG-1 und SDF-1α) had a significantly increased proangiogenic features. Cells modified in this way could therefore improve the blood supply in damaged/ ischemic tissue after transplantation.

Induced pluripotent stem cells (iPSCs) can serve as a limitless patient-specific cell source for the differentiation into a desired cell type for future therapeutic applications. For the non-integrative reprogramming of iPSCs from neonatal fibroblasts, an mRNA-based was compared with a self-replicative (sr)RNA-based approach. The generated iPSCs showed typical characteristics of stem cells and were able to differentiate in all 3 lineages: mesoderm, endoderm and ectoderm. Additionally, no residual expression of the RNA or chromosomal abnormalities was detected after the reprogramming. This renders them particularly suitable for a therapeutic use. The cells could be reprogrammed by a single srRNA transfection. Furthermore, the study showed that the srRNA was more efficient, less expensive and less work intensive than the daily transfections with multiple mRNAs. Following the successful establishment of the srRNA-based protocol for the production of iPSCs, adult cells from different donors were also reprogrammed. On the one hand, renal epithelial cells (RECs) were non-invasively extracted from urine samples and on the other hand, cells from jaw periosteum (JPCs) were isolated xeno-free. After the confirmation of pluripotency and 3-lineage differentiation potential in vitro and in vivo, as well as the elimination of srRNA, the iPSCs were differentiated into tissue-specific cells and successfully tested for their specific marker expression. The REC-iPSCs were differentiated into beating cardiomyocytes within 10 days, and a video-based analysis of Ca2+ intermediates confirmed the contractility of the generated cells. The treatment of the generated cardiomyocytes with the activator isoproterenol led to an increase in the beat rate and the inhibitor treatment with nifidipine inhibited the contractions in the cells. The JPC-iPSCs were first differentiated into induced mesenchymal stem/ stroma-like cells (iMSCs) and then further differentiated into osteogenic cells that showed a strong mineralization.

In addition, various hydrogels were investigated with regard to the release of mRNA over a longer period of time in order to prolong the transient mRNA-mediated production of proteins. Chitosan-alginate hydrogels showed the local release of synthetic mRNAs through the delayed release of cyanine 3-labeled luciferase (Cy3-hGluc) mRNAs over 3 weeks. The bioactivity of the released hGLuc mRNAs and sustained protein expression could also be confirmed by the additional integration of HEK293 cells into the mRNA hydrogels by measuring the luciferase activity over 21 days. Rheological measurements showed stable gel-like properties of the mRNA-loaded hydrogels over 3 weeks. The local protein expression in the cells could be prolonged due to the delayed release of the mRNAs from the hydrogel and the subsequent transfection of the cells, without the need of repeated application of the mRNA.

The use of non-integrative RNAs for the targeted overexpression of specific proteins for the modification of patient-specific cells opens up enormous opportunities in the field of regenerative medicine and tissue engineering. In this way, cells are modified vector-free and can safely be used as cell therapy or integrated into 3-dimensional tissue constructs, without causing a rejection reaction.

1. EINLEITUNG

1.1. Regenerative Medizin

Die Regenerative Medizin ist ein vielversprechender und stetig wachsender Bereich[1] der Translationalen Forschung und verbindet Biomaterialforschung, Tissue Engineering, sowie Molekular- und Zellbiologie (Abbildung 1), die sich mit dem Prozess des Ersetzens, Reparierens und Regenerierens menschlicher Zellen, Gewebe oder Organe zu deren funktionalen Wiederherstellung befasst. Dabei werden eine Vielzahl an Anwendungen zur Behandlung von angeborenen und erworbenen Erkrankungen, Traumata und natürliche Alterungsprozesse angestrebt.[2] Man erhofft sich durch die regenerative Medizin, die Humanmedizin zu revolutionieren. Krankheiten, die mit herkömmlichen Medikamenten und medizinischen Verfahren nur unzureichend behandelt werden können, sollen dabei nicht nur behandelt, sondern funktional geheilt werden oder mechanischen Ersatz erhalten. Dies wird durch das Rekonstruieren von Zellen, Geweben, oder Organen erreicht, die die Stimulation von körpereigenen Regenerations- und Reparaturmechanismen anregen.[3]

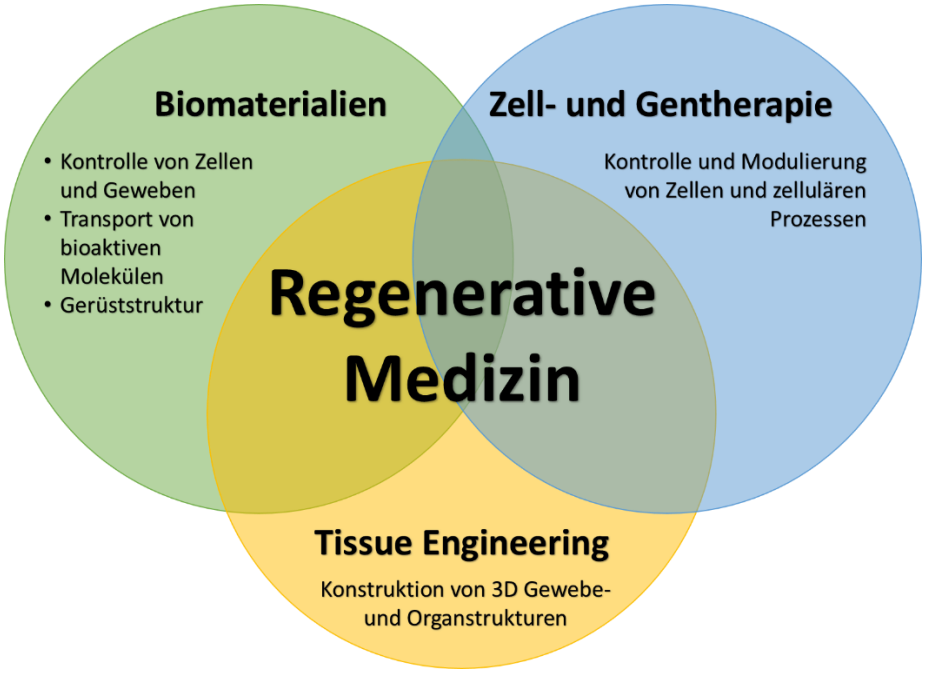

Abbildung 1: Die Regenerative Medizin ist ein multidisziplinärer Ansatz der Translationalen Medizin und vereint Bereiche der Biomaterialforschung, der Zell-basierten Therapie und des Tissue Engineering.

Die Schädigung von lebenswichtigen Organen wie Herz, Leber und Nieren sind nach wie vor eine der Hauptursachen für die weltweite Sterblichkeit.[4] Das Überleben eines Patienten, der an einer schwerwiegenden Erkrankung dieser Organe leidet kann oft nur durch den Ersatz mit einem geeigneten Spenderorgan gewährleistet werden. In der Vergangenheit wurden bereits zahlreiche Erfolge durch die Transplantationen verschiedener Organe (wie z.B. Niere, Leber, Lunge, Bauchspeicheldrüse, Herz, Knochenmark, Haut, Blutgefäße) erzielt.[5] Durch die

regenerative Medizin können theoretisch sämtliche akute und chronische Krankheiten, wie Schlaganfall, koronare Herzerkrankungen (Myokardinfarkt), Diabetes, Krebserkrankungen (Leukämie, Lymphom, Melanom), progressive neuronale Erkrankungen (Alzheimer, Parkinson), Autoimmunerkrankungen und Osteoarthritis geheilt werden.

Vor allem der Mangel an geeignetem Spendergewebe und transplantierbaren Organen, sowie dem hohen Risiko einer immunologischen Abstoßung, treibt das Bioengineering von Geweben und Organen voran.[4, 5] Menschen, die das Glück haben, eine Organtransplantation zu erhalten, müssen mit chronischer Abstoßung des implantierten Gewebes/ Organs und dem lebenslangem Einnahme von Immunsuppressiva rechnen.[2] Zelltransplantationen und das Tissue Engineering soll daher als Alternative zum Organersatz dienen, indem funktionelle Gewebe und Organe de novo im Labor konstituiert und anschließend in den Patienten implantiert werden oder Zellen zur therapeutischen Behandlung eingesetzt werden.[3]

Die Regeneration von Geweben oder Organen, die durch Verletzung, Alterung oder Krankheit des Gewebes geschädigt wurden, können durch verschiedene Ansätze durchgeführt werden. 1) Transplantation von Stammzellen oder Progenitorzellen in vivo, die sich dann lokal in den gewünschten Zelltyp differenzieren, ins Gewebe integrieren und diese mit zusätzlichen Biomolekülen versorgen. 2) Komplett- oder Teilersatz eines Organs oder Gewebes mit ex vivo kultivierten gewebespezifischen Zellen. 3) Einsatz von bioaktiven Materialien als Gerüst-/ Stützstrukturen zur Rekonstruktion der Extrazellulärmatrix (EZM) und um körpereigene regenerative Prozesse anzuregen.[4, 6, 7]

Die Kombination von unterschiedlichen Komponenten, wie einem Grundgerüst, bioaktiven Materialien und gewebetypspezifischen Zellen, um 3-dimensionale funktionelle Gewebekonstrukte herzustellen wird als Tissue Engineering bezeichnet.[2, 4] Dabei werden die Zellen auf eine geeignete Matrix aufgebracht, mit dem Ziel eine möglichst natürliche Umgebung zu simulieren. Diese können in Bioreaktoren in vitro kultiviert werden und das vitale Gewebe wird dann in Patienten implantiert, wo durch körpereigene Prozesse die endgültige Stabilität und volle Funktion erreicht wird. Als Biomaterialien werden natürliche Materialien (z. B. Kollagen und Alginat), azellularisierte Gewebe (z. B. Herzklappen) oder synthetische Polymere wie Poly(lactid-co-glycolid) (PLGA) verwendet, die biologische Prozesse und mechanische Funktion des zu ersetzenden Gewebes als künstliche EZM simulieren.[6] Sie ermöglichen auch die Abgabe von geeigneten bioaktiven Faktoren an der gewünschten Stelle im Körper.[8] Gerüste können mit bioaktiven Faktoren wie Zelladhäsionspeptiden, Wachstumsfaktoren und Zellen geladen werden, um die Funktion der Zellen (Adhäsion, Proliferation, Migration und Differenzierung) zu regulieren, so dass sich ein funktionelles Gewebe bilden kann. Am besten geeignet sind biologisch abbaubare und bioresorbierbare Materialien, die keine Entzündungsreaktion auslösen. Inkompatible Materialien können eine Entzündung hervorrufen und dies kann zu einer Nekrose und zur Abstoßung im Gewebe führen.[9]

Für eine Anwendung in verschiedenen Bereichen der Regenerativen Medizin stehen humane Zellen, wie somatische Zellen, adulte oder embryonale Stammzellen, pluripotente Stammzellen und Progenitorzellen, sowie ausdifferenzierte Zellen zur Verfügung. Dabei ist der Einsatz von autologen („körpereigenen“) Zellen von besonderem Interesse, da durch ihre Verwendung die Abstoßung verhindert wird, auf die Gabe von immunsuppressiven Medikamenten verzichtet und die Übertragung von Infektionen ausgeschlossen werden kann.[5, 7] Die größten Einschränkungen für den erfolgreichen Einsatz dieser Technologien sind Zellbeschaffung und -expansion, hohe Kosten, regulatorische Probleme und die Schwierigkeit des Hochskalierens.[3]

1.2. Zelltherapie

Bei einer Zelltherapie werden dem Patienten zelluläres Material injiziert, mit dem Ziel, einen Zellersatz in geschädigtem Gewebe zu bewirken und/ oder lösliche Faktoren wie Zytokine, Chemokine und Wachstumsfaktoren freizusetzen, um eine parakrine oder endokrine Wirkung zu erzielen.[3] Die dafür präferierten autologen Zellen werden durch eine Biopsie eines bestimmten Gewebes dem Patienten selbst entnommen, vom Gewebeverband dissoziiert bzw. isoliert und in einer in vitro Zellkultur vermehrt. Seltener werden minimal-invasiv entnommene Blutproben und nicht-invasiv gewonnene Urinproben verwendet, um daraus Zellen zu gewinnen. Anschließend können diese Zellen wieder in das gleiche Individuum zurück transplantiert werden.[10, 11] Daher hängen die meisten aktuellen Strategien für das Tissue Engineering und der Regenerativen Medizin von der Verfügbarkeit autologer Zellen aus dem Gewebe/ Organ des Patienten ab.[6]

1.2.1. Endotheliale Progenitorzellen

Endotheliale Progenitorzellen (EPCs) sind im Blutkreislauf zirkulierende Stammzellen und werden durch Chemokine aus dem Knochenmark rekrutiert. Diese werden von Zellen im verletzten Gewebe sekretiert, um die Revaskularisierung zu induzieren.[12, 13] Die EPCs homen an den Stellen vaskulärer Läsionen und differenzieren dort, um eine Gefäßreparatur durchzuführen.[14] Dadurch kann die Blutversorgung in ischämischen Geweben wieder-hergestellt, die Schädigung des Gewebes reduziert und somit die Funktion betroffener Gewebe oder Organe (z.B. nach einem Schlaganfall oder Herzinfarkt) wiederhergestellt werden.

Mehrere präklinische Studien haben bereits das vaskulogene und angiogene Potenzial von EPCs bestätigt[15, 16], sowie eine positive parakrine Wirkung von transplantierten EPCs bei der Behandlung ischämischer Erkrankungen.[17, 18] In früheren Studien wurden EPCs auch als Biomarker bei Herz-Kreislauf-Erkrankungen identifiziert und der Rückgang der Anzahl an EPCs und deren Dysfunktion wurden mit ischämische Erkrankungen in Verbindung

gebracht.[19, 20] Jedoch ist der Erfolg einer klinischen Anwendung von EPCs aufgrund der geringen Retention und des begrenzten Einwachsens nach der Transplantation, sowie das geringe Überleben der migrierten EPCs im ischämischen Gewebe, eingeschränkt.[21, 22] Weiterhin stellt auch die geringe Menge und Qualität von den isolierten autologen EPCs eine Herausforderung für klinische Anwendungen dar.[23] So konnte in mehreren Tierversuchen und klinischen Studien mit EPCs unterschiedliche Ergebnisse in ihrer Wirksamkeit bei der Behandlung ischämischer Erkrankungen nachgewiesen werden.[18, 24]

Neue Strategien beinhalten zelluläre Modifikationen, um die Anzahl und Proliferation der EPCs zu erhöhen[25] und deren Funktion und Integration ins Gewebe zu verbessern.[26] Somit können die Geweberegenerationen effizienter durchgeführt werden. Hierfür können Biomoleküle, wie z. B. rekombinante Proteine, wie Zytokine, Chemokine und Wachstumsfaktoren, oder Gentherapien eingesetzt werden, um den natürlichen Ablauf von beispielsweise angiogenetischen Prozessen zu steuern.

Ein Beispiel dafür ist der vaskuläre endotheliale Wachstumsfaktor-A (VEGF-A), der unter anderem an der Chemotaxis, Migration und Differenzierung von EPCs beteiligt ist und in früheren Studien zur verbesserten Revaskularisierung von Geweben geführt hat.[27-30] Weitere Wachstumsfaktoren und Chemokine, wie das Angiopoietin (ANG)[31, 32], der Fibroblasten-Wachstumsfaktor (FGF)[33, 34], der Stromazellen-abgeleiteter Faktor-1 (SDF-1)[35, 36] und der Hypoxie-induzierbarer Faktor-1α (HIF-1α)[37] haben ebenfalls ihre Wirksamkeit für einen proangiogene tischen Einsatz bestätigt.

1.2.2. Pluripotente Stammzellen

Im Gegensatz zu gewebespezifischen adulten Stammzellen, die meist ein limitiertes Proliferations- und Differenzierungspotenzial aufweisen, besitzen pluripotente Stammzellen (PSCs), wie humane embryonale Stammzellen (ESCs) und induziert Pluripotente Stammzellen (iPSCs) die Fähigkeiten sich selbst zu vermehren und in fast alle spezialisierten Zelltypen des Körpers zu differenzieren.[38] Sie können in vitro in alle verschiedenen Zelltypen der drei Keimbahnrichtungen (Mesoderm, Endoderm und Ektoderm) differenzieren. Daher können sie als Quelle für therapeutisch einsetzbare Zellen jeden Typs dienen. Die PSCs sind auch in der Lage sich in 3-dimensionale Zellkonstrukte zusammenzulagern, um in vitro Embryonalkörperchen (Embryoid bodies) oder in vivo Teratomas auszubilden, die alle drei embryonalen Gewebetypen enthalten. Diese Fähigkeit dient auch als Beweis für ihre Pluripotenz[39] und bedeutet, dass die Zellen für den in vivo Einsatz vollständig differenziert werden müssen. Auch die Immunogenität der ESCs, die nur allogen transplantiert werden können, stellt ein Problem für klinische Anwendungen dar, da sie eine Abstoßungsreaktion im Empfängerorganismus auslösen können.[40, 41]

Humane ESCs werden hauptsächlich aus der inneren Zellmasse von Blastozysten im Embryonalstadium isoliert.[42] Im Vergleich dazu ist die Gewinnung von iPSCs ethisch unbedenklich, da sie aus somatischen Zellen generiert werden (Abbildung 2). Die Generierung der iPSCs aus patienteneigenen Zellen, deren anschließende Differenzierung in den gewünschten Zelltyp und die Implantation dieser autologen Zellen verhindert eine Abstoßung im Empfängerorganismus. Daher ist die Induktion der Pluripotenz in somatischen Zellen, um iPSCs zu erhalten, ein wichtiges Instrument für die in vitro Erzeugung autologer Zellen.[43]

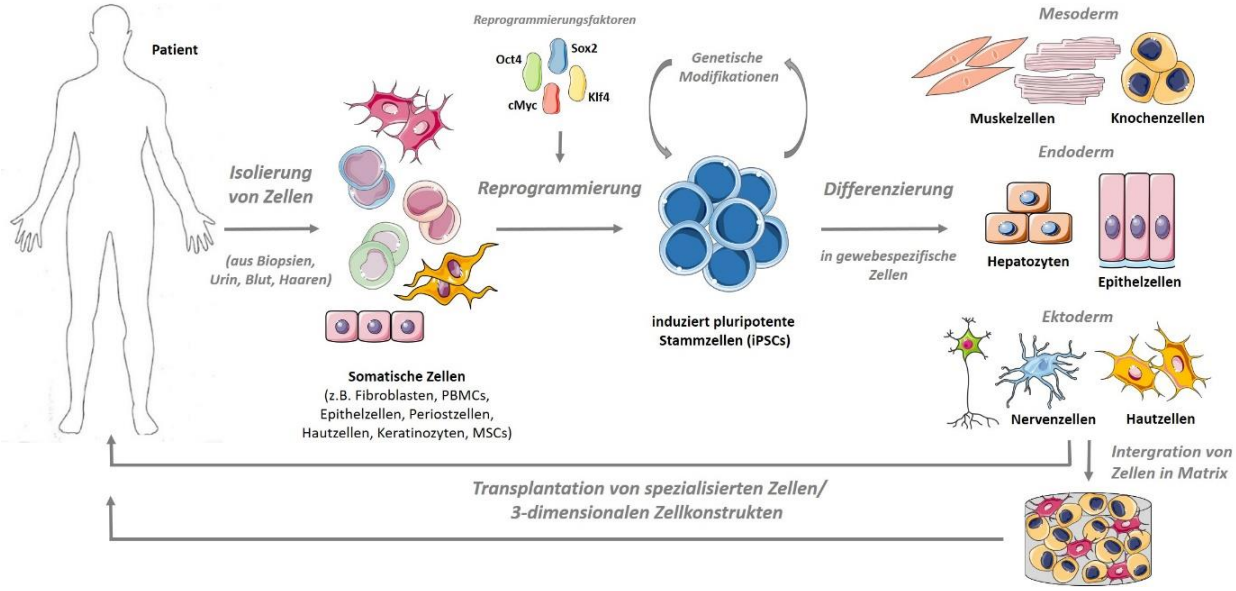

Abbildung 2: Der Einsatz von induziert pluripotenten Stammzellen (iPSCs) besitzt ein enormes Potenzial für Zell-basierte Therapien. Adulte Zellen können aus dem Patienten isoliert und mit Reprogrammierungsfaktoren behandelt werden um iPSCs zu generieren. Diese können dann in Gewebe-spezifische Zellen eines gewünschten Typs differenziert werden und für eine autologe Transplantation von Enddifferenzierten Zellen oder nach Integration in ein Matrixmaterial als 3-dimensionales Zellkonstrukt verwendet werden. Die Grafik wurde mit Hilfe von Servier Medical Art Vorlagen erstellt.

Zur Herstellung der iPSCs werden somatische Zellen reprogrammiert. Der Prozess wird durch epigenetische Regulationen, wie Chromatinumlagerungen und das Entfernen von Methylierungsmustern der DNA möglich und durch die Überexpression von Transkriptions-faktoren eingeleitet, die im embryonalen Stadium vorhanden sind. Die Kern-Transkriptionsfaktoren Oct4, Sox2 und Nanog werden dabei am höchsten exprimiert und erhalten den Pluripotenzstatus der Zellen aufrecht. Weitere intrinsische Faktoren (Expression von Interaktionspartnern wie cMyc, Klf4, Lin28) und extrinsische Faktoren (Kulturbedingungen, Wachstumsfaktoren, Zytokine usw.) spielen dabei eine Rolle.[44]

Die erfolgreiche Generierung von iPSCs aus humanen Fibroblasten wurde erstmals 2007 von Yamanaka und Takahashi anhand der Reprogrammierungsfaktoren Oct4, Sox2, Klf4 und cMyc mithilfe retroviraler Transduktion gezeigt.[45] Es zeigte sich, dass Nanog für die Induktion der Pluripotenz nicht nötig war, aber die Beteiligung der Transkriptionsfaktoren Klf4 und cMyc

für den Prozess hilfreich sind. Klf4 und cMyc stehen mit der Regulation des Zellzyklus in Verbindung und fördern das Wachstum und die Proliferation der Zellen.[46] Weitere Forschungen zeigten, dass verschiedene Reprogrammierungsstrategien und Faktoren zur Herstellung von iPSCs eingesetzt werden können, wie beispielweise die Induktion der Pluripotenz ohne cMyc[47], die Kombination der Faktoren Oct4, Sox2, Nanog und Lin28[48], oder die Überexpression der Faktoren Oct4 und Sox2[49].

In den vergangenen Jahren wurden bereits erhebliche Fortschritte mit iPSCs bei der Umsetzung von Grundlagenforschung in die klinische Anwendung gemacht. So konnte die Sicherheit von regenerativen Therapien anhand der ersten klinischen Studie mit iPSCs zur Behandlung der Makuladegeneration nachgewiesen werden, die im Jahr 2014 in Japan gestartet wurde.[50] Weiterhin bestätigten vorläufige Ergebnisse einer Studie zur Behandlung der akuten Graft-versus-Host Erkrankung, die im Jahr 2017 in Australien gestartet wurde, dass die eingesetzten iPSC-abgeleiteten mesenchymale Stamm-/ Stromazellen (MSCs) sicher sind und führten zu einer Reduktion des Schweregrads der Erkrankung bei 14 von 15 Patienten.[51]

1.2.3. Differenzierung von iPSCs in gewebespezifische Zellen

Stammzellen, wie iPSCs eignen sich aufgrund ihrer unbegrenzten Teilungsfähigkeit hervorragend zur Differenzierung in nahezu alle gewünschten Zelltypen des menschlichen Körpers (Abbildung 2). Diese Zellen können theoretisch endlos in Kultur gehalten und kryokonserviert werden und sind somit ständig in ausreichender Menge verfügbar. Für die Differenzierung in einen gewebespezifischen Zelltyp, wie beispielsweise Kardiomyozyten[52], Hepatozyten[53], Neuronen[54], MSCs[55] oder Osteoblasten[56], sind verschiedene Protokolle in der Literatur verfügbar. Die Differenzierung erfolgt durch Zugabe von Wachstumsfaktoren, Zytokinen oder chemischen Substanzen und unter bestimmten Kulturbedingungen.

Der therapeutische Einsatz von Kardiomyozyten ist von besonderem Interesse, da aufgrund ihrer extrem geringen Selbsterneuerungsfähigkeit, mit weniger als 1% pro Jahr im erwachsenen Herzen eine ausreichende physiologische Regeneration des Herzmuskels nicht möglich ist.[57] Durch die Zerstörung von Kardiomyozyten kann es jedoch zu einer Herzinsuffizienz kommen, die zu einer Verringerung der Pumpleistung, Herzrhythmus-störungen und weiteren körperlichen Beeinträchtigungen führt und im schlimmsten Fall eine Herztransplantation erfordert.[58] Dabei führt die Verletzung des Herzmuskels eher zur Vernarbung des Gewebes anstatt zur Regeneration.[59] Neben Vorhofflimmern und arterieller Hypertonie ist der Myokardinfarkt eine häufige Ursache für die Herzinsuffizienz, die derzeit zu einer der häufigsten Todesursachen zählt und weltweit 23 Millionen Menschen betrifft.[60] In früheren in vivo Studien wurde z. B. in einem porcinen Myokardmodell gezeigt, dass die Transplantation von Kardiomyozyten die Herzleistung nach einem Herzinfarkt verbessern kann.[61] Ebenso konnte durch eine Transplantation von humanen ESCs[58, 62] oder von aus iPSC

differenzierte Kardiomyozyten[63] in das Herzinfarktgewebe eine Verbesserung der Herzfunktion gezeigt werden. Weiterhin zeigten klinische Studien bereits durch den Einsatz von kardialen Vorläuferzellen (CPCs), die unter anderen in Kardiomyozyten differenzieren, einen positiven Einfluss bei der Behandlung verschiedener Herzkrankheiten.[64] Aufgrund des Mangels an geeigneten Quellen für humane Kardiomyozyten wurden in den letzten Jahren verschiedene Ansätze zur Erzeugung von regenerativ wirksamen Kardiomyozyten aus verschiedenen Zellquellen entwickelt, einschließlich Stammzellen aus dem Knochenmark[65], ESCs[66, 67], iPSCs[52, 68] und CPCs[64, 69].

Ein weiteres Beispiel für die mögliche Anwendung von gewebespezifischen Zellen ist im Bereich der Mund-, Kiefer- und Gesichtschirurgie. Dabei stellt die Rekonstruktion und Regeneration kleiner als auch großer Knochendefekte eine ständige Herausforderung dar, da die benötigte Anzahl der Zellen zur Behandlung des Patienten begrenzt ist und oft nicht ausreicht. Humane Kieferperiostzellen (JPCs) stellen eine ausgezeichnete aber begrenzte Quelle für osteogene Vorläuferzellen dar, die für die Generierung von 3-dimensionalen Knochengewebekonstruktionen verwendet werden können.[70, 71] Um ausreichende Mengen an Zellen zu erhalten, können autologe iPSCs osteogen differenziert werden. Dies kann entweder direkt oder anhand eines zweistufigen Prozesses erfolgen, indem die iPSCs zuerst in induzierte MSCs (iMSCs) und anschließend in Osteoblasten differenziert werden.[55, 56] Die Differenzierung von iPSCs in iMSCs hat den Vorteil, dass das Potenzial zur Tumorbildung im Vergleich zu Knochenmarks-MSCs verringert ist, da sie nicht den epithelial-mesenchymalen Übergang (EMT), die Invasion und die Stammzelleigenschaften von Krebszellen fördern, sowie geringere Level an Tumor-fördernden Faktoren exprimieren.[72] Ein weiterer Vorteil ist, dass die dazu verwendeten Zellkultur- und Differenzierungsprotokolle für MSCs standardisiert werden können und die Verwendung von iPSCs eine zusätzliche genetische Modifikation vereinfacht.[72]

1.3. Modifikation von Zellen zur therapeutischen Behandlung

1.3.1. Gentherapie

Die Gentherapie beruht auf der Verwendung von Nukleinsäuren als therapeutischen Wirkstoff zur Reparatur oder zum Ersatz defekter Erbinformationen für die Behandlung von Krankheiten, indem sie in die Zellen des Patienten eingebracht werden, um diese zu modifizieren. In den Zellen werden die Nukleinsäuren dann entweder in Proteine translatiert oder können eingesetzt werden, um die Expression anderer Proteine zu beeinflussen oder um genetische Mutationen zu korrigieren.[3] Im Vergleich zur Gentherapie ist die Verwendung von rekombinanten Proteinen teuer und aufgrund ihrer relativ kurzen Halbwertszeiten ist es schwierig ausreichende Proteinmengen in gewünschten Regionen des Körpers aufrechtzuerhalten.[73] Für die Initiierung der exogenen Proteinexpression in Zellen können

verschiedene DNA- oder RNA-basierte virale und nicht-virale Vektoren für den Gentransfer verwendet werden, wie Retroviren[43], Adenoviren[74], PiggyBac-Transposons[75], episomale Plasmide z. B. des Epstein-Barr-Virus[76], Sendai-Viren[77], synthetische selbst-replizierende RNA (srRNA) Replikons[78], in vitro transkribierte mRNAs[79] oder mRNA-basierte CRISPR-Cas Systeme[80]. Bei Verwendung Genom-integrierenden Vektoren, wie z. B. Retroviren, bestehen jedoch Sicherheitsbedenken bei einer klinischen Anwendung, aufgrund der Integration von Transgenen in das Wirtsgenom und dem daraus resultierenden hohen Risiko einer Mutation.[81-83] Dabei kann es zur Reaktivierung von den exogen gelieferten Transgenen und somit zu einer unerwünschten zurück bleibenden Expression kommen.[84] Die Verwendung von integrativen Vektoren[85] weist auch eine erhöhte Immunogenität auf, was den Einsatz für klinische Anwendungen weiter einschränkt.[86] Die Integration von Transgenen kann vermieden werden, indem verschiedene nichtintegrative Vektoren (z.B. RNA-Viren, zirkuläre und lineare Plasmide, RNAs) und Vektor-Exzisionsverfahren (Cre-Lox-System[87], PiggyBac-Transposons[75]) für die Proteinexpression verwendet werden. Bei nicht-integrierenden DNA-basierte Methoden, wie z. B. Adenoviren oder Adeno-assiziierten Viren (AVV) liegt die DNA als eigenständiges Element (Episom) getrennt vom Erbgut des Empfängerorganismus vor und wird somit nicht von den Zellen verdoppelt. Daher geht es über die Zeit verloren und birgt nur ein geringes Risiko der genomischen Integration.[88] Des Weiteren ist die Effizienz der AVV-vermittelte Proteinproduktion deutlich geringer als bei Integrativen und DNA-freien Vektoren.[89] Trotz der geringen Immunogenizität der AVV kann es in manchen Fällen zur Erkennung durch das Immunsystem kommen und eine Immunreaktion des Empfängers auslösen.[88] Weiterhin limitierend ist die begrenzte Verpackungskapazität (~4,5 kb) der Vektoren, die es unmöglich macht, z. B. alle Komponenten einer Reprogrammierung auf einem einzigen AAV Vektor unterzubringen.[90] Auch die Verwendung von Sendai Viren als Vektor ist integrationsfrei und unabhängig von Kernfaktoren. Es beinhaltet keine DNA-Phase, da es sich um ein RNA-Virus handelt.[91] Jedoch ist auch hier die Packungskapazität limitiert. Sendai Viren bieten eine gute Effizienz und einfache Verwendung, jedoch sind die verbleibenden Viruspartikel schwer wieder aus den Zellen zu entfernen, sodass multiple Durchgänge klonaler Expansion und Analyse erforderlich sind, um rückstandsfreie Zellen zu erhalten.[89, 91] In den letzten Jahren hat vor allem die Verwendung von synthetischer bzw. in vitro transkribierten (IVT) mRNAs, die für gewünschte Proteine kodieren, als eine vielversprechende Alternative zu viralen Vektoren an Bedeutung zugenommen. Ebenso wie bei der Verwendung von Sendai Viren muss die mRNA nicht in den Nukleus der Zelle gelangen, da die Proteinbiosynthese im Zytosol stattfindet und somit besteht keine Gefahr der Integration. Bei dieser Methode müssen auch keine Vektorrückstände entfernt werden, da die mRNA durch natürliche Abbaumechanismen der Zellen abgebaut wird.[79, 92, 93] Dies ermöglicht auch die gezielte Kontrolle der effizienten Proteinproduktion.[94]

1.3.2. Therapeutischer Einsatz von synthetischer mRNA

Die Verwendung von synthetischer mRNA zum Proteinersatz ist kein neues Konzept, es war jedoch über viele Jahre hinweg wegen der Instabilität und Immunogenität des Moleküls nicht sehr beliebt und für therapeutische Anwendungen ungeeignet. Vor fast 15 Jahren gelang Kariko und ihren Kollegen ein Durchbruch in der mRNA-Therapie, wodurch diese Problematik, durch den Einbau von (natürlich vorkommenden) modifizierten Nukleosiden in die mRNA[95], weitestgehend behoben werden konnten. Nachdem gezeigt werden konnte, dass mRNA Modifikationen und die Optimierung der Applikations- und Transportverfahren, sowie die effiziente und kostengünstige Herstellung die Verwendung von mRNAs für therapeutische Anwendungen ermöglichen, wurde auch das Engineering von Zellen mit synthetischer mRNA immer populärer.[96, 97]

Das enorme Potenzial der synthetischen mRNAs wurde bereits in verschiedenen vielversprechenden Studien in unterschiedlichen Bereichen der regenerativen Medizin bestätigt. Dazu zählen unter anderem die Reprogrammierung von somatischen Zellen zu iPSCs[79, 93], die direkte Reprogrammierung/ Konversion oder (Trans-)Differenzierung von Zellen[98-100], sowie die Verbesserung des Homing-Verhaltens von Stammzellen in bestimmte Gewebe[101] und die Produktion von Wachstumsfaktoren[94, 102, 103] (Abbildung 3). Dabei können nach einer exogenen Zuführung der synthetischen mRNAs in die Zellen auch funktionale Proteine hergestellt werden, die nicht unbedingt von den Zellen natürlicherweise hergestellt oder benötigt werden. Somit werden Zellen generiert, die ein neues Proteinexpressionsprofil aufweisen oder zelluläre Funktionen können verbessert werden.[101, 103] Darüber hinaus wird die Applikation von synthetischer mRNA auch zur Vakzinierung erfolgreich untersucht, um Tumorwachstum[104-106] und Infektionskrankheiten[107-109] zu behandeln. Dabei wird durch die mRNA-vermittelte Synthese von Tumorzell- oder pathogen-spezifischen Antigenen das erworbene Immunsystem aktiviert[105, 107, 109] und somit werden die Tumorentwicklung und Infektionskrankheiten verhindert oder es kommt zu einer verbesserten Eradikation der Tumorzellen. Weitere Studien zeigten auch, dass synthetische mRNAs für Protein-ersatztherapien genetisch bedingter Erkrankungen erfolgreich eingesetzt werden können[110-113], wenn bestimmte Proteine nicht oder nur fehlerhaft von den Zellen synthetisiert werden können.

Im Vergleich zu sämtlichen Genom-integrierenden Methoden, ist der größte Vorteil bei der Verwendung von synthetischer mRNA als Vektor zur exogenen Proteinproduktion, dass die mRNA nicht ins Genom der Empfänger-/ Hostzellen integriert, sondern im Zytoplasma verbleibt. Somit besteht durch die mRNA keine Gefahr eine Mutation oder Krebs auszulösen. Die mRNA Moleküle werden nach Transfektion der Zelle durch natürliche Abbaumechanismen nach 2-3 Tagen degradiert.[79, 92, 93] Aufgrund der relativ kurzen Halbwertszeit von mRNA ist eine Inaktivierung oder ein Herausschneiden des Vektors, wie es bei den integrativen

Methoden nötig ist, nicht erforderlich. Zusätzlich kann durch die transiente Wirkung der mRNA[114] eine einfache und präzise Kontrolle der Proteinbiosynthese erfolgen.[94] Weiterhin ist es vorteilhaft, dass mRNAs in das Zytoplasma der Zellen eingebracht werden, wo sie direkt in gewünschte Zielproteine übersetzt werden, ohne dass sie in den Zellkern gelangen müssen. Im Vergleich dazu müssen die DNA-Plasmide während des mitotischen Zustands in den Kern gelangen, um eine effektive Proteinexpression zu erhalten. Im Gegensatz dazu findet die mRNA-vermittelte Proteinexpression sowohl in sich teilenden und nicht teilenden Zellen statt. Da die DNA Plasmide größer sind als die mRNA-Moleküle können die Zellen weniger effizient transfiziert werden.[115]

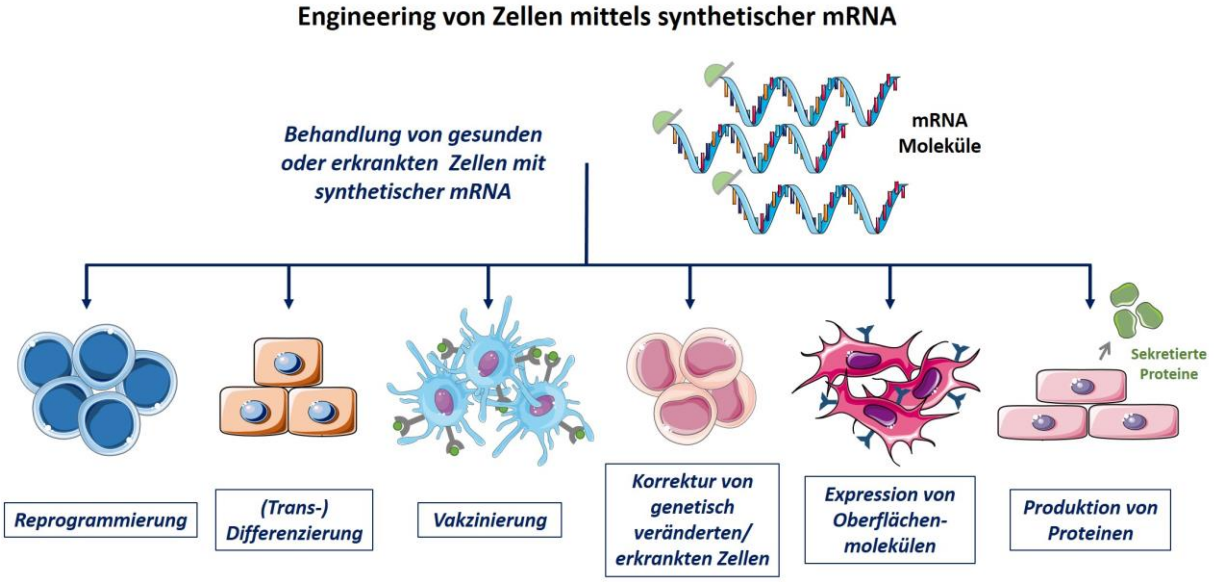

Abbildung 3: Perspektiven zum Einsatz von synthetischer mRNA für das Konstruieren von Zellen für therapeutische Zwecke. Die exogene Überexpression von Proteinen mittels von mRNA kann zur Reprogrammierung von somatischen zu induziert pluripotenten Stammzellen, zur direkten (Trans-)Differenzierung in einen gewünschten Zelltyp (z.B. Hepatozyten), zur Vakzinierung oder für die Korrektur von genetischen Defekten in erkrankten Zellen, der Expression von Oberflächenmolekülen/ Rezeptoren und für die Produktion von sekretierbaren Proteinen, eingesetzt werden. Die Grafik wurde mit Hilfe von Servier Medical Art Vorlagen erstellt.

Ein weiterer Vorteil dieser Methode ist, dass die synthetischen mRNAs von den zelleigenen Ribosomen in Proteine translatiert und vom endoplasmatischen Retikulum und Golgi-Apparat post-transnational modifiziert werden, um funktionale extrazelluläre[102, 112] und intrazelluläre Proteine[79, 114], sowie Rezeptoren[110] mit richtiger Faltung und Glykosylierungsmustern herzustellen, wodurch die Bildung von Antikörpern gegen diese Proteine durch das Immunsystem verhindert wird. Theoretisch gibt es keine Größenbeschränkung für die Erzeugung der gewünschten Ziel-mRNA-Sequenz und ihre Herstellung kann im erforderlichen Maßstab mit im Handel erhältlichen Materialien durchgeführt werden. Jedoch ist die Verwendung von synthetischer mRNA immer noch teuer, da sie für therapeutische Ansätze in großen Mengen und mit hoher Reproduzierbarkeit unter GMP-Bedingungen (Qualität unter guter Herstellungspraxis) hergestellt werden muss. Im Vergleich zu rekombinanten Proteinen,

die in eukaryotischen Zellen hergestellt werden, sind die GMP-Produktionskosten für synthetische mRNAs ungefähr fünf- bis zehnfach niedriger.[96] In den letzten Jahren wurden außerdem mRNA-Transportmethoden, Transfektionsreagenzien und das mRNA-Molekül selbst optimiert, um mehr Stabilität und eine bessere Translationseffizienz, sowie eine geringere Immunogenität zu erreichen.[79, 116, 117] Für Vakzinierungsanwendungen mit synthetischen mRNAs könnte die Induktion einer Immunantwort jedoch vorteilhaft sein, da diese zusätzlich als Adjutant wirken können, indem sie die immunologische Antwort und die Antigenpräsentation verstärken.[118]

1.3.3. Aktivierung des Immunsystems durch synthetische mRNA

In eukaryotischen Zellen sind Mustererkennungsrezeptoren (PRRs) des angeborenen Immunsystems, einschließlich der Toll-like-Rezeptoren (TLR) 3[119, 120], TLR7[121-124] und TLR8[123], sowie der Retinsäure-induzibares Gen-I (RIG-I)-ähnliche Rezeptoren (RLRs)[125] in der Lage fremde einzelsträngige (ss) oder doppelsträngige (ds) RNA-Moleküle zu erkennen und Signale zu übertragen, um eine Nuklear-faktor κB (NF-κB) und Typ I-Interferon (IFN) vermittelte virale Immunantwort zu induzieren. Die Aktivierung der zytoplasmatischen RLRs und die endosomalen Rezeptoren TLR3 und TLR7/8 führen dabei zur nachgeschalteten Regulation der Transkriptionsfaktoren Interferon-regulatorischer Faktor (IRF) 3, IRF7 und NF-κB.[126] Dadurch wird die Expression von proinflammatorischen Zytokinen (wie z. B. TNFα, IL-6, IL-1β, IL-10) und der Typ I-Interferone (IFN-α und IFN-β) ausgelöst. Die RLRs, einschließlich RIG-I[127-131], Melanom-Differenzierungsantigen 5 (MDA5)[132-134] und Labor für Genetik und Physiologie 2 (LGP2)[125] erkennen fremde RNA im Zytoplasma der meisten Zelltypen und initiieren die Signalweiterleitung, die zum Auslösen der viralen Immunantwort führt. Neben der TLR3 vermittelten Erkennung von RNA sind auch Nukleotid-Bindungsdomänen (NOD)-ähnliche Rezeptoren (NLRs)[135], vor allem NLRP3[136] und NOD2[137], an der zellulären Erkennung von RNA beteiligt. NLRs regulieren die Produktion von Caspase-1, die bei der Apoptose, dem programmierten Zelltod, eine wesentliche Rolle spielt.[136] Die Überexpression von Caspase-1 nach Applikation von synthetischer mRNA kann demnach zum verstärkten Zelltod und zu Entzündungsreaktionen führen.[135]

Bei der Interferon-abhängigen Immunantwort binden die Typ-I-IFNs an IFN-Rezeptoren der aktivierten Zellen und führen so zur Aktivierung von Signaltransduktor und Aktivator der Transkription (STAT) Komplexen und zur Erzeugung des IFN-stimuliertem Genfaktors 3 (ISGF-3), der die Transkription von mehr als 300 IFN-stimulierten Genen (ISGs) initiiert.[126] Dazu gehören Effektoren/Enzyme, die durch dsRNA Moleküle aktiviert werden, wie die dsRNA-abhängige Proteinkinase (PKR), 20-50 Oligoadenylatsynthetasen (OASs) und die RNA-spezifische Adenosindeaminase (ADAR), die zur Verstärkung der antiviralen Aktivität produziert werden.[138] Die Aktivierung der PKR durch dsRNA führt zur Hemmung der

Translation von mRNA und damit zur Reduktion der Proteinsynthese[139], die bei einer möglichen Virusinfektion zur Unterdrückung der Virusreplikation erforderlich ist. Der Transkriptionsfaktor NF-κB kann ebenfalls aktiviert werden und die durch PKR vermittelte zelluläre Apoptose induziert werden.[140] Die Aktivierung von OASs durch dsRNA führt zur Spaltung von ssRNA und somit zur Degradation der RNA[141], die dann erneut von PRRs gebunden werden können. Bei der dsRNA-abhängigen Aktivierung von ADARs kommt es zu einer RNA-Editierung durch Umwandlung von Adenosin zu Inosin. Folglich kann die Bildung von schwachen Inosin:Uridin-Fehlpaarungen die RNA destabilisieren und die Kodierungskapazität der mRNA reduzieren.[142, 143]

1.3.4. Herstellung und Modifikationen von synthetischer mRNA

Durch verschiedene Modifikationen der synthetischen mRNA kann die Immunaktivierung deutlich herabgesetzt, die Stabilität der Moleküle erhöht, sowie die Translationseffizienz verbessert und somit das Potenzial für therapeutische Anwendungen gesteigert werden.[96] In eukaryotischen Zellen tragen mRNA-Moleküle natürlicherweise am 5‘-Ende eine 7-Methylguanosin (7mG) Cap-Struktur, die durch eine 5‘5‘-Triphosphatbrücke mit der mRNA verbunden sind[144] und am 3‘-Ende eine lange Sequenz eines Polyadenylat-Anhangs, dem Poly(A)-Schwanz, die nach der Transkription der DNA hinzugefügt werden. Die Cap-Struktur und der Poly(A)-Schwanz können die Stabilität und Translation stark verbessern, daher erhält die synthetisch hergestellte in vitro transkribierte (IVT) mRNA diese Strukturen (Abbildung 4A).

Die Cap-abhängige Translation wird durch die Bindung der 5'-Cap Struktur an den Eukaryoten-Translationsinitiationsfaktor 4E (eIF4E) initiiert, der anschließend mit einer Vielzahl von Translationsinitiationsfaktoren interagiert, um den Translationsinitiationskomplex zu bilden. Dabei wird die Bindung der mRNA an die kleine ribosomale Untereinheit gefördert und somit die Halbwertszeit der mRNA im Zytoplasma verlängert, was wiederum die Translation der synthetischen mRNA in das gewünschte Protein verbessert.[145-148] Darüber hinaus schützt die 5’Cap-Struktur die mRNAs vor dem Abbau durch Exonukleasen.[149, 150] Die IVT mRNA kann durch Zugabe eines Cap-Analogs während der IVT co-transkriptionell gekappt werden oder post-transkriptionell unter Verwendung von Capping-Enzymen eines rekombinanten Vaccinia-Virus [151] (Abbildung 4B). Die enzymatische Reaktion ermöglicht eine einfache und vollständige Verkappung der 5'-Enden (m7GpppG; cap0) der IVT-mRNA. Weitere Cap-Strukturen können generiert werden, indem eine zusätzliche Methylierung an der 2′-O Position des ersten Nukleotids (m7GpppNmpN; cap1) oder sowohl des ersten als auch des zweiten Nukleotids (m7GpppNmpNm; cap2) eingefügt wird, die die Translationseffizienz weiter verbessern.[152] Die Co-transkriptionelle 5‘-End-Modifikation von IVT-mRNAs mit einem Cap-Analogon ist einfacher und kostengünstiger als das enzymatische Verkappungsverfahren und wird daher häufiger angewendet. Dabei wird während der IVT eine überschüssige Menge an verkappten

Dinukleotiden (z. B. m7GpppG) zugesetzt und an das 5‘-Ende der mRNA angehängt. Im IVT Ansatz sind für die Synthese der mRNA auch Guanosintriphosphate (GTPs) enthalten, die in Konkurrenz zu m7GpppG eingebaut bzw. angehängt werden. Dadurch werden auch nicht-gekappte mRNAs generiert, die nicht translatiert werden.[96] Zusätzlich zum unvollständigen 5’-Capping der synthetischen mRNA, kann es bei der co-transkriptionellen Modifikation auch zum Einbau der Cap-Struktur in falscher Orientierung kommen, wodurch die Initiation der Translation gestört wird. Eine Möglichkeit dieses Problem zu umgehen, ist die Verwendung eines Anti-Reverse-Cap-Analogons (ARCA, 3´-O-Me-m7G(5')ppp(5')G), bei der die 3’-OH Gruppe durch 3‘-OCH_3 ersetzt wurde. Somit wird verhindert, dass die Cap-Struktur in falscher Orientierung bindet.[145, 148]

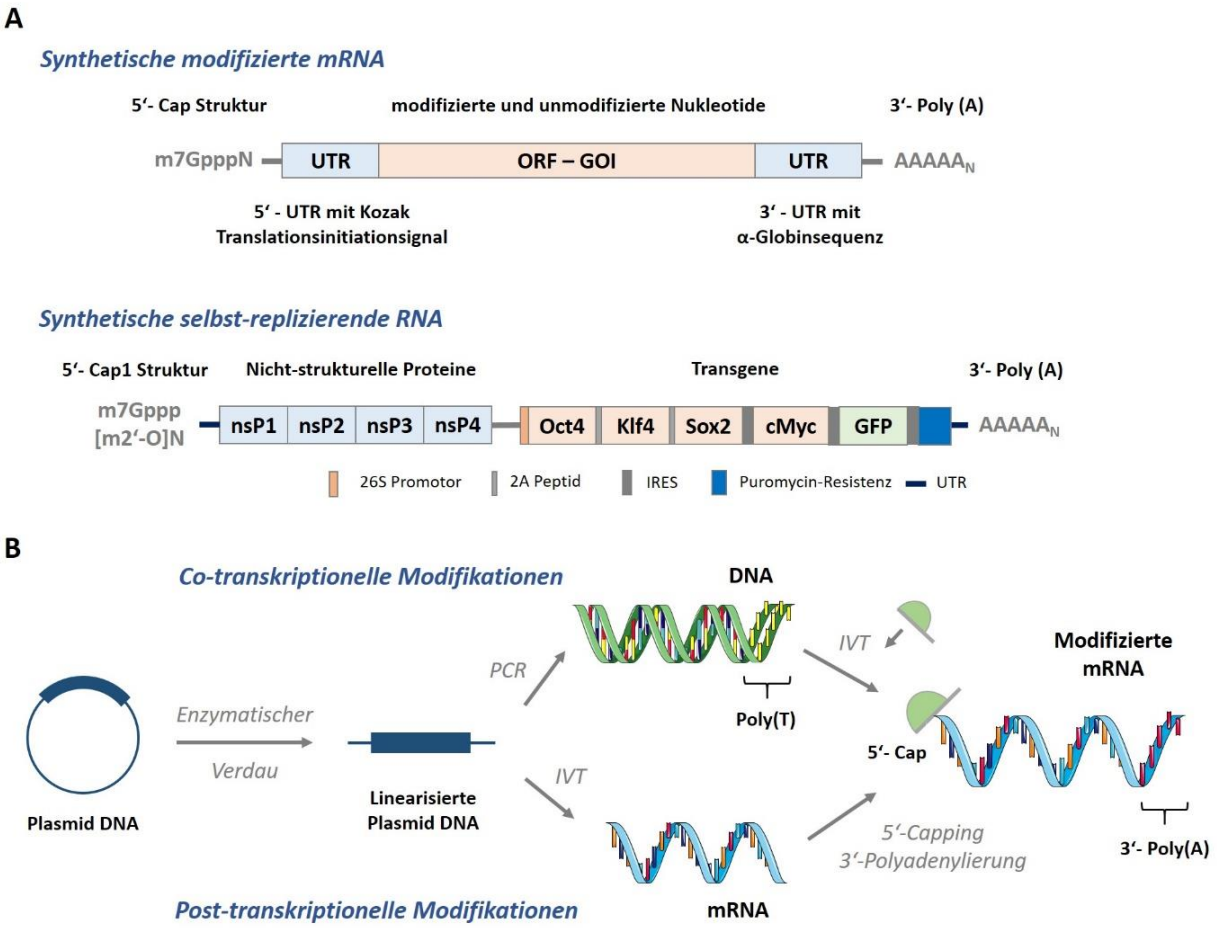

Abbildung 4: Schematischer Aufbau von synthetischer mRNA und srRNA (A). Modifikationen der UTRs, sowie das Einfügen einer 5’-Cap Struktur, eines 3‘-Poly(A) Schwanzes und modifizierten Nukleotiden erhöhen die Stabilität und Translationseffizienz der RNAs und verringern die Immunogenität. Die srRNA enthält die nicht-strukturellen Proteine (nsP1-4), die für die Replikationsmaschinerie kodieren, sowie die Reprogrammierungsgene (Oct4, Klf4, Sox, cMyc) und eine Reporter GFP Sequenz auf einem Konstrukt. Das Puromycin-Resistenzgen ermöglicht die Positivselektion von srRNA transfizierten Zellen. (B) Die Modifikation der synthetisch hergestellten RNAs kann während (co-transkriptionell) oder nach (post-transkriptionell) der mRNA Synthese mittels In-Vitro-Transkription (IVT) erfolgen. Bei der Co-transkriptionellen Modifikation wird anhand eines 3‘-Poly(T) DNA Templates der Poly(A) Schwanz angehängt und eine Cap-Struktur zur IVT Reaktion zugegeben. Bei der post-transkriptionellen Modifikation wird nach der IVT enzymatisch die 5‘-Cap Struktur und der 3’-Poly(A) Schwanz angehängt. Die Grafik wurde mit Hilfe von Servier Medical Art Vorlagen erstellt.

Neben der 5‘-terminalen Modifikation der IVT-mRNA wird das 3‘-Ende ebenfalls modifiziert, indem ein Poly(A)-Schwanz (100-250 A‘s) an der 3'-untranslatierten Region (UTR) angehängt wird, um die mRNA vor Nukleaseabbau zu schützen.[145, 153] Dabei lagert sich das Poly-(A)-bindende Protein (PABP) an den PolyA-Schwanz an, das dann mit dem Cap-Bindungskomplex interagiert und eine zirkuläre mRNA-Struktur bildet, die die mRNA schützt. Die Polyadenylierung der synthetischen mRNAs kann ebenfalls co- oder post-transkriptionell erfolgen (Abbildung 4). Mithilfe eines Poly(T) Primers bestimmter Länge kann während der Polymerase Kettenreaktion (PCR) ein Poly-Thymin (T) Anhang generiert werden. Dieser wird dann während der IVT in einen Poly(A) Schwanz transkribiert. Post-transkriptionell kann die Polyadenylierung nach der IVT anhand einer enzymatischen Reaktion mithilfe einer rekombinanten Poly(A)-Polymerase erfolgen. Dadurch können jedoch mRNAs mit unterschiedlich langen Poly(A)-Anhängen entstehen. Diese Reaktion ist somit schlechter kontrollierbar und weist eine höhere Batch-to-Batch Variabilität auf.

Weiterhin wichtig sind auch Modifikationen der synthetischen mRNAs durch den Einbau von modifizierten Nukleosiden, wie beispielsweise 5-Methylcitidin (5mC) und Pseudo-Uridin (ΨU) anstelle von Cytidin (C) und Uridin (U), die zu einer verringerten Immunogenität führen und die Stabilität der mRNA fördern.[95, 117, 139] Dadurch wird die durch die synthetischen mRNAs verursachte Toxizität und der verstärkt auftretende Zelltod reduziert, sowie die Proteinexpression erhöht. Eine erfolgreiche Anwendung von IVT-mRNAs die zu 100% mit 5mC und ΨU modifiziert wurden konnte beispielsweise für die Erzeugung von iPSCs gezeigt werden.[79]

Darüber hinaus spielen auch die kodierende Sequenz und die 5‘- und 3‘-terminalen UTRs bei der Proteinsynthese und der Stabilität der mRNA eine Rolle. Die genetische Information die in einem offenen Leserahmen (open reading frame (ORF)) für ein gewünschtes Protein kodiert kann so optimiert werden, dass die Translationseffizienz gesteigert wird. Dabei werden selten vorkommende Codons gegen häufig verwendete Codons ersetzt, die mehrfach von der entsprechenden tRNA verwendet werden und somit den Prozess beschleunigen.[96] Durch die Reduktion von UU und UA Dinukleotid-Abfolgen kann der Endonukleaseangriff verringert und somit die Stabilität der mRNA erhöht werden.[154] Eine Codon-Optimierung wird für eine bestimmte kodierende Sequenz empfohlen, außer für Proteine, die eine langsamere Translation erfordern, da schon der Austausch eines einzigen Codons die Proteinexpression, Faltung und zelluläre Funktion eines Proteins beeinflussen kann.[155] Weiterhin können die DNA Templates für die IVT durch den Einbau von 5'- und 3'- terminalen UTRs optimiert werden, wie z.B. alpha- und beta-Globin-UTRs[156, 157], um die RNA-Stabilität und die Translationseffizienz zu erhöhen. Darüber hinaus verzögern die UTRs die Abspaltung des 5‘-Cap von der mRNA und somit die Degradation der synthetischen mRNA.[96] Zusätzlich erhöht das Vorhandensein

einer starken Kozak-Translationsinitiationssequenz in der 5′-UTR Sequenz der mRNA die Translationskapazität.[158]

Trotz all dieser Modifikationen können synthetische mRNAs immer noch durch PRR-Erkennung eine Typ-I-Interferon-abhängige Immunantwort induzieren.[159-161] Ein Grund hierfür ist, dass nach der IVT neben den gewünschten einzelsträngigen modifizierten mRNAs auch unerwünschte RNA-Moleküle enthalten sein können, wie z. B. verkürzte RNAs, die aufgrund einer unvollständigen Synthese entstanden sind, nicht gekappte mRNAs oder dsRNA-Fragmente, die am 5‘-Ende ein Triphosphatgruppe (5‘ppp) tragen und zur Aktivierung von RIG-I führen können.[128, 129] Diese RNA Kontaminationen können eine Immunreaktionen verursachen und sollten daher nach der IVT Reaktion eliminiert werden. Die Aufreinigung der mRNA Produkte kann mittels Hochleistungsflüssigkeitschromatographie (HPLC) effektiv erfolgen.[116] Eine weitere Methode zur Eliminierung von dsRNA Kontaminationen ist die schnelle und Kosten-effektivere Aufreinigung mithilfe von Cellulose in einem Ethanol-enthaltenden Puffer.[162]

1.4. Generierung von iPSCs mittels RNA

Für die Herstellung von iPSCs aus humanen Fibroblasten wurden bereits vor fast 10 Jahren ein Cocktail aus synthetischen modifizierten mRNAs, die für die Reprogrammierungsfaktoren Oct4, Sox2, Klf4, cMyc und Lin28 kodieren, erfolgreich eingesetzt.[79] Da die Proteinexpression durch synthetische mRNAs transient ist, besteht ein klarer Nachteil der mRNA-basierten Reprogrammierung darin, dass mehrere mRNA-Transfektionen für eine kontinuierliche Expression der Reprogrammierungsfaktoren in einer Zelle über ungefähr zwei Wochen erforderlich sind. So müssen die somatischen Zellen täglich mit dem Cocktail aus synthetischen mRNAs und Transfektionsreagenz behandelt werden, um den Proteingehalt aufrechtzuerhalten. Dies führt jedoch zu einer wiederholten und verstärkten Aktivierung der angeborenen Immunantwort, was dann zum übermäßigen Zelltod führt. Daher sind die Unterdrückung der Immunogenität gegen exogen zugeführte mRNA und die erhöhte Stabilität der mRNA-Moleküle für eine effiziente Reprogrammierung von Zellen besonders wichtig. Die Unterdrückung dieser Immunantwort kann neben den mRNA-Modifikationen durch die Zugabe des immunsuppressiven Proteins B18R (Interferoninhibitor) erreicht werden, was die wiederholte Transfektion von Zellen und somit die iPSC-Generierung ermöglicht.[79, 163]

Bisher wurden zumeist primäre Fibroblasten anhand der mRNA Transfektion mit hoher Effizienz zu iPSCs reprogrammiert.[79, 164, 165] Dazu zählen neonatale, adulte und seltener seneszente Fibroblasten[163], aber auch Fibroblasten von erkrankten Patienten (z. B. Long-QT-Syndrom[166], Leigh’s Syndrom[167], Huntington-Krankheit[168]). Die erfolgreiche mRNA-basierte Reprogrammierung von iPSCs wurde auch aus Fettgewebezellen[169], Zahngewebe und Urin isolierten Zellen[170], sowie aus Blut isolierten EPCs[171] bestätigt.

Da die tägliche Transfektion von Zellen mit einem Cocktail aus mehreren mRNAs relativ arbeits-, zeit- und kostenintensiv ist, hat die Arbeitsgruppe um Yoshioka et al. eine selbst-replizierende RNA (srRNA) entwickelt, die die Reprogrammierungsfaktoren (Oct4, Sox2, Klf4, cMyc) in vier offenen Leserahmen auf einem RNA Konstrukt enthält und somit nach einmaliger Transfektion eine kontinuierliche Expression der Proteine über mehrere Zellteilungen hinweg erlaubt.[78] Um dieses Ziel zu erreichen, wurde ein nicht-infektiöser, nicht-packender und selbst-replizierender RNA-Virus (Venezolanischer Pferdeenzephalomyelitis Virus, VEE) als Basis verwendet[172] und modifiziert, um die ektopische Überexpression der Reprogrammierungs-faktoren zu vermitteln (Abbildung 4A). Das Genom des Virus basiert auf einer positiv-strängigen ssRNA, die zelluläre 5‘-gecappte und 3‘-polyadenylierte mRNA nachahmt.[172] Die nicht-strukturellen Proteine (nsP1-4) kodieren für den Selbstrelikationskomplex der RNA und die strukturellen Proteine (C, E1, E2) des VEE Virus wurden durch die Reprogrammierungs-gene ausgetauscht.[173] Zusätzlich wurde eine Puromyzinresistenz eingefügt, um die Positivselektion[78] transfizierter Zellen zu ermöglichen. Eine für das GFP kodierende Sequenz dient zur Analyse der Transfektionseffizienz und Translationskontrolle in den Zellen. Aufgrund der sehr langen Größe der srRNA Konstrukte (ungefähr 17500 Nukleotide) ist jedoch auch bei dieser Methode die Verwendung eines Interferoninhibitors (B18R) erforderlich, um die Immunreaktion zu unterdrücken. Durch Entzug von B18R nach erfolgreicher iPSC Generierung kann die srRNA gezielt selektiv aus den Zellen degradiert werden.[78]

1.5. Transport von mRNA-basierten Wirkstoffen in Zellen

Um eine erfolgreiche Wirkung von synthetischen mRNAs zu erzielen, ist es wichtig eine ausreichende Menge des gewünschten Proteins in einer möglichst großen Anzahl der Zielzellen herzustellen. Daher ist es zunächst erforderlich, die synthetische mRNA effektiv und sicher in das Zytoplasma der Zielzellen zu transportieren (Abbildung 5). Da Nukleinsäuren, wie mRNAs, negativ geladene hydrophile Moleküle sind, wird ihre Aufnahme in die Zelle durch die Plasmamembran erschwert. Daher sind effiziente Abgabe-/ Transportsysteme erforderlich, die mehrere Funktionen erfüllen sollten: (1) Bindung der mRNA zur Ausbildung von Transfektionskomplexen, (2) Vermittlung der Aufnahme in die Zellen, (3) Schutz der mRNA vor intrazellulärem und extrazellulärem Nuklease-Abbau und (4) Freisetzung der mRNA ins Zytoplasma.[174]

Häufig erfolgt die Transfektion von Zellen über die Erzeugung von Komplexen mit kationischen Lipid-basierten Transportvehikeln (beispielsweise Lipofectamin oder 1,2-Dioleoyl-3-trimethyl-ammoniumpropan (DOTAP))[175] und den negativ geladenen mRNA-Molekülen, die als Lipoplexe bezeichnet werden. Dadurch können positiv geladene Komplexe erzeugt werden, die die mRNA vor extrazellulärem Abbau durch RNasen schützen und den Eintritt der mRNA durch die negativ geladene Zelloberfläche in die Zelle mittels natürlicher Endozytose fördern.

Die Internalisierung von synthetischer mRNA in die Zellen findet durch die Clathrin- und Caveolin- vermittelte Endozytose und unter Beteiligung von Scavenger-Rezeptoren statt.[176, 177] Vesikel, die die mRNA-Transfektionskomplexe enthalten, werden dann zu späten Endosomen geleitet, entweichen ins Zytosol und fusionieren anschließend zu lysosomalen Vesikeln, die schließlich die mRNA freisetzen. Als weitere Transportmittel können auch andere Endozytose-vermittelnde kationische Träger wie Polyethylenimin (PEI), Poly(L-Lysin) oder Dendrimere eingesetzt werden, die sogenannte Polyplexe erzeugen und ebenfalls zur Abgabe von mRNA in Zellen verwendet werden. Eine andere übliche Methode zum Transport von mRNA in Zellen ist die Elektroporation.[178] Hier wird die Zellmembran-Lipid-Doppelschicht vorübergehend destabilisiert und die mRNAs dringen über die spontan gebildeten Poren in die Zellen ein nachdem ein starker elektrischer Feldimpuls an die Zellen angelegt wurde. Ein großer Nachteil dieser Methode ist, dass zahlreiche Zellen während dieses Verfahrens sterben. Die Elektroporation scheint jedoch für einige Zelltypen (z. B. Blutzellen) besser geeignet zu sein als die Lipid-basierte Transfektion.[96] Weiterhin werden zur mRNA Transfektion immer häufiger Lipidnanopartikel (LNPs) verwendet, die aus ionisierbaren kationischen Aminolipiden, Phospholipiden, zusammen mit hydrophobem Cholesterin und PEG zur verbesserten zellulären Aufnahme in die Zellen, hergestellt werden und sich bereits für den Transport von small interfering RNAs (siRNAs) in klinischen Studien als sicher und effizient erwiesen haben.[90, 179]

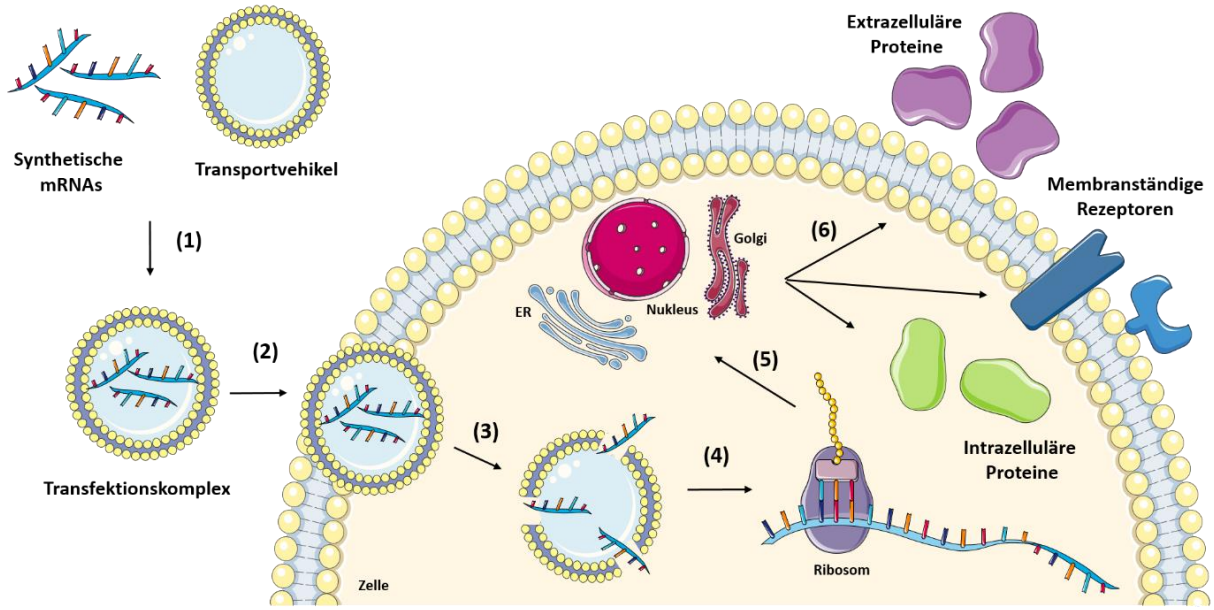

Abbildung 5: Schematische Darstellung der Transfektion von Zellen mit synthetischer mRNA und anschließende Translation. (1) Die synthetische mRNA wird zusammen mit dem Transportvehikel (kationischer Lipidkomplex) vermischt um Transfektionskomplexe zu bilden. Diese werden dann von der Zelle mittels Endozytose aufgenommen (2) und dadurch in die Zellen transfiziert. Im Zytosol werden die mRNAs freigesetzt (3) und von den Ribosomen translatiert (4). Durch post-translationale Modifikationen (Endoplasmatisches Retikulum, Golgi-Apparat) und Faltung der Polypeptidketten entstehen so funktionale intrazelluläre, extrazelluläre oder membrangebundene Proteine (5). Die Grafik wurde mit Hilfe von Servier Medical Art Vorlagen erstellt.

1.6. Hydrogel-vermittelte Applikation von mRNA

Die Applikation von synthetischer modifizierter mRNA zur zielgerichteten Belieferung in bestimmte Zellen oder Gewebe spielt für eine effiziente und gezielte Therapie eine wichtige Rolle. So sind bei einer systemischen Verabreichung (intravenös) hauptsächlich Zellen im Blutkreislauf, Leberzellen und retikuloendotheliale Zellen in der Milz und dem Knochenmark mit Trägermaterial verpackten mRNAs zu erreichen.[96] Die systemische Abgabe von IVT-mRNA an andere Gewebe wird durch nicht durchlässige Gefäßendothelien, interzelluläre Übergänge und dichte extrazelluläre Fibrillennetzwerke behindert, die die Zugänglichkeit der Zielzellen, wie Muskelzellen einschränken.[96, 180] Wirkstofffreisetzende Systeme, die kontinuierlich synthetische mRNA lokal an die umliegenden Zellen im entsprechenden Gewebe über einen erforderlichen Zeitraum abgeben, um eine anhaltende Proteinexpression zu erhalten, stellen daher eine geeignete Alternative zur wiederholten Verabreichung von synthetischer mRNA dar.

Hydrogele sind 3-dimensionale Netzwerke, die aus natürlichen oder synthetischen hydrophilen Polymerketten bestehen und physikalisch oder chemisch vernetzt werden können.[181, 182] Aufgrund ihrer hohen Biokompatibilität, biologischen Abbaubarkeit und geringen Toxizität sind natürliche Polymere im Vergleich zu synthetischen Polymeren, vor allem auch im Bereich der regenerative Medizin, von Vorteil[181, 183], um z. B. durch eine kontrollierte Freisetzung von Arzneimitteln die Regeneration von Geweben anregen.[182, 184] Die Wirksamkeit von Hydrogelen für die Freisetzung eines breiten Spektrums verschiedener Therapeutika im Gewebe, wie Wachstumsfaktoren[185-187], Insulin[188, 189] und Krebsmedikamenten[186, 190] wurde bereits durch verschiedene Studien bestätigt und ist daher auch für die langanhaltende lokale Abgabe von mRNA interessant.

Alginat und Chitosan sind sehr häufig verwendete Biomaterialien zur Erzeugung von Hydrogelen. Alginat ist ein polyanionisches Polysaccharid, das aus alternierenden Blöcken von (1-4)-gebundenen β-D-Mannuronsäure und α-L-Glucoronsäure Monomeren besteht [191] und mit zweiwertigen Kationen wie Ca^{2+} ionisch vernetzt werden, um ein 3-dimensionales Netzwerk zu erhalten.[192] Dies kann auch in vivo nach Injektion einer Alginatlösung erfolgen, indem die Ca^{2+} Ionen aus dem umliegenden Gewebe herangezogen werden.[193] Darüber hinaus wurden Alginat-Hydrogele aufgrund ihrer Ähnlichkeit mit EZM-Proteinen[194] als Träger für Zellen und zur Freisetzung von Nukleinsäuren, wie siRNA[195, 196] und Plasmid-DNA (pDNA)[196, 197] verwendet. Im Gegensatz zu Alginat ist Chitosan ein kationisches Polysaccharid und wird durch Deacetylierung von Chitin hergestellt. Die Quervernetzung einer injizierbaren Chitosanlösungen kann beispielsweise durch Zugabe von Glycerinphosphat (GP) erfolgen, die bei physikalischem pH-Wert beim Erwärmen auf Körpertemperatur die Gelbildung in situ auslöst.[198] Aufgrund ihrer Ladung sind kationische Polymere günstig für die Freisetzung anionischer Moleküle, wie Nukleinsäuren, da sie zu kationischen Polyplexen kondensieren

können und dadurch die Transfektion von Zellen erleichtern.[199] Die Freisetzung funktioneller Nukleinsäuren aus Chitosan-Hydrogelen wurde bereits für RANK (Receptor Activator of NF-κB)- und Cy3-spezifische siRNA demonstriert, was zu einem verlängerten und lokalisierten Gen-Silencing in vitro und in vivo führte und eine gute Biokompatibilität aufweiste.[199] Durch die Verwendung von BMP2 (bone morphogenetic protein 2) pDNA, integriert in Chitosan Hydrogele konnte die Regeneration von Knochengewebe zur Heilung von Knochendefekten eingesetzt werden.[200]

Durch eine Kombination von Chitosan und Alginat werden Polyelektrolytkomplexe ausgebildet, die genutzt werden können, um sogenannte Hybridhydrogele herzustellen.[201, 202] Aufgrund der elektrostatischen Wechselwirkungen der entgegengesetzt geladenen Polymere beider Biomaterialien zeigt die Hybridmaterialkombination im Vergleich zu beiden Polymeren allein eine erhöhte Stabilität.[203] Chitosan-Alginat-Hybridhydrogele unterstützen das Zellwachstum, die Wundheilung und Knochenregeneration und förderten die Bildung des Knorpelgewebes durch die verstärkte Anlagerung von Osteoblasten an die Gerüststruktur[202] und in Kombination mit MSCs und rekombinantem BMP2[204]. Weiterhin wurden Chitosan-Alginat Hydrogele erfolgreich zur regenerativen Behandlung von Myokardinfarkten und zur Induktion der Angiogenese in Versuchen mit Ratten eingesetzt.[205] Darüber hinaus konnte gezeigt werden, dass der Einsatz von kationischen pDNA-Nanopartikeln, die in ein poröses 3D-Chitosan-Alginat-Gerüst eingebaut wurden, erfolgreich freigesetzt und von Prostatakrebszellen aufgenommen wurden und zur in vitro und in vivo Expression von RFP (rot fluoreszierendes Protein) führten.[206]

2. ZIELSETZUNG

Die Regenerative Medizin ist ein breit gefächerter Bereich mit enormen Potenzial, um eine Vielzahl von seltenen genetischen und weit verbreiteten Krankheiten zu behandeln und tatsächlich zu heilen. Durch Transplantation von autologen Zellen kann die Regeneration von Geweben angeregt werden, um deren Funktion zu verbessern oder wieder herzustellen. Die Gewinnung und Anzahl der benötigten patientenspezifischen Zellen und deren Integrationsfähigkeit und das Überleben nach der Transplantation stellt dabei die größte Limitierung für den therapeutischen Einsatz von Zellen dar.

Der Einsatz von synthetischer mRNA stellt ein vielversprechenden Ansatz dar, um die Proteinexpression in Zellen zu modulieren und somit deren natürliche Funktion zu verbessern oder ihr zelluläres Schicksal zu beeinflussen/ verändern. Im Vergleich zu DNA- und Virus-basierter Vektoren, stellt die synthetische mRNA eine interessante Alternative zur endogenen Proteinexpression dar. Die synthetische mRNA wird nicht ins Genom integriert und ruft somit keine Mutationen hervor und die Expression von Proteinen ist transient, schnell und effizient.

Daher sollen im Rahmen der vorliegenden Arbeit synthetische mRNAs eingesetzt werden, die für die proangiogene tische Proteine VEGF-A, ANG-1 oder SDF-1α kodieren, um die natürliche Funktion von endothelialen Progenitorzellen (EPCs) im Hinblick auf ihr angiogenetisches Potenzial zu verbessern, um somit eine effektivere therapeutische Anwendung von EPCs gewährleisten zu können. Nach der mRNA-basierten Modifikation der EPCs sollen typische Parameter, wie die chemotaktische Migration von modifizierten EPCs gegenüber nativer EPCs, deren Wundheilungskapazität und charakteristische Merkmale der Gefäßbildung in vitro und in vivo untersucht werden.

Die Wirkung von synthetischer mRNA ist aufgrund natürlicher Abbaumechanismen in Zellen transient und daher hält die Proteinexpression nur wenige Tage an. Jedoch ist eine länger anhaltende Wirkung der synthetischen mRNA für zahlreiche Applikationen, wie z. B. für Proteinersatz-Therapien oder zur Reprogrammierung und Differenzierung von Zellen notwendig, um den gewünschten Effekt zu erzielen.

Daher war ein weiteres Ziel dieser Arbeit die Etablierung einer selbst-replizierenden RNA (srRNA) zur Reprogrammierung von somatischen Zellen für die Herstellung von integrations-freien induziert pluripotenten Stammzellen (iPSCs), die als Quelle zur Differenzierung in einen gewünschten Zelltyp für zukünftige therapeutische Applikationen dienen können. Dafür sollte zuerst die synthetische mRNA-basierte Reprogrammierung von Fibroblasten, mit der srRNA-basierten Reprogrammierung verglichen werden. Zum Nachweis der Pluripotenz sollten typische Merkmale, wie spezifische Pluripotenzmarkerexpression mittels Antikörperfärbung und mRNA Expressionsanalysen, sowie die Differenzierungsfähigkeit in alle drei Keimbahnrichtungen in vitro mittels Durchflusszytometrie und in vivo anhand eines CAM-Teratomaassays überprüft werden.

Ebenfalls angestrebt war die Generierung von iPSCs mithilfe der srRNA aus renalen Epithelzellen, die aus humanem Urin isoliert wurden und deren anschließende Differenzierung in kontraktierende Kardiomyozyten, sowie die srRNA vermittelte Reprogrammierung von iPSC aus Kieferperiostzellen und darauffolgender osteogener Differenzierung.
Weiterhin sollten injizierbare Hydrogele, basierend auf natürlichen Biomaterialien, wie Chitosan und Alginat, entwickelt werden, die die lokale Freisetzung von synthetischen mRNAs verzögern und somit die Proteinexpression in den Zellen und deren Wirkung verlängern. Dabei sollten Freisetzungskinetiken von Fluoreszenz-markierten Luziferase (hGluc) mRNAs aus den Hydrogelen und die Bioaktivität der Luziferase mRNA, sowie der rheologische Einfluss der mRNA Integration in die Hydrogele auf die Eigenschaften analysiert werden.

3. ERGEBNISSE

3.1. PUBLIKATION I

Incorporation of Synthetic mRNA in Injectable Chitosan-Alginate Hybrid Hydrogels for Local and Sustained Expression of Exogenous Proteins in Cells

Heidrun Steinle, Tudor-Mihai Ionescu, Selina Schenk, Sonia Golombek, Silju-John Kunnakattu, Melek Tutku Özbek, Christian Schlensak, HansPeter Wendel, Meltem Avci-Adali

International Journal of Molecular Science / Akzeptiert am: 25. April 2018

Zusammenfassung:

Der Einsatz von synthetischer messenger RNA (mRNA) weist im Vergleich zu viralen und DNA Vektoren verschiedene Vorteile auf. Die gewünschten Proteine werden im Zytosol der Zellen anhand der mRNA synthetisiert, wodurch eine Integration des Vektors ins Empfängergenom vermieden wird und somit die mutagenen und karzinogenen Effekte minimiert werden. Die transiente Natur der mRNA ermöglicht eine einfache und gezielte Kontrolle der Proteinbiosynthese, erfordert jedoch eine wiederholte Transfektion der Zellen, um die Expression der gewünschten Proteine zu verlängern. Eine verzögerte Freisetzung der mRNAs und länger anhaltende Transfektion der Zellen könnte durch den Einsatz von Hydrogelen zur Wirkstofffreigabe erreicht werden.

In dieser Studie wurde eine synthetische mRNA, die für die humanisierte Gaussia Luziferase (hGLuc) kodiert, in ein Alginat-, Chitosan- oder Chitosan-Alginat Hybridhydrogel integriert und die Freisetzung der mRNA aus diesen Hydrogelen analysiert. Nach 3 Wochen wurde eine Freisetzung von 79% der eingebauten hGLuc mRNA aus dem Alginat-Hydrogel, 42% der mRNA aus dem Chitosan-Hydrogel und 70% der mRNA aus dem Chitosan-Alginat Hybridhydrogel detektiert. Aufgrund dieser Ergebnisse und der guten Injizierbarkeit wurde das Chitosan-Alginat Hybridhydrogel für die weiteren Untersuchungen der Bioaktivität der integrierten hGLuc mRNA ausgewählt. Die Stabilität der mRNA-enthaltenden Hybridhydrogele wurde anhand rheologischer Untersuchungen analysiert. Für die Analyse der Bioaktivität wurden HEK293 Zellen in das Chitosan-Alginat Hybridhydrogel, das die mRNA Transfektionskomplexe enthält, integriert und die Luziferaseaktivität über 3 Wochen hinweg im Überstand gemessen. Die Ergebnisse bestätigten, dass die bioabbaubaren Chitosan-Alginat Hybridhydrogele ein vielversprechendes Transportsystem für eine anhaltende Freisetzung von synthetischen mRNAs, der mRNA Transfektion in die Zellen und die Expression von Proteinen in Zellen darstellt. Aufgrund der guten Injizierbarkeit können diese Hybridhydrogele zusammen mit Zellen und einer gewünschten synthetisch hergestellten mRNA minimal-invasiv lokal appliziert werden, um eine langanhaltende exogene Expression von gewünschten Proteinen zu vermitteln.

3.2. PUBLIKATION II

Improving the angiogenic potential of EPCs via engineering with synthetic modified mRNAs

Heidrun Steinle, Sonia Golombek, Andreas Behring, Christian Schlensak, Hans Peter Wendel, Meltem Avci-Adali

Molecular Therapy – Nucleic Acids / Akzeptiert am: 06. September 2018

Zusammenfassung:

Endotheliale Progenitorzellen (EPCs) weisen ein enormes Potenzial zur Revaskularisierung von ischämischen Geweben auf. Ischämien können beispielweise nach einen Myokardinfarkt oder Schlaganfall/ Gehirninfarkt aufgrund einer Sauerstoffunterversorgung des Gewebes entstehen. Jedoch ist bisher die erfolgreiche therapeutische Behandlung mit EPCs aufgrund ihrer geringen Retention und deren Einwachsen ins Gewebe, sowie dem begrenzten Überleben der Zellen nach der Transplantation ins ischämische Gewebe limitiert.

Um das angiogenetische Potenzial der EPCs zu verbessern, wurden in dieser Studie murine EPCs mit synthetischen mRNAs modifiziert, um die proangiogenetischen Faktoren VEGF-A, SDF-1α und ANG-1 transient in den Zellen zu produzieren. Dafür wurden zunächst die mRNAs mittels IVT hergestellt und deren Qualität und Reinheit mittels Gelelektrophorese bestätigt. Anschließend wurden die mRNAs erfolgreich in die Zellen transfiziert und ein signifikanter Anstieg der Proteinproduktion im Vergleich zu unbehandelten Zellen gemessen. Die mit mRNA modifizierten EPCs wiesen im Vergleich zu den unbehandelten Zellen eine erhöhte chemotaktische Aktivität auf und führten zum verstärkten Anlocken von nativen EPCs. Des Weiteren wurde die Wundheilungskapazität der ANG-1 mRNA transfizierten EPCs bestimmt. Hierbei konnte in einem Wundheilungstest ein verletzter Bereich schon nach 12 Stunden zu 94% geschlossen werden, im Vergleich dazu führten die Zellen ohne mRNA Behandlung zu einem 45%igen Verschluss der verletzten Region. Darüber hinaus führten die ANG-1 und SDF-1 mRNA behandelten Zellen zu einer signifikant erhöhten Gefäßbildung in vitro, die durch die gleichzeitige Modifikation der Zellen mit VEGF-A, SDF-1α und ANG-1 mRNA weiter verstärkt werden konnte. Außerdem zeigten die mit ANG-1 mRNA transfizierten EPCs im in vivo CAM Assay das stärkste angiogenetische Potenzial, das anhand verschiedener Parameter, wie z. B. der Gefäßdichte, ermittelt wurde.

In der vorliegenden Studie konnte erfolgreich gezeigt werden, dass EPCs mit synthetischen mRNAs, die für proangiogenetische Faktoren kodieren, modifiziert werden können. Somit könnte das therapeutische angiogenetische Potenzial von EPCs durch Modifikationen mit mRNA verbessert werden und ischämisches Gewebe effizient behandelt werden.

3.3. PUBLIKATION III

Generation of iPSCs by non-integrative RNA-based reprogramming techniques: Benefits of self-replicating RNA versus synthetic mRNA

Heidrun Steinle, Marbod Weber, Andreas Behring, Ulrike Mau-Holzmann, Christian Schlensak, Hans Peter Wendel, Meltem Avci-Adali

Stem Cells International / Akzeptiert am: 20. Mai 2019

Zusammenfassung:

Die Reprogrammierung von somatischen Zellen zur Herstellung von induziert pluripotenten Stammzellen (iPSCs) gewinnt immer mehr an Bedeutung für den Einsatz in den Bereichen der Regenerativen Medizin, dem Tissue Engineering und dem Modellieren von Krankheitsbildern. iPSCs haben die Fähigkeit sich in alle Zelltypen des menschlichen Körpers zu differenzieren. Eine patientenspezifische Generierung der iPSCs kann eine Abstoßung der einzusetzenden Zellen im Körper vermeiden. Für eine zukünftige klinische Anwendung müssen die Zellen mit integrationsfreien Methoden hergestellt werden, um das Risiko einer Mutagenese durch zufällige Integration des Vektors ins Hostgenom zu vermeiden und die Methode eine hohe Reprogrammierungseffizienz aufweisen.

In dieser Studie wurden zwei synthetische mRNA-basierte Methoden zur Herstellung von iPSCs miteinander verglichen: Die Verwendung von synthetisch modifizierter ‚messenger' RNAs (mRNAs) und der Einsatz von einer selbst-replizierenden RNA (srRNA). Mit beiden Methoden konnten ‚footprint'-freie iPSCs aus neonatalen Fibroblasten hergestellt werden, die keine karyotypischen Veränderungen ausweisen. Die Detektion spezifischer Pluripotenz-marker anhand von Immunfärbungen und qRT-PCR Analysen, sowie die in vitro und in vivo Differenzierungsfähigkeit in alle drei Keimbahngewebetypen (Mesoderm, Endoderm und Ektoderm) zeigten vergleichbare Ergebnisse für beide Methoden. Des Weiteren ermöglichte das Einfügen einer für das GFP kodierenden Sequenz in die srRNA ein direktes und komfortables Überwachen des Reprogrammierungsprozesses und der erfolgreichen Detektion der srRNA Translation in den transfizierten Zellen. Es stellte sich heraus, dass der Gebrauch der srRNA, durch die einmalige Applikation weniger zeitaufwändig, schneller und effizienter bei der iPSC-Generierung war als die tägliche Transfektion der Zellen mit mehreren Reprogrammierungsfaktor-kodierenden mRNAs über mindestens zwei Wochen. Nach der Reprogrammierung der Zellen wurden keine Rückstände der srRNA nachgewiesen. Daher ist davon auszugehen, dass der srRNA-basierte Ansatz zur Induktion der Pluripotenz eine effizientere Methode zur Reprogrammierung von somatischen Zellen darstellen könnte, um für den klinischen Bereich integrationsfreie iPSCs zu generieren.

3.4. PUBLIKATION IV

Reprogramming of urine-derived renal epithelial cells into iPSCs using srRNA and consecutive differentiation into beating cardiomyocytes

Heidrun Steinle*, Marbod Weber*, Andreas Behring, Ulrike Mau-Holzmann, Christiane von Ohle, Aron-Frederik Popov, Christian Schlensak, Hans Peter Wendel, Meltem Avci-Adali

* gleichermaßen beigetragen

Molecular Therapy – Nucleic Acids / Akzeptiert am: 22. Juli 2019

Zusammenfassung:

Die Herstellung von iPSCs aus patientenspezifischen somatischen Zellen und deren darauffolgende Differenzierung in gewünschte Zelltypen eröffnet eine Vielzahl an Möglichkeiten im Bereich der Herzregeneration. Aufgrund der geringen Selbsterneuerungskapazität von adulten Kardiomyozyten ist es von großer Bedeutung autologe Kardiomyozyten effizient und sicher für klinische Anwendungen herzustellen, um geschädigtes Myokardgewebe behandeln zu können.

In dieser Studie wurde mithilfe einer einzigen Applikation einer selbst-replizierenden RNA (srRNA) in renale Epithelzellen, die aus Urin gewonnen wurden, erfolgreich ‚footprint'-freie iPSCs hergestellt. Die Pluripotenz wurde anhand der Expression verschiedener Marker, wie Oct4, Nanog und TRA-1-60 mittels Antikörperfärbungen und qRT-PCR Analysen bestätigt. Das Differenzierungspotenzial in die drei Keimbahnrichtungen wurde in vitro anhand von Durchflusszytometrie durch den Nachweis verschiedener Antigene, wie CD31, AFP und TUBB3 gezeigt. Im in vivo CAM-Teratoma Assays wurde die Ausbildung von meso-, endo- und ektodermalem Gewebe mittels H&E-Färbungen von Gewebeschnitten bestätigt. Weiterhin konnten nach der Reprogrammierung der Zellen keine Rückstände der srRNA mittels qRT-PCR und spezifischer Primer, die im Bereich der nicht-strukturellen Gene der srRNA binden, detektiert werden oder chromosomale Abnormitäten mithilfe von Karyotypisierungen ermittelt werden. Die 10-tägige kardiale Differenzierung der iPSCs resultierte in autologen kontaktierenden Herzmuskelzellen, die nachweislich kardiales Troponin exprimierten. Video-basierte Analysen des Ca^{2+} Flusses in den Zellen bestätigten die Kontraktilität, die auf die Behandlung mit dem Aktivator Isoproterenol und dem Inhibitor Nifidipin ansprechbar waren.

Die nicht-invasive Entnahme von Urin zur Gewinnung von patientenspezifischen Zellen für die Reprogrammierung ist besonders geeignet, da dies einfach und schmerzfrei, sowie gleichermaßen aus gesunden und erkrankten Probanden erfolgen kann. Die Reprogrammierung dieser Zellen mittels srRNA in iPSCs kann in Zukunft die Erzeugung klinisch verwendbarer autologer Kardiomyozyten ermöglichen. Dies könnte die Regeneration von infarziertem Myokard ermöglichen, ohne die Gefahr einer Immunabstoßung zu riskieren.

3.5. PUBLIKATION V

Generation of iPSCs from jaw periosteal cells using self-replicating RNA

Felix Umrath*, Heidrun Steinle*, Marbod Weber, Hans-Peter Wendel, Siegmar Reinert, Dorothea Alexander, Meltem Avci-Adali

* gleichermaßen beigetragen

International Journal of Molecular Science – Special Issue „Cell Reprogramming, II“
Akzeptiert am: 30. März 2019

Zusammenfassung:

Kieferperiostzellen (JPCs) stellen eine geeignete Zellquelle für die Behandlung von Knochendefekten im Bereich der Mund-, Kiefer- und Gesichtschirurgie dar. Die Verfügbarkeit der JPCs ist jedoch begrenzt und so reichen die aus dem Patienten isolierten Zellzahlen oft nicht aus, um beispielweise eine Rekonstruktion des Knochengewebes bei Tumorpatienten nach einer größeren Resektion durchzuführen. Die Vermehrung dieser Zellen in vitro, um eine ausreichende Anzahl zu erhalten, stellt eine weitere Limitierung der Verwendung dar, da es in höhere Passage der Kultivierung verstärkt zu zellulärer Seneszenz und einem verminderten osteogenen Differenzierungspotenzials kommt. Der Einsatz von induziert pluripotenten Stammzellen (iPSCs) könnte dieses Problem bewältigen, da die Zellen in ausreichender Anzahl zur Generierung von Osteoblasten hergestellt werden können.
In dieser Studie wurden zum ersten Mal iPSCs aus humanen JPCs verschiedener Probanden generiert. Für die Herstellung der JPC-iPSCs wurde eine nicht-integrierende selbst-replizierende RNA (srRNA) verwendet, die die Reprogrammierungsfaktoren (Oct4, Sox2, Klf4 und cMyc) und GFP als Reporterprotein kodiert. Die Reprogrammierung wurde anhand eines ‚Feeder'-freien und xeno-freien Protokolls durchgeführt und entspricht somit den höchsten Sicherheitsstandards für zukünftige klinische Applikationen. Um iPSCs in osteogene Progenitorzellen zu differenzieren, wurden die JPC-iPSCs zuerst in induziert mesenchymale Stamm/ Stroma-ähnliche Zellen (iMSCs) differenziert und dann unter xeno-freien Bedingungen weiter zu osteogenen Zellen differenziert. Die immunologischen Färbungen und qRT-PCR Analysen zeigten, dass die produzierten iPSCs, sowie die daraus differenzierten iMSCs die für den jeweiligen Zelltyp typischen Marker exprimierten, sowie eine charakteristische Morphologie aufwiesen. Während der darauffolgenden osteogenen Differenzierung der iMSCs konnte eine starke Mineralisierung nachgewiesen werden, die charakteristisch für Knochenzellen ist. Die srRNA-basierte Reprogrammierung von JPCs generiert autologe iPSCs in klinischer Qualität, die zu iMSCs differenziert werden können und zur Erzeugung von Zellen mit einem hohen osteogenen Potential führen. Die so generierten Zellen stellen die eine vielversprechende Quelle für Knochenkonstruktionen und zur Geweberegeneration dar.

4. DISKUSSION

Im Vordergrund der Regenerativen Medizin stehen autologe Zellen, die zur Reparatur verschiedener Organe/ Gewebe eingesetzt werden. Um die Verfügbarkeit und Anzahl patientenspezifischer Zellen zu erhöhen, sowie die Retention, das Überleben und Einwachsen der Zellen nach Transplantation zu verbessern, können gezielte Modifikationen im Gewebe oder der zu transplantierenden Zellen erfolgen.

4.1. Verbesserung des angiogenetischen Potenzials von mRNA-modifizierten EPCs

Die Induktion der Angiogenese ist eine vielversprechende Maßnahme zur Verbesserung der Blutversorgung in ischämischen Geweben und in den davon betroffenen Organen. Die Revaskularisierung mithilfe von körpereigenen EPCs kann das geschädigte Gewebe regenerieren und zur Wiederherstellung der gestörten Funktion führen. Des Weiteren kann die Überexpression von proangiogenen, parakrinen Faktoren bei ischämischen Patienten besonders vorteilhaft sein, um die Mobilisierung und Rekrutierung autologer Stammzellen in den geschädigten Bereich zu fördern und somit die Heilung und Regeneration durch Verbesserung der Blutgefäßbildung zu unterstützen.

In Rahmen dieser Arbeit, wurden murine EPCs transient mit synthetischen mRNAs modifiziert, die für ANG-1, VEGF-A und SDF-1α kodieren, um das proangiogene Potenzial der EPCs zu steigern. Nach der Transfektion der EPCs mit den einzelnen synthetischen mRNAs wurde die Expression von ANG-1, VEGF-A und SDF-1α erfolgreich nachgewiesen. Die Behandlung zeigte keinen negativen Einfluss auf die Lebensfähigkeit der Zellen. Jedoch führte der Einsatz des mRNA-Cocktails bestehend aus den 3 mRNAs zu einer verringerten Proteinproduktion in den Zellen im Vergleich zu den Einzel-mRNA Transfektionen der Zellen. Grund hierfür ist vermutlich die höhere Gesamtmenge an synthetischer mRNA pro Zelle, die zu einer Überladung der Zellen und translationalen Repression führen kann.[207] In eukaryotischen Zellen durchlaufen die meisten Proteine verschiedene Reifungsschritte im Lumen des endoplasmatischen Retikulums (ER).[207] Dort werden nach der Translation entstehende Polypeptide chemisch modifiziert und zu reifen Proteinen gefaltet. Eine Anhäufung von ungefalteten Proteinen im ER Lumen kann zu einer Stressantwort in den Zellen durch die ‚ungefaltete Proteinantwort' (UPR) führen. Durch Rückkopplungsmechanismen kann es so zum selektivem Abbau von synthetischer mRNA kommen und dadurch zu einer Verringerung der produzierten Proteinmengen.[207, 208]

Im Vergleich zu unbehandelten EPCs zeigten die mit jeweils ANG-1, VEGF-A oder SDF-1α mRNA und dem mRNA Cocktail behandelten Zellen im Migrationstest eine verstärkte chemotaktische Wirkung auf native EPCs. Entgegen der Erwartung, konnte bei den mit dem

mRNA-Cocktail transfizierten EPCs keine deutliche Erhöhung der chemoattraktiven Aktivität im Vergleich zu den einzeln transfizierten mRNAs erzielt werden. Der Grund hierfür könnten die reduzierten Proteinmengen in diesen EPCs sein. Im Gegensatz dazu konnte in den mit dem mRNA Cocktail transfizierten Zellen im in vitro Angiogenesetest eine deutliche Verbesserung des angiogenetischen Potenzials festgestellt werden. Dies lässt auf einen synergetischen Effekt der gleichzeitig hergestellten ANG-1, VEGF-A und SDF-1α Proteine schließen. Dies wurde auch in vivo anhand des CAM-Assays durch einen moderaten Anstieg der verschiedenen angiogenetischen Parameter bei den Triple-mRNA Cocktail transfizierten Zellen bestätigt. Hier konnte auch eine starke Erhöhung des angiogenetischen Potenzials der mit ANG-1 mRNA modifizierten EPCs ermittelt werden. Der Wundheilungstest zeigte die höchste Migrationsgeschwindigkeit bei den ANG-1 mRNA transfizierten EPCs, sodass bereits nach 12 h 94% der Wundfläche verschlossen war. Im Vergleich dazu, konnte bei unbehandelten EPCs eine 45%ige Schließung der Wundfläche beobachtet werden. Nach 24 Stunden führte die ANG-1 mRNA Behandlung zum kompletten Verschluss des Wundbereichs und die SDF-1 mRNA Behandlung zu einer 93%igen Schließung. Es konnte auch ein leicht verbesserter Wundverschluss für die mit VEGF-A mRNA und dem mRNA Cocktail modifizierten Zellen nachgewiesen werden.

Die Ergebnisse dieser Studie zeigten, dass die Modifikation von EPCs mit der ANG-1 mRNA ausreichen könnte, um die Angiogenese zu verbessern. Die SDF-1α mRNA Transfektion zeigte ebenfalls eine Verbesserung des Wundverschlusses und der in vitro Angiogenese. Im Gegensatz dazu konnte nach der einmaligen Transfektion von EPCs mit der VEGF mRNA keine Verbesserung des angiogenetischen Potenzials festgestellt werden. In einer Studie von Zangi et al.[102] wurden 100 µg VEGF mRNA in den Herzmuskel von Mäusen injiziert, wodurch eine verstärkte Bildung von Gefäßen im Bereich der Injektion gezeigt wurde. Daher könnte die Produktion einer höheren VEGF-Menge durch die mRNA-modifizierten EPCs erforderlich sein, um die Angiogenese zu verbessern. VEGF ist der am häufigsten untersuchte Wachstumsfaktor für die Behandlung von Ischämien und Herz-Kreislauf-Erkrankungen, da es ein wichtiger Regulator der Blutgefäßbildung ist. Studien haben jedoch gezeigt, dass neu gebildete Blutgefäße, die durch VEGF induziert werden, unreif und undicht sind.[209, 210] Weiterhin konnten Su und Kollegen[209] durch die gleichzeitige Expression von ANG-1 und VEGF mittels eines Adeno-assoziierten Virus (AAV)-vermittelten Ansatzes positive Auswirkungen in einem murinen Infarktmodell zeigen. Dabei konnten sie mehr Kapillare, eine geringere Infarktgröße und eine bessere Herzfunktion ermitteln, sowie im Vergleich zur alleinigen Behandlung mit VEGF, eine weniger undichte Neovaskulatur.

In früheren Studien mit ischämischen Tiermodellen konnte durch die Überexpression von chemotaktischen Faktoren oder Wachstumsfaktoren bereits eine unterstützende Wirkung auf die Angiogenese gezeigt werden. Dazu wurden AAV- oder lentivirale Vektoren angewendet,

um ANG-1 in MSCs [32], VEGF-A in Skelettmuskelzellen [28] und SDF-1α in humanen MSCs herzustellen[35]. Außerdem wurden EPCs erfolgreich gentechnisch mit adenoviralen Vektoren, die für HIF-1α[37], FGF-1[34], und VEGF[29] kodieren oder mit lentiviralen Vektoren, die für ANG-1[31] kodieren, sowie mit retroviralen Vektoren, die SDF-1α und VEGF-A[36] kodieren verändert, um die Angiogenese in Ischämie-Tiermodellen zu verbessern. Bei der Verwendung von viralen Vektoren wird die Expression von Proteinen über einen längeren Zeitraum aufrechterhalten. Die längere Anwesenheit von exogen exprimierten Proteinen kann jedoch auch nachteilige Auswirkungen haben. Beispielsweise führte die längere Exposition von Gefäßen mit VEGF-A aufgrund eines DNA-vermittelten Gentransfers zu einer erhöhten Gefäßpermeabilität und zur Ödembildung.[102] Die mRNA-vermittelte Proteinexpression in den Zellen wird nach dem natürlichen Abbau der transfizierten synthetischen mRNA, nach ungefähr 2-3 Tagen, rückstandfrei eingestellt. Dieser Zeitrahmen sollte ausreichen, um endogene EPCs in das ischämische Gewebe zu homen und die implantierten Zellen mit den Blutgefäßen des umgebenden intakten Gewebes zu verbinden.

Die ex vivo Modifikation von EPCs mit synthetischer mRNA zur Herstellung ihrer eigenen proangiogenen Proteine könnte das angiogene Potenzial im Vergleich zur Injektion großer Mengen von mRNA in das betroffene Gewebe erhöhen. Durch die Transplantation der mit mRNA modifizierten EPCs kann nicht nur die Migration von Zellen aus dem umliegenden Gewebe und die Gefäßbildung gesteigert werden, sondern zusätzlich die Anzahl an EPCs im ischämischen Gewebe und deren Wachstumsfaktor und Zytokinausschüttung erhöht werden. Eine ausreichende Anzahl an EPCs für die Modifikationen kann durch die Isolierung aus Blut und Expansion der Zellen erhalten werden.

Die Behandlung mit synthetischen mRNAs könnte in Zukunft zur Bildung neuer Blutgefäße in beschädigten Geweben, z. B. nach einem Myokardinfarkt oder Schlaganfall eingesetzt werden. Auf dem Gebiet des Tissue Engineerings könnte dieser Ansatz außerdem dazu beitragen, die Vaskularisierung von Gerüststrukturen zu verbessern und die Gefäß-anastomose mit dem Wirtsgefäßsystem zu fördern.

4.2. RNA-basierte Reprogrammierung von iPSCs

In den letzten Jahren hat sich die Erzeugung patientenspezifischer iPSCs aus somatischen Zellen zu einem leistungsstarken Instrument im Bereich des Tissue Engineerings und der Krankheitsmodellierung entwickelt und zu großen Fortschritten bei Anwendungen in der Regenerativen Medizin geführt. Dabei spielt die integrationsfreie Generation der Zellen eine bedeutende Rolle für die therapeutische Anwendung. Die Verwendung von synthetischen RNAs für die iPSC Herstellung ist im Vergleich zu anderen nichtintegrativen Methoden wie z. B. episomalen DNA-Plasmiden aufgrund ihrer hohen Reprogrammierungseffizienz von Vorteil.[89, 102]

4.2.1. Vergleich der Reprogrammierung von neonatalen Fibroblasten mittels mRNAs und srRNA in iPSCs

In dieser Studie wurden die Reprogrammierungsmethoden basierend auf synthetischer mRNA und srRNA verglichen, um aus humanen Fibroblasten ‚footprint'-freie iPSCs zu generieren. Dabei wurden die Transfektions- und Reprogrammierungseffizienz, sowie die Gesamtkosten und der Arbeitsaufwand beider Methoden untersucht. Dazu wurden Fibroblasten entweder durch mehrmaliges tägliches Transfizieren mit einem mRNA-Cocktail bestehend aus 5 verschiedenen mRNAs, die für die Reprogrammierungsfaktoren Oct4, Sox2, Klf4, cMyc, Lin28 und eGFP kodieren[79] oder durch einmaliges Transfizieren mit srRNA, die für Oct4, Sox2, Klf4, cMyc kodiert, reprogrammiert, die eine anhaltende Expression der Reprogrammierungs-faktoren[173] ermöglicht. Um die Transfektionseffizienz und die Translation der srRNA zu überwachen, wurde zusätzliche eine IRES (interne Ribosomeneintrittsstelle)[211] und eine GFP Sequenz in das srRNA Konstrukt hinzugefügt, um die 5'-Cap-unabhängige Initiierung der Translation des GFPs zu ermöglichen.

Die erhaltenen iPSCs exprimierten typische Pluripotenzmarker und das Potential zur Differenzierungsfähigkeit in die drei embryonalen Keimblätter, Meso-, Endo- und Ektoderm, konnte in vitro und in vivo bestätigt werden. Es wurden keine genomischen Anomalien anhand von Karyogrammanalysen in den erzeugten iPSCs festgestellt. In den durch die srRNA generierten iPSCs konnten keine Rückstände der srRNA mittels qRT-PCR Analysen nachgewiesen werden. Der Vergleich beider Methoden ergab jedoch, dass die srRNA-basierte Reprogrammierung deutlich effizienter und einfacher in der Handhabung war, als die mRNA-basierte Methode. Die Kosten für die Synthese und Reinigung von einem Mikrogramm mRNA oder srRNA sind dabei vergleichbar (ca. 2,50 € / 1 µg). Ein wesentlicher Vorteil bei der Reprogrammierung mit srRNA sind die ca. 24-fach geringeren Produktionskosten durch die einmalige Transfektion von Zellen mit 1 µg srRNA (2,50 €), gegenüber der erforderlichen täglichen Transfektion von Zellen mit 1,2 µg mRNA-Cocktail mit 6 verschiedenen Nukleosid-modifizierten mRNAs für ca. 20 Tage (60 €). Darüber hinaus wurden die mit srRNA generierten iPSCs früher erhalten, als die iPSCs, die durch die mRNA-Transfektionen entstanden sind. Die srRNA enthält auch eine Sequenz, die für eine Puromyzinresistenz kodiert, um die positive Selektion von srRNA-haltigen Zellen zu ermöglichen.[78] Aufgrund dessen konnten nur Zellen überleben, die die srRNA enthielten, wodurch die Reprogrammierungseffizienz erhöht wurde. Weiterhin konnte durch die tägliche Überwachung der GFP-Expression, der Wechsel des B18R-haltigen Mediums auf einen 2-Tage-Rhythmus reduziert werden. Im Fall der synthetischen mRNA-basierten Reprogrammierung wurde ebenfalls die eGFP-Expression analysiert, um die Transfektionseffizienz und Translation der mRNA in den Zellen zu überwachen. Dazu enthielt der mRNA-Cocktail eine eGFP-kodierende mRNA, die gleichzeitig mit den Reprogrammierungsfaktor-kodierenden mRNAs in die Zellen transfiziert wurde. Hier

ist die Überwachung der Transfektionseffizienz und Translation der mRNA jedoch deutlich ungenauer als bei der srRNA Verwendung, da nicht gewährleistet werden kann, dass jede Zelle jeden Tag mit allen 6 mRNAs konstant mit der gleichen Menge versorgt werden kann. Darüber hinaus kann die srRNA, die zusätzlich eine GFP-kodierende Sequenz enthält, auch direkt zur Analyse der Transfizierbarkeit von somatischen Zellen vor Beginn der Reprogrammierung eingesetzt werden. Dadurch können für verschiedene Zelltypen das benötigte Transfektionsreagenz und die Transfektionsdauer bestimmt werden.

Die Verwendung von RNA Molekülen zur Expression von Transkriptionsfaktoren in Zellen ist integrationsfrei. Die Transfektion von synthetischer mRNA in die Zellen führt zu einer vorübergehenden Expression der gewünschten Proteine für etwa 2-3 Tage[79, 92, 93] und erfordert daher mehrere Transfektionen für die Reprogrammierung. Im Vergleich dazu, ermöglicht eine einmalige Transfektion von srRNA in die Zellen, die Expression von nicht-strukturellen Proteinen (nsP1-nsP4), die für eine Replikase kodieren und die wiederholte Replikation der RNA im Zytosol bewirkt und damit die Proteinexpression verlängert. Der rückstandsfreie Abbau der srRNA in den erhaltenen iPSCs wurde in frühen Passagen (P3-5) durch qRT-PCR Analysen, die mit nsP2- und nsP4-spezifischen Primern durchgeführt wurden, nachgewiesen. Darüber hinaus deutete die Abnahme der cMyc und Klf4 Expression ebenfalls auf den Abbau des srRNA-Konstrukts in den iPSCs hin. Diese Proteine werden während des Reprogrammierungsvorgangs benötigt. Danach sollte ihre Expression jedoch herunterreguliert werden, da die permanente Überexpression dieser Proteine mit einer erhöhten Tumorentstehung verbunden ist [212, 213] und in verschiedenen Krebstypen [214, 215] detektiert wurden.

Um somatische Zellen mit synthetischen RNAs zu reprogrammieren, ist die Verwendung des Interferoninhibitors B18R erforderlich[79, 173, 216], um die zelluläre Typ-I-Interferon-Immunantwort gegen die RNA und somit deren vorzeitigen Abbau zu verhindern.[217] Nach der Reprogrammierung der Zellen kann der Entzug von B18R aus dem Medium zum gezielten Abbau und zur Eliminierung der srRNA[173] genutzt werden. Somit bleiben keine srRNA Rückstände zurück. B18R kann entweder als rekombinantes Protein dem Medium zugesetzt werden, oder es kann ein konditioniertes Medium verwendet werden, das B18R enthält (BcM) und durch Transfektion von Fibroblasten mit B18R mRNA hergestellt wird. Die Verwendung von BcM anstelle von rekombinantem B18R Protein reduziert die Kosten und kann zusätzliche Proteine, wie z. B. FGF-2, bereitstellen, die die Reprogrammierung unterstützen können. Dies kann von Vorteil sein, wenn serumfreies Medium, z. B. Essential 8 Stammzellmedium, verwendet wird. Das Mengenverhältnis von BcM zu Kultur- oder Reprogrammierungsmedium ist einstellbar. In dieser Studie führte eine 25%ige Zugabe des BcM in Reprogrammierungs-medium (Pluriton) zur erfolgreichen Reprogrammierung der Zellen. Die BcM-Menge kann auf beispielsweise 50% erhöht werden, wenn in den transfizierten Zellen ein schwaches GFP-

Signal nachgewiesen wird. Eine weitere Möglichkeit zur Reduktion der Immunreaktion wurde in einer Studie von Angel und Yanik[159] gezeigt. Dabei konnte durch den siRNA-induzierten Knockdown der Mediatoren des angeborenen Immunsystems, Ifnb1, Eif2ak2 und Stat2, die Immunaktivierung verhindert werden und das Überleben der Zellen nach wiederholter Transfektion mit mRNAs erhöht werden. Poleganov et al.[171] untersuchten einen weiteren Ansatz zur Reduktion der Zytotoxizität und Immunogenität während der Reprogrammierung. Hierfür wurden die Reprogrammierungs-mRNAs mit mRNAs, die für E3, K3 und B18R des Vaccinia-Virus kodieren, und der Enhancer-microRNA miR302/367 kombiniert. Unter Verwendung dieses Verfahrens konnten auch mRNAs, die keine modifizierten Nukleoside enthielten und von denen bekannt ist, dass sie eine zelluläre Immunreaktion auslösen, eingesetzte werden, um Fibroblasten mit vier wiederholten Transfektionen innerhalb von 11 Tagen effektiv in iPSCs umzuwandeln.

In Rahmen dieser Arbeit wurde zur besseren Vergleichbarkeit der mRNA- und srRNA-basierten Reprogrammierung das Pluriton Reprogrammierungsmedium für beide Ansätze verwendet. Dies ist jedoch für eine erfolgreiche und effiziente Reprogrammierung mit srRNA nicht explizit erforderlich. Yoshioka et al.[173] zeigte die erfolgreiche Reprogrammierung in iPSCs unter Verwendung von srRNA und Fibroblasten-Kulturmedium anstelle von Pluriton. Darüber hinaus ist es auch möglich, die einzige tierische Komponente des Protokolls, Fötales Kälberserum (FBS) im Zellkulturmedium durch humanes Serum oder humanes Plättchenlysat (hPL) zu ersetzen, um iPSCs unter xeno-freien Bedingungen zu erzeugen.[218]

In dieser Arbeit wurden neonatale menschliche Fibroblasten reprogrammiert. Allerdings zeigten Yoshioka und Dowdy[219], dass iPSCs auch aus adulten Fibroblasten von 54- bis 77-jährigen gesunden Spendern, sowie von einem 24-jährigen Kardiomyopathie-Patienten mittels srRNA generiert werden können. Die Reprogrammierung von adulten Körperzellen mit srRNA konnte auch im Rahmen dieser Arbeit mit humanen Urinzellen[220] und Kieferperiostzellen[218] durchgeführt werden.

In früheren Studien wurden nach Reprogrammierung von neonatalen BJ-Fibroblasten mit synthetischen modifizierten mRNAs Reprogrammierungseffizienzen von 4,4% erreicht.[79, 164] In Rahmen dieser Arbeit wurde eine Reprogrammierungseffizienz von 0,8% erreicht, indem das gleiche Protokoll auf NuFFs anwendet wurde. Der Unterschied ist vermutlich auf Materialschwankungen und laborbedingte Unterschiede zurück zu führen. Darüber hinaus kann die Verwendung von Zellen mit unterschiedlichen Proliferationsraten und verschiedener Spendern variieren und zu unterschiedlichen Reprogrammierungseffizienzen führen.[79, 165, 219] Daher sollten die Reprogrammierungsprotokolle für jeden Zelltyp getestet und optimiert werden.[89] Darüber hinaus hängt die Reprogrammierungseffizienz des mRNA-basierten Ansatzes von der anfänglichen Zelldichte ab[164, 216] und wird durch hohe Zellzyklusraten gefördert [221, 222]. Dies ist auf die charakteristischen Merkmale von ESCs zurückzuführen, die

durch hohe Proliferationsraten und kurze G1-Phasen im Zellzyklus vor ihrer Differenzierung geschützt werden und ihre Selbsterneuerungsfähigkeit erhalten bleibt.[222] Durch die Verwendung von mitotisch arretierten ‚Feeder'-Zellen können niedrigere Zellzahlen für die Reprogrammierung verwendet werden. Jedoch sind Feeder-freie Protokolle von Vorteil, um eine zusätzliche Variabilität und das Risiko einer Kontamination mit xenogenem Material zu verhindern.[164]

Im Vergleich zur täglichen Transfektion von mehreren mRNAs, ermöglichte die einmalige Transfektion von srRNA in humane Fibroblasten eine zeit- und kostengünstigere Erzeugung von iPSCs ohne genomische Integration. Aufgrund des komfortablen Reprogrammierungsprozesses mit nur einer einmaligen srRNA-Verabreichung, direkter Detektion der srRNA Translation mittels GFP und höherer Reprogrammierungseffizienz ist die srRNA-basierte Reprogrammierung eine vielversprechende Methode für die integrationsfreie Reprogrammierung verschiedener somatischen Zellen.

4.2.2. srRNA-basierte Reprogrammierung von renalen Epithelzellen aus Urin und Kieferperiostzellen zu iPSCs

Für die Reprogrammierung von iPSCs werden häufig Fibroblasten als somatischen Zellen verwendet.[223] Diese werden meist durch invasive Hautbiopsien der Patienten gewonnen. Dieser Vorgang führt zur Verletzung des gesunden Gewebes, kann schmerzhaft für den Patienten sein und ist mit einem Infektionsrisiko verbunden. Die Isolierung von renalen Epithelzellen (RECs) aus humanem Urin stellt eine alternative Zellquelle für die Reprogrammierung patientenspezifischer Zellen dar. Urin von Natur aus ausgeschieden und ermöglicht eine einfache, kostengünstige und schmerzfreie Isolierung einer ausreichenden Anzahl menschlicher Körperzellen zur Reprogrammierung. Aufgrund der physiologischen Selbsterneuerung des Epithelgewebes im Harntrakt werden täglich etwa 2.000 bis 7.000 Zellen abgelöst und mit dem Urin ausgeschieden.[224] Im Urin können drei Arten von Epithelzellen gefunden werden: Nieren-, Übergangs- und Plattenepithelzellen.[225] Die RECs kleiden als einzelne Epithelschicht das Nephron der Niere aus. Eine erhöhte Anzahl der Epithelzellen im Urin kann auf eine Infektion oder Erkrankung hinweisen. Zur Gewinnung der RECs werden während einer Kultivierungszeit von 3 bis 5 Tagen in einem speziellen Proliferationsmedium die unerwünschten Übergangs- und Plattenepithelzellen entfernt, da diese nicht an der Zellkulturplatte anhaften und somit nach dem Wechsel des Mediums beseitigt werden. Typischerweise erscheinen 3 bis 6 kleine REC-Kolonien, die stetig weiter wachsen. In dieser Studie reichten 100-200 ml Urin aus, um eine ausreichende Menge an Zellen für das Reprogrammierungsverfahren nach ca. 2 Wochen Expansionsdauer zu erhalten. Durch die Verwendung einer größeren Menge an Urin könnten mehr Zellen erhalten werden, wodurch die Expansionszeit der Zellen verringert werden kann. Die Herkunft der aus

Urin isolierten Zellen wurde durch die Analyse des Epithelmarker β-Catenin und des renalen proximalen tubulären Marker CD13 bestätigt.

Eine ausgezeichnete Quelle für Zell-basierte regenerative Therapien zur Behandlung und Rekonstruktion von Knochendefekten sind osteogene Vorläuferzellen. Diese können aus dem Kieferperiost und z. B. während einer Mund- und Kieferoperation entnommen werden. Da das Material nur in begrenzter Menge vorliegt, ist es schwierig, eine ausreichende Zellzahl in guter Qualität zu erhalten. Durch die Vermehrung der Primärzellen in Zellkulturen kann es zu verstärkter Zellalterung und zur Verringerung des Differenzierungspotentials in höheren Passagen kommen.
Nichtintegrativ generierte iPSCs stellen eine vielversprechende alternative Zellquelle dar, um osteogene Zellen für regenerative Zwecke zu generieren. Darüber hinaus weisen die primären Kieferperiostzellen (JPCs), sowie auch MSCs, die aus verschiedenen Geweben stammen, eine gewisse Spendervariabilität hinsichtlich ihres Differenzierungspotenzials auf. Diese Variabilität wird durch die unterschiedlichen Anteile der einzelnen Zellpopulationen im Gewebe, wie beispielsweise dem Stammzellanteil, der verschiedenen Spender, sowie Isolierungs-, Kultivierungs- und Expansionsmethoden verursacht.[226, 227] Bekannte Oberflächemarker können zur Fluoreszenz- oder Magnetisch-aktivierten Zellsortierung (FACS, MACS) verwendet werden, um die Anreicherung der osteogenen Vorläuferzellen aus heterogenen Zellpopulationen zu ermöglichen.[228-230] Jedoch sind die Zellausbeuten aufgrund niedriger Sortierungseffizienzen und hoher Zellmortalität oft unbefriedigend. Die im Rahmen dieser Arbeit isolierten JPCs zeigten eine eindeutig mesenchymale Herkunft durch die Expression der Marker CD73, CD105 und CD44.

Die Reprogrammierung von beiden adulten Zelltypen RECs und JPCs verschiedener Probanden unterschiedlichen Alters konnte mittels srRNA erfolgreich durchgeführt werden. Wie auch bei der Generierung von iPSCs aus neonatalen Fibroblasten, konnte der Einbau der IRES-GFP Sequenz in die srRNA für die Optimierung des Protokolls und der Kontrolle der erfolgreichen Transfektion und Translation der srRNA während des Reprogrammierungs-prozesses genutzt werden. Die Reprogrammierung erfolgte hier mit normalem Kulturmedium für den jeweiligen Zelltyp und nicht unter Verwendung eines speziellen Reprogrammierungs-mediums, wie z. B. Pluriton. Für die Reprogrammierung der JPCs wurde zusätzlich die natürlich vorkommende Fettsäure Natriumbutyrat als ‚Enhancer'-Molekül dem Medium zugesetzt, um die die Effektivität der iPSC Generierung zu steigern. Dabei wird die Reprogrammierungseffizienz verbessert, indem die Acetylierung von H3 Histonen und die DNA Demethylierung gesteigert werden und somit die Expression der endogen zugeführten Pluripotenzgene verbessert wird.[231] Andere bekannte ‚Enhancer'-Moleküle, die zur Steigerung der Reprogrammierungseffizienz eingesetzt werden, sind beispielsweise Histondeacetylase-Inhibitoren, wie Valproinsäure, TGF-Inhibitoren, Vitamin C oder miRNA-367/302.[49, 89, 216, 232-234]

Die erhaltenen iPSCs zeigten ebenfalls die typischen Stammzellmerkmale, wie die Expression von Pluripotenzmarkern, die für die Aufrechterhaltung der Pluripotenz und der Selbsterneuerungskapazität von iPSCs spezifisch sind. Auch die Fähigkeit der erhaltenen iPSCs sich in Zellen der drei Keimschichten (Mesoderm, Endoderm und Ektoderm) zu differenzieren wurde anhand der gerichteten Differenzierung in vitro, sowie in vivo nach Aufbringung der iPSCs auf die CAM von bebrüteten Hühnereiern demonstriert. Im Vergleich zur Teratombildung bei Mäusen, die ca. 4 Wochen inkubiert werden, reichte die Applikation von 2×10^6 iPSCs auf die CAM und eine Inkubationszeit von 10 Tagen aus, um alle drei Keimschichten auszubilden. Nach Reprogrammierung und B18R-Entzug wurde die vollständige Entfernung der srRNA durch die Verwendung der nsP2- und nsP4-Primer in den erzeugten iPSCs in Passage drei mittels qRT-PCR gezeigt. Darüber hinaus bestätigte die Abnahme der cMyc und Klf4 Expression den Abbau des srRNA-Konstrukts in den iPSCs und es konnten keine genomischen Anomalien anhand von Karyogrammanalysen nachgewiesen werden.

Eine weitere interessante Quelle für adulte Zellen zur Reprogrammierung sind von Blut abgeleitete Zellen, wie mononukleäre Zellen des peripheren Blutes (PBMCs) oder EPCs, die durch eine minimal-invasive Blutentnahme von gesunden Spendern oder Patienten gewonnen werden können.[235] Poleganov et al.[171] berichteten über die erfolgreiche Reprogrammierung von humanen EPCs unter Verwendung eines mRNA-basierten Ansatzes. Daher könnte die Verwendung von Blutzellen für die srRNA-basierte Reprogrammierung eine weitere vielversprechende Quelle für adulte Zellen darstellen. Jedoch ist die Reprogrammierung von Zellen aus Blut problematisch, da sie eine geringe Transfektionseffizienz aufweisen und daher auch die Reprogrammierungseffizienz bisher noch relativ gering ist.[236] Unterschiede in der Transfektionseffizienz mit synthetisch modifizierten mRNAs oder srRNA werden für Zellen unterschiedlicher Herkunft aufgrund ihrer unterschiedlichen Transfektionsfähigkeit erwartet. Daher ist auch die Wahl der verwendeten Transfektionsmethode oder Reagenz wichtig und sollte vor Beginn der Reprogrammierung getestet und optimiert werden, um die beste Transfektionseffizienz für jeden Zelltyp zu erreichen.[163, 237] Weiterhin wurde auch gezeigt, dass iPSCs, die aus verschiedenen Zellquellen generiert wurden, unterschiedliche epigenetische Marker aufweisen, die Spendergewebe-abhängig sind.[238] Dies deutet darauf hin, dass die iPSCs effizienter in den ursprünglichen somatischen Zelltyp differenziert werden könnten. In der vorliegenden Arbeit wurde die erfolgreiche Differenzierung von den REC-iPSCs und JPC-iPSCs in Zellen aller drei Keimbahnrichtungen gleichermaßen gezeigt.
Im Rahmen dieser Arbeit wurde gezeigt, dass die Reprogrammierung von adulten Zellen verschiedener Probanden und Herkunftsgeweben mit srRNA eine effiziente Methode ist, um footprint- und xeno-freien iPSCs zu erhalten.

4.2.3. Differenzierung der mit srRNA generierten iPSCs in gewebespezifische Zellen

Im Rahmen dieser Arbeit wurden patientenspezifische iPSCs, die aus verschiedenen Zellquellen mittels srRNA generiert wurden in gewebespezifische Zellen differenziert.

Die iPSCs (REC-iPSCs), die aus Urin isoliert wurden, wurden in kardiale Richtung differenziert, um Kardiomyozyten zur Regeneration des verletzten Myokards, z. B. nach einem Herzinfarkt, zu produzieren. Nach bereits 7 Tagen wurden sich kontrahierende Kardiomyozyten mit einer Schlagrate von etwa 25 Schlägen pro Minute erhalten. Die Funktionalität und elektromechanische Kupplung dieser Zellen wurde anhand der Ca^{2+} Oszillation bei der Kontraktion bestätigt. Das Reaktionsvermögen der Kardiomyozyten wurde durch die Zugabe von Ca^{2+}-Antagonist Nifedipin oder β-Adrenozeptor-Agonist Isoproterenol untersucht. Dabei führte 0,1 mM Nifidipin zu einer vollständigen Hemmung der Kontraktion. Im Gegensatz dazu führte die pharmakologische Modulation der REC-iPSC-abgeleiteten Kardiomyozyten mit Isoproterenol zu einer erhöhten Kontraktion.

Die zukünftige Anwendung dieser Zellen zur Reparatur von geschädigtem Herzgewebe erfordert die Produktion einer reinen Kardiomyozytenkultur in großem Maßstab und die Auswahl der geeigneten Subpoulationen: nodale, atriale oder ventrikuläre Kardiomyozyten.[239-241] Bei einer Herzinsuffizienz oder einem Myokardinfarkt sind hauptsächlich die Ventrikel des Herzens betroffen. Daher sollte ventrikuläre Kardiomyozyten verwendet werden, um nach der Transplantation der Zellen in die Herzkammern Arrhythmien zu verhindern. Eine Zellsortierung mittels Durchflusszytometrie kann durchgeführt werden, um reine Kardiomyozytenkulturen verschiedener Subtypen zu erhalten. Ein häufig verwendeter Marker für ventrikuläre Kardiomyozyten ist die leichte Myosinkette 2v (MLC-2v). Für atriale Kardiomyozyten kann die leichte Myosinkette 2a (MLC-2a) als ein spezifischer Marker verwendet werden. Darüber hinaus sollte die vollständige Differenzierung der iPSCs in reife Kardiomyozyten sichergestellt werden.[242, 243] Dazu können die aus iPSCs generierten Kardiomyozyten z. B. mithilfe von spezifischen Antikörper und FACS angereichert werden.[244] In Rahmen dieser Studie wurden die Kardiomyozyten mit einem Medium auf Laktatbasis angereichert.[245] Aufgrund der Unterschiede im Energiestoffwechsel können Kardiomyozyten, im Vergleich zu anderen Säugetierzellen, anstelle von Glukose auch aus Laktat oder Fettsäuren Energie produzieren.[246] Die Kultivierung von Zellen in einem Lactat-haltigem Medium ohne Glukose führt daher zum Überleben von Kardiomyozyten und zur Eliminierung von undifferenzierten Zellen. Mit dieser Methode konnten patientenspezifische Kardiomyozytenkulturen generiert werden, die einen hohen Anteil (ca. 90%) an kardialem Troponin exprimierten.

Die aus Kieferperiost isolierten und mittels srRNA in iPSCs reprogrammierten Zellen (JPC-iPSCs) wurden zu osteogenen Vorläuferzellen differenziert, um diese als Quelle für das Tissue

Engineering von Knochenkonstrukten einzusetzen. Die osteogene Differenzierung von iPSCs kann mit unterschiedlichen Protokollen durchgeführt werden. In der vorliegenden Arbeit wurde ein zweistufiger Ansatz verwendet, bei dem die iPSCs zuerst in induzierte MSCs (iMSCs) differenziert wurden und in einem zweiten Schritt dann der osteogenen Differenzierung unterzogen wurden.[55, 56] In der Regel wird die Differenzierung von iPSCs zu iMSCs entweder über die Bildung von Embryonalkörperchen (EBs) erreicht oder durch die spontane Differenzierung von iPSCs, die mit einem Differenzierungsmedium behandelt und in mehreren Passagierschritte kultiviert wurden.[247] Im Rahmen dieser Arbeit wurde ein hPL-haltiges Differenzierungsmedium[248] verwendet, um die iPSCs in xeno-freie iMSCs zu differenzieren. Um den Ertrag der iMSCs zu erhöhen, wurden die iPSC-Kolonien über einen längeren Zeitraum von 10 Tagen ohne Passagieren in E8 Stammzellmedium kultiviert, bevor sie wieder als Einzelzellen ausplattiert und für 3-5 weitere Passagen in Ascorbinsäure-haltigem hPL-Medium kultiviert wurden. Die resultierenden hohen Zelldichten, die durch die 10-tägige Kultivierung der iPSCs entstanden sind, trugen wahrscheinlich zur Differenzierung in den mesenchymalen Zelltyp bei. Die Analyse der erhaltenen iMSCs mittels Durchflusszytometrie und Genexpression zeigte die Expression typischer Oberflächenmarker und entsprach dem Expressionsprofil von MSCs.[226] Anschließend wurden die iMSCs für 15-25 Tage in einem Differenzierungsmedium in osteogene Richtung differenziert. Die erhaltenen Zellen zeigten eine starke Mineralisierung und typische Morphologie. Dies zeigt, dass diese Zellen ein vielversprechendes Potenzial besitzen, um im Bereich des Knochen Tissue Engineerings eingesetzt zu werden.

Die Ergebnisse zeigten, dass die mit srRNA generierten iPSCs als potente Ausgangsquelle für die Differenzierung in verschiedene gewebespezifische Zellarten genutzt werden können, um autologe Zellen für eine personalisierte Zelltherapie zu erhalten.

4.3. Langzeitfreisetzung von mRNA aus Hydrogelen ermöglicht eine verlängerte Proteinexpression in Zellen

Hydrogele werden im Bereich der Regenerativen Medizin und des Tissue Engineerings als Gerüststrukturen zur Besiedelung mit Zellen und für die Abgabe von therapeutischen Wirkstoffen verwendet. Um die Freisetzung von synthetischen mRNAs aus verschiedenen Hydrogelen zu untersuchen, wurde eine Cy3-markiete sezernierbare humanisierte Gaussia Luziferase kodierende (hGLuc) mRNA in Alginat, Chitosan und Chitosan-Alginat Hydrogele integriert. Die injizierbaren Chitosan-Alginat Hybridhydrogele, die mit 4 µg synthetischer Cy3-hGLuc mRNA beladen wurden, zeigten eine um 70% verzögerte Freisetzung der inkorporierten mRNA, die zu einer Produktion von hGLuc über 3 Wochen führte. Damit konnte gezeigt werden, dass Hydrogele mit Zellen und zusätzlich synthetischer mRNA beladen werden können, damit die Zellen über einen längeren Zeitraum mit mRNA versorgt werden

können. Dies ermöglicht die Herstellung gewünschter Proteinen von den in Hydrogel-inkorporierten Zellen über einen längeren Zeitraum, ohne dass eine wiederholte Verabreichung von synthetischer mRNA erforderlich ist. Dieser Ansatz kann im Bereich des Tissue Engineering für die lokale Transfektion von Zellen mit therapeutisch wirkenden mRNAs direkt im Gewebe genutzt werden, um eine verlängerte Produktion von Proteinen zu erreichen.

Zur Analyse der Freisetzungskinetik von synthetischer mRNA aus verschiedenen Hydrogelen wurden Alginat-, Chitosan- und Chitosan-Alginat Hybridhydrogele hergestellt, die alle eine anhaltende mRNA Freisetzung über 21 Tage zeigten. Die langsamste Freisetzung wurde in Chitosan-Hydrogelen (42%) und die schnellste Freisetzung in Alginat-Hydrogelen nachgewiesen (79%). Im Vergleich dazu führte die Verwendung des Chitosan-Alginat-Hybridhydrogels zu einer schnelleren mRNA-Freisetzung als aus dem Chitosan-Hydrogel und zu einer langsameren Freisetzung als aus dem Alginat-Hydrogel. Dafür verantwortlich könnten Wechselwirkungen zwischen dem positiv geladenen Chitosan mit der negativ geladenen mRNA sein, die zu einer erhöhten Retention der mRNA in den Hydrogelen führen. Im Gegensatz dazu könnten aufgrund elektrostatischer Abstoßungskräfte zwischen dem negativ geladenen Alginat und der ebenfalls negativ geladenen synthetischen mRNA die Freisetzung aus den Alginat-Hydrogelen schneller erfolgen als aus Chitosan-Hydrogelen. Krebs et al. zeigten auch eine verzögerte Freisetzung von siRNA nach der Zugabe der positiv geladenen Polymere, Polyethylenimin (PEI) oder Chitosan zu Calcium-vernetzen Alginat-Hydrogelen.[195] In einer weiteren Studie wurde die Zugabe von Chitosan zu einem Alginat-Hydrogel untersucht, das ebenfalls zu einer langsameren Freisetzung von einem negativ geladenen Molekül (Sphingosin-1-Phosphat), im Vergleich zu Alginat-Hydrogele allein, führte.[249] In einer Studie von Ma und Kollegen wurden siRNA-beladene Chitosan-Hydrogele subkutan in Mäuse injiziert und untersucht. Im Vergleich zur siRNA, die ohne Chitosan injiziert wurde und weniger als 1 Tag nachweisbar war, konnte die Cy5-markierte siRNA im Chitosan-Hydrogel für bis zu 7 Tage detektiert werden.[199] Die unterschiedliche Freisetzungskinetik der synthetischen mRNAs durch Verwendung unterschiedlicher Biomaterialien, zeigte, dass die Freisetzung der mRNA z. B. mit unterschiedlichen Ladungen moduliert werden kann.

Weiterhin konnte gezeigt werden, dass die ins Chitosan-Alginat-Hydrogel inkorporierte hGLuc-mRNA erfolgreich von HEK293-Zellen im Hydrogel aufgenommen werden kann und zur Herstellung von hGLuc über 21 Tage führte. Die schnelle Freisetzung und Aufnahme der hohen mRNA Menge nach dem ersten Inkubationstag könnte der Grund für die Produktion der höchsten hGLuc-Menge am zweiten Inkubationstag sein. Danach nahm die Produktion von hGLuc mit der Zeit ab. Dies könnte mit Abbauprozessen des Hydrogels zusammenhängen, sowie mit dem Abbau der komplexierten mRNA. Zusätzlich wurde gezeigt, dass die Erzeugung von mRNA-Transfektionskomplexen erforderlich ist, um die mRNA effizient in die Zellen zu transportieren und kontinuierlich das gewünschte Protein zu produzieren. Durch das

Verpacken mit Transfektionsreagenzien können die mRNA-Moleküle effizienter in die Zellen aufgenommen werden. Des Weiteren können die mRNA-Moleküle vor Nukleasen geschützt werden und dadurch kann die Stabilität verbessert werden.[250] Messungen der Viabilität über 3 Tage zeigten, dass der Einbau der synthetischen hGLuc mRNA in die Chitosan-Alginat Hybridhydrogele keinen negativen Einfluss auf die Zellen hat.

Diese Studie bestätigt, dass Hydrogele mit synthetischen mRNAs beladen werden können und zu einer verlängerten Abgabe von mRNA-Transfektionskomplexen geeignet sind. Dies könnte für die lokale und kontinuierliche mRNA-Abgabe an Zellen für diverse Anwendungen von großem Nutzen sein, wie z. B. zur Produktion von Wachstums- oder Differenzierungsfaktoren für die Geweberegeneration.

5. AUSBLICK

Während dieser Arbeit gelang es, den Grundstein für RNA-basierte Modifikationen von Zellen zu legen, die für therapeutische Applikationen in der regenerativen Medizin verwendet werden können. Es war möglich verschiedene synthetische mRNAs und eine srRNA herzustellen und damit Zellen so zu modifizieren, dass entweder ihr natürliches funktionelles Potenzial verbessert oder ihr zelluläres Schicksal verändert wurde. Die proangiogenen mRNAs, die für ANG-1, VEGF-A und SDF-1α kodieren, erhöhten das angiogenetische Potenzial von EPCs und iPSCs konnten mit srRNA effektiv und rückstandsfrei aus verschiedenen Ausgangszellen hergestellt und zu gewebespezifischen Zellen differenziert werden. Darüber hinaus wurde ein injizierbares mRNA-Hydrogel aus den natürlichen Komponenten Chitosan und Alginat entwickelt, das eine verzögerte Freisetzung von synthetischer mRNA gewährleistet.

Bevor eine therapeutische Anwendung von mRNA bzw. srRNA modifizierten Zellen am Patienten stattfinden kann, muss die Sicherheit und Wirksamkeit dieser Zellen weiter in vivo untersucht werden. Dazu ist eine genaue Charakterisierung und Sortierung der gewünschten Zellen mithilfe von z. B. Oberflächenmolekülen mittels FACS nötig, um die Reinheit der Kardiomyozyten zu gewährleisten und somit negative Effekte nach einer Transplantation zu vermeiden. Weiterhin ist für Zelltherapien auch die Art der Applikation von Bedeutung, um einerseits die Zellen effektiv und schonend mit der RNA zu modifizieren und andererseits die modifizierten Zellen an den gewünschten Ort im Körper einzubringen. Daher spielt auch die Transfektionsmethode für das Überleben der Zellen eine große Rolle und somit auch für den Erfolg einer Zelltransplantation und sollte daher gründlich untersucht werden. Interessant sind dabei Methoden für einen schnellen Gentransfer, wie z. B. die Elektroporation von Zellen mit mRNA. Dabei können die Zellen direkt nach der Transfektion in ein Gewebe injiziert werden und somit kann die Überlebensfähigkeit und Integrationsfähigkeit der Zellen im Gewebe optimiert werden und zur verbesserten Geweberegeneration führen. Dies würde auch die zukünftige individualisierte Behandlung des Patienten beschleunigen und würde somit zur Einsparungen von Kosten, die zur weiteren in vitro Kultivierung der Zellen nötig sind, führen. Um die regenerative Wirksamkeit, sowie die Integrations- und Überlebensfähigkeit der mit mRNA modifizierten Zellen zu bestätigen, müssen diese in geeigneten In-vivo-Modellen, wie Ischämie- oder Infarkt-Modellen, untersucht werden.

Die RNA-basierte Modifikation von Zellen stellt eine vielseitige Methode für therapeutische Anwendungen im Bereich der Regenerativen Medizin dar, da diese Zellen durch die mRNA vermittelte Expression von gewünschten Proteinen rückstandsfrei und transient verändert werden können. Darüber hinaus könnte dieses Verfahren für die Behandlung verschiedener Krankheiten individuell eingesetzt werden, indem der gewünschte Zelltyp mit einer oder mehreren mRNAs bzw. srRNA modifiziert wird, um die Produktion von therapeutischen Proteinen in vivo zu produzieren oder einen gewünschten Zelltyp zu generieren

6. LITERATURVERZEICHNIS

1. Kaltenbach, TR, S.; Eulenstein, B.; Hosseini, M.; Erharter, M.; Besteman, K.; Boesch, L. (2017). Regenerative medicine - The next generation of therapeutic products is set to shake up the pharmaceutical world.
2. Atala, A, Kasper, FK, and Mikos, AG (2012). Engineering complex tissues. *Science translational medicine* 4: 160rv112.
3. Baptista, PM, and Atala, A (2016). Chapter 1 - Regenerative Medicine: The Hurdles and Hopes. In: Laurence, J (ed). *Translating Regenerative Medicine to the Clinic*. Academic Press: Boston. pp 3-7.
4. Langer, R, and Vacanti, JP (1993). Tissue engineering. *Science* 260: 920-926.
5. Vacanti, JP, Otte, J-B, and Wertheim, JA (2014). Chapter 1 - Introduction: Regenerative Medicine and Solid Organ Transplantation from a Historical Perspective. In: Orlando, G, Lerut, J, Soker, S and Stratta, RJ (eds). *Regenerative Medicine Applications in Organ Transplantation*. Academic Press: Boston. pp 1-15.
6. Atala, A (2012). Tissue engineering of reproductive tissues and organs. *Fertility and sterility* 98: 21-29.
7. Atala, A (2012). Regenerative medicine strategies. *J Pediatr Surg* 47: 17-28.
8. Kim, BS, and Mooney, DJ (1998). Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends Biotechnol* 16: 224-230.
9. Bergsma, JE, Rozema, FR, Bos, RR, Boering, G, de Bruijn, WC, and Pennings, AJ (1995). In vivo degradation and biocompatibility study of in vitro pre-degraded as-polymerized polyactide particles. *Biomaterials* 16: 267-274.
10. Amiel, GE, Komura, M, Shapira, O, Yoo, JJ, Yazdani, S, Berry, J*, et al.* (2006). Engineering of blood vessels from acellular collagen matrices coated with human endothelial cells. *Tissue Eng* 12: 2355-2365.
11. Atala, A (2001). Bladder regeneration by tissue engineering. *BJU Int* 88: 765-770.
12. Takahashi, T, Kalka, C, Masuda, H, Chen, D, Silver, M, Kearney, M*, et al.* (1999). Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nature medicine* 5: 434-438.
13. Asahara, T, Masuda, H, Takahashi, T, Kalka, C, Pastore, C, Silver, M*, et al.* (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circulation research* 85: 221-228.
14. Asahara, T, Kawamoto, A, and Masuda, H (2011). Concise Review: Circulating Endothelial Progenitor Cells for Vascular Medicine. *STEM CELLS* 29: 1650-1655.
15. Kawamoto, A, Gwon, HC, Iwaguro, H, Yamaguchi, JI, Uchida, S, Masuda, H*, et al.* (2001). Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation* 103: 634-637.
16. Minami, Y, Nakajima, T, Ikutomi, M, Morita, T, Komuro, I, Sata, M*, et al.* (2015). Angiogenic potential of early and late outgrowth endothelial progenitor cells is dependent on the time of emergence. *Int J Cardiol* 186: 305-314.
17. Fazel, S, Cimini, M, Chen, L, Li, S, Angoulvant, D, Fedak, P*, et al.* (2006). Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *The Journal of clinical investigation* 116: 1865-1877.
18. Chong, MSK, Ng, WK, and Chan, JKY (2016). Concise Review: Endothelial Progenitor Cells in Regenerative Medicine: Applications and Challenges. *STEM CELLS Translational Medicine* 5: 530-538.
19. Sen, S, McDonald, Stephen P, Coates, PToby H, and Bonder, Claudine S (2011). Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease. *Clinical Science* 120: 263-283.
20. Kachamakova-Trojanowska, N, Bukowska-Strakova, K, Zukowska, M, Dulak, J, and Jozkowicz, A (2015). The real face of endothelial progenitor cells – Circulating angiogenic cells as endothelial prognostic marker? *Pharmacological Reports* 67: 793-802.

21. Terrovitis, JV, Smith, RR, and Marbán, E (2010). Assessment and Optimization of Cell Engraftment after Transplantation into the Heart. *Circulation research* 106: 479-494.
22. Lipiec, P, Krzeminska-Pakula, M, Plewka, M, Kusmierek, J, Plachcinska, A, Szuminski, R*, et al.* (2009). Impact of intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction on left ventricular perfusion and function: a 6-month follow-up gated 99mTc-MIBI single-photon emission computed tomography study. *European journal of nuclear medicine and molecular imaging* 36: 587-593.
23. Sukmawati, D, and Tanaka, R (2015). Introduction to next generation of endothelial progenitor cell therapy: a promise in vascular medicine. *American Journal of Translational Research* 7: 411-421.
24. Zhang, H, van Olden, C, Sweeney, D, and Martin-Rendon, E (2014). Blood vessel repair and regeneration in the ischaemic heart. *Open Heart* 1: e000016.
25. Wadajkar, AS, Santimano, S, Tang, L, and Nguyen, KT (2014). Magnetic-based multi-layer microparticles for endothelial progenitor cell isolation, enrichment, and detachment. *Biomaterials* 35: 654-663.
26. Chen, J, Xiao, X, Chen, S, Zhang, C, Chen, J, Yi, D*, et al.* (2013). Angiotensin-converting enzyme 2 priming enhances the function of endothelial progenitor cells and their therapeutic efficacy. *Hypertension (Dallas, Tex : 1979)* 61: 681-689.
27. Takeshita, S, Zheng, LP, Brogi, E, Kearney, M, Pu, LQ, Bunting, S*, et al.* (1994). Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *Journal of Clinical Investigation* 93: 662-670.
28. Shimpo, M, Ikeda, U, Maeda, Y, Takahashi, M, Miyashita, H, Mizukami, H*, et al.* (2002). AAV-mediated VEGF gene transfer into skeletal muscle stimulates angiogenesis and improves blood flow in a rat hindlimb ischemia model. *Cardiovasc Res* 53: 993-1001.
29. Iwaguro, H, Yamaguchi, J, Kalka, C, Murasawa, S, Masuda, H, Hayashi, S*, et al.* (2002). Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation* 105: 732-738.
30. Sun, X-T, Ding, Y-T, Yan, X-G, Wu, L-Y, Li, Q, Cheng, N*, et al.* (2004). Angiogenic synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in an in vitro quantitative microcarrier-based three-dimensional fibrin angiogenesis system. *World Journal of Gastroenterology : WJG* 10: 2524-2528.
31. Wang, YQ, Song, JJ, Han, X, Liu, YY, Wang, XH, Li, ZM*, et al.* (2014). Effects of angiopoietin-1 on inflammatory injury in endothelial progenitor cells and blood vessels. *Current gene therapy* 14: 128-135.
32. Sun, L, Cui, M, Wang, Z, Feng, X, Mao, J, Chen, P*, et al.* (2007). Mesenchymal stem cells modified with angiopoietin-1 improve remodeling in a rat model of acute myocardial infarction. *Biochem Biophys Res Commun* 357: 779-784.
33. Baffour, R, Berman, J, Garb, JL, Rhee, SW, Kaufman, J, and Friedmann, P (1992). Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. *Journal of vascular surgery* 16: 181-191.
34. Chen, SY, Wang, F, Yan, XY, Zhou, Q, Ling, Q, Ling, JX*, et al.* (2009). Autologous transplantation of EPCs encoding FGF1 gene promotes neovascularization in a porcine model of chronic myocardial ischemia. *Int J Cardiol* 135: 223-232.
35. Liang, X, Su, Y-P, Kong, P-Y, Zeng, D-F, Chen, X-H, Peng, X-G*, et al.* (2010). Human bone marrow mesenchymal stem cells expressing SDF-1 promote hematopoietic stem cell function of human mobilised peripheral blood CD34+ cells in vivo and in vitro. *International Journal of Radiation Biology* 86: 230-237.
36. Yu, JX, Huang, XF, Lv, WM, Ye, CS, Peng, XZ, Zhang, H*, et al.* (2009). Combination of stromal-derived factor-1alpha and vascular endothelial growth factor gene-modified endothelial progenitor cells is more effective for ischemic neovascularization. *Journal of vascular surgery* 50: 608-616.

37. Jiang, M, Wang, B, Wang, C, He, B, Fan, H, Shao, Q*, et al.* (2008). In vivo enhancement of angiogenesis by adenoviral transfer of HIF-1α-modified endothelial progenitor cells (Ad-HIF-1α-modified EPC for angiogenesis). *The International Journal of Biochemistry & Cell Biology* 40: 2284-2295.
38. Brivanlou, AH, Gage, FH, Jaenisch, R, Jessell, T, Melton, D, and Rossant, J (2003). Stem cells. Setting standards for human embryonic stem cells. *Science* 300: 913-916.
39. Itskovitz-Eldor, J, Schuldiner, M, Karsenti, D, Eden, A, Yanuka, O, Amit, M*, et al.* (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6: 88-95.
40. Ben-David, U, and Benvenisty, N (2011). The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nature reviews Cancer* 11: 268-277.
41. Laurent, LC, Ulitsky, I, Slavin, I, Tran, H, Schork, A, Morey, R*, et al.* (2011). Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. *Cell stem cell* 8: 106-118.
42. Thomson, JA, Itskovitz-Eldor, J, Shapiro, SS, Waknitz, MA, Swiergiel, JJ, Marshall, VS*, et al.* (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1147.
43. Takahashi, K, and Yamanaka, S (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126: 663-676.
44. Bieberich, E, and Wang, G (2013). *Molecular Mechanisms Underlying Pluripotency*.
45. Takahashi, K, Tanabe, K, Ohnuki, M, Narita, M, Ichisaka, T, Tomoda, K*, et al.* (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872.
46. Hanna, J, Carey, BW, and Jaenisch, R (2008). Reprogramming of somatic cell identity. *Cold Spring Harbor symposia on quantitative biology* 73: 147-155.
47. Nakagawa, M, Koyanagi, M, Tanabe, K, Takahashi, K, Ichisaka, T, Aoi, T*, et al.* (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature biotechnology* 26: 101-106.
48. Yu, J, Vodyanik, MA, Smuga-Otto, K, Antosiewicz-Bourget, J, Frane, JL, Tian, S*, et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318: 1917-1920.
49. Huangfu, D, Osafune, K, Maehr, R, Guo, W, Eijkelenboom, A, Chen, S*, et al.* (2008). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nature biotechnology* 26: 1269-1275.
50. Takahashi, M (2016). [Retinal Cell Therapy Using iPS Cells]. *Nippon Ganka Gakkai Zasshi* 120: 210-224; discussion 225.
51. Cynata Therapeutics Limited (2017). A Study of CYP-001 for the Treatment of Steroid-Resistant Acute Graft Versus Host Disease.
52. Burridge, PW, and Zambidis, ET (2013). Highly efficient directed differentiation of human induced pluripotent stem cells into cardiomyocytes. *Methods Mol Biol* 997: 149-161.
53. Mallanna, SK, and Duncan, SA (2013). Differentiation of hepatocytes from pluripotent stem cells. *Current protocols in stem cell biology* 26: 1G.4.1-1G.4.
54. Salimi, A, Nadri, S, Ghollasi, M, Khajeh, K, and Soleimani, M (2014). Comparison of different protocols for neural differentiation of human induced pluripotent stem cells. *Molecular biology reports* 41: 1713-1721.
55. Luzzani, CD, and Miriuka, SG (2017). Pluripotent Stem Cells as a Robust Source of Mesenchymal Stem Cells. *Stem Cell Rev* 13: 68-78.
56. Wu, Q, Yang, B, Hu, K, Cao, C, Man, Y, and Wang, P (2017). Deriving Osteogenic Cells from Induced Pluripotent Stem Cells for Bone Tissue Engineering. *Tissue Eng Part B Rev* 23: 1-8.
57. Bergmann, O, Zdunek, S, Felker, A, Salehpour, M, Alkass, K, Bernard, S*, et al.* (2015). Dynamics of Cell Generation and Turnover in the Human Heart. *Cell* 161: 1566-1575.
58. Xin, M, Olson, EN, and Bassel-Duby, R (2013). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nature reviews Molecular cell biology* 14: 529-541.

59. Muraoka, N, and Ieda, M (2014). Direct reprogramming of fibroblasts into myocytes to reverse fibrosis. *Annual review of physiology* 76: 21-37.
60. Roger, VL (2013). Epidemiology of heart failure. *Circulation research* 113: 646-659.
61. Watanabe, E, Smith, DM, Delcarpio, JB, Sun, J, Smart, FW, Van Meter, CH*, et al.* (1998). Cardiomyocyte transplantation in a porcine myocardial infarction model. *Cell Transplantation* 7: 239-246.
62. Xiao, YF (2003). Cardiac application of embryonic stem cells. *Sheng li xue bao : [Acta physiologica Sinica]* 55: 493-504.
63. Rojas, SV, Kensah, G, Rotaermel, A, Baraki, H, Kutschka, I, Zweigerdt, R*, et al.* (2017). Transplantation of purified iPSC-derived cardiomyocytes in myocardial infarction. *PloS one* 12: e0173222.
64. Amini, H, Rezaie, J, Vosoughi, A, Rahbarghazi, R, and Nouri, M (2017). Cardiac progenitor cells application in cardiovascular disease. *J Cardiovasc Thorac Res* 9: 127-132.
65. Valarmathi, MT, Fuseler, JW, Goodwin, RL, Davis, JM, and Potts, JD (2011). The mechanical coupling of adult marrow stromal stem cells during cardiac regeneration assessed in a 2-D co-culture model. *Biomaterials* 32: 2834-2850.
66. Caspi, O, Itzhaki, I, Kehat, I, Gepstein, A, Arbel, G, Huber, I*, et al.* (2009). In Vitro Electrophysiological Drug Testing Using Human Embryonic Stem Cell Derived Cardiomyocytes. *Stem Cells and Development* 18: 161-172.
67. Mummery, C, Ward-van Oostwaard, D, Doevendans, P, Spijker, R, van den Brink, S, Hassink, R*, et al.* (2003). Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 107: 2733-2740.
68. Zhang, J, Wilson, G, and Soerens, A (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circulation research* 104: e30-e41.
69. Sebastião, MJ, Serra, M, Pereira, R, Palacios, I, Gomes-Alves, P, and Alves, PM (2019). Human cardiac progenitor cell activation and regeneration mechanisms: exploring a novel myocardial ischemia/reperfusion in vitro model. *Stem Cell Research & Therapy* 10: 77.
70. Alexander, D, Hoffmann, J, Munz, A, Friedrich, B, Geis-Gerstorfer, J, and Reinert, S (2008). Analysis of OPLA scaffolds for bone engineering constructs using human jaw periosteal cells. *Journal of Materials Science: Materials in Medicine* 19: 965-974.
71. Ferretti, C, and Mattioli-Belmonte, M (2014). Periosteum derived stem cells for regenerative medicine proposals: Boosting current knowledge. *World J Stem Cells* 6: 266-277.
72. Zhao, Q, Gregory, CA, Lee, RH, Reger, RL, Qin, L, Hai, B*, et al.* (2015). MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. *Proc Natl Acad Sci U S A* 112: 530-535.
73. Gupta, R, Tongers, J, and Losordo, DW (2009). Human studies of angiogenic gene therapy. *Circulation research* 105: 724-736.
74. Stadtfeld, M, Nagaya, M, Utikal, J, Weir, G, and Hochedlinger, K (2008). Induced pluripotent stem cells generated without viral integration. *Science* 322: 945-949.
75. Woltjen, K, Michael, IP, Mohseni, P, Desai, R, Mileikovsky, M, Hamalainen, R*, et al.* (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 458: 766-770.
76. Yu, J, Hu, K, Smuga-Otto, K, Tian, S, Stewart, R, Slukvin, II*, et al.* (2009). Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324: 797-801.
77. Seki, T, Yuasa, S, Oda, M, Egashira, T, Yae, K, Kusumoto, D*, et al.* (2010). Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell stem cell* 7: 11-14.
78. Yoshioka, N, Gros, E, Li, HR, Kumar, S, Deacon, DC, Maron, C*, et al.* (2013). Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell stem cell* 13: 246-254.

79. Warren, L, Manos, PD, Ahfeldt, T, Loh, YH, Li, H, Lau, F*, et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell stem cell* 7: 618-630.
80. Weltner, J, Balboa, D, Katayama, S, Bespalov, M, Krjutskov, K, Jouhilahti, EM*, et al.* (2018). Human pluripotent reprogramming with CRISPR activators. *Nature communications* 9: 2643.
81. Rao, MS, and Malik, N (2012). Assessing iPSC Reprogramming Methods for Their Suitability in Translational Medicine. *Journal of cellular biochemistry* 113: 3061-3068.
82. Steichen, C, Luce, E, Maluenda, J, Tosca, L, Moreno-Gimeno, I, Desterke, C*, et al.* (2014). Messenger RNA- versus retrovirus-based induced pluripotent stem cell reprogramming strategies: analysis of genomic integrity. *Stem Cells Transl Med* 3: 686-691.
83. Mallon, BS, Hamilton, RS, Kozhich, OA, Johnson, KR, Fann, YC, Rao, MS*, et al.* (2014). Comparison of the molecular profiles of human embryonic and induced pluripotent stem cells of isogenic origin. *Stem Cell Research* 12: 376-386.
84. Okita, K, Ichisaka, T, and Yamanaka, S (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448: 313-317.
85. Hu, K (2014). All roads lead to induced pluripotent stem cells: the technologies of iPSC generation. *Stem Cells Dev* 23: 1285-1300.
86. Zhao, T, Zhang, ZN, Rong, Z, and Xu, Y (2011). Immunogenicity of induced pluripotent stem cells. *Nature* 474: 212-215.
87. Kaji, K, Norrby, K, Paca, A, Mileikovsky, M, Mohseni, P, and Woltjen, K (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 458: 771-775.
88. Mingozzi, F, and High, KA (2011). Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nature Reviews Genetics* 12: 341-355.
89. Schlaeger, TM, Daheron, L, Brickler, TR, Entwisle, S, Chan, K, Cianci, A*, et al.* (2015). A comparison of non-integrating reprogramming methods. *Nature biotechnology* 33: 58-63.
90. Kaczmarek, JC, Kowalski, PS, and Anderson, DG (2017). Advances in the delivery of RNA therapeutics: from concept to clinical reality. *Genome Med* 9: 60.
91. Hu, K (2014). Vectorology and factor delivery in induced pluripotent stem cell reprogramming. *Stem Cells Dev* 23: 1301-1315.
92. Yakubov, E, Rechavi, G, Rozenblatt, S, and Givol, D (2010). Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. *Biochem Biophys Res Commun* 394: 189-193.
93. Plews, JR, Li, J, Jones, M, Moore, HD, Mason, C, Andrews, PW*, et al.* (2010). Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach. *PloS one* 5: e14397.
94. Kariko, K, Muramatsu, H, Keller, JM, and Weissman, D (2012). Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. *Molecular therapy : the journal of the American Society of Gene Therapy* 20: 948-953.
95. Kariko, K, Buckstein, M, Ni, H, and Weissman, D (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23: 165-175.
96. Sahin, U, Kariko, K, and Tureci, O (2014). mRNA-based therapeutics--developing a new class of drugs. *Nat Rev Drug Discov* 13: 759-780.
97. Steinle, H, Behring, A, Schlensak, C, Wendel, HP, and Avci-Adali, M (2017). Concise Review: Application of In Vitro Transcribed Messenger RNA for Cellular Engineering and Reprogramming: Progress and Challenges. *Stem Cells* 35: 68-79.
98. Goparaju, SK, Kohda, K, Ibata, K, Soma, A, Nakatake, Y, Akiyama, T*, et al.* (2017). Rapid differentiation of human pluripotent stem cells into functional neurons by mRNAs encoding transcription factors. *Sci Rep* 7: 42367.

99. Kim, B-E, Choi, SW, Shin, J-H, Kim, J-J, Kang, I, Lee, B-C*, et al.* (2018). Single-Factor SOX2 Mediates Direct Neural Reprogramming of Human Mesenchymal Stem Cells via Transfection of In Vitro Transcribed mRNA. *Cell Transplantation* 27: 1154-1167.
100. Simeonov, KP, and Uppal, H (2014). Direct Reprogramming of Human Fibroblasts to Hepatocyte-Like Cells by Synthetic Modified mRNAs. *PloS one* 9: e100134.
101. Levy, O, Zhao, W, Mortensen, LJ, Leblanc, S, Tsang, K, Fu, M*, et al.* (2013). mRNA-engineered mesenchymal stem cells for targeted delivery of interleukin-10 to sites of inflammation. *Blood* 122: e23-32.
102. Zangi, L, Lui, KO, von Gise, A, Ma, Q, Ebina, W, Ptaszek, LM*, et al.* (2013). Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nature biotechnology* 31: 898-907.
103. Steinle, H, Golombek, S, Behring, A, Schlensak, C, Wendel, HP, and Avci-Adali, M (2018). Improving the Angiogenic Potential of EPCs via Engineering with Synthetic Modified mRNAs. *Molecular therapy Nucleic acids* 13: 387-398.
104. Diken, M, Kranz, LM, Kreiter, S, and Sahin, U (2017). mRNA: A Versatile Molecule for Cancer Vaccines. *Curr Issues Mol Biol* 22: 113-128.
105. Perche, F, Benvegnu, T, Berchel, M, Lebegue, L, Pichon, C, Jaffres, PA*, et al.* (2011). Enhancement of dendritic cells transfection in vivo and of vaccination against B16F10 melanoma with mannosylated histidylated lipopolyplexes loaded with tumor antigen messenger RNA. *Nanomedicine : nanotechnology, biology, and medicine* 7: 445-453.
106. Benteyn, D, Anguille, S, Van Lint, S, Heirman, C, Van Nuffel, AM, Corthals, J*, et al.* (2013). Design of an Optimized Wilms' Tumor 1 (WT1) mRNA Construct for Enhanced WT1 Expression and Improved Immunogenicity In Vitro and In Vivo. *Molecular therapy Nucleic acids* 2: e134.
107. Garcia, F, Plana, M, Climent, N, Leon, A, Gatell, JM, and Gallart, T (2013). Dendritic cell based vaccines for HIV infection: the way ahead. *Human vaccines & immunotherapeutics* 9: 2445-2452.
108. Brazzoli, M, Magini, D, Bonci, A, Buccato, S, Giovani, C, Kratzer, R*, et al.* (2016). Induction of Broad-Based Immunity and Protective Efficacy by Self-amplifying mRNA Vaccines Encoding Influenza Virus Hemagglutinin. *J Virol* 90: 332-344.
109. Petsch, B, Schnee, M, Vogel, AB, Lange, E, Hoffmann, B, Voss, D*, et al.* (2012). Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. *Nature biotechnology* 30: 1210-1216.
110. Kormann, MS, Hasenpusch, G, Aneja, MK, Nica, G, Flemmer, AW, Herber-Jonat, S*, et al.* (2011). Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nature biotechnology* 29: 154-157.
111. Bangel-Ruland, N, Tomczak, K, Fernandez Fernandez, E, Leier, G, Leciejewski, B, Rudolph, C*, et al.* (2013). Cystic fibrosis transmembrane conductance regulator-mRNA delivery: a novel alternative for cystic fibrosis gene therapy. *The journal of gene medicine* 15: 414-426.
112. Michel, T, Kankura, A, Salinas Medina, ML, Kurz, J, Behring, A, Avci-Adali, M*, et al.* (2015). In Vitro Evaluation of a Novel mRNA-Based Therapeutic Strategy for the Treatment of Patients Suffering from Alpha-1-Antitrypsin Deficiency. *Nucleic acid therapeutics* 25: 235-244.
113. Ramaswamy, S, Tonnu, N, Tachikawa, K, Limphong, P, Vega, JB, Karmali, PP*, et al.* (2017). Systemic delivery of factor IX messenger RNA for protein replacement therapy. *Proc Natl Acad Sci U S A* 114: E1941-e1950.
114. Mays, LE, Ammon-Treiber, S, Mothes, B, Alkhaled, M, Rottenberger, J, Muller-Hermelink, ES*, et al.* (2013). Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. *The Journal of clinical investigation* 123: 1216-1228.
115. Rettig, L, Haen, SP, Bittermann, AG, von Boehmer, L, Curioni, A, Kramer, SD*, et al.* (2010). Particle size and activation threshold: a new dimension of danger signaling. *Blood* 115: 4533-4541.
116. Kariko, K, Muramatsu, H, Ludwig, J, and Weissman, D (2011). Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves

translation of nucleoside-modified, protein-encoding mRNA. *Nucleic acids research* 39: e142.
117. Kariko, K, Muramatsu, H, Welsh, FA, Ludwig, J, Kato, H, Akira, S*, et al.* (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Molecular therapy : the journal of the American Society of Gene Therapy* 16: 1833-1840.
118. Hoerr, I, Obst, R, Rammensee, HG, and Jung, G (2000). In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. *European journal of immunology* 30: 1-7.
119. Alexopoulou, L, Holt, AC, Medzhitov, R, and Flavell, RA (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413: 732-738.
120. Kariko, K, Ni, H, Capodici, J, Lamphier, M, and Weissman, D (2004). mRNA is an endogenous ligand for Toll-like receptor 3. *The Journal of biological chemistry* 279: 12542-12550.
121. Diebold, SS, Kaisho, T, Hemmi, H, Akira, S, and Reis e Sousa, C (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529-1531.
122. Diebold, SS, Massacrier, C, Akira, S, Paturel, C, Morel, Y, and Reis e Sousa, C (2006). Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *European journal of immunology* 36: 3256-3267.
123. Heil, F, Hemmi, H, Hochrein, H, Ampenberger, F, Kirschning, C, Akira, S*, et al.* (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303: 1526-1529.
124. Lorenzi, JC, Trombone, AP, Rocha, CD, Almeida, LP, Lousada, RL, Malardo, T*, et al.* (2010). Intranasal vaccination with messenger RNA as a new approach in gene therapy: use against tuberculosis. *BMC biotechnology* 10: 77.
125. Loo, YM, and Gale, M, Jr. (2011). Immune signaling by RIG-I-like receptors. *Immunity* 34: 680-692.
126. Devoldere, J, Dewitte, H, De Smedt, SC, and Remaut, K (2016). Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger. *Drug discovery today* 21: 11-25.
127. Hornung, V, Ellegast, J, Kim, S, Brzozka, K, Jung, A, Kato, H*, et al.* (2006). 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994-997.
128. Schlee, M, Roth, A, Hornung, V, Hagmann, CA, Wimmenauer, V, Barchet, W*, et al.* (2009). Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 31: 25-34.
129. Schmidt, A, Schwerd, T, Hamm, W, Hellmuth, JC, Cui, S, Wenzel, M*, et al.* (2009). 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc Natl Acad Sci U S A* 106: 12067-12072.
130. Schuberth-Wagner, C, Ludwig, J, Bruder, AK, Herzner, AM, Zillinger, T, Goldeck, M*, et al.* (2015). A Conserved Histidine in the RNA Sensor RIG-I Controls Immune Tolerance to N1-2'O-Methylated Self RNA. *Immunity* 43: 41-51.
131. Devarkar, SC, Wang, C, Miller, MT, Ramanathan, A, Jiang, F, Khan, AG*, et al.* (2016). Structural basis for m7G recognition and 2'-O-methyl discrimination in capped RNAs by the innate immune receptor RIG-I. *Proc Natl Acad Sci U S A* 113: 596-601.
132. Kato, H, Takeuchi, O, Mikamo-Satoh, E, Hirai, R, Kawai, T, Matsushita, K*, et al.* (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *The Journal of experimental medicine* 205: 1601-1610.
133. Pichlmair, A, Schulz, O, Tan, CP, Rehwinkel, J, Kato, H, Takeuchi, O*, et al.* (2009). Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J Virol* 83: 10761-10769.
134. Zust, R, Cervantes-Barragan, L, Habjan, M, Maier, R, Neuman, BW, Ziebuhr, J*, et al.* (2011). Ribose 2'-O-methylation provides a molecular signature for the distinction of

self and non-self mRNA dependent on the RNA sensor Mda5. *Nature immunology* 12: 137-143.
135. Andries, O, De Filette, M, De Smedt, SC, Demeester, J, Van Poucke, M, Peelman, L*, et al.* (2013). Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mRNA to respiratory cells. *Journal of controlled release : official journal of the Controlled Release Society* 167: 157-166.
136. Kanneganti, TD, Body-Malapel, M, Amer, A, Park, JH, Whitfield, J, Franchi, L*, et al.* (2006). Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *The Journal of biological chemistry* 281: 36560-36568.
137. Sabbah, A, Chang, TH, Harnack, R, Frohlich, V, Tominaga, K, Dube, PH*, et al.* (2009). Activation of innate immune antiviral responses by Nod2. *Nature immunology* 10: 1073-1080.
138. Sadler, AJ, and Williams, BR (2008). Interferon-inducible antiviral effectors. *Nat Rev Immunol* 8: 559-568.
139. Nallagatla, SR, and Bevilacqua, PC (2008). Nucleoside modifications modulate activation of the protein kinase PKR in an RNA structure-specific manner. *Rna* 14: 1201-1213.
140. Garcia, MA, Meurs, EF, and Esteban, M (2007). The dsRNA protein kinase PKR: virus and cell control. *Biochimie* 89: 799-811.
141. Silverman, RH (2007). Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J Virol* 81: 12720-12729.
142. Samuel, CE (2012). ADARs: viruses and innate immunity. *Curr Top Microbiol Immunol* 353: 163-195.
143. George, CX, John, L, and Samuel, CE (2014). An RNA editor, adenosine deaminase acting on double-stranded RNA (ADAR1). *J Interferon Cytokine Res* 34: 437-446.
144. Ramanathan, A, Robb, GB, and Chan, SH (2016). mRNA capping: biological functions and applications. *Nucleic acids research* 44: 7511-7526.
145. Mockey, M, Goncalves, C, Dupuy, FP, Lemoine, FM, Pichon, C, and Midoux, P (2006). mRNA transfection of dendritic cells: synergistic effect of ARCA mRNA capping with Poly(A) chains in cis and in trans for a high protein expression level. *Biochem Biophys Res Commun* 340: 1062-1068.
146. Grudzien-Nogalska, E, Kowalska, J, Su, W, Kuhn, AN, Slepenkov, SV, Darzynkiewicz, E*, et al.* (2013). Synthetic mRNAs with superior translation and stability properties. *Methods Mol Biol* 969: 55-72.
147. Grudzien-Nogalska, E, Jemielity, J, Kowalska, J, Darzynkiewicz, E, and Rhoads, RE (2007). Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells. *Rna* 13: 1745-1755.
148. Stepinski, J, Waddell, C, Stolarski, R, Darzynkiewicz, E, and Rhoads, RE (2001). Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogs 7-methyl(3'-O-methyl)GpppG and 7-methyl (3'-deoxy)GpppG. *Rna* 7: 1486-1495.
149. Nagarajan, VK, Jones, CI, Newbury, SF, and Green, PJ (2013). XRN 5'-->3' exoribonucleases: structure, mechanisms and functions. *Biochim Biophys Acta* 1829: 590-603.
150. Chang, JH, Xiang, S, Xiang, K, Manley, JL, and Tong, L (2011). Structural and biochemical studies of the 5'-->3' exoribonuclease Xrn1. *Nat Struct Mol Biol* 18: 270-276.
151. Yisraeli, JK, and Melton, DA (1989). Synthesis of long, capped transcripts in vitro by SP6 and T7 RNA polymerases. *Methods Enzymol* 180: 42-50.
152. Zhao, Y, Moon, E, Carpenito, C, Paulos, CM, Liu, X, Brennan, AL*, et al.* (2010). Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res* 70: 9053-9061.
153. Goldstrohm, AC, and Wickens, M (2008). Multifunctional deadenylase complexes diversify mRNA control. *Nature reviews Molecular cell biology* 9: 337-344.

154. Al-Saif, M, and Khabar, KS (2012). UU/UA dinucleotide frequency reduction in coding regions results in increased mRNA stability and protein expression. *Molecular therapy : the journal of the American Society of Gene Therapy* 20: 954-959.
155. Plotkin, JB, and Kudla, G (2011). Synonymous but not the same: the causes and consequences of codon bias. *Nat Rev Genet* 12: 32-42.
156. Malone, RW, Felgner, PL, and Verma, IM (1989). Cationic liposome-mediated RNA transfection. *Proc Natl Acad Sci U S A* 86: 6077-6081.
157. Asrani, KH, Farelli, JD, Stahley, MR, Miller, RL, Cheng, CJ, Subramanian, RR*, et al.* (2018). Optimization of mRNA untranslated regions for improved expression of therapeutic mRNA. *RNA biology* 15: 756-762.
158. Kozak, M (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic acids research* 15: 8125-8148.
159. Angel, M, and Yanik, MF (2010). Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. *PloS one* 5: e11756.
160. Drews, K, Tavernier, G, Demeester, J, Lehrach, H, De Smedt, SC, Rejman, J*, et al.* (2012). The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts. *Biomaterials* 33: 4059-4068.
161. Rautsi, O, Lehmusvaara, S, Salonen, T, Hakkinen, K, Sillanpaa, M, Hakkarainen, T*, et al.* (2007). Type I interferon response against viral and non-viral gene transfer in human tumor and primary cell lines. *The journal of gene medicine* 9: 122-135.
162. Baiersdorfer, M, Boros, G, Muramatsu, H, Mahiny, A, Vlatkovic, I, Sahin, U*, et al.* (2019). A Facile Method for the Removal of dsRNA Contaminant from In Vitro-Transcribed mRNA. *Molecular therapy Nucleic acids* 15: 26-35.
163. Kogut, I, McCarthy, SM, Pavlova, M, Astling, DP, Chen, X, Jakimenko, A*, et al.* (2018). High-efficiency RNA-based reprogramming of human primary fibroblasts. *Nature communications* 9: 745.
164. Warren, L, Ni, Y, Wang, J, and Guo, X (2012). Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. *Sci Rep* 2: 657.
165. McGrath, PS, Diette, N, Kogut, I, and Bilousova, G (2018). RNA-based Reprogramming of Human Primary Fibroblasts into Induced Pluripotent Stem Cells. *Journal of visualized experiments : JoVE*.
166. Ma, D, Wei, H, Zhao, Y, Lu, J, Li, G, Sahib, NBE*, et al.* (2013). Modeling type 3 long QT syndrome with cardiomyocytes derived from patient-specific induced pluripotent stem cells. *International Journal of Cardiology* 168: 5277-5286.
167. Grace, HE, III, PG, Lesnefsky, EJ, West, DFD, and Iyer, DS. mRNA reprogramming of T8993G Leigh's syndrome fibroblast cells to create induced pluripotent stem cell models for mitochondrial disorders. *Stem Cells and Development* 0: null.
168. Arnold, A, Naaldijk, YM, Fabian, C, Wirth, H, Binder, H, Nikkhah, G*, et al.* (2012). Reprogramming of Human Huntington Fibroblasts Using mRNA. *ISRN Cell Biology* 2012: 12.
169. Heng, BC, Heinimann, K, Miny, P, Iezzi, G, Glatz, K, Scherberich, A*, et al.* (2013). mRNA transfection-based, feeder-free, induced pluripotent stem cells derived from adipose tissue of a 50-year-old patient. *Metab Eng* 18: 9-24.
170. Gaignerie, A, Lefort, N, Rousselle, M, Forest-Choquet, V, Flippe, L, Francois–Campion, V*, et al.* (2018). Urine-derived cells provide a readily accessible cell type for feeder-free mRNA reprogramming. *Scientific Reports* 8: 14363.
171. Poleganov, MA, Eminli, S, Beissert, T, Herz, S, Moon, JI, Goldmann, J*, et al.* (2015). Efficient Reprogramming of Human Fibroblasts and Blood-Derived Endothelial Progenitor Cells Using Nonmodified RNA for Reprogramming and Immune Evasion. *Hum Gene Ther* 26: 751-766.
172. Petrakova, O, Volkova, E, Gorchakov, R, Paessler, S, Kinney, RM, and Frolov, I (2005). Noncytopathic Replication of Venezuelan Equine Encephalitis Virus and Eastern Equine Encephalitis Virus Replicons in Mammalian Cells. *Society* 79: 7597-7608.

173. Yoshioka, N, Gros, E, Li, HR, Kumar, S, Deacon, DC, Maron, C*, et al.* (2013). Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell* 13: 246-254.
174. Loomis, K, Kirschman, J, Bhosle, S, Bellamkonda, R, and Santangelo, P (2015). *Strategies for modulating innate immune activation and protein production of in vitro transcribed mRNAs*.
175. Hawley-Nelson, P, Ciccarone, V, and Moore, ML (2008). Transfection of cultured eukaryotic cells using cationic lipid reagents. *Curr Protoc Mol Biol* Chapter 9: Unit 9 4.
176. Lorenz, C, Fotin-Mleczek, M, Roth, G, Becker, C, Dam, TC, Verdurmen, WP*, et al.* (2011). Protein expression from exogenous mRNA: uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway. *RNA biology* 8: 627-636.
177. Bire, S, Gosset, D, Jegot, G, Midoux, P, Pichon, C, and Rouleux-Bonnin, F (2013). Exogenous mRNA delivery and bioavailability in gene transfer mediated by piggyBac transposition. *BMC biotechnology* 13: 75.
178. Van Tendeloo, VF, Ponsaerts, P, and Berneman, ZN (2007). mRNA-based gene transfer as a tool for gene and cell therapy. *Curr Opin Mol Ther* 9: 423-431.
179. Pardi, N, Tuyishime, S, Muramatsu, H, Kariko, K, Mui, BL, Tam, YK*, et al.* (2015). Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *Journal of controlled release : official journal of the Controlled Release Society* 217: 345-351.
180. Wang, T, Upponi, JR, and Torchilin, VP (2012). Design of multifunctional non-viral gene vectors to overcome physiological barriers: dilemmas and strategies. *International journal of pharmaceutics* 427: 3-20.
181. Hoare, TR, and Kohane, DS (2008). Hydrogels in drug delivery: Progress and challenges. *Polymer* 49: 1993-2007.
182. Tan, H, and Marra, KG (2010). Injectable, Biodegradable Hydrogels for Tissue Engineering Applications. *Materials* 3: 1746.
183. Li, X, Kong, X, Zhang, Z, Nan, K, Li, L, Wang, X*, et al.* (2012). Cytotoxicity and biocompatibility evaluation of N,O-carboxymethyl chitosan/oxidized alginate hydrogel for drug delivery application. *Int J Biol Macromol* 50: 1299-1305.
184. Guan, X, Avci-Adali, M, Alarcin, E, Cheng, H, Kashaf, SS, Li, Y*, et al.* (2017). Development of hydrogels for regenerative engineering. *Biotechnol J* 12.
185. Hao, X, Silva, EA, Mansson-Broberg, A, Grinnemo, KH, Siddiqui, AJ, Dellgren, G*, et al.* (2007). Angiogenic effects of sequential release of VEGF-A165 and PDGF-BB with alginate hydrogels after myocardial infarction. *Cardiovasc Res* 75: 178-185.
186. Ishihara, M, Fujita, M, Obara, K, Hattori, H, Nakamura, S, Nambu, M*, et al.* (2006). Controlled Releases of FGF-2 and Paclitaxel from Chitosan Hydrogels and their Subsequent Effects on Wound Repair, Angiogenesis, and Tumor Growth. *Current Drug Delivery* 3: 351-358.
187. Lee, KW, Yoon, JJ, Lee, JH, Kim, SY, Jung, HJ, Kim, SJ*, et al.* (2004). Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. *Transplant Proc* 36: 2464-2465.
188. Ghasemi Tahrir, F, Ganji, F, Mani, AR, and Khodaverdi, E (2016). In vitro and in vivo evaluation of thermosensitive chitosan hydrogel for sustained release of insulin. *Drug Deliv* 23: 1038-1046.
189. Khodaverdi, E, Tafaghodi, M, Ganji, F, Abnoos, K, and Naghizadeh, H (2012). In vitro insulin release from thermosensitive chitosan hydrogel. *AAPS PharmSciTech* 13: 460-466.
190. Bastiancich, C, Danhier, P, Preat, V, and Danhier, F (2016). Anticancer drug-loaded hydrogels as drug delivery systems for the local treatment of glioblastoma. *Journal of controlled release : official journal of the Controlled Release Society* 243: 29-42.
191. Augst, AD, Kong, HJ, and Mooney, DJ (2006). Alginate hydrogels as biomaterials. *Macromol Biosci* 6: 623-633.
192. Guarino, V, Caputo, T, Altobelli, R, and Ambrosio, L (2015). Degradation properties and metabolic activity of alginate and chitosan polyelectrolytes for drug delivery and tissue engineering applications. *AIMS Materials Science* 2: 497-502.

193. Ito, T, Saito, M, Uchino, T, Senna, M, Iafisco, M, Prat, M*, et al.* (2012). Preparation of injectable auto-forming alginate gel containing simvastatin with amorphous calcium phosphate as a controlled release medium and their therapeutic effect in osteoporosis model rat. *Journal of Materials Science: Materials in Medicine* 23: 1291-1297.
194. Rowley, JA, Madlambayan, G, and Mooney, DJ (1999). Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* 20: 45-53.
195. Krebs, MD, Jeon, O, and Alsberg, E (2009). Localized and sustained delivery of silencing RNA from macroscopic biopolymer hydrogels. *Journal of the American Chemical Society* 131: 9204-9206.
196. Mao, S, Sun, W, and Kissel, T (2010). Chitosan-based formulations for delivery of DNA and siRNA. *Advanced Drug Delivery Reviews* 62: 12-27.
197. Krebs, MD, Salter, E, Chen, E, Sutter, KA, and Alsberg, E (2010). Calcium phosphate-DNA nanoparticle gene delivery from alginate hydrogels induces in vivo osteogenesis. *Journal of Biomedical Materials Research Part A* 92A: 1131-1138.
198. Supper, S, Anton, N, Seidel, N, Riemenschnitter, M, Curdy, C, and Vandamme, T (2014). Thermosensitive chitosan/glycerophosphate-based hydrogel and its derivatives in pharmaceutical and biomedical applications. *Expert Opin Drug Deliv* 11: 249-267.
199. Ma, Z, Yang, C, Song, W, Wang, Q, Kjems, J, and Gao, S (2014). Chitosan hydrogel as siRNA vector for prolonged gene silencing. *Journal of nanobiotechnology* 12: 23.
200. Li, H, Ji, Q, Chen, X, Sun, Y, Xu, Q, Deng, P*, et al.* (2017). Accelerated bony defect healing based on chitosan thermosensitive hydrogel scaffolds embedded with chitosan nanoparticles for the delivery of BMP2 plasmid DNA. *Journal of biomedical materials research Part A* 105: 265-273.
201. Khong, TT, Aarstad, OA, Skjak-Braek, G, Draget, KI, and Varum, KM (2013). Gelling concept combining chitosan and alginate-proof of principle. *Biomacromolecules* 14: 2765-2771.
202. Li, Z, Ramay, HR, Hauch, KD, Xiao, D, and Zhang, M (2005). Chitosan-alginate hybrid scaffolds for bone tissue engineering. *Biomaterials* 26: 3919-3928.
203. Rafiee, A, Alimohammadian, MH, Gazori, T, Riazi-rad, F, Fatemi, SMR, Parizadeh, A*, et al.* (2014). Comparison of chitosan, alginate and chitosan/alginate nanoparticles with respect to their size, stability, toxicity and transfection. *Asian Pacific Journal of Tropical Disease* 4: 372-377.
204. Park, DJ, Choi, BH, Zhu, SJ, Huh, JY, Kim, BY, and Lee, SH (2005). Injectable bone using chitosan-alginate gel/mesenchymal stem cells/BMP-2 composites. *J Craniomaxillofac Surg* 33: 50-54.
205. Deng, B, Shen, L, Wu, Y, Shen, Y, Ding, X, Lu, S*, et al.* (2015). Delivery of alginate-chitosan hydrogel promotes endogenous repair and preserves cardiac function in rats with myocardial infarction. *Journal of biomedical materials research Part A* 103: 907-918.
206. Wang, K, Kievit, FM, Florczyk, SJ, Stephen, ZR, and Zhang, M (2015). 3D Porous Chitosan-Alginate Scaffolds as an In Vitro Model for Evaluating Nanoparticle-Mediated Tumor Targeting and Gene Delivery to Prostate Cancer. *Biomacromolecules* 16: 3362-3372.
207. Ron, D (2006). Stressed Cells Cope with Protein Overload. *Science* 313: 52-53.
208. Hollien, J, and Weissman, JS (2006). Decay of Endoplasmic Reticulum-Localized mRNAs During the Unfolded Protein Response. *Science* 313: 104-107.
209. Su, H, Takagawa, J, Huang, Y, Arakawa-Hoyt, J, Pons, J, Grossman, W*, et al.* (2009). Additive effect of AAV-mediated angiopoietin-1 and VEGF expression on the therapy of infarcted heart. *Int J Cardiol* 133: 191-197.
210. Dvorak, HF, Nagy, JA, Feng, D, Brown, LF, and Dvorak, AM (1999). Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr Top Microbiol Immunol* 237: 97-132.
211. Hellen, CU, and Sarnow, P (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes & development* 15: 1593-1612.

212. Okita, K, Ichisaka, T, and Yamanaka, S (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448: 313-317.
213. Yu, F, Li, J, Chen, H, Fu, J, Ray, S, Huang, S*, et al.* (2011). Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. *Oncogene* 30: 2161-2172.
214. Foster, KW, Ren, S, Louro, ID, Lobo-Ruppert, SM, McKie-Bell, P, Grizzle, W*, et al.* (1999). Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* 10: 423-434.
215. Foster, KW, Frost, AR, McKie-Bell, P, Lin, CY, Engler, JA, Grizzle, WE*, et al.* (2000). Increase of GKLF messenger RNA and protein expression during progression of breast cancer. *Cancer Res* 60: 6488-6495.
216. Kogut, I, McCarthy, SM, Pavlova, M, Astling, DP, Chen, X, Jakimenko, A*, et al.* (2018). High-efficiency RNA-based reprogramming of human primary fibroblasts. *Nat Commun* 9: 745.
217. Kim, YG, Baltabekova, AZ, Zhiyenbay, EE, Aksambayeva, AS, Shagyrova, ZS, Khannanov, R*, et al.* (2017). Recombinant Vaccinia virus-coded interferon inhibitor B18R: Expression, refolding and a use in a mammalian expression system with a RNA-vector. *PloS one* 12: e0189308.
218. Umrath, F, Steinle, H, Weber, M, Wendel, H-P, Reinert, S, Alexander, D*, et al.* (2019). Generation of iPSCs from Jaw Periosteal Cells Using Self-Replicating RNA. *International Journal of Molecular Sciences* 20: 1648.
219. Yoshioka, N, and Dowdy, SF (2017). Enhanced generation of iPSCs from older adult human cells by a synthetic five-factor self-replicative RNA. *PloS one* 12: e0182018.
220. Steinle, H, Weber, M, Behring, A, Mau-Holzmann, U, von Ohle, C, Popov, AF*, et al.* (2019). Reprogramming of Urine-Derived Renal Epithelial Cells into iPSCs Using srRNA and Consecutive Differentiation into Beating Cardiomyocytes. *Molecular therapy Nucleic acids* 17: 907-921.
221. Guo, S, Zi, X, Schulz, VP, Cheng, J, Zhong, M, Koochaki, SH*, et al.* (2014). Nonstochastic reprogramming from a privileged somatic cell state. *Cell* 156: 649-662.
222. Ruiz, S, Panopoulos, AD, Herrerías, A, Bissig, K-D, Lutz, M, Berggren, WT*, et al.* (2011). A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity. *Current biology : CB* 21: 45-52.
223. Raab, S, Klingenstein, M, Liebau, S, and Linta, L (2014). A Comparative View on Human Somatic Cell Sources for iPSC Generation. *Stem Cells International* 2014: 12.
224. Ingelfinger, JR (2002). Nephrogenic adenomas as renal tubular outposts. *The New England journal of medicine* 347: 684-686.
225. Ringsrud, KM (2001). Cells in the Urine Sediment. *Laboratory Medicine* 32: 153-155.
226. Dominici, M, Le Blanc, K, Mueller, I, Slaper-Cortenbach, I, Marini, F, Krause, D*, et al.* (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8: 315-317.
227. McLeod, CM, and Mauck, RL (2017). On the origin and impact of mesenchymal stem cell heterogeneity: new insights and emerging tools for single cell analysis. *Eur Cell Mater* 34: 217-231.
228. Alexander, D, Schafer, F, Olbrich, M, Friedrich, B, Buhring, HJ, Hoffmann, J*, et al.* (2010). MSCA-1/TNAP Selection of Human Jaw Periosteal Cells Improves their Mineralization Capacity. *Cell Physiol Biochem* 26: 1073-1080.
229. Olbrich, M, Rieger, M, Reinert, S, and Alexander, D (2012). Isolation of osteoprogenitors from human jaw periosteal cells: a comparison of two magnetic separation methods. *PloS one* 7: e47176.
230. Umrath, F, Thomalla, C, Poschel, S, Schenke-Layland, K, Reinert, S, and Alexander, D (2018). Comparative Study of MSCA-1 and CD146 Isolated Periosteal Cell Subpopulations. *Cell Physiol Biochem* 51: 1193-1206.
231. Mali, P, Chou, BK, Yen, J, Ye, Z, Zou, J, Dowey, S*, et al.* (2010). Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic

remodeling and the expression of pluripotency-associated genes. *Stem Cells* 28: 713-720.
232. Anokye-Danso, F, Trivedi, CM, Juhr, D, Gupta, M, Cui, Z, Tian, Y*, et al.* (2011). Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell stem cell* 8: 376-388.
233. Maherali, N, and Hochedlinger, K (2009). Tgfbeta signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc. *Current biology : CB* 19: 1718-1723.
234. Esteban, MA, Wang, T, Qin, B, Yang, J, Qin, D, Cai, J*, et al.* (2010). Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell stem cell* 6: 71-79.
235. Loh, Y-H, Agarwal, S, Park, I-H, Urbach, A, Huo, H, Heffner, GC*, et al.* (2009). Generation of induced pluripotent stem cells from human blood. *Blood* 113: 5476-5479.
236. Kim, Y, Rim, YA, Yi, H, Park, N, Park, S-H, and Ju, JH (2016). The Generation of Human Induced Pluripotent Stem Cells from Blood Cells: An Efficient Protocol Using Serial Plating of Reprogrammed Cells by Centrifugation. *Stem Cells International* 2016: 9.
237. Avci-Adali, M, Behring, A, Keller, T, Krajewski, S, Schlensak, C, and Wendel, HP (2014). Optimized conditions for successful transfection of human endothelial cells with in vitro synthesized and modified mRNA for induction of protein expression. *Journal of biological engineering* 8: 8.
238. Kim, K, Doi, A, Wen, B, Ng, K, Zhao, R, Cahan, P*, et al.* (2010). Epigenetic memory in induced pluripotent stem cells. *Nature* 467: 285-290.
239. Später, D, Hansson, EM, Zangi, L, and Chien, KR (2014). How to make a cardiomyocyte. *Development* 141: 4418-4431.
240. Cyganek, L, Tiburcy, M, Sekeres, K, Gerstenberg, K, Bohnenberger, H, Lenz, C*, et al.* (2018). Deep phenotyping of human induced pluripotent stem cell-derived atrial and ventricular cardiomyocytes. *JCI Insight* 3.
241. Schweizer, PA, Darche, FF, Ullrich, ND, Geschwill, P, Greber, B, Rivinius, R*, et al.* (2017). Subtype-specific differentiation of cardiac pacemaker cell clusters from human induced pluripotent stem cells. *Stem Cell Res Ther* 8: 229.
242. Besser, RR, Ishahak, M, Mayo, V, Carbonero, D, Claure, I, and Agarwal, A (2018). Engineered Microenvironments for Maturation of Stem Cell Derived Cardiac Myocytes. *Theranostics* 8: 124-140.
243. Di Baldassarre, A, Cimetta, E, Bollini, S, Gaggi, G, and Ghinassi, B (2018). Human-Induced Pluripotent Stem Cell Technology and Cardiomyocyte Generation: Progress and Clinical Applications. *Cells* 7.
244. Dubois, NC, Craft, AM, Sharma, P, Elliott, DA, Stanley, EG, Elefanty, AG*, et al.* (2011). SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nature biotechnology* 29: 1011-1018.
245. Tohyama, S, Hattori, F, Sano, M, Hishiki, T, Nagahata, Y, Matsuura, T*, et al.* (2013). Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell stem cell* 12: 127-137.
246. Lopaschuk, GD, and Jaswal, JS (2010). Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. *J Cardiovasc Pharmacol* 56: 130-140.
247. Steens, J, and Klein, D (2018). Current Strategies to Generate Human Mesenchymal Stem Cells In Vitro. *Stem cells international* 2018: 6726185-6726185.
248. Luzzani, C, Neiman, G, Garate, X, Questa, M, Solari, C, Fernandez Espinosa, D*, et al.* (2015). A therapy-grade protocol for differentiation of pluripotent stem cells into mesenchymal stem cells using platelet lysate as supplement. *Stem Cell Res Ther* 6: 6.
249. Williams, PA, Campbell, KT, Gharaviram, H, Madrigal, JL, and Silva, EA (2017). Alginate-Chitosan Hydrogels Provide a Sustained Gradient of Sphingosine-1-Phosphate for Therapeutic Angiogenesis. *Annals of biomedical engineering* 45: 1003-1014.
250. Guan, S, and Rosenecker, J (2017). Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. *Gene therapy* 24: 133-143.

7. ANHANG

7.1. Abkürzungsverzeichnis

Abkürzung	Deutsch	Englisch
ADAR	RNA-spezifische Adenosindeaminase	RNA-specific adenosine deaminase
ANG-1	Angiopoientin-1	angiopoientin-1
ARCA	Anti-Reverses Cap-Analogon	anti-reverse cap analog
BcM	B18R konditioniertes Medium	B18R conditioned Medium
BMP2	knochenmorphogenetischen Protein 2	bone morphogenetic protein 2
CAM	Chorioallantoinmembran	chorioallantoic membrane
CLSM	Konfokale Laser Scanning Mikroskopie	confocal laser scanning microscopy
CPCs	Kardiale Vorläuferzellen	cardiac progenitor cells
DNA	Desoxyribonukleinsäure	desoxyribonucleic acid
dsRNA	Doppelsträngige RNA	double stranded RNA
cMyc	zelluläres Myelocytomatose-Gen	celluar myelocytosis gen
EPCs	endotheliale Progenitorzellen	endothelial progenitor cells
eGFP	Verbessertes grün fluoreszierendes Protein	enhanced green fluorecent protein
FGF	Fibroblasten-Wachstumsfaktor	fibroblast growth factor
β-GP	β-Glycerolphosphat	β-glycerophosphat
GMP	Gute Herstellungspraxis	good manufacturing practice
hGluc	Humanisierte Gaussia Luziferase	humanized Gaussia luciferase
hPL	Humanes Plättchenlysat	Human platelet lysate
HPLC	Hochleistungsflüssigkeits-chromatographie	high performance liquid chromatography
IFN	Interferon	interferon
IL	Interleukin	interleukin
iPSCs	Induziert pluripotente Stammzellen	induced pluripotent stem cells
IRES	Internale Ribosomen Eintrittsstelle	internal ribosom entry site
IRF	Interferon-regulatorischer Faktor	Interferon-regulatory factor
ISG	IFN-stimuliertes Gen	IFN-stimulated gen
ISGF-3	IFN-stimulierter Genfaktor 3	IFN-stimulated gen factor 3
IVT	In vitro Transkription	in vitro transcription
JPCs	Kieferperiostzellen	jaw periostal cells
Klf4	Krüppel-ähnlicher Faktor 4	Krüppel-like factor 4
LPG2	Labor für Genetik und Physiologie 2	laboratory of genetics and physiology 2
5-mCTP	5'-Methylcytidintriphosphat	5'-methylcytidine triphosphate
MDA5	Melanoma-Differenzierungsantigen 5	melanoma differentiation-associated protein 5
MSCs	Mesenchymale Stamm-/ Stromazellen	mesenchymal stem/ stroma cells
mRNA	Boten-RNA	messenger RNA
NF-κB	Nuklear Faktor κB	nuclear factor κB
NLR	NOD-ähnliche Rezeptoren	NOD-like receptors
NOD	Nukleotid-bindende Domäne	nucleotide-binding domain
NuFF	Vorhaut-Fibroblasten gewonnen von Neugeborenen	newborn foreskin fibroblasts
OAS	Oligoadenylatsynthetase	oligoadenylate synthetases
Oct4	Oktamer-bindender Transkriptionsfaktor4	octamer-binding transcription factor 4
PCR	Polymerase Kettenreaktion	polymerase chain reaction
pDNA	Plasmid DNA	plasmid DNA

PKR	dsRNA-abhängige Proteinkinase	dsRNA-dependent protein kinase
PRR	Mustererkennungsrezeptoren	pattern recognition receptors
PSCs	Pluripotenete Stammzellen	pluripotent stem cells
RECs	Renale Epithelzellen	renal epithelial cells
RIG-I	Retinsäure-induzierbares Gen I	retinoic acid inducible gene I
RLR	RIG-I-ähnliche Rezeptor	RIG-I-like receptor
RNA	Ribonukleinsäure	ribonucleic acid
SDF-1α	Stromazellen-abgeleiteter Faktor 1 alpha	stromal cell-derived factor 1 alpha
Sox2	geschlechtsbestimmende Region Y-Box 2	SRY(sex determing region Y)-Box 2
srRNA	selbst-replizierende RNA	self-replicating RNA
siRNA	kleine eingreifende RNA	small interfering RNA
ssRNA	Einzelsträngige RNA	single stranded RNA
STAT	Signaltransduktor und Aktivator der Transkription	signal transductor and activator of transcription
TLR	Toll-ähnlicher Rezeptor	Toll-like receptor
TNF-α	Tumornekrosefaktor-α	tumor necrosis factor-α
UPR	Ungefaltetes Protein-Antwort	unfolded protein response
Ψ-UTP	Pseudo-Uridintriphosphat	Pseudouridine-triphosphate
UTR	Untranslatierbare Bereiche	untranslated region
VEE	Venezolanische Pferdeenzephalomyelitis	Venezuelan Equine Encephalitis
VEGF	Vaskulärer endothelialer Wachstumsfaktor	vascular endothelial growth factor

7.2. Eigenanteil der wissenschaftlichen Veröffentlichungen

Publikation I: Incorporation of Synthetic mRNA in Injectable Chitosan-Alginate Hybrid Hydrogels for Local and Sustained Expression of Exogenous Proteins in Cells

Heidrun Steinle:	Schreiben des Manuskripts, Durchführung von Experimenten, Auswertung und Evaluierung von Daten, Konzention und Design der Studie
Tudor-Mihai Ionescu:	Durchführung und Auswertung von Vorversuchen und Experimenten
Selina Schenk:	Durchführung von Vorversuchen und Experimenten
Sonia Golombek:	mRNA Produktion, Korrekturlesen des Manuskripts
Siliju John Kunnakattu:	Methodenethablierung von rheologische Experimenten, Auswerten und Evaluieren von Daten
Melek Tutku Özbek:	Durchführung von rheologische Experimenten
Christian Schlensak:	Korrekturlesen des Manuskripts
Hans Peter Wendel:	Korrekturlesen des Manuskripts, Supervision der Studie
Meltem Avci-Adali:	Konzept, Design und Supervision der Studie, Daten Evaluierung, Schreiben und Korrekturlesen des Manuskripts

<u>Publikation II</u>: Improving the Angiogenic Potential of EPCs via Engineering with Synthetic Modified mRNAs

Heidrun Steinle:	Schreiben des Manuskripts, Studien Konzept und Design, Durchführung von Experimenten, Auswertung von Daten
Sonia Golombek:	Unterstützung bei der mRNA Produktion und Analyse, Korrekturlesen des Manuskripts
Andreas Behring:	Durchführung und Auswertung von Experimenten, Korrekturlesen des Manuskripts
Christian Schlensak:	Korrekturlesen des Manuskripts
Hans Peter Wendel:	Evaluierung von Daten, Korrekturlesen des Manuskripts, Supervision der Studie
Meltem Avci-Adali:	Konzeption, Design und Supervision der Studie, Daten Evaluierung, Schreiben and Korrekturlesen des Manuskripts

<u>Publikation III</u>: Generation of iPSCs by non-integrative RNA-based reprogramming techniques: Benefits of self-replicating RNA versus synthetic mRNA

Heidrun Steinle:	Methodenethablierung, Studien Design und Konzept, Durchführung und Auswertung von Experimenten, Interpretation von Daten, Schreiben des Manuskripts
Marbod Weber:	Durchführung und Auswertung von Experimenten
Andreas Begring:	Methodenethablierung, Durchführung und Analyse von Vorversuchen
Ulrike Mau-Holzmann:	Supervision und Evaluierung von Karyogrammanalysen
Christian Schlensak:	Korrekturlesen des Manuskripts
Hans Peter Wendel:	Korrekturlesen des Manuskripts, Supervision der Studie
Meltem Avci-Adali:	Design und Supervision der Studie, Datenevaluation, Manuskriptentwurf und -korrektur

<u>Publikation IV</u>: Reprogramming of urine-derived renal epithelial cells into iPSCs using srRNA and consecutive differentiation into beating cardiomyocytes

Heidrun Steinle*:	Studien Design und Konzept, Methodenethablierung, Durchführung und Auswertung von Experimenten, Auswertung und Evaluierung von Daten, Schreiben des Manuskripts
Marbod Weber*:	Durchführung und Auswertung von Experimenten, Schreiben des Manuskripts, Auswertung und Evaluierung von Daten
Sonia Golombek:	Korrekturlesen des Manuskripts

Andreas Begring:	Methodenethablierung
Ulrike Mau-Holzmann:	Supervision und Interpretation von Karyogrammanalysen, Korrekturlesen des Manuskripts
Christiane von Ohle:	Supervision von CSLM Experimenten
Christian Schlensak:	Korrekturlesen des Manuskripts
Hans Peter Wendel:	Korrekturlesen des Manuskripts, Supervision der Studie
Meltem Avci-Adali:	Konzention und Supervision der Studie, Daten Evaluierung, Korrekturlesen des Manuskripts

* *gleichermaßen beigetragen*

Publikation V: Generation of iPSCs from jaw periosteal cells using self-replicating RNA

Felix Umrath*:	Methodenethablierung, Durchführung von Experimenten, Auswertung und Evaluierung von Daten, Schreiben des Manuskripts
Heidrun Steinle*:	Methodenethablierung, Durchführung von Experimenten, Auswertung und Evaluierung von Daten, Schreiben des Manuskripts
Marbod Weber:	Durchführung von Experimenten, Auswertung und Evaluierung von Daten
Hans Peter Wendel:	Korrekturlesen des Manuskripts, Supervision der Studie
Siegmar Reinert:	Korrekturlesen des Manuskripts, Supervision der Studie
Dorothea Alexander:	Konzeption und Supervision der Studie, Korrekturlesen des Manuskripts
Meltem Avci-Adali:	Konzeption und Supervision der Studie, Korrekturlesen des Manuskripts

* *gleichermaßen beigetragen*

7.3. Liste der wissenschaftlichen Veröffentlichungen

Steinle, H.*, M. Weber*, A. Behring, U. Mau-Holzmann, C. von Ohle, A.-F. Popov, C. Schlensak, H. P. Wendel and M. Avci-Adali (2019). "Reprogramming of urine-derived renal epithelial cells into iPSCs using srRNA and consecutive differentiation into beating cardiomyocytes." Mol Ther Nucleic Acids 17:907-921.

* *gleichermaßen beteiligt*

Steinle, H., M. Weber, A. Behring, U. Mau-Holzmann, C. Schlensak, H. P. Wendel and M. Avci-Adali (2019). "Generation of iPSCs by Nonintegrative RNA-Based Reprogramming Techniques: Benefits of Self-Replicating RNA versus Synthetic mRNA." Stem Cells International 2019: 16.

Michel, T., Golombek, S., **Steinle, H**., Hann, L., Velic, A., Macek, B., Stefanie Krajewski, Christian Schlensak, Hans Peter Wendel, Avci-Adali, M. (2019). Efficient reduction of synthetic mRNA induced immune activation by simultaneous delivery of B18R encoding mRNA. Journal of Biological Engineering, 13(1), 40.

Umrath, F.*, **H. Steinle***, M. Weber, H. P. Wendel, S. Reinert, D. Alexander and M. Avci-Adali (2019). "Generation of iPSCs from Jaw Periosteal Cells Using Self-Replicating RNA." Int J Mol Sci 20(7).

Steinle, H., S. Golombek, A. Behring, C. Schlensak, H. P. Wendel and M. Avci-Adali (2018). "Improving the Angiogenic Potential of EPCs via Engineering with Synthetic Modified mRNAs." Mol Ther Nucleic Acids 13: 387-398.

Steinle, H., T. M. Ionescu, S. Schenk, S. Golombek, S. J. Kunnakattu, M. T. Ozbek, C. Schlensak, H. P. Wendel and M. Avci-Adali (2018). "Incorporation of Synthetic mRNA in Injectable Chitosan-Alginate Hybrid Hydrogels for Local and Sustained Expression of Exogenous Proteins in Cells." Int J Mol Sci 19(5).

Golombek, S., M. Pilz, **H. Steinle**, E. Kochba, Y. Levin, D. Lunter, C. Schlensak, H. P. Wendel and M. Avci-Adali (2018). "Intradermal Delivery of Synthetic mRNA Using Hollow Microneedles for Efficient and Rapid Production of Exogenous Proteins in Skin." Mol Ther Nucleic Acids 11: 382-392.

Weber, M., **H. Steinle**, S. Golombek, L. Hann, C. Schlensak, H. P. Wendel and M. Avci-Adali (2018). "Blood-Contacting Biomaterials: In Vitro Evaluation of the Hemocompatibility." Front Bioeng Biotechnol 6: 99.

Steinle, H., A. Behring, C. Schlensak, H. P. Wendel and M. Avci-Adali (2017). "Concise Review: Application of In Vitro Transcribed Messenger RNA for Cellular Engineering and Reprogramming: Progress and Challenges." Stem Cells 35(1): 68-79.

Stoll, H., **H. Steinle**, K. Stang, S. Kunnakattu, L. Scheideler, B. Neumann, J. Kurz, I. Degenkolbe, N. Perle, C. Schlensak, H. P. Wendel and M. Avci-Adali (2017). "Generation of Large-Scale DNA Hydrogels with Excellent Blood and Cell Compatibility." Macromol Biosci 17(4).

Stoll, H.*, **H. Steinle***, N. Wilhelm, L. Hann, S. J. Kunnakattu, M. Narita, C. Schlensak, H. P. Wendel and M. Avci-Adali (2017). "Rapid Complexation of Aptamers by Their Specific Antidotes." Molecules 22(6).

Avci-Adali, M., L. Hann, T. Michel, **H. Steinle**, S. Stoppelkamp, K. Stang, M. Narita, C. Schlensak and H. P. Wendel (2015). "In vitro test system for evaluation of immune activation potential of new single-stranded DNA-based therapeutics." Drug Test Anal 7(4): 300-308.

Avci-Adali, M., A. Behring, **H. Steinle**, T. Keller, S. Krajeweski, C. Schlensak and H. P. Wendel (2014). "In vitro synthesis of modified mRNA for induction of protein expression in human cells." J Vis Exp(93): e51943.

Avci-Adali, M., **H. Steinle**, T. Michel, C. Schlensak and H. P. Wendel (2013). "Potential capacity of aptamers to trigger immune activation in human blood." PLoS One 8(7): e68810.

8. WISSENSCHAFTLICHE VERÖFFENTLICHUNGEN IM ORIGINAL

8.1. PUBLIKATION I

Incorporation of synthetic mRNA in injectable chitosan-alginate hybrid hydrogels for local and sustained expression of exogenous proteins in cells

International Journal of *Molecular Sciences*

MDPI

Article

Incorporation of Synthetic mRNA in Injectable Chitosan-Alginate Hybrid Hydrogels for Local and Sustained Expression of Exogenous Proteins in Cells

Heidrun Steinle, Tudor-Mihai Ionescu, Selina Schenk, Sonia Golombek, Silju-John Kunnakattu, Melek Tutku Özbek, Christian Schlensak, Hans Peter Wendel and Meltem Avci-Adali *

Department of Thoracic and Cardiovascular Surgery, University Hospital Tuebingen, Calwerstraße 7/1, 72076 Tuebingen, Germany; heidi.steinle@googlemail.com (H.S.); tudor_mihai.ionescu@yahoo.de (T.-M.I.); schenkse@hs-albsig.de (S.S.); sonia.golombek@klinikum.uni-tuebingen.de (S.G.); silju_j1984@yahoo.de (S.-J.K.); mtutkuozbek@gmail.com (M.T.O.); Christian.Schlensak@med.uni-tuebingen.de (C.S.); hans-peter.wendel@med.uni-tuebingen.de (H.P.W.)

* Correspondence: meltem.avci-adali@uni-tuebingen.de

Received: 3 March 2018; Accepted: 25 April 2018; Published: 27 April 2018

check for updates

Abstract: The application of synthetic messenger RNA (mRNA) exhibits various advantages, such as expression of desired proteins in cells without genomic integration. In the field of tissue engineering, synthetic mRNAs could be also used to modulate the protein expression in implanted cells. Therefore, in this study, we incorporated synthetic humanized Gaussia luciferase (hGLuc) mRNA into alginate, chitosan, or chitosan-alginate hybrid hydrogels and analyzed the release of hGLuc mRNA from these hydrogels. After 3 weeks, 79% of the incorporated mRNA was released from alginate hydrogels, approximately 42% was released from chitosan hydrogels, and about 70% was released from chitosan-alginate hydrogels. Due to the injectability, chitosan-alginate hybrid hydrogels were selected for further investigation of the bioactivity of embedded hGLuc mRNA and the stability of these hydrogels was examined after the incorporation of synthetic mRNA by rheometric analysis. Therefore, HEK293 cells were incorporated into chitosan-alginate hydrogels containing mRNA transfection complexes and the luciferase activity in the supernatants was detected for up to 3 weeks. These results showed that the biodegradable chitosan-alginate hybrid hydrogels are promising delivery systems for sustained delivery of synthetic mRNAs into cells. Since chitosan-alginate hybrid hydrogels are injectable, the hydrogels can be simultaneously loaded with cells and the desired synthetic mRNA for exogenous protein synthesis and can be administered by minimally invasive local injection for tissue engineering applications.

Keywords: synthetic mRNA; injectable hydrogels; mRNA delivery; production of exogenous proteins

1. Introduction

In recent years, the application of synthetic messenger RNAs (mRNAs) for the production of therapeutic proteins has gained interest due to their advantageous properties over the use of DNA-based methods, including a safe, easy, and efficient translation of proteins [1,2]. The synthetic mRNA does not integrate into the host genome, it does not need to enter the nucleus for the translation of proteins, and after reaching the cytosol, delivered mRNA is immediately translated by ribosomes into proteins. So far, several applications with therapeutic mRNAs have been investigated, including vaccination, treatment of cancer, infectious diseases, protein deficiency disorders, inflammation, as well as production of growth factors, cellular reprogramming and differentiation [3–18].

Hydrogels are three-dimensional (3D) networks composed of natural or synthetic hydrophilic polymer chains, which can be cross-linked physically or chemically [19–21]. Especially, due to their

high biocompatibility, biodegradability, and low toxicity, natural polymers are beneficial compared to synthetic polymers [19,22]. Thus, hydrogels have attracted increased attention especially in tissue engineering applications and regenerative medicine, e.g., for the regeneration and repair of tissues or the controlled release of drugs [20,23,24]. Multiple studies demonstrated the applicability of hydrogels for the release of a broad range of different therapeutic agents, such as growth factors [25–29], insulin [30–32], and anti-cancer drugs [27,33–36].

Alginate and chitosan are very frequently used biomaterials for the generation of hydrogels. Alginate is a polyanionic polysaccharide, which consists of alternating blocks of (1–4)-linked β-D-mannuronic acid (M) and α-L-glucoronic acid (G) monomers [37]. The polymers can be ionically crosslinked using bivalent cations, such as Ca^{2+}, to obtain a three-dimensional network [38]. Alginate solutions can also be injected in vivo and hydrogels can then be generated by Ca^{2+} ions that are present in the surrounding tissue [39]. Furthermore, due to their similarity to extracellular matrix proteins, alginate hydrogels have been used as a carrier for cells, and have proven to promote the release of nucleic acids, such as small interfering RNA (siRNA) and plasmid DNA (pDNA) [40–43]. In contrast to alginate, chitosan is a cationic polysaccharide and it is produced by the deacetylation of chitin. The gelation of chitosan can be performed by adding glycerol phosphate (GP). Thereby, injectable chitosan solutions can be generated, which are thermo-responsive at physical pH and enable in situ formation of gels upon warming to body temperature [44]. Due to their charge, cationic polymers are favorable for delivery of anionic molecules. Thus, chitosan is able to condense nucleic acids into cationic polyplexes and thereby, facilitate transfection [45]. The release of functional nucleic acids from chitosan hydrogels was demonstrated for siRNA, which resulted in a prolonged and localized gene silencing, and for pDNA, which promoted tissue regeneration [43,45–47].

Chitosan and alginate are known to form polyelectrolyte complexes and have been combined to create hybrid hydrogels [48,49]. Due to electrostatic interactions of the opposite charged polymers, this hybrid material combination demonstrates an increased stability compared to both polymers alone [50]. In previous studies, chitosan-alginate hybrid hydrogels supported cell growth, wound healing, bone regeneration, cartilage tissue engineering, and they were used for the treatment of myocardial infarction and to induce angiogenesis [49,51–54]. Furthermore, cationic pDNA-nanoparticles incorporated into a 3D chitosan-alginate porous scaffold demonstrated gene delivery in prostate cancer cells in vitro and in vivo [55].

Drug-releasing systems continuously delivering synthetic mRNA locally to the cells during the required period could represent a suitable alternative to repeated administration of synthetic mRNA, especially for in vivo applications. To obtain a sustained expression of an exogenous protein, we developed a local delivery approach based on mRNA embedded injectable hydrogels. Here, we analyzed for the first time, the applicability of synthetic mRNA in hydrogels for the extended transfection of cells incorporated into hydrogels. Therefore, synthetic secretable humanized Gaussia luciferase (hGLuc) mRNA was loaded into alginate, chitosan, or chitosan-alginate hybrid hydrogels and the release characteristics of synthetic mRNA was investigated over a period of 21 days. Afterwards, the bioactivity of incorporated mRNA and the transfectability of cells were determined.

2. Results

2.1. Release of Cy3-Labeled hGLuc mRNA from Hydrogels

In order to investigate the release kinetics of synthetic mRNA from alginate, chitosan, and chitosan -alginate hybrid hydrogels, 4 µg of complexed Cy3-labeled hGLuc mRNA was incorporated into the hydrogels. After 4 h, 1, 2, 3, 7, 14, and 21 days of incubation at 37 °C, the relative fluorescence intensity (RFU) of Cy3-labeled mRNA was measured in 50 µL supernatant in triplicates. The amount of released mRNA was calculated by using a standard curve ranging from 31 to 1000 ng Cy3-labeled mRNA. According to the measurements, all hydrogels demonstrated a sustained release of Cy3-labeled hGLuc mRNA over 21 days (Figure 1). At 21 days, alginate hydrogels (1.3% alginate, 19.9 mM $CaCl_2$)

released 79.0% of the incorporated Cy3-mRNA. Chitosan hydrogels (1.9% chitosan, 4.2% GP) showed compared to alginate hydrogels a slower mRNA release and only 41.8% of the incorporated mRNA was released during 21 days. The incorporation of mRNA into a hybrid hydrogel containing 1.4% chitosan, 0.6% alginate and 4% GP resulted in slower release compared to alginate hydrogels. After 21 days of incubation, 69.6% of the incorporated Cy3-mRNA was released.

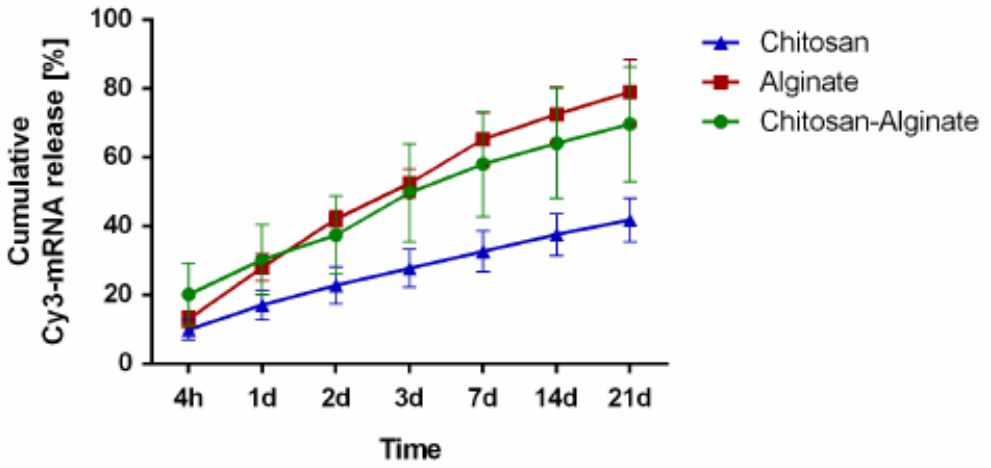

Figure 1. Cumulative release profile of Cy3-labeled hGLuc mRNA from alginate, chitosan, and chitosan -alginate hybrid hydrogels. Hydrogels consisting of alginate (1.3% alginate and 19.9 mM $CaCl_2$), chitosan (1.9% chitosan, 4.2% glycerol phosphate (GP)), or chitosan-alginate (1.4% chitosan, 4% GP, and 0.6% alginate) were prepared and loaded with 4 µg of Cy3-hGLuc mRNA transfection complexes. The release of Cy3-mRNA was determined after 4 h, 1, 2, 3, 7, 14, and 21 days (d). The total amount of released mRNA after 21 days was 79% for alginate, 42% for chitosan, and 70% for chitosan-alginate hybrid hydrogels. Data are shown as mean ± SEM ($n = 3$).

2.2. *Rheological Characterization of mRNA-Loaded Chitosan-Alginate Hydrogels*

Due to the injectability of chitosan-alginate hydrogels compared to chitosan hydrogels and slower mRNA release compared to alginate hydrogels, chitosan-alginate hydrogels were selected for further analysis. To assess hydrogel characteristics and the stability of hydrogels over several days in vitro, chitosan-alginate hydrogels were loaded without or with 4 µg of complexed hGLuc mRNA. As a control, hydrogels with transfection reagent were generated. Rheological measurements were performed directly after gelation (day 0) and after the incubation of hydrogels with 0.5 mL Dulbecco's phosphate-buffered saline (DPBS) for 1, 7, 14, and 21 days at 37 °C.

Over the whole frequency range (10–100 Hz) and incubation time, the hydrogels demonstrated higher storage (G′) modulus compared to the loss (G″) modulus, which indicated stable gel-like properties of the chitosan-alginate hydrogels (Figure 2). After the addition of DPBS to the gels (day 1), mRNA-loaded chitosan-alginate hydrogels showed significantly higher elastic G′ and viscous G″ values (187 ± 79 Pa G′ and 185 ± 86 Pa G″) compared to hydrogels without mRNA (54 ± 19 Pa G′ and 38 ± 16 Pa G″), representing higher mechanical strength and stiffness of the mRNA-loaded hydrogel. Although after 7, 14, and 21 days, the mRNA containing hydrogels showed higher G′ values than the hydrogels without mRNA, only the value after 14 days of incubation was statistically significant (Figure 2B). A slight decrease of G′ and G″ was detected in all hydrogels from day 1 to day 7, which could be the result of hydrogel degradation.

Int. J. Mol. Sci. **2018**, *19*, 1313

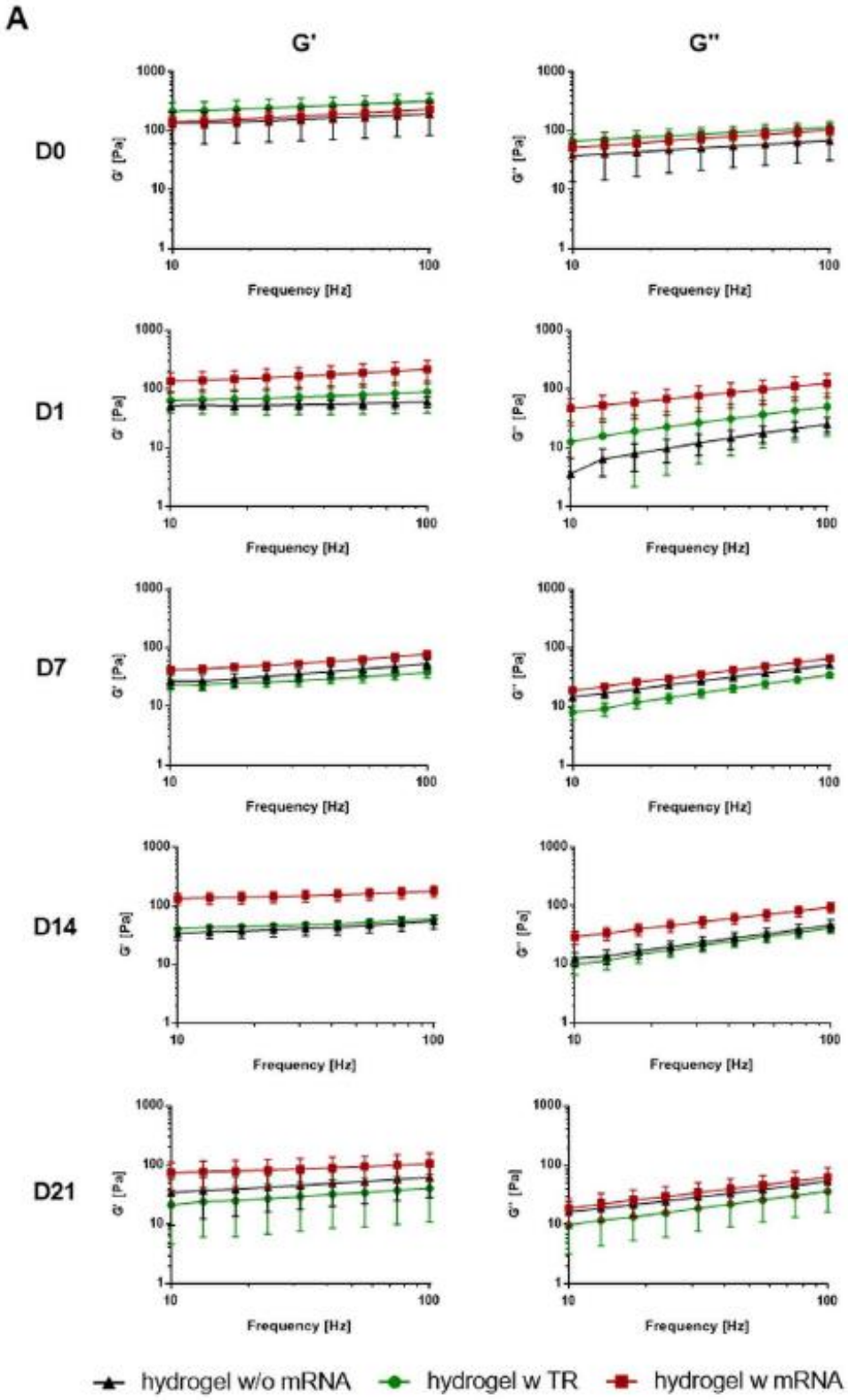

Figure 2. *Cont.*

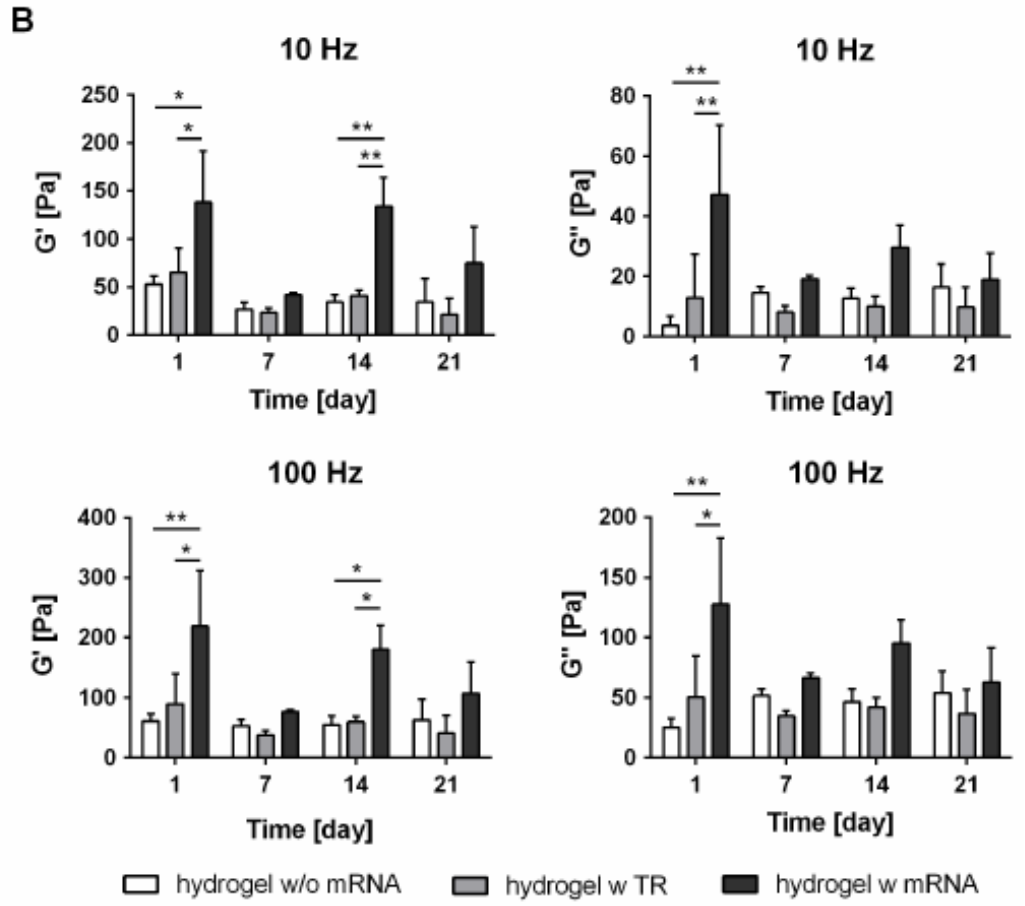

Figure 2. Rheological characterization of mRNA containing chitosan-alginate hybrid hydrogels over time. (**A**) Hydrogels without and with 4 µg mRNA and hydrogels containing only the transfection reagent (TR) were generated. Storage (G′) and loss moduli (G″) were analyzed without adding Dulbecco's phosphate-buffered saline (DPBS) (D0) and after the addition of DPBS and incubation for 1 (D1), 7 (D7), 14 (D14), and 21 (D21) days using oscillatory squeeze-flow rheometer with a frequency scan of 10–100 Hz. Hydrogels without and with mRNA showed higher G′ than G″ modulus after different time points of incubation in DPBS, which indicates stable gel-like properties over time; (**B**) Statistical analysis of G′ and G″ values at 10 and 100 Hz. Results are shown as mean ± SD ($n = 3$). Statistical differences were determined using repeated measures two-way ANOVA with Bonferroni's multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$).

2.3. Bioactivity of hGLuc mRNA Incorporated into Chitosan-Alginate Hydrogels

Chitosan-alginate hydrogels containing 1.5×10^5 HEK293 cells were generated and 4 µg hGLuc mRNA with or without complexation with transfection reagent was incorporated into the hydrogels. Additionally, hydrogels without mRNA and transfection reagent or only with transfection reagent were produced as negative controls. The ability of hGLuc mRNA to mediate protein expression in cells was analyzed over a period of 21 days by detection of luciferase activity in the supernatant (Figure 3). During 21 days, significantly increased amount of hGLuc was detected in hydrogels containing synthetic hGLuc mRNA transfection complexes compared to control hydrogels. The highest luciferase activity was measured after 48 h of incubation. Afterwards, a continuous decrease of luciferase activity was determined. Due to the ability of chitosan to generate complexes with negatively charged oligonucleotides, we further investigated if the HEK293 cells can be transfected without using the transfection reagent. Although hGLuc protein could be detected until 7 days, the measured values showed large fluctuations and the differences were statistically not significant. Clearly, less protein was

produced when the mRNA was incorporated without transfection reagent compared to the hydrogels with complexed mRNA (Figure 3). After 9 days, the protein expression dropped to the levels of control hydrogels without mRNA. These results demonstrated that the generation of mRNA transfection complexes are required to efficiently transfect cells and to continuously produce a desired protein in cells. The hGLuc expression of cells after a single transfection with 4 µg hGLuc mRNA over 4 days is shown in Supplementary Figure S1.

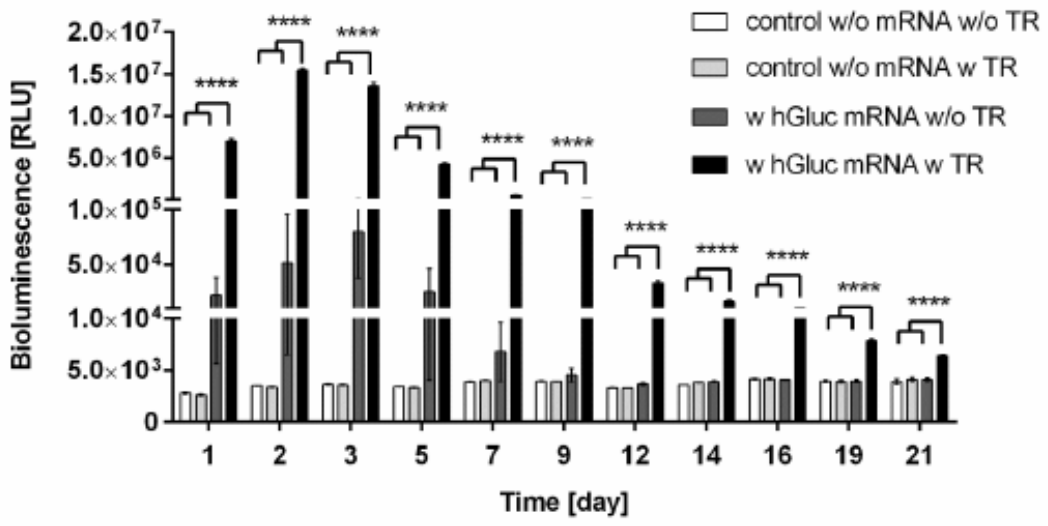

Figure 3. Bioactivity of hGLuc mRNA incorporated into chitosan-alginate hybrid hydrogels. Chitosan-alginate hydrogels with 4 µg hGLuc mRNA complexed with or without transfection reagent (TR) were generated and 1.5×10^5 HEK293 cells were incorporated. Additionally, hydrogels containing HEK293 cells without mRNA and TR or with TR were produced. The production of hGLuc was determined from 1 to 21 days in medium using luciferase assay. Data are shown as mean ± SEM, ($n = 4$). Statistical differences were determined using one-way ANOVA with Bonferroni's multiple comparisons test. (**** $p < 0.0001$).

2.4. Influence of hGLuc mRNA Loading into Chitosan-Alginate Hybrid Hydrogels on Cell Viability

To assess the influence of synthetic mRNA loading in chitosan-alginate hydrogels on cell viability, 1.5×10^5 HEK293 cells were incorporated into the gels. The cell viability was measured 24, 48, and 72 h after the cultivation at 37 °C using PrestoBlue assay (Figure 4). The same number of cells was also seeded in one well of a 12-well plate without hydrogel. No statistically significant differences in cell viability were detected in hydrogels containing the hGLuc mRNA, transfection reagent, or only OptiMEM compared to the adherent growing cells without hydrogel. After 48 h of cultivation, a small reduction of cell viability to approximately 70% was detected in HEK293 cells cultivated in hydrogels compared to the adherent growing cells. This could probably be caused by adaptation differences between the cells encapsulated in hydrogels and adhesive growing cells on the cell culture plate. However, the decrease of cell viability was statistically not significant.

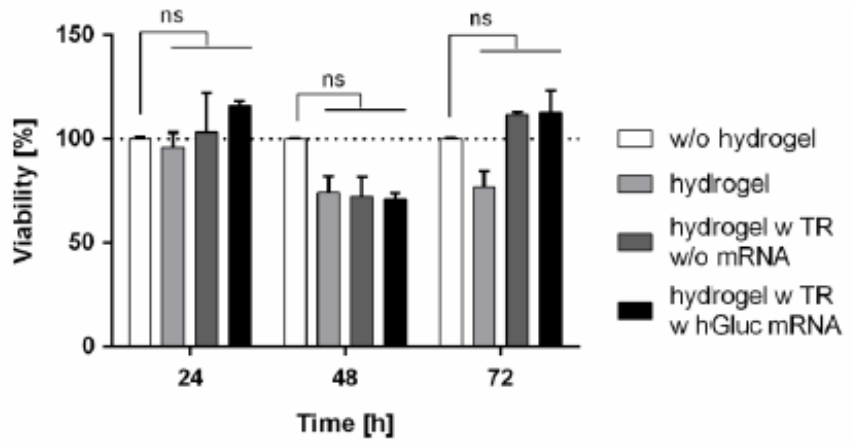

Figure 4. Analysis of the influence of hGLuc mRNA loading into chitosan-alginate hybrid hydrogels on cell viability using PrestoBlue® assay. 1.5×10^5 HEK293 cells were incorporated into chitosan-alginate hybrid hydrogels containing 4 µg hGLuc mRNA complexed with transfection reagent (TR). Furthermore, hydrogels without mRNA and TR or without TR were generated. PrestoBlue cell viability assay was performed 24, 48, and 72 h after incubation of hydrogels at 37 °C. 1.5×10^5 HEK293 cells cultivated in 12-well plates were analyzed as positive controls. The viability of cells without hydrogel was set to 100% and the viability of cells in hydrogels was expressed relative to cells without hydrogel. Data are shown as mean ± SEM, ($n = 3$). Statistical differences were analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. (ns: non-significant).

3. Discussion

Hydrogels are promising scaffolds for the delivery of drugs as well as for the cultivation of cells. In this study, different hydrogels were tested for the incorporation of synthetic mRNA. Injectable chitosan-alginate hybrid hydrogels loaded with synthetic modified hGLuc mRNA demonstrated the sustained release of the incorporated Cy3 hGLuc mRNA over 21 days. The incorporated hGLuc mRNA was bioactive and led to the production of hGLuc for up to 3 weeks. These results demonstrated that hydrogels containing cells can be additionally loaded with synthetic mRNA and applied for simultaneous delivery of mRNA to implanted cells for tissue engineering applications. Thereby, desired proteins can be produced by the incorporated cells over an extended period without the need of repeated administration of synthetic mRNA.

In order to analyze the release kinetic of synthetic mRNA from different hydrogels, alginate, chitosan, and chitosan-alginate hybrid hydrogels were generated. All hydrogels showed sustained release of mRNA over 21 days. The slowest release was detected in chitosan hydrogels and the fastest release was detected in alginate hydrogels. In comparison, the use of a chitosan-alginate hybrid hydrogel resulted in a faster release of mRNA than from the chitosan hydrogel and a slower release than from the alginate hydrogel. Chitosan is a positively charged polymer, thus, the interaction of the negatively charged mRNA with chitosan could lead to increased retention of the mRNA in hydrogels. In contrast, alginate is a negatively charged polymer and due to electrostatic repulsive forces, the synthetic mRNA could be released much faster from alginate hydrogels than from chitosan hydrogels. Krebs et al. demonstrated that the release of siRNA was delayed after the addition of positively charged polymers, such as polyethylenimine (PEI) or chitosan, to the calcium crosslinked alginate hydrogels [41]. In a recent study, the addition of chitosan to an alginate-based hydrogel resulted also in slower release of negatively charged sphingosine-1-phospate compared to alginate hydrogels alone [56]. In another study, Ma and colleagues subcutaneously injected siRNA containing chitosan hydrogels in mice and they were able to detect the Cy5-labeled siRNA for up to 7 days compared to less than one day for siRNA alone [45].

The results of our study showed that the release kinetics of the synthetic mRNAs can be modulated by using different biomaterials e.g., with different charges. Furthermore, the production of hGLuc over 21 days by HEK293 cells incorporated into hGLuc mRNA containing chitosan-alginate hydrogels demonstrated that the incorporated hGLuc mRNA can be successfully uptaken into cells. The burst release and the uptake of synthetic mRNA after the first day of incubation could be the reason for the production of the highest hGLuc amount in HEK293 cells at the second day of incubation. After the second day, the production of hGLuc decreased over time. This could be related to the degradation processes, such as the degradation of hydrogels as well as possible degradation of complexed mRNA. Additionally, it was demonstrated that the generation of mRNA transfection complexes is required to efficiently deliver the mRNA into the cells and to continuously produce the desired protein in cells. Using transfection reagents, mRNA molecules can be protected from nucleases and thereby, the stability can be improved and the uptake into the cells can be increased [57]. In addition, cell viability measurements over 3 days revealed that the incorporation of synthetic hGLuc mRNA into chitosan-alginate hydrogels has no negative influence on the viability of cells.

4. Materials and Methods

4.1. In Vitro mRNA Synthesis

The plasmid pEX-A2 containing the hGLuc encoding sequence was produced by Eurofins Genomics (Ebersberg, Germany). The DNA template for the synthesis of hGLuc mRNA was generated by PCR and transcribed into mRNA using an in vitro transcription (IVT) reaction as previously described [58]. Briefly, the insert containing the coding sequence of hGLuc was amplified by PCR using the forward primer: 5′-TTGGACCCTCGTACAGAAGCTAATACG-3′ and reverse primer: 5′-T_{120}CTTCCTACTCAGGCTTTATTCAAAGACCA-3′ (Ella Biotech, Martinsried, Germany) and the HotStar HiFidelity polymerase kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR product was purified using MinElute PCR purification kit (Qiagen). Subsequently, IVT reaction was performed using MEGAscript® T7 kit (Ambion, Glasgow, Scotland, UK) according to manufacturer's instructions. The IVT reaction mixture contained 7.5 mM ATP and 1.875 mM GTP, and to improve the stability and the translation of mRNA and to reduce the immunogenicity, UTP and CTP were completely substituted by 7.5 mM pseudoruridine-5′-triphosphate (pseudo-UTP) and 7.5 mM 5-methylcytidine-5′-triphosphate (5-methyl-CTP) (TriLink Biotech, San Diego, CA, USA). Furthermore, 2.5 mM 3′-0-Me-m7G(5′)ppp(5′)G RNA Cap Structure Analog (ARCA, New England Biolabs, Frankfurt, Germany) was used for 5′-end capping, and the mRNA was dephosphorylated using 5 U Antarctic phosphatase (New England Biolabs, Frankfurt am Main, Germany). Additionally, the IVT reaction mixture contained 40 U RiboLock RNAse inhibitor (Thermo Scientific, Waltham, MA, USA) to prevent mRNA degradation. After the IVT and dephosphorylation, the mRNA was purified using RNeasy kit (Qiagen) and nuclease-free water was used for the elution of mRNA. The purity and specific length of generated PCR and mRNA product was analyzed using 1% agarose gel electrophoresis. Samples were run at 100 V for 45 min and the gel was stained with 1× GelRed™ (Biotium, Fremont, CA, USA) in 1× TBE buffer.

4.2. Fluorescent Labeling of mRNA

To determine the released mRNA from hydrogels, cyanine 3 (Cy3) labeled hGLuc mRNA was generated by Cu(I)-free dibenzocyclooctyne (DBCO) click chemistry. Therefore, the IVT mixture contained instead of 7.5 mM pseudo-UTP, 1.9 mM 5-azido-C_3-UTP (Jena Bioscience, Jena, Germany) and 5.6 mM pseudo-UTP. Thereby, 25% of the total amount of pseudo-UTP was replaced by 5-azido-C_3-UTP. Afterwards, 5-fold amount of DBCO-sulfo-Cy3 (Jena Bioscience) was added to the 5-azido-C3-UTP containing mRNA and the volume was adjusted with DPBS (w/o Ca^{2+}/Mg^{2+}) to 40 µL, vortexed for 10 s and incubated for 60 min at 37 °C. In order to remove the remaining

unbound DBCO-sulfo-Cy3 molecules, the labeling mixture was purified using RNeasy kit according to manufacturer's instructions.

4.3. Generation of mRNA Transfection Complexes and Incorporation into Hydrogels

Transfection complexes were generated by incubation of 4 µg hGLuc mRNA with 4 µL of the transfection reagent from the GenaxxoFect transfection kit and 8 µL of the dilution buffer (Genaxxon Bioscience, Ulm, Germany) for 15 min at room temperature (RT). Subsequently, the generated transfection complexes were filled with nuclease-free water up to 30 µL and added to the hydrogels, mixed, and incubated for 30 min at 37 °C to achieve complete gelation of hydrogels. Hydrogels with only transfection reagent or nuclease-free water were also prepared as negative controls.

4.4. Preparation of Hydrogels

4.4.1. Alginate Hydrogels

A solution containing 3% (*w*/*v*) alginate was prepared by gradual addition of 300 mg alginate (Sigma-Aldrich, St. Louis, MO, USA) to 10 mL DPBS. After 1 h of constant stirring at 300 rpm and RT, alginate was completely dissolved and sterilized using UV light for 30 min. An aqueous solution containing 100 mM $CaCl_2$ was prepared and filtered through a 0.22 µm sterile filter. Afterwards, $CaCl_2$ solution was diluted in DPBS to 49 mM. For the incorporation of mRNA into the hydrogels, 88.7 µL of 3% alginate solution was added to 30 µL mRNA transfection complex mixture and mixed thoroughly. After adding 81.3 µL of 49 mM $CaCl_2$ solution to the mRNA containing alginate solution, hydrogels consisting of 1.3% alginate and 19.9 mM $CaCl_2$ were obtained.

4.4.2. Chitosan Hydrogels

Chitosan with a deacetylation degree of 75–85% was purchased from Sigma-Aldrich and autoclaved. To obtain a solution with 3% (*w*/*v*) chitosan, 300 mg of the sterilized chitosan was dissolved in 10 mL DPBS containing 0.12 M hydrochloric acid. Chitosan was completely dissolved by initial stirring at 100 rpm for 30 min at RT and the following stirring for 30 min at 4 °C. Thermosensitive hydrogels were then produced by dropwise adding of 3.3 mL 20% sterile-filtered cold GP (Sigma-Aldrich, St. Louis, MO, USA) solution in DPBS. After stirring for another 60 min at about 300–350 rpm and 4 °C, the transfection complexes (30 µL) were added to the hydrogel (170 µL). The resulting final concentration was 1.9% chitosan and 4.2% GP.

4.4.3. Chitosan-Alginate Hybrid Hydrogel

To generate the chitosan-alginate hybrid hydrogels, 3% (*w*/*v*) chitosan solution was prepared as described above. The chitosan solution (10 mL) was mixed at 4 °C with 4.4 mL 20% GP solution by stirring at 600 rpm for 5–10 s and then at 100 rpm for 1 h. Subsequently, 4.14 mL of 3% alginate solution was added and stirred at 350 rpm for 1 h. After mixing the gel (170 µL) with the mRNA transfection complexes (30 µL), the final concentration was 1.4% chitosan, 0.6% alginate, and 4.0% GP.

4.4.4. Detection of Released Cy3-Labeled hGLuc mRNA from Hydrogels

To perform release experiments, 200 µL of each hydrogel, containing 30 µL of the transfection complex mixture (4 µg of Cy3-labeled mRNA in 18 µL nuclease-free water, 4 µL of GenaxxoFect transfection reagent, and 8 µL of dilution buffer) was generated per well of 48-well plates. Negative controls were prepared by adding the required amount of nuclease-free water, transfection reagent and dilution buffer or only nuclease-free water and dilution buffer. After complete gelation of the hydrogels, 150 µL DPBS was added to each well and incubated at 37 °C. The supernatant was collected after 4 h, 1, 2, 3, 7, 10, 14, and 21 days. After each time point, 150 µL fresh DPBS was carefully pipetted onto the hydrogels and further incubated. The mRNA, released from the hydrogels, was analyzed in duplicates (2 × 50 µL) using the Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad,

Germany). Fluorescence intensity was measured using the excitation/emission wavelength of 495 nm/540 nm. To quantify the released mRNA amount, a standard curve of Cy3-labeled mRNA with concentrations ranging from 31.25 ng to 1000 ng per 50 µL DPBS was used.

4.5. Rheological Characterization of Chitosan-Alginate Hybrid Hydrogels Containing hGLuc mRNA

To assess hydrogel characteristics and the stability of hydrogels over several days in vitro, hydrogels were produced as mentioned above and 200 µL of hydrogel without and with 4 µg mRNA was transferred into 1.5 mL reaction tube and allowed to solidify for 1 h at 37 °C. Then, the first rheological measurement (day 0) was performed after removing air bubbles by centrifugation at 100× *g* for 1 min. Next, 0.5 mL DPBS was added to the gels followed by further incubation at 37 °C. The rheological characterization of the hydrogels was then performed after 1, 7, 14, and 21 days of incubation at 37 °C using a squeeze-flow rheometer, called piezo axial vibrator (PAV) [59]. The storage G′ (elastic) and loss storage G″ (viscous) moduli of chitosan-alginate hydrogels were assessed by oscillatory squeeze-flow measurements using a gap size of 100 µm. Hydrogels of 200 µL were placed on the bottom plate of the PAV and the lid was carefully fixed with a defined pressure. The experiments were performed at RT with a frequency scan of 10–100 Hz.

4.6. Cultivation of HEK293 Cells

HEK293 cells were cultivated in DMEM with high glucose and L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin (all from Life Technologies, Darmstadt, Germany). Cells were kept at 37 °C with 5% CO_2 and the medium was changed every 3 days. Cells were rinsed with 1 mL DPBS and detached using 0.04% trypsin/0.03% EDTA and 0.05% trypsin inhibitor in 0.1% BSA (both from PromoCell, Heidelberg, Germany). After centrifugation for 5 min at 300× *g*, the cell pellet was resuspended in culture medium.

4.7. Influence of hGLuc mRNA Loading in Chitosan-Alginate Hydrogels on Cell Viability

Chitosan-alginate hydrogels containing 1.5 × 10^5 HEK293 cells and 4 µg hGLuc mRNA were generated and transferred into one well of a 12-well plate. Then, hydrogels were incubated at 37 °C with 1 mL cell culture medium. The cell viability was measured after 24, 48, and 72 h using PrestoBlue® assay (Invitrogen, Carlsbad, CA, USA). Therefore, 110 µL PrestoBlue® cell viability reagent was added to 1 mL cell culture medium per well and incubated for 1.5 h at 37 °C. The fluorescence intensity of 50 µL supernatant was measured in triplicates at excitation wavelength of 530 nm and emission wavelength of 600 nm using multimode microplate reader (Mithras LB 940, Berthold Technologies).

4.8. Analyses of the Bioactivity of Synthetic hGLuc mRNA Incorporated in Chitosan-Alginate Hydrogels

To analyze the transfectability and the functionality of the incorporated hGLuc mRNA in chitosan-alginate hydrogels, 1.5 × 10^5 HEK293 cells were added in 100 µL cell culture medium to 500 µL chitosan-alginate hydrogels containing 4 µg hGLuc mRNA and carefully mixed. Hydrogels were then transferred into 12-well plates. After thermal gelation for about 30 min at 37 °C, 1 mL cell culture medium was added to the gels and incubated at 37 °C with 5% CO_2. The production of secreted hGLuc by HEK293 cells was determined over 21 days in medium using luciferase assay.

4.9. Luciferase Assay

To measure the luciferase activity in the supernatants, DPBS containing 20 µg/mL coelenterazine (Carl Roth, Karlsruhe, Germany) was prepared. During the measurement, 100 µL coelenterazine solution was automatically injected into each well of a 96-well plate containing 40 µL supernatant in triplicates. The resulting bioluminescence was immediately detected as relative light units (RLU) using multimode microplate reader (Mithras LB 940).

4.10. Statistical Analysis

Data are shown as mean ± standard deviation (SD) or standard error of mean (SEM). One or two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was performed to compare the means. All statistical analyses were performed double-tailed using GraphPad Prism version 6.01 (GraphPad Software, La Jolla, CA, USA). Differences of $p < 0.05$ were considered significant.

5. Conclusions

This study confirmed the potency of chitosan-alginate hydrogels for prolonged delivery of mRNA transfection complexes until 21 days in vitro. Above all, these promising results demonstrated for the first time that hydrogels can be loaded with synthetic mRNAs for tissue engineering applications. A schematic representation of the future clinical application of injectable chitosan-alginate hybrid hydrogels loaded with synthetic mRNAs is displayed in Figure 5. The mRNA-loaded hydrogels could be applied for local and continuous synthetic mRNA delivery to cells over weeks for diverse approaches, e.g., to produce growth or differentiation factors to improve tissue regeneration.

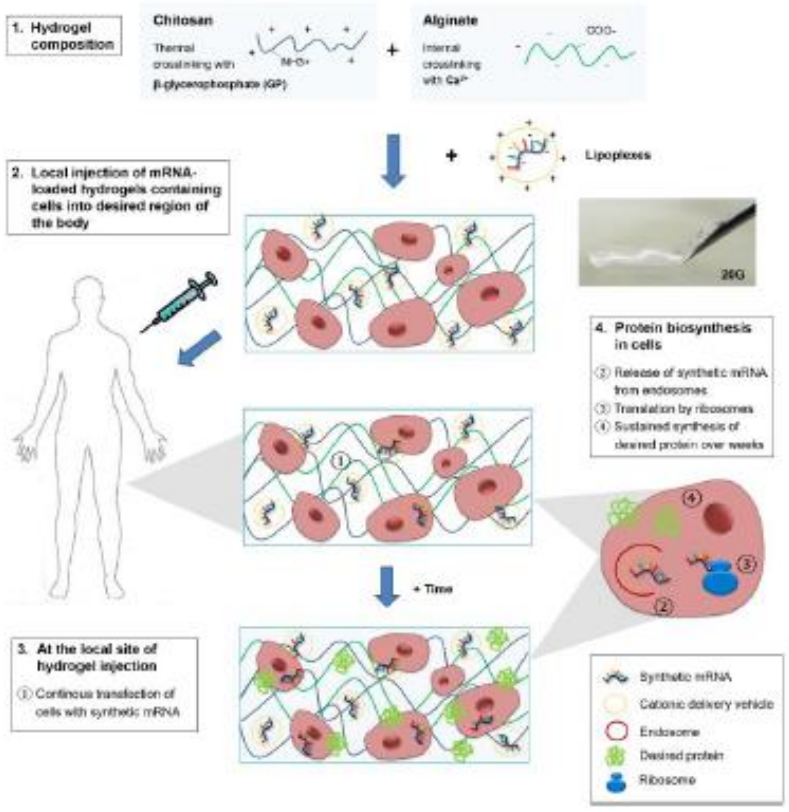

Figure 5. Schematic representation of the future clinical application of synthetic mRNA-loaded injectable chitosan-alginate hybrid hydrogels for local and sustained delivery of synthetic mRNAs. (**1**) The chitosan-alginate hybrid hydrogel is made of opposite charged polymers chitosan and alginate; (**2**) After the addition of mRNA containing transfection complexes (lipoplexes) and cells, the obtained hydrogels are injectable through a 20 G needle. Due to the use of β-glycerophosphate (GP) for thermal activated crosslinking and an internal crosslinking of alginate by Ca^{2+}, the gel can solidify inside patient's body at a desired position; (**3**) At the local site of injection, the lipoplexes can lead to the continuous transfection of cells with synthetic mRNA; (**4**) After the release of mRNA from endosomes into the cytosol, the ribosomal translation of mRNA into proteins starts and results in sustained synthesis of desired proteins.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/5/1313/s1.

Author Contributions: H.S. and M.A.-A. conceived and designed the experiments; H.S., T.-M.I., S.S., S.-J.K., and M.T.O. performed the experiments; H.S., T.-M.I., S.S., S.-J.K., S.G., M.A.-A., and H.P.W. performed data analysis and interpretation; H.P.W. and C.S. contributed reagents/materials/analysis tools; H.S., T.-M.I., and M.A.-A. wrote the paper.

Acknowledgments: This study was funded by the European Social Funds in Baden-Wuerttemberg, Germany and the MWK-BW, Germany. We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of University of Tübingen.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rabinovich, P.M.; Weissman, S.M. Cell engineering with synthetic messenger RNA. *Methods Mol. Biol.* **2013**, *969*, 3–28. [PubMed]
2. Tavernier, G.; Andries, O.; Demeester, J.; Sanders, N.N.; de Smedt, S.C.; Rejman, J. mRNA as gene therapeutic: How to control protein expression. *J. Control Release* **2011**, *150*, 238–247. [CrossRef] [PubMed]
3. García, F.; Plana, M.; Climent, N.; León, A.; Gatell, J.M.; Gallart, T. Dendritic cell based vaccines for HIV infection: The way ahead. *Hum. Vaccines Immunother.* **2013**, *9*, 2445–2452. [CrossRef]
4. Pardi, N.; Hogan, M.J.; Pelc, R.S.; Muramatsu, H.; Andersen, H.; DeMaso, C.R.; Dowd, K.A.; Sutherland, L.L.; Scearce, R.M.; Parks, R.; et al. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. *Nature* **2017**, *543*, 248–251. [CrossRef] [PubMed]
5. Petsch, B.; Schnee, M.; Vogel, A.B.; Lange, E.; Hoffmann, B.; Voss, D.; Schlake, T.; Thess, A.; Kallen, K.J.; Stitz, L.; et al. Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. *Nat. Biotechnol.* **2012**, *30*, 1210–1216. [CrossRef] [PubMed]
6. Kormann, M.S.; Hasenpusch, G.; Aneja, M.K.; Nica, G.; Flemmer, A.W.; Herber-Jonat, S.; Huppmann, M.; Mays, L.E.; Illenyi, M.; Schams, A.; et al. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat. Biotechnol.* **2011**, *29*, 154–157. [CrossRef] [PubMed]
7. Mays, L.E.; Ammon-Treiber, S.; Mothes, B.; Alkhaled, M.; Rottenberger, J.; Muller-Hermelink, E.S.; Grimm, M.; Mezger, M.; Beer-Hammer, S.; von Stebut, E.; et al. Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. *J. Clin. Investig.* **2013**, *123*, 1216–1228. [CrossRef] [PubMed]
8. Bangel-Ruland, N.; Tomczak, K.; Fernandez Fernandez, E.; Leier, G.; Leciejewski, B.; Rudolph, C.; Rosenecker, J.; Weber, W.M. Cystic fibrosis transmembrane conductance regulator-mRNA delivery: A novel alternative for cystic fibrosis gene therapy. *J. Gene Med.* **2013**, *15*, 414–426. [CrossRef] [PubMed]
9. Michel, T.; Kankura, A.; Salinas Medina, M.L.; Kurz, J.; Behring, A.; Avci-Adali, M.; Nolte, A.; Schlensak, C.; Wendel, H.P.; Krajewski, S. In Vitro evaluation of a novel mrna-based therapeutic strategy for the treatment of patients suffering from alpha-1-antitrypsin deficiency. *Nucleic Acid Ther.* **2015**, *25*, 235–244. [CrossRef] [PubMed]
10. Abraham, M.K.; Nolte, A.; Reus, R.; Behring, A.; Zengerle, D.; Avci-Adali, M.; Hohmann, J.D.; Peter, K.; Schlensak, C.; Wendel, H.P.; et al. In vitro study of a novel stent coating using modified CD39 messenger rna to potentially reduce stent angioplasty-associated complications. *PLoS ONE* **2015**, *10*, e0138375. [CrossRef] [PubMed]
11. Levy, O.; Zhao, W.; Mortensen, L.J.; Leblanc, S.; Tsang, K.; Fu, M.; Phillips, J.A.; Sagar, V.; Anandakumaran, P.; Ngai, J.; et al. mRNA-engineered mesenchymal stem cells for targeted delivery of interleukin-10 to sites of inflammation. *Blood* **2013**, *122*, e23–e32. [CrossRef] [PubMed]
12. Weide, B.; Carralot, J.P.; Reese, A.; Scheel, B.; Eigentler, T.K.; Hoerr, I.; Rammensee, H.G.; Garbe, C.; Pascolo, S. Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *J. Immunother.* **2008**, *31*, 180–188. [CrossRef] [PubMed]
13. Kreiter, S.; Diken, M.; Selmi, A.; Tureci, O.; Sahin, U. Tumor vaccination using messenger RNA: Prospects of a future therapy. *Curr. Opin. Immunol.* **2011**, *23*, 399–406. [CrossRef] [PubMed]
14. Stadler, C.R.; Bahr-Mahmud, H.; Celik, L.; Hebich, B.; Roth, A.S.; Roth, R.P.; Kariko, K.; Tureci, O.; Sahin, U. Elimination of large tumors in mice by mRNA-encoded bispecific antibodies. *Nat. Med.* **2017**, *23*, 815–817. [CrossRef] [PubMed]

15. Zangi, L.; Lui, K.O.; von Gise, A.; Ma, Q.; Ebina, W.; Ptaszek, L.M.; Spater, D.; Xu, H.; Tabebordbar, M.; Gorbatov, R.; et al. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat. Biotechnol.* **2013**, *31*, 898–907. [CrossRef] [PubMed]
16. Goparaju, S.K.; Kohda, K.; Ibata, K.; Soma, A.; Nakatake, Y.; Akiyama, T.; Wakabayashi, S.; Matsushita, M.; Sakota, M.; Kimura, H.; et al. Rapid differentiation of human pluripotent stem cells into functional neurons by mRNAs encoding transcription factors. *Sci. rep.* **2017**, *7*, 42367. [CrossRef] [PubMed]
17. Preskey, D.; Allison, T.F.; Jones, M.; Mamchaoui, K.; Unger, C. Synthetically modified mRNA for efficient and fast human iPS cell generation and direct transdifferentiation to myoblasts. *Biochem. Biophys. Res. Commun.* **2016**, *473*, 743–751. [CrossRef] [PubMed]
18. Warren, L.; Manos, P.D.; Ahfeldt, T.; Loh, Y.H.; Li, H.; Lau, F.; Ebina, W.; Mandal, P.K.; Smith, Z.D.; Meissner, A.; et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **2010**, *7*, 618–630. [CrossRef] [PubMed]
19. Hoare, T.R.; Kohane, D.S. Hydrogels in drug delivery: Progress and challenges. *Polymer* **2008**, *49*, 1993–2007. [CrossRef]
20. Tan, H.; Marra, K.G. Injectable, biodegradable hydrogels for tissue engineering applications. *Materials* **2010**, *3*, 1746–1787. [CrossRef]
21. Yang, J.-A.; Yeom, J.; Hwang, B.W.; Hoffman, A.S.; Hahn, S.K. In situ-forming injectable hydrogels for regenerative medicine. *Prog. Polym. Sci.* **2014**, *39*, 1973–1986. [CrossRef]
22. Li, X.; Kong, X.; Zhang, Z.; Nan, K.; Li, L.; Wang, X.; Chen, H. Cytotoxicity and biocompatibility evaluation of N,O-carboxymethyl chitosan/oxidized alginate hydrogel for drug delivery application. *Int. J. Biol. Macromol.* **2012**, *50*, 1299–1305. [CrossRef] [PubMed]
23. Van Vlierberghe, S.; Dubruel, P.; Schacht, E. Biopolymer-Based Hydrogels as Scaffolds for Tissue Engineering Applications: A Review. *Biomacromolecules* **2011**, *12*, 1387–1408. [CrossRef] [PubMed]
24. Guan, X.; Avci-Adali, M.; Alarcin, E.; Cheng, H.; Kashaf, S.S.; Li, Y.; Chawla, A.; Jang, H.L.; Khademhosseini, A. Development of hydrogels for regenerative engineering. *Biotechnol. J.* **2017**, *12*. [CrossRef] [PubMed]
25. Hao, X.; Silva, E.A.; Mansson-Broberg, A.; Grinnemo, K.H.; Siddiqui, A.J.; Dellgren, G.; Wardell, E.; Brodin, L.A.; Mooney, D.J.; Sylven, C. Angiogenic effects of sequential release of VEGF-A165 and PDGF-BB with alginate hydrogels after myocardial infarction. *Cardiovasc. Res.* **2007**, *75*, 178–185. [CrossRef] [PubMed]
26. Lee, K.W.; Yoon, J.J.; Lee, J.H.; Kim, S.Y.; Jung, H.J.; Kim, S.J.; Joh, J.W.; Lee, H.H.; Lee, D.S.; Lee, S.K. Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. *Transpl. Proc.* **2004**, *36*, 2464–2465. [CrossRef] [PubMed]
27. Ishihara, M.; Fujita, M.; Obara, K.; Hattori, H.; Nakamura, S.; Nambu, M.; Kiyosawa, T.; Kanatani, Y.; Takase, B.; Kikuchi, M.; et al. Controlled releases of FGF-2 and paclitaxel from chitosan hydrogels and their subsequent effects on wound repair, angiogenesis, and tumor Growth. *Curr. Drug Deliv.* **2006**, *3*, 351–358. [CrossRef] [PubMed]
28. Feng, G.; Zhang, J.; Li, Y.; Nie, Y.; Zhu, D.; Wang, R.; Liu, J.; Gao, J.; Liu, N.; He, N.; et al. IGF-1 C Domain-Modified Hydrogel Enhances Cell Therapy for AKI. *J. Am. Soc. Nephrol.* **2016**, *27*, 2357–2369. [CrossRef] [PubMed]
29. Fang, R.; Qiao, S.; Liu, Y.; Meng, Q.; Chen, X.; Song, B.; Hou, X.; Tian, W. Sustained co-delivery of BIO and IGF-1 by a novel hybrid hydrogel system to stimulate endogenous cardiac repair in myocardial infarcted rat hearts. *Int. J. Nanomed.* **2015**, *10*, 4691–4703. [CrossRef] [PubMed]
30. Ghasemi Tahrir, F.; Ganji, F.; Mani, A.R.; Khodaverdi, E. In vitro and in vivo evaluation of thermosensitive chitosan hydrogel for sustained release of insulin. *Drug Deliv.* **2016**, *23*, 1038–1046. [PubMed]
31. Khodaverdi, E.; Tafaghodi, M.; Ganji, F.; Abnoos, K.; Naghizadeh, H. In vitro insulin release from thermosensitive chitosan hydrogel. *AAPS PharmSciTech* **2012**, *13*, 460–466. [CrossRef] [PubMed]
32. Zhang, N.; Li, J.; Jiang, W.; Ren, C.; Li, J.; Xin, J.; Li, K. Effective protection and controlled release of insulin by cationic beta-cyclodextrin polymers from alginate/chitosan nanoparticles. *Int. J. Pharm.* **2010**, *393*, 212–218. [CrossRef] [PubMed]
33. Nayak, P.L.; Sahoo, D. Chitosan-alginate composites blended with cloisite 30B as a novel drug delivery system for anticancer drug paclitaxel. *Int. J. Plast. Technol.* **2011**, *15*, 68–81. [CrossRef]
34. Bastiancich, C.; Danhier, P.; Preat, V.; Danhier, F. Anticancer drug-loaded hydrogels as drug delivery systems for the local treatment of glioblastoma. *J. Control Release* **2016**, *243*, 29–42. [CrossRef] [PubMed]

35. St'astny, M.; Plocova, D.; Etrych, T.; Ulbrich, K.; Rihova, B. HPMA-hydrogels result in prolonged delivery of anticancer drugs and are a promising tool for the treatment of sensitive and multidrug resistant leukaemia. *Eur. J. Cancer* **2002**, *38*, 602–608. [CrossRef]
36. Wu, H.; Liu, S.; Xiao, L.; Dong, X.; Lu, Q.; Kaplan, D.L. Injectable and pH-Responsive silk nanofiber hydrogels for sustained anticancer drug delivery. *ACS Appl. Mater. Interfaces* **2016**, *8*, 17118–17126. [CrossRef] [PubMed]
37. Augst, A.D.; Kong, H.J.; Mooney, D.J. Alginate hydrogels as biomaterials. *Macromol. Biosci.* **2006**, *6*, 623–633. [CrossRef] [PubMed]
38. Guarino, V.; Caputo, T.; Altobelli, R.; Ambrosio, L. Degradation properties and metabolic activity of alginate and chitosan polyelectrolytes for drug delivery and tissue engineering applications. *AIMS Mater. Sci.* **2015**, *2*, 497–502. [CrossRef]
39. Ito, T.; Saito, M.; Uchino, T.; Senna, M.; Iafisco, M.; Prat, M.; Rimondini, L.; Otsuka, M. Preparation of injectable auto-forming alginate gel containing simvastatin with amorphous calcium phosphate as a controlled release medium and their therapeutic effect in osteoporosis model rat. *J. Mater. Sci. Mater. Med.* **2012**, *23*, 1291–1297. [CrossRef] [PubMed]
40. Rowley, J.A.; Madlambayan, G.; Mooney, D.J. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* **1999**, *20*, 45–53. [CrossRef]
41. Krebs, M.D.; Jeon, O.; Alsberg, E. Localized and sustained delivery of silencing RNA from macroscopic biopolymer hydrogels. *J. Am. Chem. Soc.* **2009**, *131*, 9204–9206. [CrossRef] [PubMed]
42. Krebs, M.D.; Salter, E.; Chen, E.; Sutter, K.A.; Alsberg, E. Calcium phosphate-DNA nanoparticle gene delivery from alginate hydrogels induces in vivo osteogenesis. *J. Biomed. Mater. Res. Part A* **2010**, *92A*, 1131–1138.
43. Mao, S.; Sun, W.; Kissel, T. Chitosan-based formulations for delivery of DNA and siRNA. *Adv. Drug Deliv. Rev.* **2010**, *62*, 12–27. [CrossRef] [PubMed]
44. Supper, S.; Anton, N.; Seidel, N.; Riemenschnitter, M.; Curdy, C.; Vandamme, T. Thermosensitive chitosan/glycerophosphate-based hydrogel and its derivatives in pharmaceutical and biomedical applications. *Expert Opin. Drug Deliv.* **2014**, *11*, 249–267. [CrossRef] [PubMed]
45. Ma, Z.; Yang, C.; Song, W.; Wang, Q.; Kjems, J.; Gao, S. Chitosan hydrogel as siRNA vector for prolonged gene silencing. *J. Nanobiotechnol.* **2014**, *12*, 23. [CrossRef] [PubMed]
46. Han, H.D.; Mora, E.M.; Roh, J.W.; Nishimura, M.; Lee, S.J.; Stone, R.L.; Bar-Eli, M.; Lopez-Berestein, G.; Sood, A.K. Chitosan hydrogel for localized gene silencing. *Cancer Biol. Ther.* **2011**, *11*, 839–845. [CrossRef] [PubMed]
47. Li, H.; Ji, Q.; Chen, X.; Sun, Y.; Xu, Q.; Deng, P.; Hu, F.; Yang, J. Accelerated bony defect healing based on chitosan thermosensitive hydrogel scaffolds embedded with chitosan nanoparticles for the delivery of BMP2 plasmid DNA. *J. Biomed. Mater. Res. Part A* **2017**, *105*, 265–273. [CrossRef] [PubMed]
48. Khong, T.T.; Aarstad, O.A.; Skjak-Braek, G.; Draget, K.I.; Varum, K.M. Gelling concept combining chitosan and alginate-proof of principle. *Biomacromolecules* **2013**, *14*, 2765–2771. [CrossRef] [PubMed]
49. Li, Z.; Ramay, H.R.; Hauch, K.D.; Xiao, D.; Zhang, M. Chitosan-alginate hybrid scaffolds for bone tissue engineering. *Biomaterials* **2005**, *26*, 3919–3928. [CrossRef] [PubMed]
50. Rafiee, A.; Alimohammadian, M.H.; Gazori, T.; Riazi-rad, F.; Fatemi, S.M.R.; Parizadeh, A.; Haririan, I.; Havaskary, M. Comparison of chitosan, alginate and chitosan/alginate nanoparticles with respect to their size, stability, toxicity and transfection. *Asian Pac. J. Trop. Dis.* **2014**, *4*, 372–377. [CrossRef]
51. Park, D.J.; Choi, B.H.; Zhu, S.J.; Huh, J.Y.; Kim, B.Y.; Lee, S.H. Injectable bone using chitosan-alginate gel/mesenchymal stem cells/BMP-2 composites. *J. Cranio-Maxillofac. Surg.* **2005**, *33*, 50–54. [CrossRef] [PubMed]
52. Baysal, K.; Aroguz, A.Z.; Adiguzel, Z.; Baysal, B.M. Chitosan/alginate crosslinked hydrogels: Preparation, characterization and application for cell growth purposes. *Int. J. Biol. Macromol.* **2013**, *59*, 342–348. [CrossRef] [PubMed]
53. Li, Z.; Zhang, M. Chitosan-alginate as scaffolding material for cartilage tissue engineering. *J. Biomed. Mater. Res. Part A* **2005**, *75*, 485–493. [CrossRef] [PubMed]
54. Deng, B.; Shen, L.; Wu, Y.; Shen, Y.; Ding, X.; Lu, S.; Jia, J.; Qian, J.; Ge, J. Delivery of alginate-chitosan hydrogel promotes endogenous repair and preserves cardiac function in rats with myocardial infarction. *J. Biomed. Mater. Res. Part A* **2015**, *103*, 907–918. [CrossRef] [PubMed]

55. Wang, K.; Kievit, F.M.; Florczyk, S.J.; Stephen, Z.R.; Zhang, M. 3D porous chitosan-alginate scaffolds as an in vitro model for evaluating nanoparticle-mediated tumor targeting and gene delivery to prostate cancer. *Biomacromolecules* **2015**, *16*, 3362–3372. [CrossRef] [PubMed]
56. Williams, P.A.; Campbell, K.T.; Gharaviram, H.; Madrigal, J.L.; Silva, E.A. Alginate-Chitosan Hydrogels Provide a Sustained Gradient of Sphingosine-1-Phosphate for Therapeutic Angiogenesis. *Ann. Biomed. Eng.* **2017**, *4*, 1003–1014. [CrossRef] [PubMed]
57. Guan, S.; Rosenecker, J. Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. *Gene Ther.* **2017**, *24*, 133–143. [CrossRef] [PubMed]
58. Avci-Adali, M.; Behring, A.; Steinle, H.; Keller, T.; Krajeweski, S.; Schlensak, C.; Wendel, H.P. In vitro synthesis of modified mRNA for induction of protein expression in human cells. *J. Vis. Exp.* **2014**, e51943. [CrossRef] [PubMed]
59. Crassous, J.J.; Régisser, R.; Ballauff, M.; Willenbacher, N. Characterization of the viscoelastic behavior of complex fluids using the piezoelastic axial vibrator. *J. Rheol.* **2005**, *49*, 851–863. [CrossRef]

8.2. PUBLIKATION II

Improving the angiogenic potential of EPCs via engineering with synthetic modified mRNA

Molecular Therapy
Nucleic Acids
Original Article

Improving the Angiogenic Potential of EPCs via Engineering with Synthetic Modified mRNAs

Heidrun Steinle,[1] Sonia Golombek,[1] Andreas Behring,[1] Christian Schlensak,[1] Hans Peter Wendel,[1] and Meltem Avci-Adali[1]

[1]University Hospital Tuebingen, Department of Thoracic and Cardiovascular Surgery, Calwerstraße 7/1, 72076 Tuebingen, Germany

The application of endothelial progenitor cells (EPCs) for the revascularization of ischemic tissues, such as after myocardial infarction, stroke, and acute limb ischemia, has a huge clinical potential. However, the low retention and engraftment of EPCs as well as the poor survival of migrated stem cells in ischemic tissues still hamper the successful clinical application. Thus, in this study, we engineered, for the first time, murine EPCs with synthetic mRNAs to transiently produce proangiogenic factors vascular endothelial growth factor-A (VEGF-A), stromal cell-derived factor-1α (SDF-1α), and angiopoietin-1 (ANG-1). After the transfection of cells with synthetic mRNAs, significantly increased VEGF-A, SDF-1α, and ANG-1 protein levels were detected compared to untreated EPCs. Thereby, mRNA-engineered EPCs showed significantly increased chemotactic activity versus untreated EPCs and resulted in significantly improved attraction of EPCs. Furthermore, ANG-1 mRNA-transfected EPCs displayed a strong wound-healing capacity. Already after 12 hr, 94% of the created wound area in the scratch assay was closed compared to approximately 45% by untreated EPCs. Moreover, the transfection of EPCs with ANG-1 or SDF-1α mRNA also significantly improved the *in vitro* tube formation capacity; however, the strongest effect could be detected with EPCs simultaneously transfected with VEGF-A, SDF-1α, and ANG-1 mRNA. In the *in vivo* chicken chorioallantoic membrane (CAM) assay, EPCs transfected with ANG-1 mRNA revealed the strongest angiogenetic potential with significantly elevated vessel density and total vessel network length. In conclusion, this study demonstrated that EPCs can be successfully engineered with synthetic mRNAs encoding proangiogenic factors to improve their therapeutic angiogenetic potential in patients experiencing chronic or acute ischemic disease.

INTRODUCTION

The insufficient perfusion of tissues or organs with blood results in ischemia and often leads to conditions such as stroke, myocardial infarction, or acute limb ischemia. During ischemia, the affected tissues are damaged due to hypoxia and the lack of nutrient supply and waste removal. Thus, the formation of new blood vessels by angiogenesis and vasculogenesis is required. During angiogenesis, new blood vessels are formed from pre-existing vessels and, during vasculogenesis, *de novo* blood vessel formation occurs through bone marrow-derived endothelial progenitor cells (EPCs).[1] Thereby, the blood supply can be restored to ischemic tissues, the tissue damage can be reduced, and, as a result, the function of affected tissues or organs can be improved.

EPCs are circulating stem cells in the bloodstream, and they are recruited by chemokines, which are secreted by injured and activated cells at the injury site to induce revascularization.[2] EPCs settle and differentiate at sites of vascular lesions, and they appear to participate in vascular repair and homeostasis.[3] Previous studies even identified EPCs as biomarkers in cardiovascular diseases, and a decline of EPC numbers and dysfunction were related to ischemic diseases.[4,5] Several preclinical studies have demonstrated the vasculogenic and angiogenic potential,[6,7] as well as beneficial paracrine effects, of transplanted EPCs in the treatment of ischemic diseases.[8,9] However, the success of clinical applications is limited due to low retention and engraftment of EPCs as well as the poor survival of migrated EPCs in ischemic tissues.[10,11] Furthermore, the low quantity and quality of isolated autologous EPCs is challenging for clinical applications.[12] Thus, multiple animal and clinical studies with EPCs demonstrated different results in their effectiveness to treat ischemic diseases.[9,13] Consequently, novel strategies are needed to enhance the number and function of EPCs and to obtain a successful autologous EPC therapy for a more efficient tissue regeneration.

In previous studies, various growth factors, such as vascular endothelial growth factor (VEGF)[14] or fibroblast growth factor (FGF),[15] were applied to improve the revascularization of tissues.[16] Local levels of proangiogenic proteins were upregulated by delivering recombinant proteins[17,18] or genes[19,20] using nano- or microparticles,[17,18] direct injection into the target tissue,[19] ultrasound-targeted microbubble destruction (UTMD),[20,21] or sustained release from implants.[22] The use of recombinant proteins is costly and it is difficult to maintain adequate protein levels in the ischemic regions due to their relatively short half-lives.[23] Therefore, gene therapy with viral and non-viral vectors was used as an alternative strategy to express the desired

Received 12 May 2018; accepted 6 September 2018;
https://doi.org/10.1016/j.omtn.2018.09.005.

Correspondence: Meltem Avci-Adali, PhD, Department of Thoracic and Cardiovascular Surgery, University Hospital Tuebingen, Calwerstraße 7/1, 72076 Tuebingen, Germany.
E-mail: meltem.avci-adali@uni-tuebingen.de

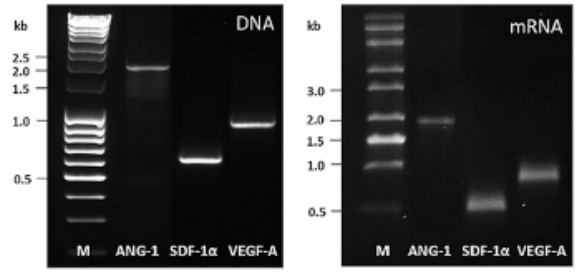

Figure 1. Quality Control of the Generated PCR Products and *In Vitro*-Transcribed mRNAs

ANG-1-, SDF-1α-, and VEGF-A-encoding mRNAs were synthesized and modified with 3′-PolyA$_{120}$ tail, 5′-ARCA, 100% m5CTP, 100% Ψ-UTP, and post-transcriptional phosphatase treatment. The specific lengths of the amplified DNA and the synthetic mRNA were detected using 1% agarose gel electrophoresis and GelRed staining at approximately 1.8 kb for ANG-1, 0.6 kb for SDF-1α, and 0.9 kb for VEGF-A. The 0.08- to 10-kb range mix DNA ladder and the 0.5- to 10-kb RNA ladder were used as length markers (Ms).

proangiogenic proteins, and it has shown to be promising, for example, for the treatment of myocardial ischemia.[13,23]

In recent years, the expression of exogenous proteins by the delivery of synthetic mRNAs has gained great importance as an alternative strategy to the viral vector or plasmid-based gene delivery methods.[24] In contrast to genome-integrating gene delivery methods, the application of synthetic mRNA is a non-integrating method with no risk of insertional mutagenesis. Since the mRNA does not need to enter the nucleus, the synthetic mRNA can be efficiently delivered in dividing as well as non-dividing cells. The transfection of synthetic mRNA leads to the transient production of exogenous proteins in the cells, and, after the natural degradation of mRNA, no footprint is left in the cells.

The most intensively studied growth factor for the induction of vascularization and angiogenesis is the vascular endothelial growth factor-A (VEGF-A). It is involved in the chemotaxis, migration, and differentiation of progenitor cells; endothelial cell (EC) survival and proliferation; as well as the sprouting of vessels and vessel permeability.[25] Among alternative splice variants of VEGF-A, VEGF-A$_{165}$ is the quantitatively and qualitatively most important variant for angiogenesis.[26] VEGF-A binds and activates VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2) expressed on vascular ECs and EPCs. VEGFR-2 has approximately 10-fold higher kinase activity than VEGFR-1, and the major proangiogenic signal is generated from the ligand-activated VEGFR-2.[26] Angiopoietin-1 (ANG-1) is produced by peri-ECs and platelets.[27] It binds to Tie2 receptors on ECs and maintains endothelial integrity and reduces the effects of inflammation,[28] which prevents vascular leakage and stabilizes vessels. ANG-1 is further involved in EC migration and the reorganization of ECs.[29] Stromal cell-derived factor-1α (SDF-1α, also known as CXCL12) is a chemokine that mediates the mobilization and recruitment of bone marrow-derived progenitor cells that express CXCR4 receptor on the cell surface, such as EPCs. Additionally, SDF-1α attenuates EC apoptosis and stimulates new vessel capillary tube formation, and the expression of SDF-1α is regulated by hypoxia.[25,30,31]

In this study, for the first time, murine EPCs were engineered by the exogenous delivery of synthetic mRNAs to increasingly express the proangiogenic proteins VEGF-A, SDF-1α, and ANG-1. The ability of EPCs to produce the desired proteins was analyzed after the transfection with synthetic mRNAs using ELISA. Next, the migration behavior of mRNA-engineered EPCs was investigated by chemotactic and wound-healing migration assay and compared with non-engineered EPCs *in vitro*. Then, the *in vitro* and *in vivo* angiogenic potential of mRNA-engineered EPCs was analyzed using tube formation and chick chorioallantoic membrane (CAM) assay.

RESULTS

Generation of Synthetic mRNA Encoding ANG-1, SDF-1α, and VEGF-A

The specific length and purity of the generated PCR products and the *in vitro*-transcribed modified mRNAs were analyzed by agarose gel electrophoresis. After the gel electrophoresis, single bands without misamplifications and expected lengths were detected for the PCR products ANG-1 (1,794 bp), SDF-1α (567 bp), and VEGF-A (873 bp) and the resulting *in vitro*-transcribed mRNA containing the coding sequence, 3′ and 5′ UTR regions, and PolyA$_{120}$ tail (Figure 1).

Expression of ANG-1, SDF-1α, and VEGF-A after the Transfection of EPCs with Synthetic Modified mRNAs

After the successful production of synthetic mRNAs, the expression of specific proteins was determined. Therefore, single-mRNA transfections with 1.6 μg ANG-1, 0.8 μg VEGF-A, or 0.5 μg SDF-1α or transfections with an mRNA cocktail consisting of 1.6 μg ANG-1, 0.8 μg VEGF-A, and 0.5 μg SDF-1α were performed. The concentrations of produced proteins were measured in supernatants 24 hr after the transfections using ELISA (Figure 2). The transfection of EPCs with ANG-1, VEGF-A, or SDF-1α mRNA resulted in significantly increased production of ANG-1 (112 ± 6.1 ng/mL), VEGF-A (142 ± 6.7 ng/mL), and SDF-1α (17 ± 1.4 ng/mL) compared to the cells incubated with medium or medium containing the transfection reagent (Figure 2A). The transfection of EPCs with the mRNA cocktail also resulted in significantly increased protein synthesis (Figure 2B), and approximately 70 ± 7.1 ng/mL ANG-1, 58 ± 2.7 ng/mL VEGF-A, and 8 ± 0.4 ng/mL SDF-1α were detected. Here, the amount of produced ANG-1 was 1.6-fold, VEGF-A 2.4-fold, and SDF-1α 2.1-fold less than after the transfection of EPCs with single mRNAs. In the used mRNA cocktail, the total amount of mRNAs was 2.9 μg.

To test the influence of higher mRNA amounts on protein synthesis, the cells were transfected with each single mRNA and EGFP mRNA, which was used as a filler mRNA, to obtain a total mRNA amount of 2.9 μg. Especially, after the co-transfection of ANG-1 mRNA with EGFP mRNA, a significant reduction of ANG-1 protein amount could be detected (Figure S1). In the case of VEGF-A mRNA, no influence could be detected. A slightly higher amount of SDF-1α was

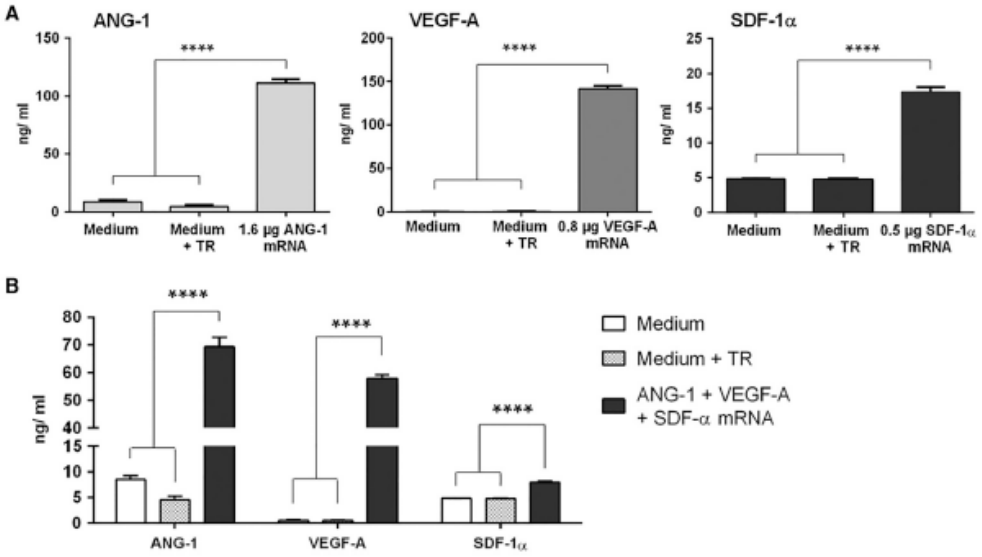

Figure 2. Expression of ANG-1, VEGF-A, and SDF-1α after the Transfection of Synthetic mRNA into Murine EPCs

1×10^5 EPCs were seeded and transfected the next day with (A) 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with (B) an mRNA cocktail containing 1.6 µg ANG-1, 0.8 µg VEGF-A, and 0.5 µg SDF-1α mRNA. The protein expression was analyzed in supernatants 24 hr after the transfection using ELISA. Cells treated with only medium or medium and transfection reagent (TR) served as negative controls. Results are shown as mean + SEM (n = 4). Statistical differences were determined using one-way ANOVA followed by Bonferroni multiple comparison test (****$p < 0.0001$).

detected after the simultaneous transfection of cells with SDF-1α and EGFP mRNA. Furthermore, double combinations of ANG-1, VEGF-A, and SDF-1α mRNA transfections were tested (Figure S2). Here, the simultaneous transfection of cells with ANG-1 and VEGF-A or SDF-1α mRNA also resulted in a reduction in ANG-1 protein expression. These results indicate that the higher mRNA amount has an influence on ANG-1 production.

Influence of mRNA Engineering on the Viability of EPCs

The influence of mRNA transfections on the viability of EPCs was analyzed using PrestoBlue cell viability assay. 1×10^5 EPCs were cultivated overnight and transfected with 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with a triple mRNA cocktail. As a control, cells were treated with Opti-MEM containing the maximal amount of Lipofectamine 2000 (4 µL), which was used for the generation of transfection complexes. The viability of cells incubated with Opti-MEM (medium) was set to 100%, and the viability of samples was displayed relative to these cells. As shown in Figure 3, 24 hr post-transfection, the transfection of cells with synthetic mRNAs resulted in no significant differences in cell viability compared to the controls, cells incubated with medium or medium containing transfection reagent.

Chemotactic Migration of EPCs toward mRNA-Engineered EPCs

The chemotactic activity of mRNA-engineered EPCs was determined using a migration assay. Transwell inserts seeded with 5×10^4 untreated EPCs were placed in wells containing 1×10^5 mRNA-transfected or untransfected EPCs. After 6 hr, the migrated cells were stained with 1 µg/mL DAPI and counted. The mRNA-transfected EPCs were able to attract significantly more EPCs compared to EPCs without mRNA transfection (ANG-1 mRNA, 405 ± 36 cells; VEGF-A mRNA, 426 ± 41 cells; SDF-1α mRNA, 400 ± 12 cells; and mRNA cocktail, 464 ± 27 cells versus EPCs without mRNA transfection [medium], 200 ± 10 cells; Figure 4). Compared to single-mRNA-transfected EPCs, the transfection of EPCs with the mRNA cocktail resulted in a slight increase of migrated cell numbers; however, this increase was not statistically significant. The treatment of cells with the transfection reagent also had no statistically significant influence on the migration behavior compared to the cells treated with medium. Interestingly, all EPCs transfected with a single mRNA or with the mRNA cocktail showed comparable migration activity. Overall, these results demonstrated an improvement of EPC migration toward EPCs transfected with proangiogenic mRNAs.

Migration Capacity of mRNA-Engineered EPCs in Wound Scratch Migration Assay

The *in vitro* wound-healing assay, which mimics the *in vivo* wound-healing process, was used to analyze the migration behavior of mRNA-engineered EPCs growing in a monolayer culture. Therefore, 28,000 EPCs with or without mRNA transfection were seeded in each chamber of Culture-Insert 3 wells. After reaching confluence, the culture inserts were removed from the dish to generate an open wound field. To quantify the closed wound area, pictures were taken

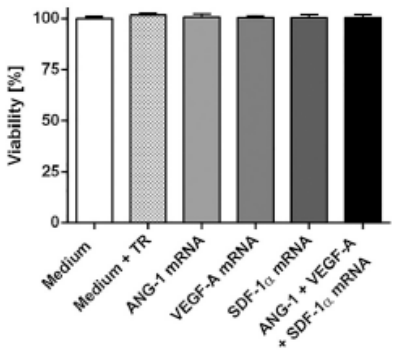

Figure 3. Influence of mRNA Transfection on the Viability of Murine EPCs

1×10^5 EPCs were transfected with 1.6 μg ANG-1, 0.8 μg VEGF-A, or 0.5 μg SDF-1α mRNA or with an mRNA cocktail containing 1.6 μg ANG-1, 0.8 μg VEGF-A, and 0.5 μg SDF-1α mRNA. Viability was determined 24 hr post-transfection using PrestoBlue assay. The viability of cells incubated with Opti-MEM (medium) was set to 100%, and the viability of samples was expressed relative to these cells. The data are shown as mean + SEM ($n = 3$). No statistically significant differences were determined using one-way ANOVA followed by Bonferroni multiple comparison test.

immediately after removing the insert (0 hr) and after 12, 24, and 36 hr. After 36 hr, the wound areas were closed for each sample (Figure 5). EPCs transfected with ANG-1 mRNA showed fastest closure of the wound area, with 94% already after 12 hr. In comparison, a wound closure area of about 45% was detected with EPCs treated only with medium and 38% with EPCs treated with the medium containing transfection reagent. After 24 hr, 100% of the wound area was closed in ANG-1 mRNA-transfected EPCs and 93% wound area was closed in SDF-1α mRNA-transfected EPCs. The closure of the wound area was significantly higher in these EPCs compared to the medium control. Especially, the transfection of EPCs with ANG-1 mRNA accelerated the migration process so that the wound area was completely closed already after 12 hr. In comparison, EPCs transfected with the mRNA cocktail showed significantly less wound closure area. Furthermore, the treatment of the cells with the transfection reagent had no significant influence on the migration and the speed of wound closure compared to untreated cells (medium control).

In Vitro Angiogenic Potential of mRNA-Engineered EPCs

The tube formation assay was performed to assess the angiogenic potential of mRNA-engineered EPCs *in vitro*. Therefore, 1×10^4 EPCs transfected with 1.6 μg ANG-1, 0.8 μg VEGF-A, or 0.5 μg SDF-1α mRNA or with a triple mRNA cocktail were seeded on Matrigel. Already within 4 hr, EPCs spontaneously initiated vascular morphogenesis and formed multicellular tubular networks (Figure 6). Using Angiogenesis Analyzer ImageJ plugin, the number of segments (elements, which are delimited by two junctions), number of master segments (segments, which are none exclusively implicated with one branch), number of nodes (pixels with at least 3 neighboring elements, corresponding to a bifurcation), total segment length (sum of lengths of the segments in the analyzed area), the total mesh areas (sum of areas enclosed by the segments or master segments), and the branching interval (the mean distance separating two branches [total segment length/number of branches]) were obtained. The unit of area and length is the pixel (px).

EPCs engineered to simultaneously produce ANG-1, VEGF-A, and SDF-1α demonstrated highly increased network formation, compared to the untreated EPCs (medium). Thereby, the networks formed by EPCs transfected with the mRNA cocktail displayed higher numbers of nodes (545 ± 29 versus 205 ± 52), segments (185 ± 14 versus 52 ± 20), and master segments (110 ± 12 versus 24 ± 10), with an increase in total segment length (11,343 ± 660 px versus 3,786 ± 1750 px), total mesh area (569,094 ± 47,325 px versus 36,107 ± 24,228 px), as well as branching interval (116,306 ± 12,322 px versus 49,573 ± 21,438 px). The single transfection of EPCs either with ANG-1 or SDF-1α mRNA also led to an increase of parameters related to network formation. However, the increase was in the case of SDF-1α mRNA-transfected EPCs, statistically significant for number of nodes, segments, and total segment length, and in the case of ANG-1 mRNA-transfected EPCs, statistically significant for number of segments. In the case of VEGF-A, no increase could be detected compared to untreated EPCs. The overall results demonstrated an improvement of network formation characteristics in triple-mRNA-transfected EPCs, which led to a denser, highly branched tubular network with increased numbers of tubes, suggesting an improved induction of angiogenesis.

In Vivo Angiogenic Potential of mRNA-Engineered EPCs

The *in vivo* angiogenic potential of mRNA-engineered EPCs was analyzed using the CAM assay. Therefore, 1×10^5 EPCs were seeded and transfected the next day with 1.6 μg ANG-1, 0.8 μg VEGF-A, or 0.5 μg SDF-1α mRNA or with a triple mRNA cocktail. After 24 hr, cells were detached, and 4×10^5 cells were mixed in Matrigel and placed within a silicone ring on the CAM. As controls, EPCs incubated with medium or medium containing transfection reagent were also applied to the CAMs. For evaluation and quantification of angiogenesis, the area of sample application (within the Ø 0.8-mm silicone ring) of the fixed CAMs was photographed and analyzed using Wimasis WimCAM web-based service (Figure 7). Compared to the mRNA untreated cells (medium), EPCs transfected with the ANG-1 mRNA resulted in an augmented angiogenesis/vascularization determined by the significant increase in numbers of total branching points (701 ± 85 versus 337 ± 37), a higher vessel density (44.5% ± 0.6% versus 32% ± 2%), and increases in total segments (1,285 ± 195 px versus 659 ± 96 px) and total vessel network length (73,101 ± 5,986 px versus 46,551 ± 6908 px) in the analyzed area. Although EPCs transfected with the mRNA cocktail also showed an improvement of parameters regarding network formation on CAMs, the differences were not significantly different from untransfected EPCs. EPCs treated with only SDF-1α mRNA or VEGF-A showed no improved effect on the formation of new vasculature compared to the controls.

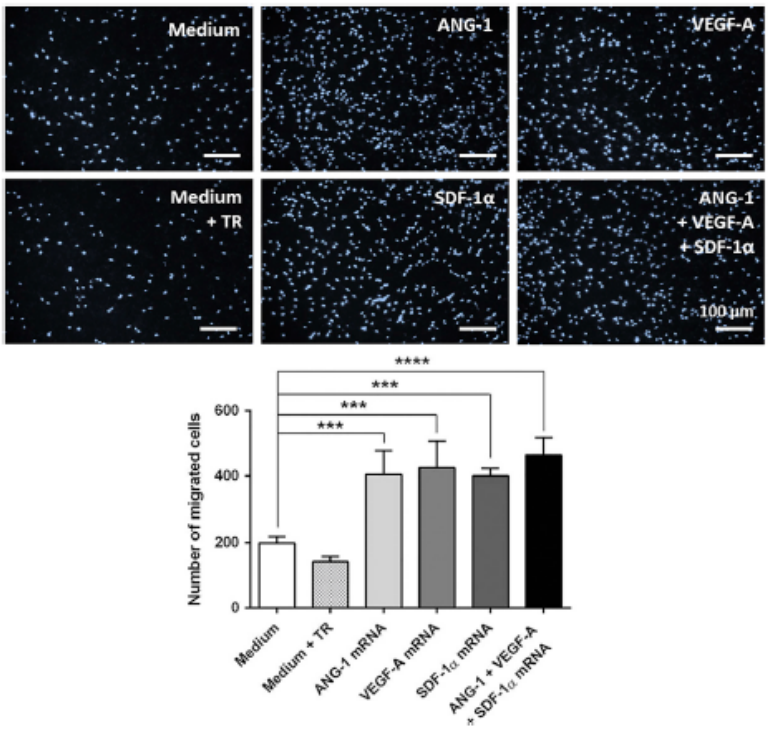

Figure 4. Chemotactic Migration of EPCs toward mRNA-Engineered EPCs

1×10^5 murine EPCs were cultivated overnight, and then they were transfected with 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with an mRNA cocktail containing 1.6 µg ANG-1, 0.8 µg VEGF-A, and 0.5 µg SDF-1α mRNA. Migration behavior of untreated EPCs (5×10^4) seeded on transwell inserts toward mRNA-transfected EPCs was analyzed using a chemotactic migration assay. As a control, EPCs incubated with medium or medium containing transfection reagent (TR) were used. After 6 hr, migrated EPCs through 8-µm transwell inserts were stained with DAPI, and cell numbers were calculated using ImageJ software. Scale bars represent 100 µm. Results are shown as mean + SEM (n = 4). Statistical differences were determined using one-way ANOVA followed by Bonferroni multiple comparison test (***$p < 0.001$ and ****$p < 0.0001$).

DISCUSSION

The improvement of angiogenic potential of EPCs by the use of synthetic modified mRNA represents a promising strategy for the revascularization of ischemic tissues. In this study, we transiently modified murine EPCs with synthetic mRNAs encoding ANG-1, VEGF-A, and SDF-1α to augment the proangiogenic potential. Thereby, tissue repair and regeneration can be improved in the affected regions, first by the local production of growth factors improving the proliferation and maintenance of implanted EPCs, and second by the local production of chemokines attracting other EPCs to the required location.

The transfection of EPCs with synthetic mRNAs showed no influence on cell viability, and, after the transfection of EPCs with single synthetic mRNAs, the expression of ANG-1, VEGF-A, and SDF-1α was demonstrated. However, the transfection of EPCs with a triple mRNA cocktail resulted in a reduced production of proteins compared to single-mRNA transfections. Especially, the production of ANG-1 protein was significantly reduced due to the increased mRNA amount in the triple mRNA cocktail-transfected cells. In eukaryotic cells, most secreted and membrane proteins undergo maturation steps in the lumen of the endoplasmic reticulum (ER).[32] There, after the translation, nascent polypeptides are chemically modified and folded into mature proteins, and an accumulation of unfolded proteins in the ER lumen can lead to an unfolded protein response (UPR) stress. Thus, cells can selectively degrade mRNAs encoding secreted proteins.[32,33] Presumably, the higher total amount of synthetic mRNA per cell compared to single-mRNA transfection could lead to an overload and to a translational repression. Using EGFP mRNA as a filler mRNA, no negative influence of mRNA amount on the production of VEGF-A and SDF-1α was detected. However, in triple-mRNA-transfected cells, feedback mechanisms due to produced protein combinations could lead to the translational repression or degradation of synthetic mRNA and, thereby, result in a reduction of produced protein amounts.

In the chemotactic migration assay, all of the mRNA-engineered EPCs were able to attract non-modified EPCs. Contrary to the expectation that EPCs transfected with the mRNA cocktail could have an augmented chemoattractant activity, this could be not demonstrated. The reason, therefore, could be the production of reduced protein amounts in the mRNA cocktail-transfected EPCs compared to the single-mRNA-transfected EPCs. However, the tube formation assay demonstrated significantly improved angiogenic potential of EPCs transfected with the mRNA cocktail, which could be caused by the synergistic effects of simultaneously produced ANG-1, VEGF-A, and SDF-1α. EPCs transfected with SDF-1α or ANG-1 mRNA, or simultaneously with SDF-1α and VEGF-A mRNA (Figure S3), also showed an improved effect on tube formation.

In the wound scratch migration assay, EPCs transfected with ANG-1 mRNA showed the fastest closure of the wound area with 94% already after 12 hr. After 24 hr, the ANG-1 mRNA-transfected EPCs resulted in 100% closure of the wound area, which was significantly higher than that in EPCs transfected with the mRNA cocktail that also contained the ANG-1 mRNA. An improved wound closure compared to

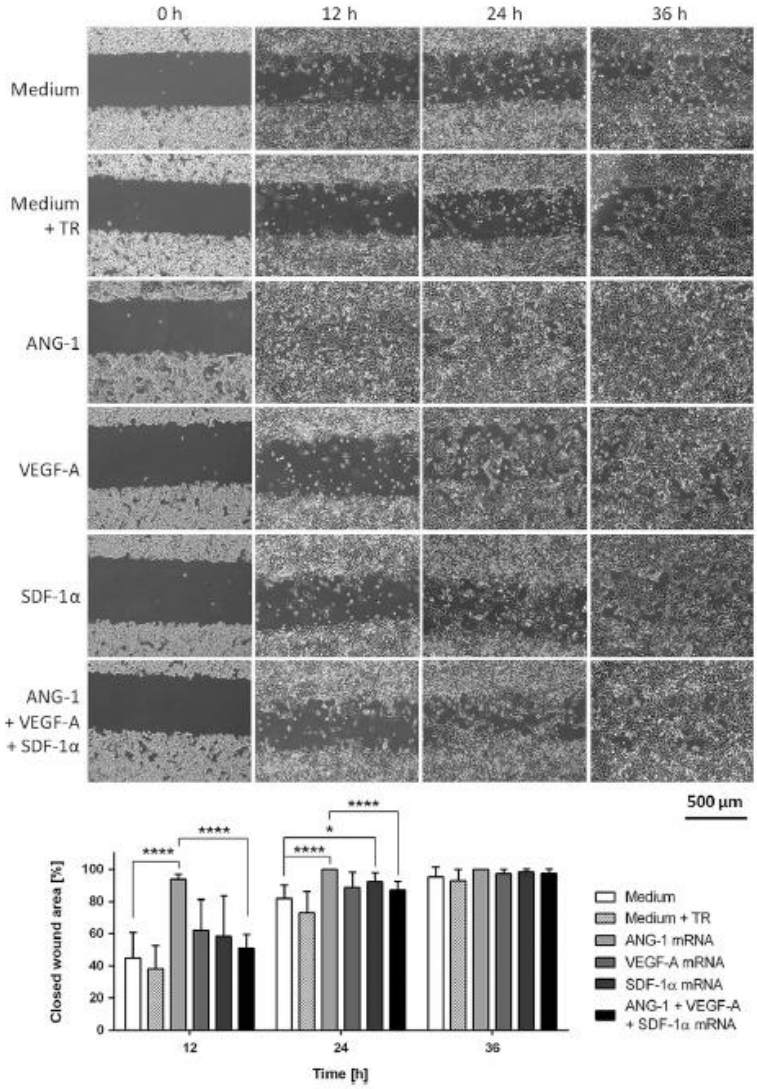

Figure 5. Analysis of Wound-Healing Capacity of mRNA-Engineered EPCs via Wound Scratch Migration Assay

1×10^5 murine EPCs were cultivated overnight, and then they were transfected with 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with an mRNA cocktail containing 1.6 µg ANG-1, 0.8 µg VEGF-A, and 0.5 µg SDF-1α mRNA. The next day, cells were detached, and 28,000 EPCs with or without mRNA transfection were seeded in each chamber of Culture-Insert 3 wells in µ-dishes. After 5 hr, when the cells completely attached and covered the surface, an open wound field was generated. Immediately after the generation of wound areas (0 hr) and after 12, 24, and 36 hr, phase-contrast images were taken and closed wound areas were calculated using Tscratch software. Scale bar represents 500 µm. Results are shown as mean + SD ($n = 8$). Statistical differences were determined using one-way ANOVA followed by Bonferroni multiple comparison test (*$p < 0.05$ and ****$p < 0.0001$).

the untreated cells was also detected after 24 hr with SDF-1α mRNA-transfected EPCs. In the *in vivo* CAM assay, the highest angiogenic potential was obtained by the ANG-1 mRNA engineering of EPCs. Here, EPCs transfected with the mRNA cocktail also showed an improvement of angiogenesis-related parameters, however, this increase was not statistically significant.

VEGF is the most investigated growth factor for the treatment of cardiovascular diseases and ischemic conditions, since it is a key regulator of blood vessel formation. However, studies demonstrated that newly formed blood vessels induced by VEGF are immature and leaky.[34,35] Su and colleagues[35] demonstrated that the simultaneous expression of ANG-1 and VEGF delivered by an adeno-associated virus (AAV)-mediated gene delivery approach has positive effects on the therapy of infarcted mouse hearts, with more capillaries, a smaller infarct size, and better cardiac function. Moreover, the neovasculature was less leaky compared to VEGF alone treatment.

The results of this study proved that the modification of EPCs only with ANG-1 mRNA could be sufficient for the improvement of angiogenesis. SDF-1α mRNA transfection also showed an improvement of wound closure and *in vitro* tube formation. In contrast, after the single transfection of EPCs with VEGF mRNA, an improvement in angiogenic potential could not be detected. In their study, Zangi et al.[36] injected 100 µg VEGF mRNA into cardiac muscle of mice and demonstrated increased formation of systemically perfused vessels in the area of injection. Thus, the production of higher VEGF amounts by the mRNA-modified EPCs could be required to improve the angiogenesis.

Compared to the *in vivo* injection of synthetic mRNA into the affected tissue, using the approach described in this study, increased numbers of EPCs can be obtained by the isolation and/or expansion of EPCs. The *ex vivo* modification of these cells by synthetic mRNA to produce their own proangiogenic proteins, after the *in vivo* injection for a limited time, could further increase the angiogenic potential compared to the injection of high amounts of VEGF mRNA into the affected tissue. In this study, due to high transfection efficiency

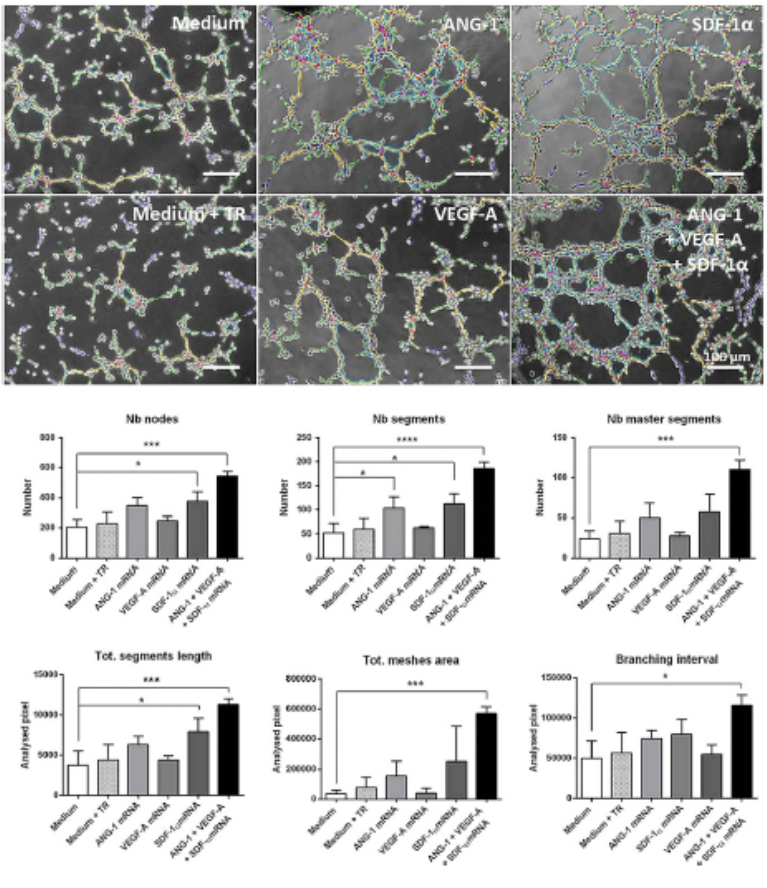

Figure 6. Analysis of Angiogenic Potential of mRNA-Engineered EPCs by Tube Formation Assay

1×10^5 murine EPCs were cultivated overnight, and then they were transfected with 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with an mRNA cocktail containing 1.6 µg ANG-1, 0.8 µg VEGF-A, and 0.5 µg SDF-1α mRNA. After 24 hr, EPCs were detached and 1×10^4 EPCs were seeded on Matrigel-coated angiogenesis slides. After 4 hr of incubation at 37°C, the formation of tubes was examined by phase-contrast microscopy. Microscopic images were analyzed using NIH ImageJ software with Angiogenesis Analyzer plugin, and segments are shown in magenta, master segments in orange, branches in green, and meshes in blue. The numbers (Nb) of nodes, segments, and master segments and the total (Tot.) segment length, total mesh area, and branching interval were quantified and compared to the medium control. The unit of area and length is pixel (px). Scale bars represent 100 µm. Results are shown as mean + SD ($n = 3$). Statistical differences were determined using one-way ANOVA followed by Bonferroni multiple comparison test (*$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$).

and cell compatibility, Lipofectamine 2000 was used as a transfection reagent; however, the quick delivery of synthetic mRNA into the cells, for example, by using methods such as electroporation, could further significantly improve the *in vivo* results. Thereby, the cells could be immediately injected into the tissue after the mRNA transfection. However, care should be taken that the selected transfection method does not harm the cells.

The overexpression of paracrine factors in ischemic patients could be especially beneficial to increase the mobilization and recruitment of autologous stem cells to the damaged area to support healing and regeneration by improving revascularization. In previous studies, AAV or lentiviral vectors were applied to produce ANG-1 in rat mesenchymal stem cells (MSCs),[37] VEGF-A in rat skeletal muscles,[38] and SDF-1α in human MSCs.[39] Furthermore, EPCs were genetically modified with adenoviral vectors encoding hypoxia-induced factor-1α (HIF-1α),[40] FGF-1,[41] and VEGF;[42] lentiviral vectors encoding ANG-1;[43] and retroviral vectors encoding SDF-1α and VEGF-A[44] to improve angiogenesis in animal models of ischemia. Using these vectors, the expression of proteins is maintained for an extended period of time. However, the prolonged presence of exogenously expressed proteins can have adverse effects. For example, the prolonged exposure of vessels to VEGF-A due to DNA-mediated gene transfer can result in edema due to an increased vessel permeability.[36] The synthetic mRNA-mediated protein expression in the cells ceases after the natural degradation of delivered synthetic mRNA, which is approximately after 2–3 days,[45] and no footprint is left. We suggest that this time frame could be sufficient for further homing of endogenous EPCs to the ischemic tissue and the rapid connection of implanted EPCs to the blood vessels of the surrounding intact tissue.

Conclusions

In this study, for the first time, we demonstrated that the angiogenic potential of EPCs can be improved by the transfection of cells with synthetic mRNAs encoding proangiogenic factors. The strongest *in vivo* angiogenic potential could be detected with ANG-1 mRNA-transfected cells. *In vitro*, EPCs transfected with the mRNA cocktail showed significantly improved tube formation. Thus, this promising synthetic mRNA-based treatment method could be beneficial in conditions associated with insufficient formation of new blood vessels in damaged tissues, e.g., after myocardial infarction, stroke, or limb ischemia, or in the field of tissue engineering to improve the vascularization of scaffolds and to promote vessel anastomosis with the host vasculature. Furthermore, this method could be applied to the treatment of various diseases by transfecting the desired cell type with one or multiple mRNAs to modify cell activity and cell fate, in order to improve cellular functions for sustained clinical outcomes.

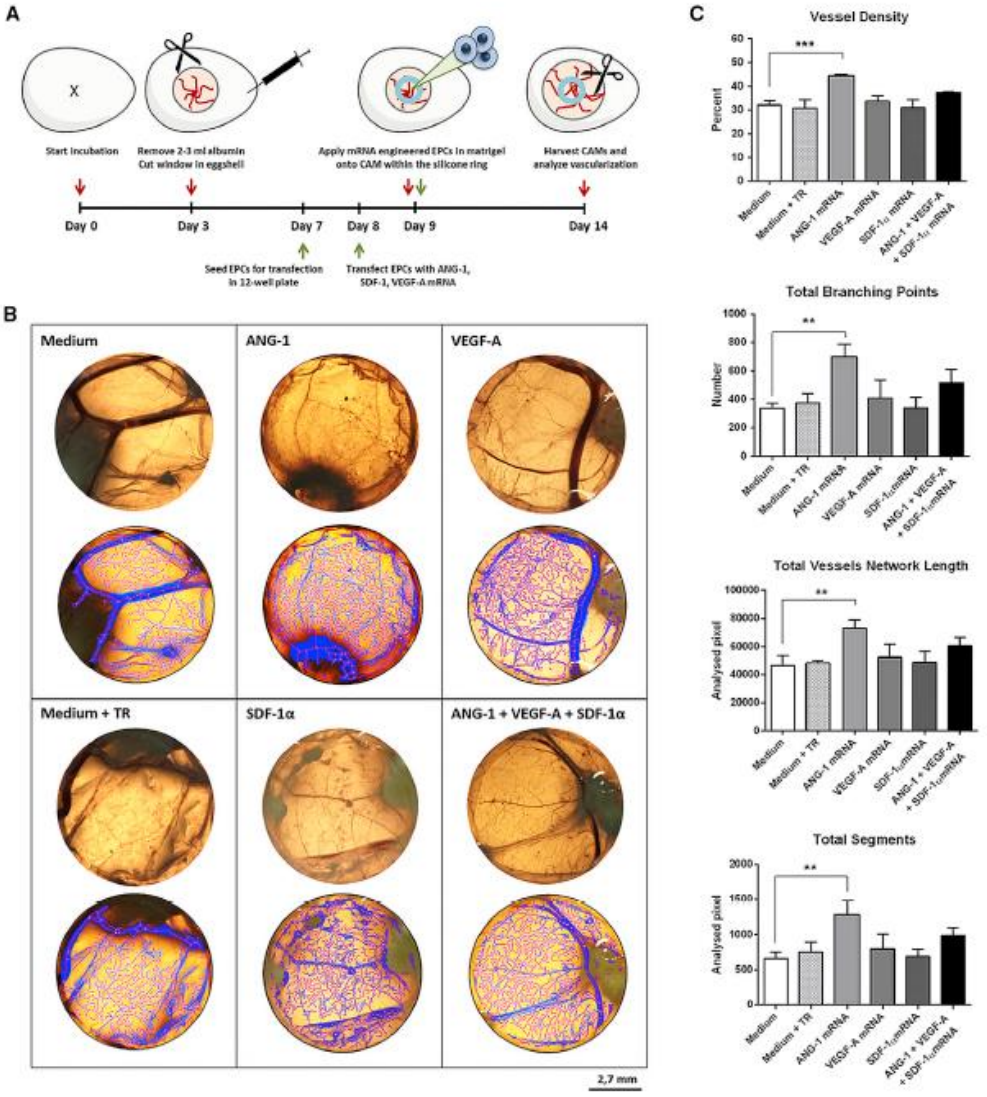

Figure 7. Analysis of *In Vivo* Angiogenetic Potential of mRNA-Engineered EPCs in Chick Embryo Chorioallantoic Membrane Assay

(A) Schematic representation of the chorioallantoic membrane (CAM) assay. ANG-1, SDF-1α, and VEGF-A mRNA-transfected and untreated cells (medium or medium containing transfection reagent [TR]) were applied in silicone rings (8-mm inner diameter) onto the CAMs at the ninth day of incubation at 37°C and 60% humidity. At the fourth day of incubation, the CAMs were fixed and excised. (B) Analysis of the region of the inner ring circle with Wimasis WimCAM image software to quantify angiogenesis. Scale bar of photographs (upper) and analyzed pictures (lower) represents 2.7 mm. (C) Quantification of angiogenesis using Wimasis WimCAM web-based service. The vessel density, total branching points, total vessel network length, and total segments were quantified and compared to the medium control. Results are shown as mean + SD ($n = 3$). Statistical differences were determined using one-way ANOVA followed by Bonferroni multiple comparison test (**$p < 0.01$ and ***$p < 0.001$).

MATERIALS AND METHODS

In Vitro mRNA Synthesis

The pcDNA 3.3 plasmid containing the coding sequences for SDF-1α or VEGF-A_{165} (referred to as VEGF-A) was produced by Aldevron (Fargo, ND, USA), and pEX-K4 plasmid containing ANG-1 was produced by Eurofins Genomics (Ebersberg, Germany). To synthesize the mRNA, first of all, DNA templates were generated by PCR using 50–100 ng plasmid DNA, 0.7 μM forward primer (5′-TTGG ACCCTCGTACAGAAGCTAATACG-3′) and 0.7 μM reverse primer (5′-T_{120}CTTCCTACTCAGGCTTTATTCAAAGACCA-3′)

(Ella Biotech, Martinsried, Germany), and HotStar HiFidelity Polymerase Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. PCR products were generated using the following amplification protocol: initial denaturation step at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Afterward, PCR products were purified using MinElute PCR purification kit (QIAGEN).

Next, the mRNA was produced by *in vitro* transcription (IVT) using MEGAscript T7 Kit (Ambion, Glasgow, Scotland), according to the manufacturer's instructions. The IVT reaction contained 1.5 µg template DNA, 7.5 mM ATP, 1.875 mM guanosine triphosphate (GTP), 7.5 mM pseudoruridine-5′-triphosphate (Ψ-UTP), and 7.5 mM 5-methylcytidine-5′-triphosphate (m5CTP) (TriLink Biotech, San Diego, CA, USA). Furthermore, 2.5 mM 3′-0-Me-m7G(5′)ppp(5′)G RNA Cap Structure Analog (New England Biolabs, Frankfurt, Germany) was used for 5′ end capping, and the mRNA was dephosphorylated using 5 U/mL Antarctic phosphatase (New England Biolabs, Frankfurt am Main, Germany). Additionally, the IVT reaction mixture contained 40 U RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) to prevent mRNA degradation. After each reaction step, the mRNA was purified using RNeasy kit (QIAGEN), and the mRNA concentration was adjusted to 100 ng/µL in nuclease-free water. The purity and specific length of generated DNA templates and mRNA products were analyzed using 1% agarose gel electrophoresis at 100 V for 45 min and staining with 1× GelRed (Biotium, Fremont, CA, USA) in 1× Tris-borate-EDTA (TBE) buffer.

Cultivation of Murine EPCs

In this study, murine embryonal EPCs (T17b cells), which were previously isolated and characterized by Hatzopoulos et al.,[46] were used. These cells can differentiate into mature ECs and form vascular structures *in vitro* and *in vivo*.[47] EPCs were cultivated in DMEM with high glucose and L-glutamine supplemented with 20% fetal bovine serum (FBS), 1× minimum essential medium (MEM) non-essential amino acids (NEAA), 100 µM 2-mercaptoethanol, and 1% penicillin and streptomycin. All these cell culture reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Cells were cultivated at 37°C with 5% CO_2 and medium was changed every 2–3 days. Cells were detached at about 70% confluency using 0.04% trypsin/0.03% EDTA, and trypsin was neutralized using trypsin-neutralizing solution (TNS, 0.05% trypsin inhibitor in 0.1% BSA; PromoCell, Heidelberg, Germany). Afterward, cells were centrifuged for 5 min at 300 × *g* and seeded on 0.1% gelatin- (Sigma-Aldrich Chemie, Steinheim, Germany) coated tissue flasks or cell culture plates.

Transfection of EPCs with Synthetic mRNAs

To perform the transfection of EPCs with synthetic mRNAs, 1×10^5 EPCs were seeded per well of a 12-well plate coated with 0.1% gelatin and incubated overnight at 37°C. The next day, lipoplexes were generated by incubation of mRNAs with Lipofectamine 2000 (Thermo Fisher Scientific) in 0.5 mL Opti-MEM I reduced serum medium (Opti-MEM, Thermo Fisher Scientific). The transfections were performed with equimolar amounts of mRNAs. Thus, EPCs were transfected with 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with all mRNAs together (called triple-mRNA cocktail). To perform single-mRNA transfections, transfection complexes were generated by using 2 µL Lipofectamine 2000 for VEGF-A_{165} and SDF-1α mRNA and 4 µL for ANG-1 mRNA. To generate transfection complexes with the mRNA cocktail, 4 µL Lipofectamine 2000 was used. The mRNAs were incubated in Opti-MEM for 15 min at room temperature (RT) with the transfection reagent, and then Opti-MEM containing lipoplexes were added to the cells. After 4 hr of incubation, transfection medium was discarded and 1 mL fresh cell culture medium was added for further overnight incubation at 37°C. As controls, cells were incubated with only Opti-MEM or Opti-MEM containing transfection reagent.

Detection of Protein Expression Using ELISA

After the transfection of EPCs with synthetic mRNAs, the expression of proteins was analyzed using ELISA. Therefore, supernatants of transfected cells were collected and diluted 1:100 in Dulbecco's PBS (DPBS)/1% BSA. The concentrations of ANG-1, VEGF-A, and SDF-1α were determined as duplicate in 100 µL using human ANG-1, CXCL-12/SDF-1α, and VEGF-A DuoSet ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Eon Synergy 2, BioTek Instruments) at 450 nm, with the correction wavelength set at 540 nm.

Influence of mRNA Engineering on EPC Viability

The influence of mRNA treatment on the viability of EPCs was assessed using PrestoBlue assay (Invitrogen, Carlsbad, CA, USA). Therefore, 1×10^5 EPCs were transfected with 1.6 µg ANG-1, 0.8 µg VEGF-A, or 0.5 µg SDF-1α mRNA or with a triple-mRNA cocktail containing 1.6 µg ANG-1, 0.8 µg VEGF-A, and 0.5 µg SDF-1α mRNA for 4 hr at 37°C. Afterward, transfection complexes were discarded and 1 mL cell culture medium was added per well of a 12-well plate. After 24 hr, 110 µL PrestoBlue cell viability reagent was added per well and incubated for 1.5 hr at 37°C. Fluorescence intensity of 50 µL supernatant was measured in triplicates at an excitation wavelength of 530 nm and an emission wavelength of 600 nm, using a multimode microplate reader (Mithras LB 940, Berthold Technologies).

Chemotactic Migration Assay

Migration of unmodified EPCs toward mRNA-engineered EPCs was analyzed using chemotactic migration assay. After the transfection of 1×10^5 EPCs with mRNAs, lipoplexes were removed and serum-reduced cell culture medium containing 1% FBS was added for overnight cultivation to the cells. The next day, 5×10^4 untreated EPCs were seeded onto transwell inserts with 8-µm pores coated with 0.1% gelatin. Subsequently, transwell inserts were transferred into wells of a 12-well plate containing the mRNA-transfected or untransfected EPCs and incubated for 6 hr at 37°C. Afterward, the transwell inserts were rinsed with DPBS containing Ca^{2+}/Mg^{2+} ($DPBS^+$) and fixed for 10 min with ice-cold methanol (AnalaR NORMAPUR,

VWR, Darmstadt, Germany). After an additional washing of transwell inserts with $DPBS^+$, the cells at the bottom of the transwell were stained for 10 min at RT with 1 μg/mL DAPI (Sigma-Aldrich) in $DPBS^+$. The migrated cells were detected using fluorescence microscopy (Axiovert135, Carl Zeiss, Oberkochen, Germany), and the numbers of cells that migrated through the transwell inserts were determined at 3 different regions of each insert membrane using ImageJ 1.51 software.[48]

Wound Scratch Migration Assay

1×10^5 EPCs were transfected in 12-well plates with synthetic mRNAs and cultivated at 37°C and 5% CO_2 overnight. Then, cells were detached, and 28,000 cells were seeded in 70 μL cell culture medium into each chamber of the 0.1% gelatin-coated Culture-Insert 3 wells in μ-$Dish^{35mm,\ high}$ (ibidi, Martinsried, Germany). Cells were cultivated at 37°C and 5% CO_2 for 5 hr. After allowing the cells to form a confluent monolayer, a 500-μm open wound field between the cells was generated by removing the culture insert from the dish. Subsequently, 1.5 mL fresh medium was added to each μ-dish. After 0, 12, 24, and 36 hr, the wound fields were documented using a phase-contrast microscope (Axiovert 135). The percentage of closed wound area was calculated using Tscratch software.[49]

Tube Formation Assay

Tube formation capacity of mRNA-engineered EPCs was analyzed after the transfection with synthetic mRNAs. Therefore, 1×10^5 EPCs were transfected in 12-well plates with synthetic mRNAs and cultivated at 37°C and 5% CO_2 overnight in serum-reduced culture medium containing 1% FBS. Each well of the μ-slides for angiogenesis (ibidi) was coated with 10 μL Matrigel (hESC qualified, Corning, Corning, NY, USA) solution (1:5 diluted in DMEM) for 1 hr at 37°C. Then, transfected and untransfected EPCs were detached, and 50 μL cell suspension containing 10,000 EPCs was added to the Matrigel-coated wells. Tube formation was assessed after 4 hr of incubation at 37°C using a light microscope (Axiovert 135) equipped with a digital camera AxioCam MRm (Carl Zeiss). Pictures were analyzed using Angiogenesis Analyzer plugin of ImageJ 1.51 software.[48]

Chicken Embryo CAM Assay

Fresh fertilized chicken eggs of the Lohmann White × White Rock breed chicken variety were purchased from the breeding facility Matthias Sittig (Buchholz, Germany). The eggs were incubated at 37°C and 60% relative humidity in an egg incubator (Heka-Brutgeräte, Rietberg-Varensell, Germany) and completely rotated twice a day. At day 3 of incubation, 2–3 mL albumen was aspirated by inserting an 18G needle at the tip of the egg without harming the yolk. Next, a semi-permeable adhesive tape Suprasorb F (Lohmann & Rauscher, Rengsdorf, Germany) was stuck to the eggshell, and a circular window (Ø 1–1.5 cm) was cut into the shell. Unfertilized eggs showing no vasculature or heart beating were removed. Then, the window was sealed using the adhesive tape to prevent dehydration and to minimize the risk of infection. The eggs were then incubated without rotation.

At day 9 of incubation, EPCs transfected with 1.6 μg ANG-1, 0.8 μg VEGF-A, or 0.5 μg SDF-1α mRNA or with a triple mRNA cocktail were applied to the CAM. Per egg, 4×10^5 EPCs were resuspended in 50 μL cell culture medium and mixed with 50 μL Matrigel (hESC qualified, Corning). A silicone ring (inner diameter: 0.8 cm) (neoLab, Leonberg, Germany) was carefully placed onto the CAM, and 100 μL matrigel and cell suspension was applied into the inner circle of the ring. The eggs were sealed and further incubated. At day 14, eggs were kept at RT for 3 hr and CAMs were then excised and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 24 hr at 4°C. After washing with DPBS, color photographs of the circular application area were taken using a photomacroscope (Wild Heerbrugg M400) and a digital camera (Canon EOS 550D). The analysis of angiogenesis-associated parameters, such as vessel density, branching points, and segment length, was performed using Wimasis image analysis web-based system.

Statistical Analysis

Data are shown as mean + SD or SEM. One-way ANOVA for repeated measurements followed by Bonferroni multiple comparison test was performed to compare the means. All statistical analyses were performed double-tailed using GraphPad Prism version 6.01. Differences of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.09.005.

AUTHOR CONTRIBUTIONS

H.S. and M.A.-A. conceived and designed the experiments. H.S. performed the experiments with support from S.G., A.B., M.A.-A., and H.P.W. and analyzed the data. H.P.W. and C.S. contributed reagents, materials, and analysis tools. H.S. and M.A.-A. wrote the paper. M.A.-A. supervised the project.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This study was funded by the European Social Funds in Baden-Wuerttemberg, Germany and the Ministry of Science, Research, and the Arts of the State of Baden-Wuerttemberg (MWK-BW). We thank Prof. Antonis K. Hatzopoulos for generously providing the embryonal murine T17b endothelial progenitor cells.

REFERENCES

1. Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J.M., and Asahara, T. (1999). Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat. Med. 5, 434–438.

2. Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M., and Isner, J.M. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ. Res. 85, 221–228.

3. Asahara, T., Kawamoto, A., and Masuda, H. (2011). Concise review: Circulating endothelial progenitor cells for vascular medicine. Stem Cells *29*, 1650–1655.
4. Sen, S., McDonald, S.P., Coates, P.T., and Bonder, C.S. (2011). Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease. Clin. Sci. (Lond.) *120*, 263–283.
5. Kachamakova-Trojanowska, N., Bukowska-Strakova, K., Zukowska, M., Dulak, J., and Jozkowicz, A. (2015). The real face of endothelial progenitor cells - Circulating angiogenic cells as endothelial prognostic marker? Pharmacol. Rep. *67*, 793–802.
6. Kawamoto, A., Gwon, H.C., Iwaguro, H., Yamaguchi, J.I., Uchida, S., Masuda, H., Silver, M., Ma, H., Kearney, M., Isner, J.M., and Asahara, T. (2001). Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation *103*, 634–637.
7. Minami, Y., Nakajima, T., Ikutomi, M., Morita, T., Komuro, I., Sata, M., and Sahara, M. (2015). Angiogenic potential of early and late outgrowth endothelial progenitor cells is dependent on the time of emergence. Int. J. Cardiol. *186*, 305–314.
8. Fazel, S., Cimini, M., Chen, L., Li, S., Angoulvant, D., Fedak, P., Verma, S., Weisel, R.D., Keating, A., and Li, R.K. (2006). Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. J. Clin. Invest. *116*, 1865–1877.
9. Chong, M.S.K., Ng, W.K., and Chan, J.K.Y. (2016). Concise Review: Endothelial Progenitor Cells in Regenerative Medicine: Applications and Challenges. Stem Cells Transl. Med. *5*, 530–538.
10. Terrovitis, J.V., Smith, R.R., and Marbán, E. (2010). Assessment and optimization of cell engraftment after transplantation into the heart. Circ. Res. *106*, 479–494.
11. Lipiec, P., Krzemińska-Pakuła, M., Plewka, M., Kuśmierek, J., Płachcińska, A., Szumiński, R., Robak, T., Korycka, A., and Kasprzak, J.D. (2009). Impact of intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction on left ventricular perfusion and function: a 6-month follow-up gated 99mTc-MIBI single-photon emission computed tomography study. Eur. J. Nucl. Med. Mol. Imaging *36*, 587–593.
12. Sukmawati, D., and Tanaka, R. (2015). Introduction to next generation of endothelial progenitor cell therapy: a promise in vascular medicine. Am. J. Transl. Res. *7*, 411–421.
13. Zhang, H., van Olden, C., Sweeney, D., and Martin-Rendon, E. (2014). Blood vessel repair and regeneration in the ischaemic heart. Open Heart *1*, e000016.
14. Takeshita, S., Zheng, L.P., Brogi, E., Kearney, M., Pu, L.Q., Bunting, S., Ferrara, N., Symes, J.F., and Isner, J.M. (1994). Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J. Clin. Invest. *93*, 662–670.
15. Baffour, R., Berman, J., Garb, J.L., Rhee, S.W., Kaufman, J., and Friedmann, P. (1992). Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. J. Vasc. Surg. *16*, 181–191.
16. Sun, X.-T., Ding, Y.-T., Yan, X.-G., Wu, L.-Y., Li, Q., Cheng, N., Qiu, Y.D., and Zhang, M.Y. (2004). Angiogenic synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in an in vitro quantitative microcarrier-based three-dimensional fibrin angiogenesis system. World J. Gastroenterol. *10*, 2524–2528.
17. Wang, J.H., Xu, Y.M., Fu, Q., Song, L.J., Li, C., Zhang, Q., and Xie, M.K. (2013). Continued sustained release of VEGF by PLGA nanospheres modified BAMG stent for the anterior urethral reconstruction of rabbit. Asian Pac. J. Trop. Med. *6*, 481–484.
18. Chappell, J.C., Song, J., Burke, C.W., Klibanov, A.L., and Price, R.J. (2008). Targeted delivery of nanoparticles bearing fibroblast growth factor-2 by ultrasonic microbubble destruction for therapeutic arteriogenesis. Small *4*, 1769–1777.
19. Baumgartner, I., Pieczek, A., Manor, O., Blair, R., Kearney, M., Walsh, K., and Isner, J.M. (1998). Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation *97*, 1114–1123.
20. Kuliszewski, M.A., Kobulnik, J., Lindner, J.R., Stewart, D.J., and Leong-Poi, H. (2011). Vascular gene transfer of SDF-1 promotes endothelial progenitor cell engraftment and enhances angiogenesis in ischemic muscle. Mol. Ther. *19*, 895–902.
21. Leong-Poi, H., Kuliszewski, M.A., Lekas, M., Sibbald, M., Teichert-Kuliszewska, K., Klibanov, A.L., Stewart, D.J., and Lindner, J.R. (2007). Therapeutic arteriogenesis by ultrasound-mediated VEGF165 plasmid gene delivery to chronically ischemic skeletal muscle. Circ. Res. *101*, 295–303.
22. Swanson, N., Hogrefe, K., Javed, Q., Malik, N., and Gershlick, A.H. (2003). Vascular endothelial growth factor (VEGF)-eluting stents: in vivo effects on thrombosis, endothelialization and intimal hyperplasia. J. Invasive Cardiol. *15*, 688–692.
23. Gupta, R., Tongers, J., and Losordo, D.W. (2009). Human studies of angiogenic gene therapy. Circ. Res. *105*, 724–736.
24. Sahin, U., Karikó, K., and Türeci, Ö. (2014). mRNA-based therapeutics–developing a new class of drugs. Nat. Rev. Drug Discov. *13*, 759–780.
25. Adams, R.H., and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. Nat. Rev. Mol. Cell Biol. *8*, 464–478.
26. Shibuya, M. (2011). Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. Genes Cancer *2*, 1097–1105.
27. Milam, K.E., and Parikh, S.M. (2015). The angiopoietin-Tie2 signaling axis in the vascular leakage of systemic inflammation. Tissue Barriers *3*, e957508.
28. Brouwers, J., Noviyanti, R., Fijnheer, R., de Groot, P.G., Trianty, L., Mudaliana, S., Roest, M., Syafruddin, D., van der Ven, A., and de Mast, Q. (2013). Platelet activation determines angiopoietin-1 and VEGF levels in malaria: implications for their use as biomarkers. PLoS ONE *8*, e64850.
29. Brindle, N.P.J., Saharinen, P., and Alitalo, K. (2006). Signaling and functions of angiopoietin-1 in vascular protection. Circ. Res. *98*, 1014–1023.
30. Ho, T.K., Tsui, J., Xu, S., Leoni, P., Abraham, D.J., and Baker, D.M. (2010). Angiogenic effects of stromal cell-derived factor-1 (SDF-1/CXCL12) variants in vitro and the in vivo expressions of CXCL12 variants and CXCR4 in human critical leg ischemia. J. Vasc. Surg. *51*, 689–699.
31. Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat. Med. *10*, 858–864.
32. Ron, D. (2006). Cell biology. Stressed cells cope with protein overload. Science *313*, 52–53.
33. Hollien, J., and Weissman, J.S. (2006). Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science *313*, 104–107.
34. Dvorak, H.F., Nagy, J.A., Feng, D., Brown, L.F., and Dvorak, A.M. (1999). Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr. Top. Microbiol. Immunol. *237*, 97–132.
35. Su, H., Takagawa, J., Huang, Y., Arakawa-Hoyt, J., Pons, J., Grossman, W., and Kan, Y.W. (2009). Additive effect of AAV-mediated angiopoietin-1 and VEGF expression on the therapy of infarcted heart. Int. J. Cardiol. *133*, 191–197.
36. Zangi, L., Lui, K.O., von Gise, A., Ma, Q., Ebina, W., Ptaszek, L.M., Später, D., Xu, H., Tabebordbar, M., Gorbatov, R., et al. (2013). Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. Nat. Biotechnol. *31*, 898–907.
37. Sun, L., Cui, M., Wang, Z., Feng, X., Mao, J., Chen, P., Kangtao, M., Chen, F., and Zhou, C. (2007). Mesenchymal stem cells modified with angiopoietin-1 improve remodeling in a rat model of acute myocardial infarction. Biochem. Biophys. Res. Commun. *357*, 779–784.
38. Shimpo, M., Ikeda, U., Maeda, Y., Takahashi, M., Miyashita, H., Mizukami, H., Urabe, M., Kume, A., Takizawa, T., Shibuya, M., et al. (2002). AAV-mediated VEGF gene transfer into skeletal muscle stimulates angiogenesis and improves blood flow in a rat hindlimb ischemia model. Cardiovasc. Res. *53*, 993–1001.
39. Liang, X., Su, Y.-P., Kong, P.-Y., Zeng, D.-F., Chen, X.-H., Peng, X.-G., Zou, Z.M., and Xu, H. (2010). Human bone marrow mesenchymal stem cells expressing SDF-1 promote hematopoietic stem cell function of human mobilised peripheral blood CD34+ cells in vivo and in vitro. Int. J. Radiat. Biol. *86*, 230–237.
40. Jiang, M., Wang, B., Wang, C., He, B., Fan, H., Shao, Q., Gao, L., Liu, Y., Yan, G., and Pu, J. (2008). In vivo enhancement of angiogenesis by adenoviral transfer of HIF-1α-modified endothelial progenitor cells (Ad-HIF-1α-modified EPC for angiogenesis). Int. J. Biochem. Cell Biol. *40*, 2284–2295.

41. Chen, S.Y., Wang, F., Yan, X.Y., Zhou, Q., Ling, Q., Ling, J.X., Rong, Y.Z., and Li, Y.G. (2009). Autologous transplantation of EPCs encoding FGF1 gene promotes neovascularization in a porcine model of chronic myocardial ischemia. Int. J. Cardiol. *135*, 223–232.

42. Iwaguro, H., Yamaguchi, J., Kalka, C., Murasawa, S., Masuda, H., Hayashi, S., Silver, M., Li, T., Isner, J.M., and Asahara, T. (2002). Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation *105*, 732–738.

43. Wang, Y.Q., Song, J.J., Han, X., Liu, Y.Y., Wang, X.H., Li, Z.M., and Tzeng, C.M. (2014). Effects of angiopoietin-1 on inflammatory injury in endothelial progenitor cells and blood vessels. Curr. Gene Ther. *14*, 128–135.

44. Yu, J.X., Huang, X.F., Lv, W.M., Ye, C.S., Peng, X.Z., Zhang, H., Xiao, L.B., and Wang, S.M. (2009). Combination of stromal-derived factor-1alpha and vascular endothelial growth factor gene-modified endothelial progenitor cells is more effective for ischemic neovascularization. J. Vasc. Surg. *50*, 608–616.

45. Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., et al. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell *7*, 618–630.

46. Hatzopoulos, A.K., Folkman, J., Vasile, E., Eiselen, G.K., and Rosenberg, R.D. (1998). Isolation and characterization of endothelial progenitor cells from mouse embryos. Development *125*, 1457–1468.

47. Bleiziffer, O., Horch, R.E., Hammon, M., Arkudas, A., Naschberger, E., Rath, S., Pryymachuk, G., Beier, J.P., Hatzopoulos, A.K., Stürzl, M., and Kneser, U. (2009). T17b murine embryonal endothelial progenitor cells can be induced towards both proliferation and differentiation in a fibrin matrix. J. Cell. Mol. Med. *13*, 926–935.

48. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671–675.

49. Gebäck, T., Schulz, M.M., Koumoutsakos, P., and Detmar, M. (2009). TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques *46*, 265–274.

8.3. PUBLIKATION III

Generation of iPSCs by non-integrative RNA-based reprogramming techniques: Benefits of self-replicating RNA versus synthetic mRNA

Hindawi
Stem Cells International
Volume 2019, Article ID 7641767, 16 pages
https://doi.org/10.1155/2019/7641767

Research Article

Generation of iPSCs by Nonintegrative RNA-Based Reprogramming Techniques: Benefits of Self-Replicating RNA versus Synthetic mRNA

Heidrun Steinle,[1] Marbod Weber,[1] Andreas Behring,[1] Ulrike Mau-Holzmann,[2] Christian Schlensak,[1] Hans Peter Wendel,[1] and Meltem Avci-Adali[1]

[1]*University Hospital Tübingen, Department of Thoracic and Cardiovascular Surgery, Calwerstraße 7/1, 72076 Tübingen, Germany*
[2]*University Hospital Tübingen, Institute of Medical Genetics and Applied Genomics, Calwerstraße 7, 72076 Tübingen, Germany*

Correspondence should be addressed to Meltem Avci-Adali; meltem.avci-adali@uni-tuebingen.de

Received 15 March 2019; Accepted 20 May 2019; Published 19 June 2019

Academic Editor: James Adjaye

The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) is gaining in importance in the fields of regenerative medicine, tissue engineering, and disease modeling. Patient-specific iPSCs have as an unlimited cell source a tremendous potential for generating various types of autologous cells. For the future clinical applicability of these iPSC-derived cells, the generation of iPSCs via nongenome integrating methods and the efficient reprogramming of patients' somatic cells are required. In this study, 2 different RNA-based footprint-free methods for the generation of iPSCs were compared: the use of synthetic modified messenger RNAs (mRNAs) or self-replicating RNAs (srRNAs) encoding the reprogramming factors and GFP. Using both RNA-based methods, integration-free iPSCs without genomic alterations were obtained. The pluripotency characteristics identified by specific marker detection and the in vitro and in vivo trilineage differentiation capacity were comparable. Moreover, the incorporation of a GFP encoding sequence into the srRNA enabled a direct and convenient monitoring of the reprogramming procedure and the successful detection of srRNA translation in the transfected cells. Nevertheless, the use of a single srRNA to induce pluripotency was less time consuming, faster, and more efficient than the daily transfection of cells with synthetic mRNAs. Therefore, we believe that the srRNA-based approach might be more appropriate and efficient for the reprogramming of different types of somatic cells for clinical applications.

1. Introduction

The reprogramming of a patient's somatic cells into induced pluripotent stem cells (iPSCs) is mediated by the exogenous delivery of the "Yamanaka" factors Oct4, Klf4, Sox2, and cMyc, and it allows the generation of an unlimited stem cell source for tissue regeneration [1–3]. In the first studies, retroviral vectors were used to deliver the reprogramming factors into cells. However, the therapeutic application of cells derived from these iPSCs is hampered due to the risks associated with the random integration of viral vectors into the host genome.

In recent years, various nonintegrative reprogramming methods have been successfully established to induce pluripotency in different somatic cell types [4–8]. One of the most promising approaches is the use of a synthetic modified mRNA for reprogramming [6, 9–11]. After the delivery of synthetic mRNA into the cytosol, the mRNA is immediately translated by ribosomes into proteins and the entry into the nucleus is not required. The synthesis of reprogramming factors ceases after the degradation of mRNA, and no footprints are left. Furthermore, during the in vitro transcription (IVT), the synthetic mRNA can be modified with a cap structure, poly(A) tail, and modified nucleosides to improve the stability and the translation of proteins [12–17]. Previous studies showed that modified nucleosides, e.g., pseudouridine (Pseudo-UTP) and 5-methylcytidine (5mCTP), can be incorporated into the synthetic mRNA to substitute cytidine and uridine to abrogate the innate immune response. However, despite the great advances in the development of synthetic

mRNA-based reprogramming approaches, one of the main obstacles is still the induction of an innate immune response following multiple daily mRNA transfections, resulting in increased cellular stress and severe cytotoxicity [18, 19]. Thus, to prevent interferon-response induced cell death, the reprogramming medium needs to contain the interferon inhibitor B18R derived from vaccinia virus [6, 20, 21].

Another alternative to synthetic mRNA-based reprogramming is the use of self-replicating RNA (srRNA) [22]. The srRNA contains the coding sequences of the "Yamanaka" transcription factors Oct4, Klf4, Sox2, and cMyc and four nonstructural proteins (nsP1 to nsP4), which encode the RNA replication complex of Venezuelan equine encephalitis (VEE) virus [22–24]. The srRNA is a single-stranded RNA that mimics cellular 5′-capped and 3′-polyadenylated mRNA. The application of srRNA enables an extended duration of protein expression. To date, no risk for genomic integration has been reported by the generation of DNA intermediates [23, 25]. However, the presence of B18R protein is also required during the srRNA-based reprogramming as in synthetic mRNA-based reprogramming.

In this work, we compared the synthetic mRNA- and srRNA-based reprogramming methods to generate iPSCs from human neonatal fibroblasts. The one-time delivery of 1 μg srRNA significantly improved the reprogramming efficiency of fibroblasts compared to the daily transfection of cells with 1.2 μg mRNAs for at least 2 weeks. The srRNA-based reprogramming enhanced the reprogramming of somatic cells and resulted in increased numbers of iPSCs compared to synthetic mRNA-based reprogramming. Furthermore, the incorporation of the GFP encoding sequence to the srRNA enabled the monitoring of the reprogramming procedure and optimization of the culture conditions.

2. Materials and Methods

2.1. mRNA Synthesis. The pcDNA 3.3 plasmids containing the coding sequence for either Klf4, cMyc, Oct4, Sox2, Lin28, or eGFP [6] were purchased from Addgene (LGC Standards, Teddington, UK). DNA templates for the in vitro transcription (IVT) of mRNAs were generated with a polyT_{120} sequence. Subsequently, the mRNA synthesis and modifications were performed according to the previously published methods by Avci-Adali et al. [26, 27]. Briefly, to generate DNA templates, PCR was performed using 50-100 ng plasmid DNA and a forward primer (5′-TTGGAC CCTCGTACAGAAGCTAATACG-3′) and reverse primer (5′-T_{120}CTTCCTACTCAGGCTTTATTCAAAGACCA-3′). During the IVT reaction, 1.5 μg DNA, ATP, GTP, pseudouridine-5′-triphosphate (Pseudo-UTP), 5-methylcytidine-5′-triphosphate (5mCTP), and 3′-0-Me-m7G(5′)ppp(5′)G RNA Cap Structure Analog was used. The incubation was performed at 37°C for 4 h. After dephosphorylation, the mRNA was purified and the concentration was adjusted to 100 ng/μl in nuclease-free water. Subsequently, produced mRNAs were analyzed using 1% agarose gel electrophoresis and gels were stained with GelRed™ in 1x TBE buffer.

2.2. srRNA Synthesis. The T7-VEE-OKS-iM plasmids containing the coding sequences for Oct4, Sox2, Klf4, and cMyc [22] were purchased from Addgene (LGC Standards). For monitoring the transfection and reprogramming efficiency, an IRES (internal ribosome entry site)-GFP reporter encoding sequence was inserted by Aldevron (Fargo, USA) into the plasmid (Figure 1(b)). Thereby, the T7-VEE-OKS-iMG plasmid was obtained. To multiply the T7-VEE-OKS-iMG plasmid, competent *E. coli* cells (α-select chemically competent cells from Bioline GmbH, Luckenwalde, Germany) were transformed with 100 ng plasmid DNA and cultivated in LB medium supplemented with 50 μg/ml ampicillin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The isolation of plasmids was performed using the QIAprep Spin Miniprep Kit (Qiagen). Linearized DNA templates were generated using the FastDigest MluI restriction enzyme (Thermo Fisher Scientific). Therefore, 36 μg plasmid was incubated for 3 h at 37°C with 5 U enzyme, 20 μl 1x reaction buffer, and nuclease-free water in a total volume of 200 μl. Afterwards, linearized DNA was purified using the ISOLATE II PCR and Gel Kit (Bioline) and the complete linearization, purity, and specific length were analyzed by 1% agarose gel electrophoresis. Next, IVT was performed using the RiboMAX Large-Scale Production System T7 Kit (Promega, Madison, USA) according to the manufacturer's instructions. The IVT reaction was prepared with 10 μg template DNA and contained 40 U of the RNAse Inhibitor (Thermo Fisher Scientific) to prevent the degradation of srRNA. Afterwards, 5′-end capping (Cap1) was performed using the ScriptCap Cap1 Capping System followed by 3′-end polyadenylation with the A-Plus Poly(A) Polymerase Tailing Kit (both from CELLSCRIPT, Madison, USA) according to the manufacturer's instructions. Following each reaction step, the srRNA was purified using the ISOLATE II RNA Mini Kit (Bioline). The specific length and purity of the generated srRNA products was analyzed by 1% agarose gel electrophoresis containing 2.2 M formaldehyde in 1x MOPS (3-(N-morpholino)propanesulfonic acid) buffer at 100 V for 60 min. The gels were stained using 1x GelRed™ (Biotium, Fremont, USA) in 1x MOPS buffer.

2.3. Cultivation of Fibroblasts. Neonatal human foreskin fibroblasts (NuFFs, untreated, passage 9, Amsbio, Milton Park, UK) were cultivated in DMEM high glucose supplemented with 10% FBS, 1x GlutaMAX, 10 mM HEPES, and 50 μg/ml gentamicin B. These cell culture reagents were obtained from Thermo Fisher Scientific. Cells were cultivated at 37°C with 5% CO_2 (normoxia), and medium was changed every 2-3 days. Cells were detached at about 70% confluency using 0.04% trypsin/0.03% EDTA, and then trypsin neutralizing solution (TNS, 0.05% trypsin inhibitor in 0.1% BSA, PromoCell, Heidelberg, Germany) was added. Afterwards, cells were centrifuged for 5 min at 300 x g, resuspended in culture medium, and seeded at the desired cell density for reprogramming.

To generate inactivated feeder cells, NuFFs and mouse embryonic fibroblasts (MEFs, CF-1, untreated, passage 3, Amsbio) were treated with 10 mg/ml mitomycin C (Merck, Darmstadt, Germany) and frozen in 10% DMSO containing

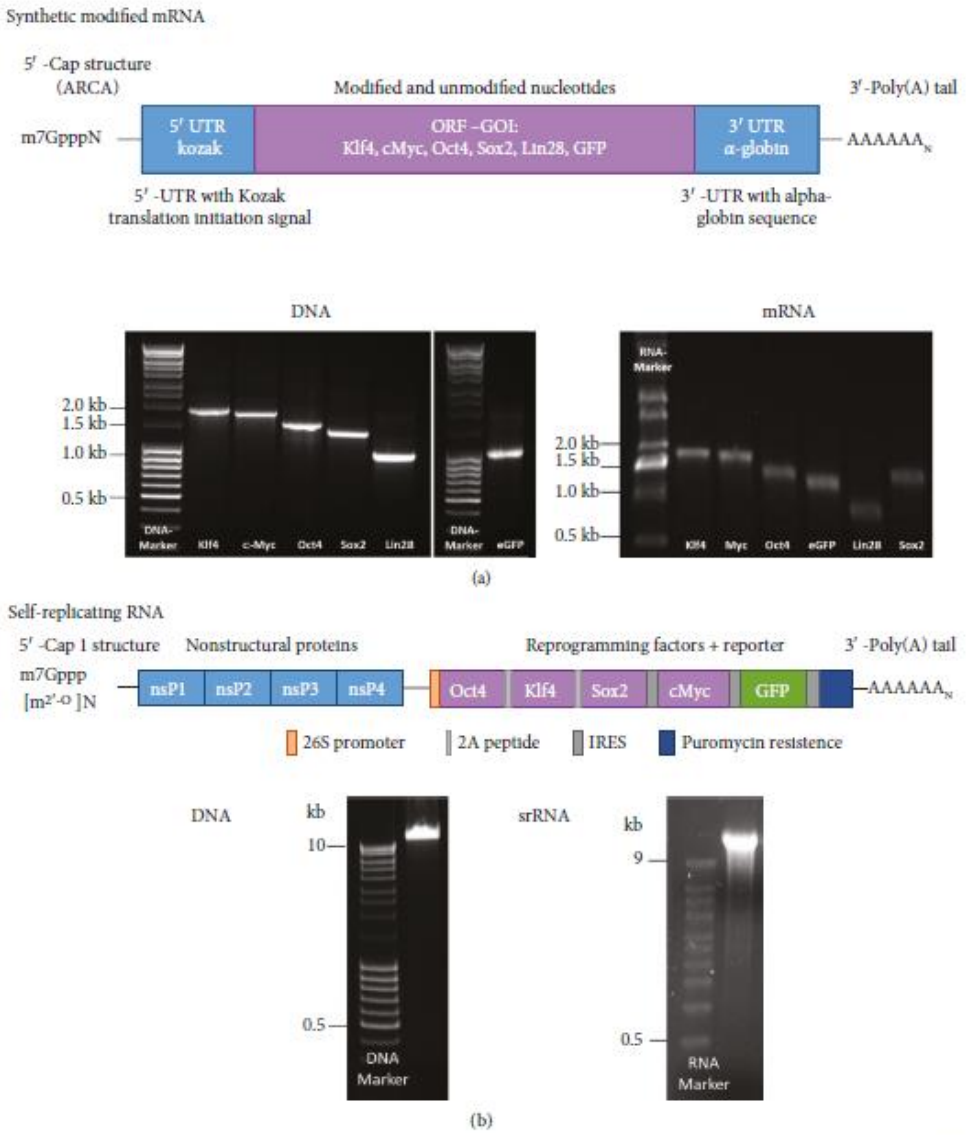

FIGURE 1: Schematic representation of RNA constructs and quality control of DNA templates and synthesized RNA. The srRNA and synthetic modified mRNAs were synthesized using in vitro transcription (IVT). (a) Agarose gel electrophoresis showed the expected lengths for mRNAs encoding the reprogramming factors (Klf4: 1.6 kb; cMyc: 1.5 kb; Oct4: 1.3 kb; Sox2: 1.1 kb; and Lin28: 0.8 kb) and eGFP (0.9 kb). (b) The srRNA contains encoding sequences for the nonstructural proteins (nsP1-4); the reprogramming factors Oct4, Klf4, Sox2, and cMyc; and GFP. Agarose gel electrophoresis showed the expected length (17.7 kb) of linearized DNA and srRNA.

cell culture medium. The wells of 6-well plates were coated with 0.1% gelatin (Sigma-Aldrich Chemie GmbH) in fibroblast culture medium for 4 h at 37°C. For the mRNA-based reprogramming 2.5×10^5 inactivated NuFFs or for the cultivation of primarily picked mRNA-iPSCs 1.5×10^5 inactivated MEFs were seeded per well of a 0.1% gelatin coated 6-well plate and cultivated overnight.

2.4. Reprogramming of Cells Using Synthetic mRNA. To perform synthetic mRNA-mediated reprogramming, 2×10^4 fibroblasts were seeded per well of a 6-well plate, which was preseeded with 2.5×10^5 inactivated NuFF feeder cells in cultivation medium. The next day, Pluriton reprogramming medium (Stemgent, Cambridge, USA) was equilibrated at hypoxia (5% O_2 and 5% CO_2, at 37°C) for 2 h and supplemented with 200 ng/ml B18R interferon inhibitor protein (Thermo Fisher Scientific) to repress synthetic mRNA-mediated immune activation. Then, the mRNA transfection cocktail was prepared with a molar ratio of 3 : 1 : 1 : 1 : 1 : 1 for Oct4, Klf4, cMyc, Sox2, Lin28, and eGFP mRNA,

respectively. For each transfection, 1.2 μg (100 ng/μl) mRNA cocktail and 4 μl Lipofectamine 2000 (Thermo Fisher Scientific) were incubated in 120 μl Opti-MEM™ I Reduced Serum Medium (Opti-MEM, Thermo Fisher Scientific) for 15 min at room temperature (RT) to form lipoplexes. The transfection complexes were then added dropwise to the cells and incubated for 4 h at hypoxia. Afterwards, the complexes were aspirated and 2 ml B18R containing Pluriton medium was added to the transfected fibroblasts and incubated for 24 h at hypoxia. The transfection of cells was performed daily for 20 days. At day 6, the medium was changed to NuFF-conditioned Pluriton medium. NuFF-conditioned medium was obtained by seeding 4×10^6 inactivated NuFFs in T75 cell culture flasks and incubating cells with 25 ml of Pluriton medium supplemented with 4 ng/ml bFGF (PeproTech, Hamburg, Germany). The medium was collected 6x after overnight incubation, pooled, and sterile-filtered using a 0.2 μm filter.

2.5. Reprogramming of Cells Using srRNA. To reprogram NuFFs using synthetic srRNAs, 5×10^4 NuFFs (passage 12) were seeded per well of a 6-well plate coated with 0.1% gelatin and incubated overnight at 37°C in fibroblast culture medium. The next day, cells were incubated at hypoxia with B18R-conditioned Pluriton medium (BcM) for 45-60 min. To generate BcM, 2×10^6 NuFFs were seeded in a T75 cell culture flask and cultivated overnight to reach a confluency of 70%. Then, cells were transfected with 7.5 μg B18R mRNA, 15 μl Lipofectamine 2000, and 7 ml Opti-MEM for 4 h at 37°C. Afterwards, the mRNA complexes were aspirated from the cells and 15 ml fibroblast culture medium was added and incubated overnight. The BcM was collected 3x after overnight incubation, pooled, and sterile-filtered using a 0.2 μm filter. Before application, the collected medium (100% BcM) was diluted 1 : 4 with either Pluriton or E8 medium (resulting in 25% BcM). For the transfection of cells, lipoplexes were generated by the incubation of 1 μg srRNA for 15 min at RT with 3 μl Lipofectamine MessengerMAX (Thermo Fisher Scientific) in 1 ml Opti-MEM. Then, medium was aspirated and Opti-MEM containing lipoplexes were added to the cells. After 4 h of incubation, the transfection medium was discarded and 2 ml Pluriton medium containing 25% BcM was added for further incubation at hypoxia for 24 h. The next day, medium was replaced by 2 ml Pluriton medium containing 25% BcM. To select the srRNA-transfected cells 1 or 2 days posttransfection, when the cells reached confluency, 0.8 μg/ml puromycin (Sigma-Aldrich Chemie GmbH) was added to the medium to eliminate srRNA negative cells. After 2-3 days of puromycin treatment, Pluriton medium containing 25% BcM was changed every 1-2 days, depending on cell density and viability. At day 7, the medium was changed to a stem cell medium (E8) containing 25% BcM/E8. After the first iPSC colonies appeared (days 12-14), the E8 medium without BcM (B18R) was used.

2.6. Cultivation of iPSCs. Primary iPSC colonies were stained with mouse anti-human StainAlive™ SSEA-4 DyLight™ 550 antibody (Stemgent). iPSCs obtained by the synthetic mRNA delivery were picked manually and transferred into one well of a 12-well plate preseeded with 1.5×10^5 iMEF feeder cells. The cultivation was performed in a standard stem cell medium containing DMEM/F12 high glucose supplemented with 20% KnockOut™ Serum Replacement, 1x GlutaMAX, 1x minimum essential medium (MEM) nonessential amino acids (NEAA), 20 ng/ml bFGF (PeproTech), 100 μM 2-mercaptoethanol, and 1x penicillin/streptomycin. After 2-3 passages, iPSCs were adapted to feeder-free conditions by cultivation on surfaces coated with 0.5 mg/cm^2 truncated recombinant human vitronectin (VTN-N) in Essential 8 (E8) medium. Unless otherwise indicated, all cell culture reagents were obtained from Thermo Fisher Scientific. iPSCs obtained by the srRNA delivery were also picked manually and cultivated on 0.5 mg/cm^2 VTN-N coated 12-well plates in E8 medium. Passaging of iPSCs was performed every 5-7 days at a split ratio of 1 : 10 with 0.5 mM EDTA in Dulbecco's phosphate-buffered saline (DPBS) for 5-10 min at RT. After the aspiration of ETDA solution, colonies were detached using the E8 medium supplemented with a 10 μM Y-27632 ROCK inhibitor (Enzo Life Science, Lörrach, Germany) to increase single cell survival.

2.7. Immunocytochemistry. iPSCs (passage 4 to 7) were seeded onto VTN-N-coated glass slides in 12-well plates and cultivated at normoxia until reaching confluency of about 60% in E8 medium. Then, the cells were washed twice with 1 ml DPBS and fixated with 500 μl fixation solution (R&D Systems, Minneapolis, USA) for 15 min at RT. Next, cells were washed again 2 times with 1 ml DPBS and blocked with 500 μl 5% BSA in wash buffer (Permeabilization/Wash Buffer I, R&D Systems) for 2 h at RT or overnight at 4°C. Primary antibodies were incubated overnight at 4°C in 500 μl 1% BSA in wash buffer, according to the manufacturer's instructions. The following unlabelled and fluorescently labelled primary antibodies were used: rabbit anti-human POU5F1 (Oct4) antibody (Sigma-Aldrich Chemie GmbH), rabbit anti-mouse/human Sox2 antibody (Stemgent), mouse anti-human LIN28A monoclonal antibody (6D1F9) (Thermo Fisher Scientific), mouse anti-human PE Nanog antibody (BD Biosciences, Franklin Lakes, USA), mouse anti-human StainAlive™ TRA-1-60 antibody (DyLight™ 488) (Stemgent), and mouse anti-human StainAlive™ SSEA-4 antibody (DyLight™ 550) (Stemgent). After washing 3x for 5 min with 0.5 ml wash buffer, iPSCs stained with unlabelled primary antibodies were incubated with fluorescently labelled secondary antibodies, sheep anti-mouse FITC-labelled IgG (whole molecule) antibody (Sigma-Aldrich Chemie GmbH), and Cy3-labelled goat anti-rabbit IgG cross-adsorbed secondary antibody (Thermo Fisher Scientific) in 500 μl of 1% BSA/wash buffer and incubated for 1-2 h at RT in the dark. Then, the cells were washed twice with 0.5 ml wash buffer and rinsed with 0.5 ml DPBS. After mounting the glass slide with a coverslip and Fluoroshield mounting medium containing DAPI (Abcam, Cambridge, UK), fluorescence images were taken using an Axiovert135 microscope and AxioVision 4.8.2 software (Carl Zeiss, Oberkochen, Germany).

Table 1: List of primer sequences used for qRT-PCR analysis.

Gene	Forward primer ($5' \rightarrow 3'$)	Reverse primer ($5' \rightarrow 3'$)
Pluripotency marker		
GAPDH	TCAACAGCGACACCCACTCC	TGAGGTCCACCACCCTGTTG
Oct4 [3]	AGCGAACCAGTATCGAGAAC	TTACAGAACCACACTCGGAC
Sox2 [3]	AGCTACAGCATGATGCAGGA	GGTCATGGAGTTGTACTGCA
Nanog [3]	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG
Lin28	CTTCTTCTCCGAACCAACC	CAGCCACCTGCAAACTG
E-cadherin	TATACCCTGGTGGTTCAAGC	CACCTGACCCTTGTACGTG
Klf4 [3]	TCTCAAGGCACACCTGCGAA	TAGTGCCTGGTCAGTTCATC
cMyc [3]	ACTCTGAGGAGGAACAAGAA	TGGAGACGTGGCACCTCTT
srRNA-specific marker		
nsP2	TCCACAAAAGCATCTCTCGCCG	TTTGCAACTGCTTCACCCACCC
nsP4	TTTTCAAGCCCCAAGGTCGCAG	TGTTCTGGATCGCTGAAGGCAC

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Oct4: octamer binding transcription factor 4; Sox2: sex-determining region Y-box 2; E-cadherin: epithelial cadherin; Klf4: Krüppel-like factor 4; cMyc: cellular myelocytomatosis; nsP: nonstructural protein.

2.8. Gene Expression Analysis. To analyze the quantitative expression of pluripotency genes and the presence of remaining srRNA, 1×10^6 iPSCs were cultivated in 6-well plates until reaching 80-90% confluency. Total RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad, Munich, Germany). For the qRT-PCR analysis, 300 ng RNA was reverse transcribed into complementary DNA (cDNA) using the iScript Kit (Bio-Rad) according to the manufacturer's instructions. Primer pairs were obtained from Ella Biotech GmbH (Martinsried, Germany) and used at a final concentration of 300 nM. Examined genes as well as used primer sequences are shown in Table 1. Real-time qRT-PCR reactions were run in a CFX Connect Real-Time PCR Detection System with the iQ™ SYBR® Green Supermix (Bio-Rad). PCR amplification of cDNA was performed under the following conditions: 10 min at 95°C for one cycle, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All PCR reactions were performed in triplicates with a total volume of 15 μl/well. Gene expression was normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.9. Trilineage Differentiation of iPSCs. To demonstrate pluripotency, iPSCs (passages 4 to 10) were differentiated using the StemMACS™ Trilineage Differentiation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions into the three embryonal germ layers: ectoderm, mesoderm, and ectoderm. Therefore, iPSCs were seeded with different cell numbers in VTN-N-coated wells of a 12-well plate: 1×10^5 iPSCs for mesoderm differentiation, 2×10^5 iPSCs for endoderm differentiation, and 1.5×10^5 iPSCs for ectoderm differentiation. After 7 days of differentiation, cells were washed with DPBS and detached with 0.04% trypsin/0.03% EDTA and TNS (PromoCell) and centrifuged at 400 x g for 5 min. Afterwards, cells were washed with DPBS and fixated for 10 min at RT in FC Fixation Buffer (R&D Systems, Minneapolis, USA). After washing with DPBS, cells were suspended in wash buffer and stained with 5 μl germ layer-specific fluorescence antibodies in 200 μl of the cell suspension. Mesoderm differentiation was detected using PE-labelled mouse anti-human CD31 antibody (BD Biosciences, Franklin Lakes, USA) and Alexa Fluor 488-labelled anti-human α-smooth muscle actin (α-SMA) antibody (R&D Systems). PE-labelled anti-human α-fetoprotein (AFP) antibody (R&D Systems) and PE-labelled anti-human C-X-C chemokine receptor type 4 (CXCR4) antibody (R&D Systems) were used to detect endodermal differentiation. Ectodermal differentiation was demonstrated using PE-labelled anti-human paired box gene 6 (Pax6) antibody (Miltenyi Biotec) and Alexa Fluor 488-labelled anti-human neuron-specific class III β-tubulin (Tuj1) antibody (BD Biosciences). After incubation for 45 min at RT, cells were washed with 500 μl wash buffer and suspended in 200 μl CellFIX (BD Biosciences).

2.10. Teratoma Formation of iPSCs Using Chicken Embryo Chorioallantoic Membrane (CAM) Assay. The in vivo formation of teratomas and the trilineage differentiation potential of iPSCs were further investigated using the CAM assay. We followed the adapted protocol previously described by Steinle et al. [27]. After a 7-day incubation of the fertilized chicken embryos at 37°C, 2×10^6 iPSCs were suspended in 50 μl E8 medium containing 10 μM Y-27632 ROCK inhibitor, mixed with 50 μl Matrigel® (hECS qualified, Corning) and the suspension was carefully applied onto the CAM. Then, the eggs were sealed and further incubated for 10 days. At day 17, the cell aggregates on CAMs were excised around the application area and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) overnight at 4°C. The specimens were washed with water, dehydrated with an ascending ethanol series, and embedded in paraffin for sectioning at 8 μm thickness. Sections were stained with hematoxylin and eosin (H&E, Morphisto GmbH, Frankfurt, Germany).

2.11. Genomic Stability. The genomic stability of mRNA- and srRNA-derived iPSCs was analyzed by karyotyping. Therefore, fibroblasts and RNA-derived iPSCs (passages 5 to 12)

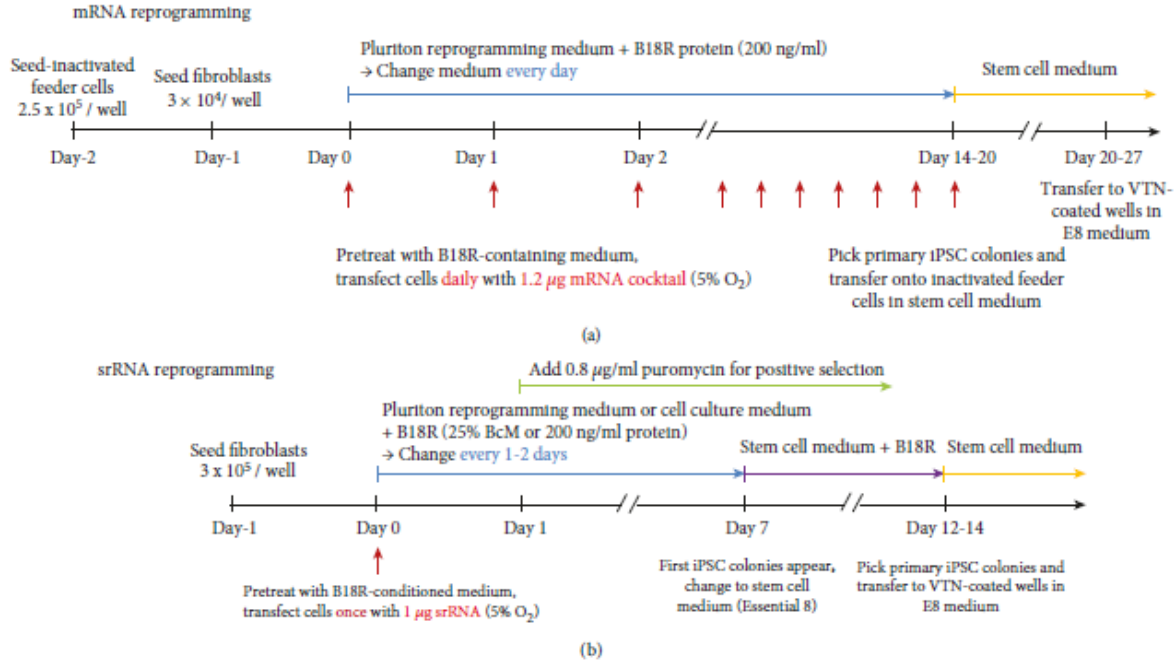

FIGURE 2: RNA-based iPSC reprogramming of fibroblasts: srRNA vs. mRNA. Timeline of (a) mRNA- or (b) srRNA-mediated reprogramming.

were cultivated to reach about 50% confluency. Then, the cells were treated for 1 h with colcemid (Merck), incubated with 0.075 M KCl for 30 min at 37°C, and harvested in fresh fixative containing 25% acetic acid and 75% methanol. Karyotyping was performed on G-banded metaphase chromosomes using standard cytogenetic procedures. After GTG banding, about 15 metaphases were counted and 5 of them were structurally evaluated by G banding (banding quality of 400-500 bp).

2.12. Statistical Analysis. Data are shown as mean + standard deviation (SD) or standard error of the mean (SEM). Paired *t*-test or one-way analysis of variance (ANOVA) for repeated measurements followed by Bonferroni's multiple comparison test was performed to compare the means. Statistical analyses were performed double tailed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA). Differences of $p < 0.05$ were considered significant.

3. Results

3.1. RNA Synthesis. The first step for the successful reprograming of cells is the production of high-quality synthetic RNA molecules encoding the reprogramming factors. To perform the mRNA-based reprogramming, synthetic modified mRNAs containing each gene of interest (GOI) and UTRs were produced (Figure 1(a)). The mRNAs were generated using the modified nucleotides 5mCTP and Pseudo-UTP to completely replace cytosine and uridine. Furthermore, a 5′-cap structure (ARCA) and a 3′-end poly(A_{120}) tail were added. After purification of the PCR product and the IVT, the products were analyzed using agarose gel electrophoresis to determine the specific length and purity. The detected bands showed the expected lengths of DNA templates and mRNAs (Klf4: 1.6 kb; cMyc: 1.5 kb; Oct4: 1.3 kb; Sox2: 1.1 kb; Lin28: 0.8 kb; and GFP: 0.9 kb).

An IRES-GFP encoding sequence was inserted into the plasmid containing nsP1 to nsP4 sequences of the VEE virus and the reprogramming factors [22] to enable the verification of transfection efficiency and the successful translation of the transfected srRNA in the cells during the reprogramming procedure (Figure 1(b)). After the linearization of the plasmid and the generation of srRNA, agarose gel electrophoresis was performed and bands at the expected length of about 17.7 kb were detected.

3.2. srRNA-Based Reprogramming Results in More Efficient and Straightforward iPSC Generation Compared to mRNA-Based Reprogramming. The reprogramming of fibroblasts with synthetic mRNAs required the daily transfection of the cells to maintain a constant level of reprogramming factor expression over 1 to 2 weeks. Thus, the repeated transfection of cells can cause stress and harm the cell viability, which in turn can decrease the reprogramming efficiency. Using the srRNA-based reprogramming, only one transfection was sufficient to maintain the expression level of reprogramming factors to generate iPSCs. The schedules for performing the reprogramming of cells with synthetic mRNA or srRNA are shown in Figure 2. Both RNA-based reprogramming

approaches were performed at hypoxic conditions (5% O_2) to improve efficiency [28].

Already one day (D1) after the transfection of fibroblasts with the mRNA cocktail, which contained besides the reprogramming factor-encoding mRNAs also eGFP mRNA, a strong eGFP expression was detected (Figure 3(a)), which indicated a rapid translation of synthetic mRNA and an efficient delivery of mRNA into the cells. After 24 h, approximately 65% of the cells were eGFP positive and after the second transfection, about 90% of the cells expressed eGFP. The high eGFP expression could be sustained constantly over the period of the daily transfections (D1-D14). The first primary iPSC colonies appeared after 14-19 days, and the treatment with B18R protein was discontinued. After 14 days (D14), the cell morphology changed to an embryonic stem cell-like cell type and the eGFP expression in reprogrammed cells was diminished in tightly packed colonies, while the surrounding fibroblasts still strongly expressed eGFP. In the following days, iPSC colonies were expanded in the stem cell medium (D16, D19) without B18R to generate stable colonies. Live cell staining was performed with DL550-labelled SSEA-4 and DL488-labelled TRA-1-60 antibodies, 3 days after the last transfection. Cells exhibiting both markers were then picked for further cultivation on inactivated MEF feeder cells to support iPSC growth (Figure 3(b)). After 19-21 days of reprogramming, 20-25 iPSC colonies per well were obtained when 3×10^4 fibroblasts were seeded per well of a 6-well plate (on 2.5×10^5 inactivated NuFF feeders), which corresponds to a reprogramming efficiency of 0.8%.

Since in the synthetic mRNA-based reprogramming approach, the eGFP mRNA was cotransfected alongside with the reprogramming factor-encoding mRNAs, it can be not ensured that all cells are transfected with the same amount of eGFP mRNA or reprogramming factor-encoding mRNAs. In contrast, the GFP expression in srRNA-based reprogramming is directly comparable with the transfection efficiency, since all exogenously delivered RNA sequences are located on the same RNA construct.

Fluorescence microscopy revealed that 1 day (D1) after the transfection of 3×10^5 cells with srRNA, only a few (1-3%) of the seeded cells were mostly expressing low levels of GFP. At the second day posttransfection (D2), the number of GFP-expressing cells increased to approximately 15% (Figure 3(a)). The increase of GFP-positive cells could be explained due to cell division and transfer of RNA to daughter cells. Furthermore, the delivered srRNA amount in the cells could be increased, which can lead to the detection of GFP in previously seemingly negative cells. After reaching confluency (D2-D3), puromycin was added for 3-7 days to the medium for positive selection of srRNA-containing cells. In general, after 2-3 days of puromycin treatment, all cells without srRNA died and only GFP-expressing cells were visible (Figure 3(a), D5). After 7 days of transfection, the morphology of most of the fibroblasts changed to an epithelial-like cell shape and the medium was changed to a stem cell medium (E8) containing 25% BcM. The daily microscopic monitoring of GFP expression in the cells showed that the change of the B18R-containing medium every two days is sufficient to maintain the srRNA in the cells. A daily medium change was performed when an increased number of dead cells were observed, e.g., after the puromycin treatment. The first colonies with typical iPSC morphology and positive SSEA-4 staining were obtained after 12 days of reprogramming (D12) (Figures 3(a) and 3(b)), while little or no GFP expression was detected in densely packed reprogrammed cells. Further cultivation and withdrawal of B18R resulted in the emergence of multiple iPSC colonies, which grew together until days 19 to 20 (D19-20) and covered most of the well surface. Thus, after 20 days of treatment, compared to synthetic mRNA-based reprogramming more iPSCs could be obtained after a single transfection with srRNA (Figure 3(a), D20). However, exact reprogramming efficiency cannot be determined, since at that time point, it cannot be distinguished whether the iPSC colonies are derived from a single parenteral cell or from their daughter cells.

3.3. Expression of Pluripotent Stem Cell-Specific Markers. The expression of pluripotent stem cell-specific markers was analyzed using specific antibodies and fluorescence microscopy. iPSCs obtained by both srRNA- and mRNA-based reprogramming showed a strong expression of Nanog, Oct4, SSEA-4, TRA-1-60, and Lin28 (Figure 4(a)). Additionally, Nanog and TRA-1-60 expression in iPSCs was analyzed by flow cytometry (Figure 4(b)). Both proteins were highly expressed in almost all iPSCs generated by mRNA- or srRNA-based reprogramming. In srRNA-iPSCs, $90 \pm 4\%$ of the cells expressed Nanog and $98 \pm 1\%$ of the cells were positive for TRA-1-60. In mRNA-iPSCs, $88 \pm 3\%$ of the cells were Nanog positive and $92 \pm 5\%$ of cells expressed TRA-1-60. Furthermore, the expression of Oct4, Sox2, Nanog, Lin28, and E-cadherin was analyzed using qRT-PCR (Figure 4(c)). A significantly higher expression of Oct4, Sox2, Nanog, Lin28, and E-cadherin was detected in iPSCs generated by mRNA as well as srRNA compared to initial fibroblasts. However, the expression of Oct4 and Sox2 in mRNA-iPSCs was higher compared to srRNA-iPSCs (Oct4: 1956-fold versus 341-fold, Sox2: 1727-fold versus 163-fold). In contrast, E-cadherin expression in srRNA-iPSCs was higher than in mRNA-iPSCs (62453-fold versus 2964-fold). The expression of Nanog and Lin28 in srRNA-iPSCs was similar to the expression in mRNA-iPSCs.

3.4. In Vitro and In Vivo Differentiation Potential of Obtained iPSCs

3.4.1. In Vitro Trilineage Differentiation. To analyze the ability of iPSCs to differentiate into all three primary germ layers, mesoderm, endoderm and ectoderm, a directed 7-day differentiation protocol was performed. The obtained cells were examined by specific antibody staining and flow cytometry. After 4 to 5 days of differentiation, cells exhibiting the typical morphological structures of the mesodermal, endodermal, and ectodermal lineages were detected (Figure 5). The mesoderm induction led to the generation of elongated endothelial-like cells as well as smooth muscle-like cells. The endoderm differentiation resulted in the detection of cells similar to early hepatocyte-like cells. Cells arranged in

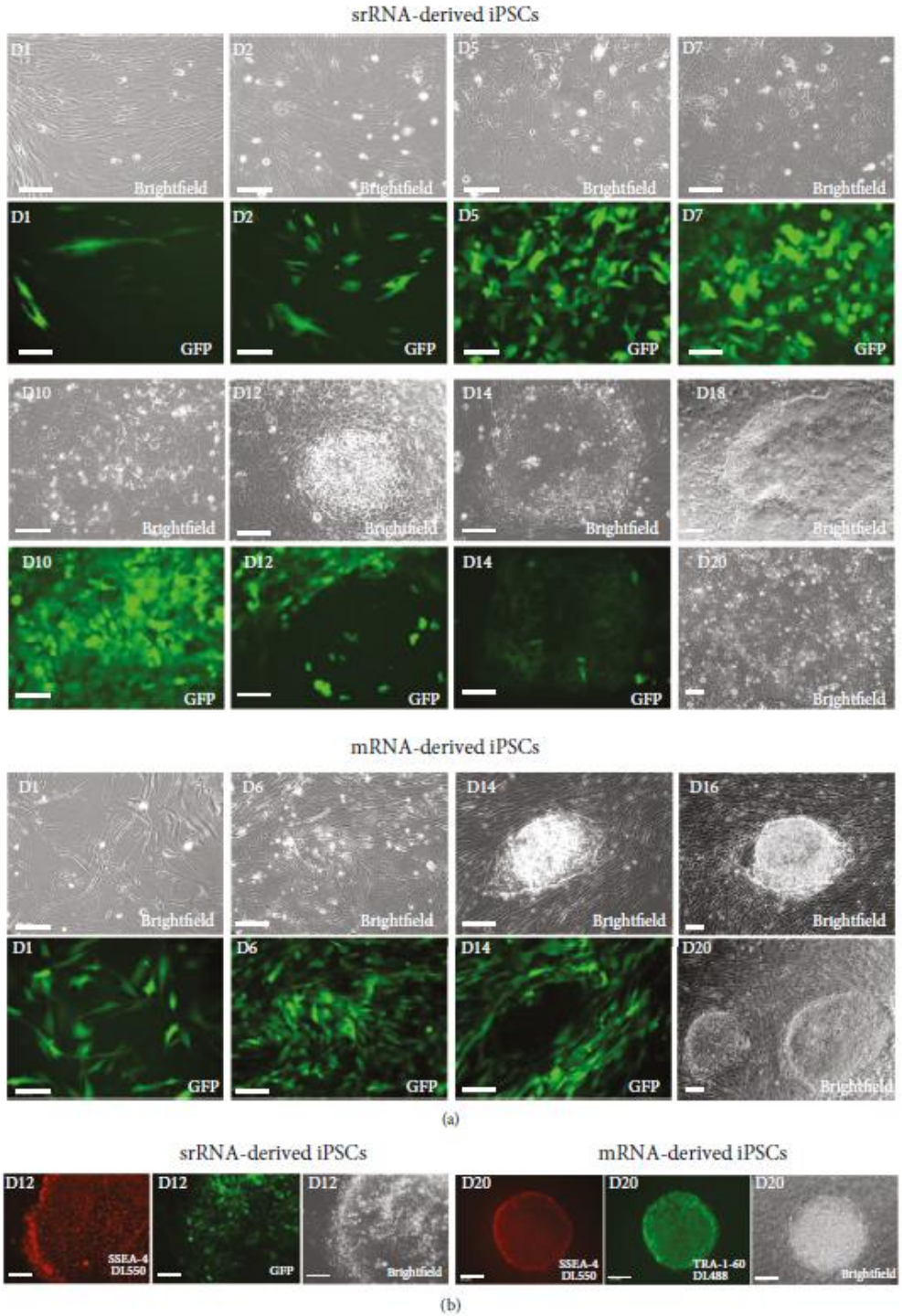

FIGURE 3: RNA-based iPSC reprogramming of fibroblasts: srRNA vs. mRNA. (a) Emerging of iPSCs over time and detection of GFP-expressing cells during the reprogramming period. Phase contrast and fluorescence microscopic pictures are shown. (b) Live-antibody staining of obtained iPSC colonies. The iPSCs obtained after 20 days of reprogramming by mRNAs were positively stained with DL550 SSEA-4 and DL488 TRA-1-60 antibodies. iPSCs obtained after 12 days of reprogramming by srRNA were positive for SSEA-4 and showed only partial GFP expression within the iPSC colony. Scale bars represent 100 μm.

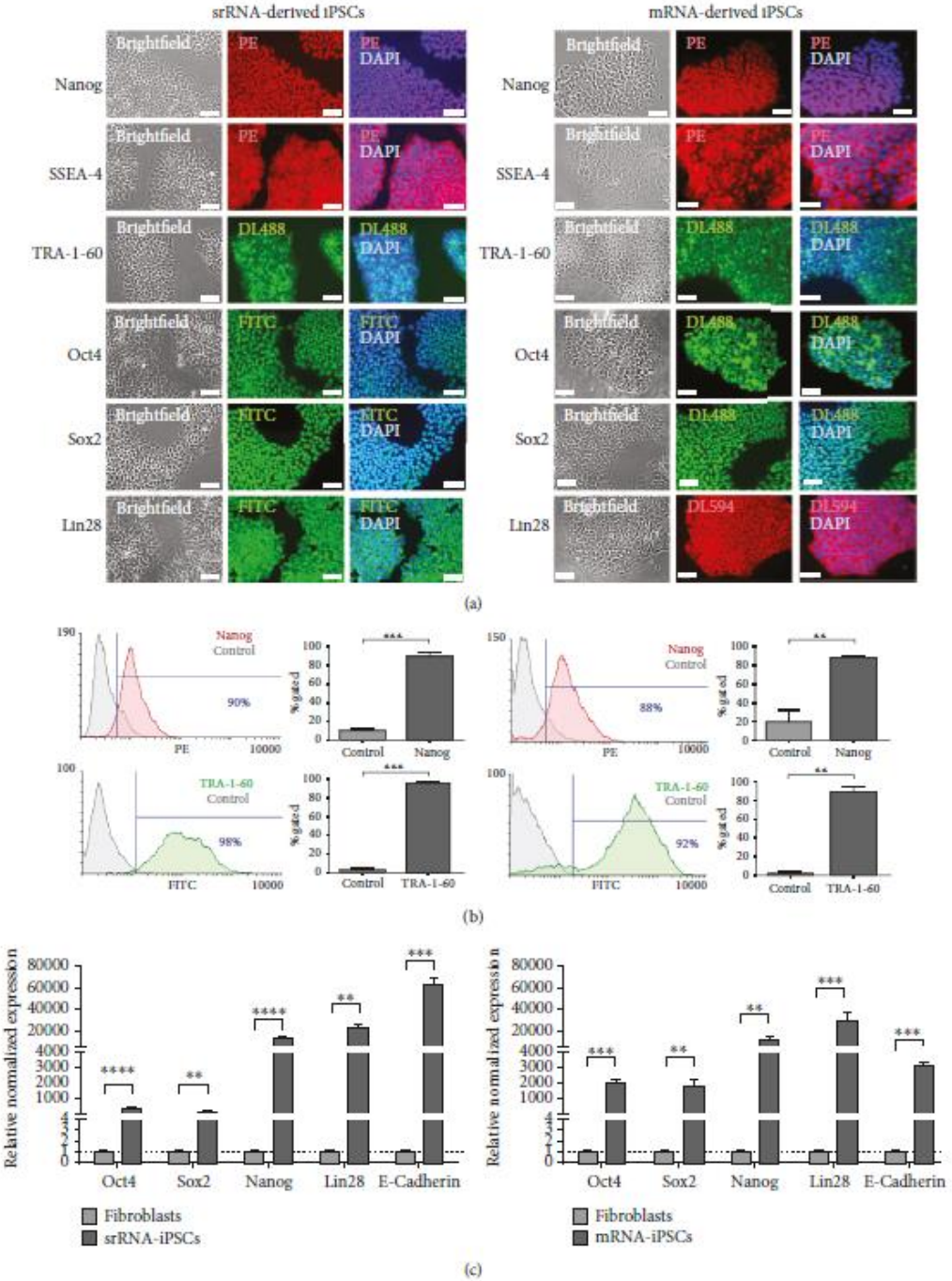

FIGURE 4: Analysis of pluripotency markers in iPSCs generated by mRNA or srRNA delivery. (a) iPSCs stained with antibodies specific for pluripotent stem cell markers (Nanog, SSEA-4, TRA-1-60, Oct4, Sox2, and Lin28) showed a strong protein expression ($n = 3$). Scale bars represent 50 μm. (b) Analysis of Nanog and TRA-1-60 expression by flow cytometry. Data are shown as mean + SD ($n = 3$). Statistical differences were determined using a paired t-test (**$p < 0.01$, ***$p < 0.001$). (c) Analysis of Oct4, Sox2, Nanog, Lin28, and E-cadherin expression in iPSCs using qRT-PCR. mRNA levels were normalized to GAPDH mRNA levels, and the expression is presented relative to the expression levels in fibroblasts. Data are shown as mean + SEM ($n = 3$). Statistical differences were determined using a paired t-test (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

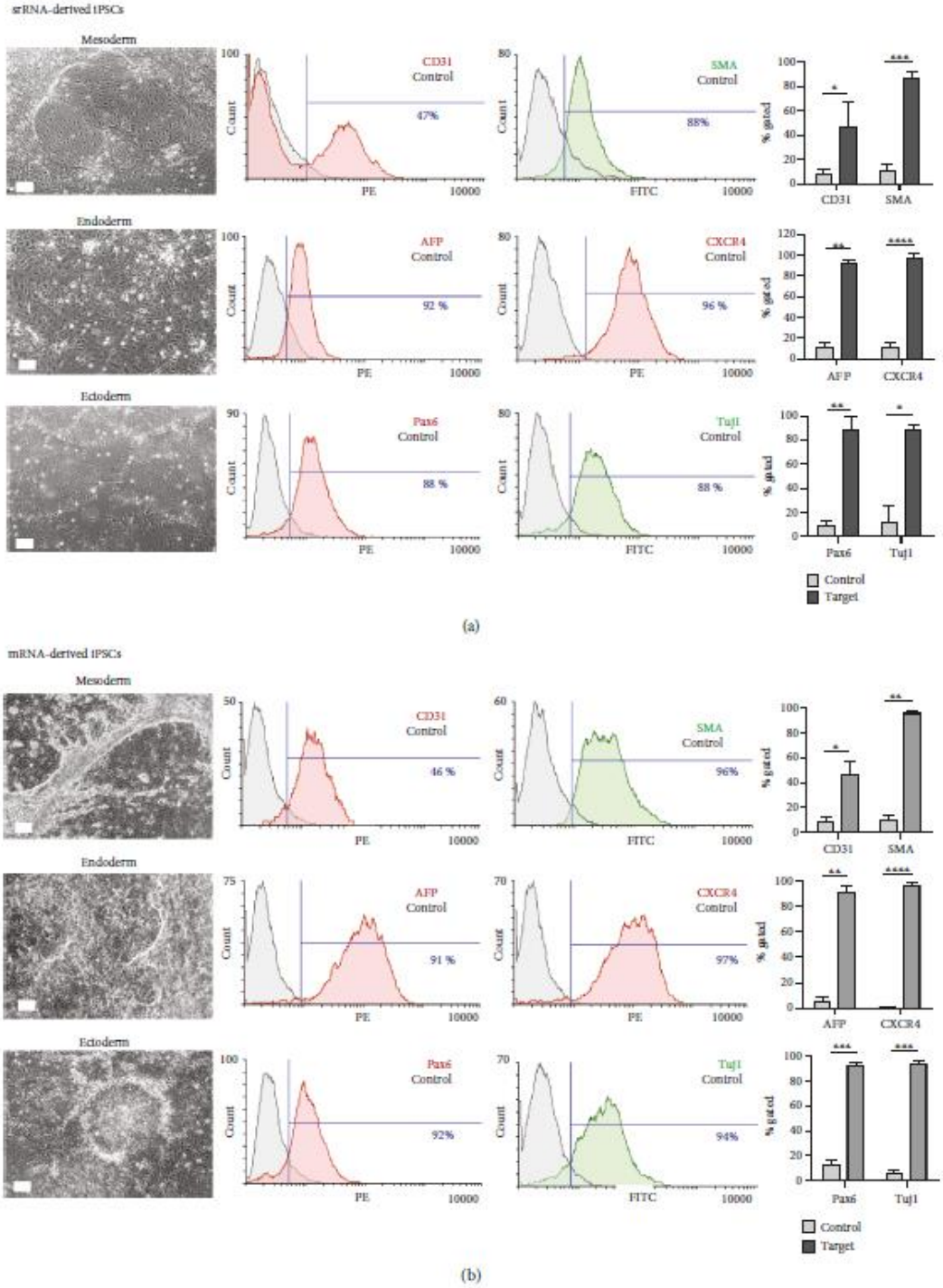

FIGURE 5: In vitro differentiation capacity of mRNA- or srRNA-iPSCs into the three germ layers: mesoderm, endoderm, and ectoderm. Microscopic images were taken 4 to 5 days after the differentiation of iPSCs, and cells with different morphologies were obtained depending on specific lineage differentiation. After 7 days of differentiation, flow cytometry analyses were performed with two specific antibodies for each lineage (mesoderm: CD31 and α-SMA; endoderm: AFP and CXCR4; ectoderm: Pax6 and Tuj1) and compared to the untreated control. Scale bars represent 100 μm. The results are shown as mean + SD ($n = 3$). Statistical differences were determined using a paired t-test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$).

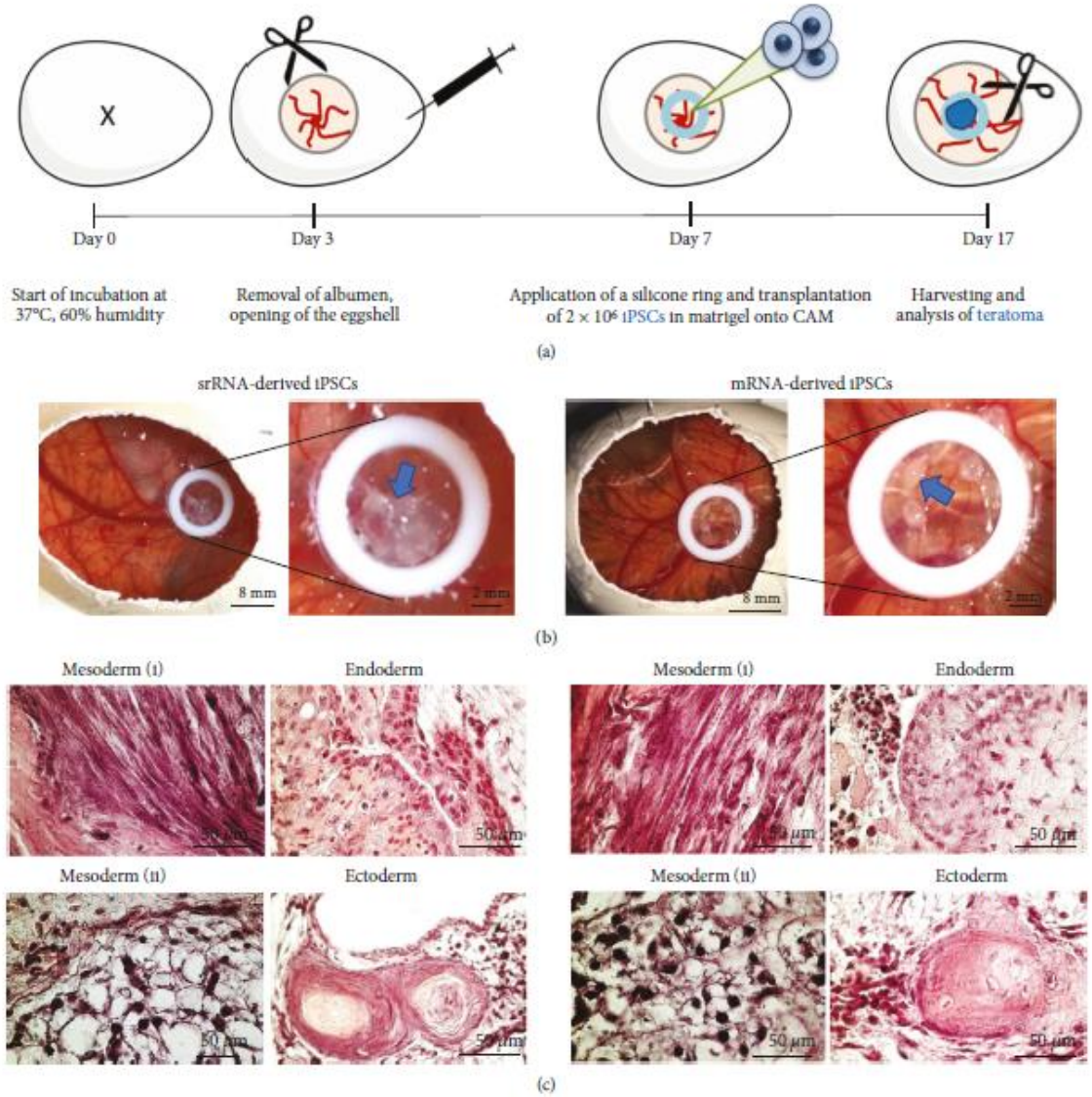

FIGURE 6: Analysis of teratoma formation after the application of mRNA- or srRNA-iPSCs onto the chicken embryo chorioallantoic membrane (CAM). (a) Schematic representation of the teratoma formation analysis on CAM. On day 7 of incubation, 2×10^6 iPSCs were applied into the inner area of a silicone ring placed on CAM. (b) After 10 days (day 17), teratoma were formed on the CAM (indicated by arrows), excised, and embedded in paraffin. (c) Representative microscopic pictures of H&E-stained teratoma sections show the in vivo differentiation of iPSCs into cells of all three germ layers: mesoderm (i: striated musde fibers, ii: adipocyte tissue), endoderm (glandular epithelium), and ectoderm (squamous epithelium).

neural rosettes were detected after the endoderm induction of iPSCs. Flow cytometric analysis of the cells obtained from srRNA-iPSCs (Figure 5(a)) revealed that 47 ± 20% of the cells were CD31 positive and 88 ± 5% SMA positive (mesoderm), 92 ± 3% were AFP positive and 96 ± 4% CXCR4 positive (endoderm), and 88 ± 11% of the cells were Pax6 positive and 88 ± 4% tubulin (Tuj1) positive (ectoderm). Cells derived from mRNA-iPSCs (Figure 5(b)) showed comparable results as srRNA-iPSC-derived cells: 46 ± 11% of the cells were CD31 positive and 96 ± 2% SMA positive (mesoderm), 91 ± 5% were AFP positive and 97 ± 1% CXCR4 positive (endoderm), and 92 ± 2% of the cells were Pax6 positive and 94 ± 3% Tuj1 positive (ectoderm).

3.4.2. In Vivo Teratoma Formation. The potential of iPSCs to differentiate into cell types of all the three germ layers was further analyzed in vivo. Therefore, iPSCs were applied 7 days after the incubation of fertilized eggs onto the CAM of chicken embryos (Figure 6(a)). After 10 days, small tumor-like cell masses were formed within the application area

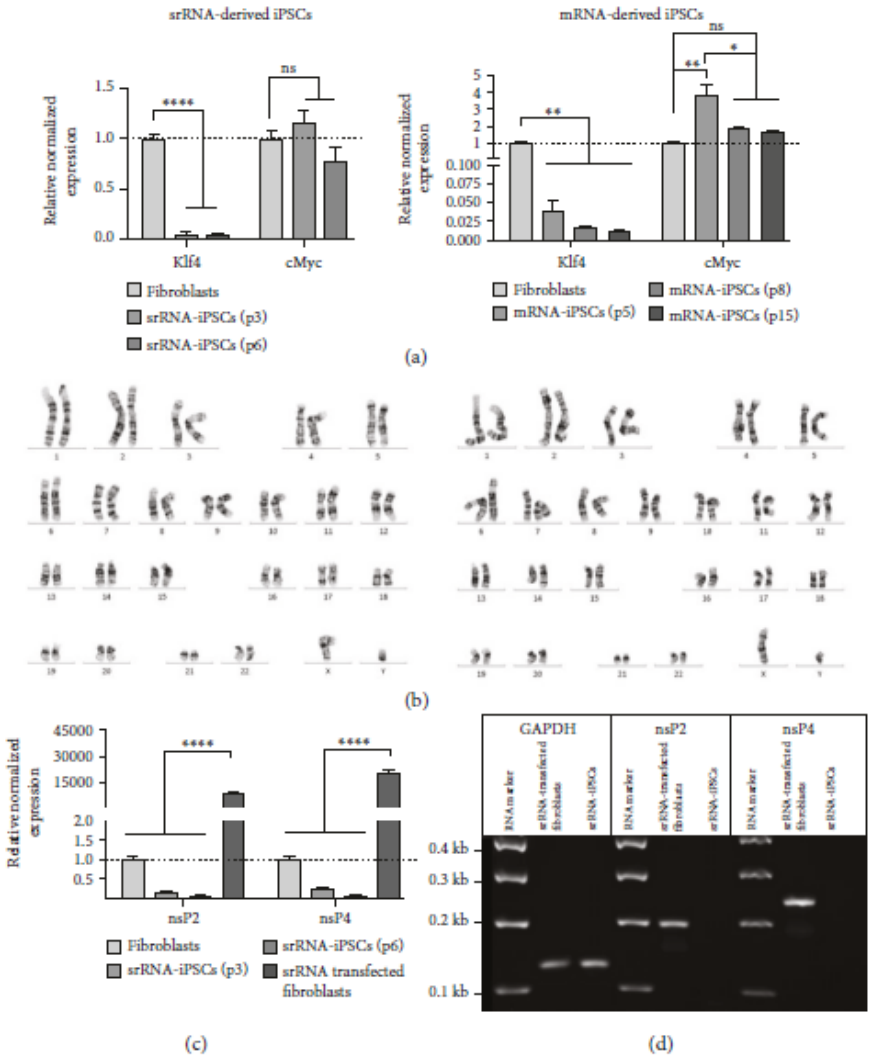

FIGURE 7: Analysis of genomic abnormalities, expression of prooncogenic factors, and the presence of srRNA residues in srRNA-iPSCs. (a) Detection of Klf4 and cMyc oncogene expression in iPSCs generated by srRNA and mRNA delivery using qRT-PCR. Results are shown as mean + SEM ($n = 3$). (b) Representative karyograms of iPSCs generated using srRNA or mRNA. (c) Detection of residual srRNA in obtained srRNA-iPSCs by performing qRT-PCR using nsP2- and nsP4-specific primers. Results are shown as mean + SEM ($n = 3$). (d) Analysis of specific PCR product lengths using 1% agarose gel electrophoresis. nsP2: 192 bases; nsP4: 238 bases; GAPDH: 126 bases. Statistical differences were determined using one-way ANOVA followed by Bonferroni's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$).

(silicone ring) as shown in Figure 6(b). H&E staining was performed with sections of 7 mm to detect the generated tissue structures. The mesodermal differentiation was confirmed by the formation of striated muscle fibers and adipocyte tissue (Figure 6(c)). Endodermal differentiation was demonstrated by the generation of the glandular epithelium and the ectodermal lineage differentiation was shown by the presence of the squamous epithelium.

3.5. Analysis of Genomic Abnormalities and Presence of srRNA Residues in srRNA-iPSCs. To examine possible genetic alterations in the iPSCs due to the reprogramming procedure, karyotyping was performed. The continued elevated expression of the oncogenes Klf4 and cMyc are associated with an increased tumorigenesis [29]; therefore, the expression of Klf4 and cMyc was also determined after the reprogramming using qRT-PCR. As shown in Figure 7(a), Klf4 expression was even significantly decreased in srRNA- and mRNA-iPSCs (different passages) compared to initial fibroblasts. In srRNA-iPSCs, already at passage 3, the expression of cMyc was not significantly different from the expression in the initial fibroblasts. However, in mRNA-iPSCs, the

expression of cMyc was still elevated in the 5th passage, but decreased after further cultivation (passages 8 and 15) to the levels as in the initial fibroblasts.

The genetic stability/integrity of iPSCs was tested by the karyotyping of iPSCs and the initial fibroblasts. No changes regarding morphological structure such as size, centromere position, and band patterning were determined after the reprogramming procedure (Figure 7(b)). Both srRNA- and mRNA-iPSCs showed a normal male karyotype (46, XY), free of any discernible abnormalities.

After the appearance of the first srRNA-iPSC colonies, B18R was withdrawn from the medium to eliminate the reprogramming srRNA from the cells. To prove that the iPSCs are not containing residual srRNA, qRT-PCR analyses were performed using nsP1- and nsP4-specific primers. As a positive control, fibroblasts were transfected with srRNA; after 48 h, both the presence of nsP2 and the presence of nsP4 were demonstrated (Figure 7(c)). As expected, a 8826-fold higher nsP2 expression and a 20318-fold higher nsP4 expression were detected in srRNA-transfected fibroblasts, compared to those in untransfected fibroblasts. In srRNA-iPSCs (passages 3 and 6), no residual srRNA expression was measured compared to fibroblasts. In addition, PCR products were analyzed using 1% agarose gel electrophoresis (Figure 7(d)) and amplicons with expected lengths for GAPDH (126 bases), nsP2 (192 bases), and nsP4 (238 bases) were detected. Solely in positive control samples, nsP2- and nsP4-specific PCR products were visible.

4. Discussion

In recent years, the generation of patient-specific iPSCs from adult somatic cells has become a powerful tool in the field of tissue engineering and disease modeling and has led to great advances in regenerative medicine applications. In this study, we compared synthetic mRNA- and srRNA-based methods to generate footprint-free iPSCs from human fibroblasts regarding transfection and reprogramming efficiency, as well as overall workload and costs. Therefore, fibroblasts were reprogrammed either by multiple daily transfections with an mRNA cocktail consisting of 5 different reprogramming factor-encoding mRNAs and an eGFP mRNA [6], or by a single transfection with srRNA, which enables the sustained expression of reprogramming factors [22]. To monitor the transfection efficiency and the translation of srRNA, an additional sequence encoding an IRES [30] and GFP was added to allow the cap-independent initiation of translation. During the reprogramming process, the treatment of cells with B18R suppresses the cellular type I interferon immune response to the srRNA [31] and prevents the premature degradation of the srRNA. After the reprogramming of cells, the withdrawal of B18R from the medium leads to the degradation and elimination of srRNA [22].

The obtained iPSCs showed the expression of typical pluripotency markers and the potential to differentiate into the cells of the three germ layers in vitro and in vivo. The generated iPSCs showed no genomic abnormalities, and no residual srRNA could be found in the iPSCs generated by srRNA. However, the comparison of both methods clearly revealed that the srRNA-based reprogramming is more efficient and convenient than the synthetic mRNA-based method (Table 2). The costs for the synthesis and purification of one microgram of mRNA or srRNA are comparable (approximately 2.5€/1 μg). A key advantage of this method is the about 24 times lower production costs due to the one-shot transfection of cells with 1 μg srRNA (2.5€) compared to the required daily transfection of cells with 1.2 μg mRNA cocktail containing 6 different nucleoside-modified mRNAs for about 20 days (60€). Furthermore, using the srRNA, iPSCs were obtained earlier than after the mRNA transfection. The srRNA also contains an open reading frame for puromycin resistance to enable the positive selection of srRNA-containing cells. Due to the positive selection with puromycin during the early time point of reprogramming, only cells containing the srRNA could survive; therefore, the reprogramming efficiency was increased.

In this work, an IRES-GFP sequence was added to the reprogramming factor-encoding srRNA, which allowed the direct control of successful transfection and translation of the srRNA during the reprogramming of cells. Additionally, the absence of B18R in the medium led to the decrease of fluorescence intensity, which indicated the degradation of srRNA. Through daily monitoring of GFP expression, we were able to adjust the medium replacement schedule of the B18R-containing medium to a 2-day rhythm. In the case of the synthetic mRNA-based application, the eGFP expression was also used to monitor the transfection efficiency and the translation of synthetic mRNAs in the cells. Therefore, the mRNA cocktail contained eGFP-encoding mRNA, which was simultaneously transfected into the cells with the reprogramming factor-encoding mRNAs. But, compared to the srRNA-containing GFP encoding sequence, the monitoring of synthetic mRNA-transfected cells was less precise, since the delivery of each single mRNA amount can differ in each cell and the consistent supply of all 6 individual mRNAs into the same cells cannot be ensured every day in the same manner. Furthermore, prior to starting with the reprogramming, the transfectability of somatic cells can be analyzed using the srRNA-containing GFP encoding sequence. Thereby, the required transfection reagent and the duration of transfection can be determined for different types of cells.

The use of RNA-based molecules for the expression of transcription factors in the cells is integration free. The delivery of synthetic mRNA into the cells leads to the transient expression of desired proteins for commonly about 2-3 days in the cells [6, 9, 10]. After the uptake of srRNA into the cells, the expression of nonstructural proteins (nsP1-nsP4) enables repeated replication of RNA in the cytosol and thereby a prolonged protein expression. The degradation of srRNA in the obtained iPSCs can be proven by qRT-PCR using nsP2- and nsP4-specific primers. In our studies, in early passage iPSCs (passages 3-5), no residual srRNA could be detected after the reprogramming. Furthermore, the decrease of cMyc and Klf4 expression also indicates the degradation of the srRNA construct in the iPSCs. These proteins are required during the reprogramming procedure; however, afterwards, their expression should be downregulated since the permanent overexpression of these proteins is linked with an increased

Table 2: Advantages and disadvantages of srRNA- or mRNA-based reprogramming of fibroblasts to obtain footprint-free iPSCs.

	srRNA reprogramming	mRNA reprogramming
Advantages of srRNA		
RNA generation	No modified nucleotides*	Modified nucleotides*
	Identical RNA molecules	Multiple mRNAs
RNA transfection	Once (1 μg)	Daily (1.2 μg) for 14-20 days
Transfection efficiency	GFP reporter on the same srRNA construct	Additional transfection with GFP mRNA for monitoring
First iPSCs emerged after	12 days	14 days
Reprogramming efficiency	Very high efficiency after positive selection	High efficiency (0.8%)
Reprogramming costs of RNA**	~2.5€ (1 μg srRNA once)	~60€ (~2.5€/1 μg mRNA for 20 days)
Transgene-free iPSCs	Yes (total elimination of srRNA was demonstrated after p3)	Yes (no integration of mRNA into the host genome)
Disadvantages of srRNA		
RNA modification	Posttranscriptional enzymatic 5′-capping and 3′-polyadenylation	Cotranscriptional 5′-capping and 3′-polyadenylation
Immune system activation counteraction	Interferon inhibitor B18R required***	Interferon inhibitor B18R required***
Transgene expression	Check for residual srRNA expression (VEE virus-derived RNA construct)	Natural degradation of mRNA in cells after 2-3 days

*Modified nucleotides (e.g., 5mCTP, Pseudo-UTP, and N1-methylpseudo-UTP) can improve the translation of proteins but are also expensive to purchase. **Costs for the synthesis and purification of RNA (with commercially available kits, without plasmid generation) needed for one reprogramming experiment. ***B18R-containing medium (BcM) can vary from batch to batch; therefore, the functionality of B18R has to be assessed before use, for example, by the determination of the positive transfection of fibroblasts with GFP mRNA with 25% BcM compared to the transfection without B18R. To date, there are no commercially available antibodies against B18R for specific analysis of the B18R content. For a constant quality, B18R can also be purchased as a recombinant protein, but this is much more expensive than the use of BcM.

tumorigenesis [29, 32] and can be found in different types of cancer [33, 34].

To reprogram somatic cells with synthetic RNAs, the use of interferon inhibitor B18R is required [6, 21, 22]. Thus, the medium can be supplemented either with 200 μg/ml recombinant B18R protein or a conditioned medium containing B18R (BcM) can be used. The use of BcM instead of B18R recombinant protein reduces the costs, and the conditioned medium can further provide additional proteins, e.g., fibroblast growth factors (FGF-2), which can support the reprogramming procedure. This can be beneficial when serum-free medium, e.g., E8 stem cell medium, is used. The ratio of BcM to culture or reprogramming medium is adjustable. In this study, medium containing fresh 25% BcM resulted in successful reprogramming, but the BcM amount can be increased to, for example, 50%, if a weak GFP signal is detected in transfected cells.

In this study, for a better comparability with the mRNA reprogramming approach, Pluriton reprogramming medium was used during the srRNA-based reprogramming. However, this is not explicitly required for successful and efficient reprogramming with srRNA. Yoshioka et al. generated iPSCs by using fibroblast culture medium instead of Pluriton [22]. Furthermore, it is also possible to substitute the only animal component FBS in the cell culture medium with human serum or platelet lysate, to generate iPSCs under xeno-free conditions from different cell types [35].

In this work, the reprogramming of newborn human fibroblasts was performed. However, Yoshioka and Dowdy also successfully generated iPSCs from adult human fibroblasts of 54- to 77-year-old healthy donors and from a 24-year-old cardiomyopathy patient using srRNA encoding the reprogramming factors Oct4, Sox2, Klf4, Glis1, and cMyc [24]. In our recent study, we could also demonstrate the successful reprogramming of human adult jaw periosteal cells into iPSCs using srRNA encoding Oct4, Klf4, Sox2, and cMyc [35]. These studies demonstrated that also adult somatic cells can be reprogrammed using srRNA. Another interesting source of adult cells for reprogramming are blood-derived cells, such as peripheral blood mononuclear cells (PBMCs) or endothelial progenitor cells (EPCs), which can be obtained by minimally invasive blood collection from healthy donors or patients. Poleganov et al. reported the successful reprogramming of human blood-outgrowth EPCs using an mRNA-based approach [36]. Therefore, the use of blood-derived cells to generate iPSCs by srRNA-based reprogramming would represent another promising cell source for adult cells.

In previous studies, a reprogramming efficiency of 4.4% was achieved after the reprogramming of BJ fibroblasts with synthetic modified mRNAs [6, 20]. In our studies, we obtained a reprogramming efficiency of 0.8% by applying the same protocol to NuFFs, this was probably caused by lab-to-lab or material variabilities. Furthermore, the use of adult fibroblasts or fibroblasts from diseased patients as well as the use of other somatic cells can result in other reprogramming efficiencies [6, 24, 37]. Therefore, reprogramming protocols should be tested and optimized for each cell type [7]. Moreover, the reprogramming efficiency of the mRNA-based approach depends on the initial cell density [20, 21],

and it is promoted by high cell cycling rates [38]. By using mitotically arrested feeder cells, lower target cell counts can be used for initial seeding, thereby promoting the reprogramming process. However, feeder-free protocols are beneficial to circumvent additional variabilities and the risk of contamination with xenogeneic material [20].

Recently, Kogut et al. established another RNA-based feeder-free protocol for the reprogramming of neonatal, adult, and senescent human fibroblasts [21]. Here, a different set of synthetic modified mRNAs encoding M3O (OCT4 fused with the MyoD transactivation domain), Sox2, Klf4, cMyc, Lin28A, Nanog, and miRNA-367/302s were applied. Using this method and only 500 primary cell neonatal fibroblasts, the reprogramming efficiency was highly increased and the RNA transfection was reduced to every 2 days with 0.6 μg mRNA cocktail. These results would suggest that the reprogramming efficiency of the srRNA-based reprogramming method could be further improved by the addition of reprogramming enhancers/modulators, such as valproic acid, TGF-β inhibitors, vitamin C, butyrate, or miRNAs [7, 8, 21, 39–42].

5. Conclusions

The footprint-free iPSCs obtained by srRNA- and synthetic mRNA-based reprogramming are promising cells to generate desired cell types for clinical application. However, the single-shot application of srRNA allowed a more time- and cost-efficient generation of unlimited numbers of iPSCs without any genomic integration compared to the daily transfection of multiple reprogramming factor-encoding mRNAs. We believe that this method holds great promise for the integration-free reprogramming of any somatic cells, due the comfortable experimental setup with only one srRNA administration, direct GFP monitoring, and higher reprogramming efficiency. The highly efficient generation of footprint-free iPSCs and the efficient differentiation into desired cells will increase the potential of this technology in translational research, therapy, and disease modeling.

Data Availability

The data used to support the findings of this study are included within the article and are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) through AV 133/7-1. Furthermore, we acknowledge the support by DFG and the Open Access Publishing Fund of the Eberhard Karls University of Tübingen.

References

[1] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[2] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[3] I. H. Park, R. Zhao, J. A. West et al., "Reprogramming of human somatic cells to pluripotency with defined factors," *Nature*, vol. 451, no. 7175, pp. 141–146, 2008.

[4] M. Stadtfeld, M. Nagaya, J. Utikal, G. Weir, and K. Hochedlinger, "Induced pluripotent stem cells generated without viral integration," *Science*, vol. 322, no. 5903, pp. 945–949, 2008.

[5] J. Yu, K. Hu, K. Smuga-Otto et al., "Human induced pluripotent stem cells free of vector and transgene sequences," *Science*, vol. 324, no. 5928, pp. 797–801, 2009.

[6] L. Warren, P. D. Manos, T. Ahfeldt et al., "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA," *Cell Stem Cell*, vol. 7, no. 5, pp. 618–630, 2010.

[7] T. M. Schlaeger, L. Daheron, T. R. Brickler et al., "A comparison of non-integrating reprogramming methods," *Nature Biotechnology*, vol. 33, no. 1, pp. 58–63, 2015.

[8] F. Anokye-Danso, C. M. Trivedi, D. Juhr et al., "Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency," *Cell Stem Cell*, vol. 8, no. 4, pp. 376–388, 2011.

[9] J. R. Plews, J. L. Li, M. Jones et al., "Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach," *PLoS One*, vol. 5, no. 12, article e14397, 2010.

[10] E. Yakubov, G. Rechavi, S. Rozenblatt, and D. Givol, "Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors," *Biochemical and Biophysical Research Communications*, vol. 394, no. 1, pp. 189–193, 2010.

[11] G. Tavernier, K. Wolfrum, J. Demeester, S. C. de Smedt, J. Adjaye, and J. Rejman, "Activation of pluripotency-associated genes in mouse embryonic fibroblasts by non-viral transfection with in vitro-derived mRNAs encoding Oct4, Sox2, Klf4 and cMyc," *Biomaterials*, vol. 33, no. 2, pp. 412–417, 2012.

[12] J. Stepinski, C. Waddell, R. Stolarski, E. Darzynkiewicz, and R. E. Rhoads, "Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogs 7-methyl(3′-O-methyl)GpppG and 7-methyl(3′-deoxy)GpppG," *RNA*, vol. 7, no. 10, pp. 1486–1495, 2001.

[13] E. Grudzien-Nogalska, J. Jemielity, J. Kowalska, E. Darzynkiewicz, and R. E. Rhoads, "Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells," *RNA*, vol. 13, no. 10, pp. 1745–1755, 2007.

[14] A. C. Goldstrohm and M. Wickens, "Multifunctional deadenylase complexes diversify mRNA control," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 4, pp. 337–344, 2008.

[15] K. Karikó, M. Buckstein, H. Ni, and D. Weissman, "Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA," *Immunity*, vol. 23, no. 2, pp. 165–175, 2005.

[16] K. Kariko and D. Weissman, "Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development," *Current*

Opinion in Drug Discovery & Development, vol. 10, no. 5, pp. 523–532, 2007.

[17] O. Andries, S. Mc Cafferty, S. C. de Smedt, R. Weiss, N. N. Sanders, and T. Kitada, "N(1)-Methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice," *Journal of Controlled Release*, vol. 217, pp. 337–344, 2015.

[18] M. Angel and M. F. Yanik, "Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins," *PLoS One*, vol. 5, no. 7, article e11756, 2010.

[19] K. Drews, G. Tavernier, J. Demeester et al., "The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts," *Biomaterials*, vol. 33, no. 16, pp. 4059–4068, 2012.

[20] L. Warren, Y. Ni, J. Wang, and X. Guo, "Feeder-free derivation of human induced pluripotent stem cells with messenger RNA," *Scientific Reports*, vol. 2, no. 1, p. 657, 2012.

[21] I. Kogut, S. M. McCarthy, M. Pavlova et al., "High-efficiency RNA-based reprogramming of human primary fibroblasts," *Nature Communications*, vol. 9, no. 1, p. 745, 2018.

[22] N. Yoshioka, E. Gros, H. R. Li et al., "Efficient generation of human iPSCs by a synthetic self-replicative RNA," *Cell Stem Cell*, vol. 13, no. 2, pp. 246–254, 2013.

[23] O. Petrakova, E. Volkova, R. Gorchakov, S. Paessler, R. M. Kinney, and I. Frolov, "Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in mammalian cells," *Society*, vol. 79, no. 12, pp. 7597–7608, 2005.

[24] N. Yoshioka and S. F. Dowdy, "Enhanced generation of iPSCs from older adult human cells by a synthetic five-factor self-replicative RNA," *PLoS One*, vol. 12, no. 7, article e0182018, 2017.

[25] R. P. Deering, S. Kommareddy, J. B. Ulmer, L. A. Brito, and A. J. Geall, "Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines," *Expert Opinion on Drug Delivery*, vol. 11, no. 6, pp. 885–899, 2014.

[26] M. Avci-Adali, A. Behring, H. Steinle et al., "*In vitro* synthesis of modified mRNA for induction of protein expression in human cells," *Journal of Visualized Experiments*, no. 93, article e51943, 2014.

[27] H. Steinle, S. Golombek, A. Behring, C. Schlensak, H. P. Wendel, and M. Avci-Adali, "Improving the angiogenic potential of EPCs via engineering with synthetic modified mRNAs," *Molecular Therapy - Nucleic Acids*, vol. 13, pp. 387–398, 2018.

[28] Y. Yoshida, K. Takahashi, K. Okita, T. Ichisaka, and S. Yamanaka, "Hypoxia enhances the generation of induced pluripotent stem cells," *Cell Stem Cell*, vol. 5, no. 3, pp. 237–241, 2009.

[29] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells," *Nature*, vol. 448, no. 7151, pp. 313–317, 2007.

[30] C. U. Hellen and P. Sarnow, "Internal ribosome entry sites in eukaryotic mRNA molecules," *Genes & Development*, vol. 15, no. 13, pp. 1593–1612, 2001.

[31] Y. G. Kim, A. Z. Baltabekova, E. E. Zhiyenbay et al., "Recombinant vaccinia virus-coded interferon inhibitor B18R: expression, refolding and a use in a mammalian expression system with a RNA-vector," *PLoS One*, vol. 12, no. 12, article e0189308, 2017.

[32] F. Yu, J. Li, H. Chen et al., "Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion," *Oncogene*, vol. 30, no. 18, pp. 2161–2172, 2011.

[33] K. W. Foster, S. Ren, I. D. Louro et al., "Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF," *Cell Growth & Differentiation*, vol. 10, no. 6, pp. 423–434, 1999.

[34] K. W. Foster, A. R. Frost, P. McKie-Bell et al., "Increase of GKLF messenger RNA and protein expression during progression of breast cancer," *Cancer Research*, vol. 60, no. 22, pp. 6488–6495, 2000.

[35] F. Umrath, H. Steinle, M. Weber et al., "Generation of iPSCs from jaw periosteal cells using self-replicating RNA," *International Journal of Molecular Sciences*, vol. 20, no. 7, p. 1648, 2019.

[36] M. A. Poleganov, S. Eminli, T. Beissert et al., "Efficient reprogramming of human fibroblasts and blood-derived endothelial progenitor cells using nonmodified RNA for reprogramming and immune evasion," *Human Gene Therapy*, vol. 26, no. 11, pp. 751–766, 2015.

[37] P. S. McGrath, N. Diette, I. Kogut, and G. Bilousova, "RNA-based reprogramming of human primary fibroblasts into induced pluripotent stem cells," *Journal of Visualized Experiments*, no. 141, article e58687, 2018.

[38] S. Guo, X. Zi, V. P. Schulz et al., "Nonstochastic reprogramming from a privileged somatic cell state," *Cell*, vol. 156, no. 4, pp. 649–662, 2014.

[39] D. Huangfu, R. Maehr, W. Guo et al., "Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds," *Nature Biotechnology*, vol. 26, no. 7, pp. 795–797, 2008.

[40] N. Maherali and K. Hochedlinger, "Tgfbeta signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc," *Current Biology*, vol. 19, no. 20, pp. 1718–1723, 2009.

[41] M. A. Esteban, T. Wang, B. Qin et al., "Vitamin C enhances the generation of mouse and human induced pluripotent stem cells," *Cell Stem Cell*, vol. 6, no. 1, pp. 71–79, 2010.

[42] P. Mali, B. K. Chou, J. Yen et al., "Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes," *Stem Cells*, vol. 28, no. 4, pp. 713–720, 2010.

8.4. PUBLIKATION IV

Reprogramming of urine-derived renal epithelial cells into iPSCs using srRNA and consecutive differentiation into beating cardiomyocytes

Molecular Therapy
Nucleic Acids
Original Article

Reprogramming of Urine-Derived Renal Epithelial Cells into iPSCs Using srRNA and Consecutive Differentiation into Beating Cardiomyocytes

Heidrun Steinle,[1,4] Marbod Weber,[1,4] Andreas Behring,[1] Ulrike Mau-Holzmann,[2] Christiane von Ohle,[3] Aron-Frederik Popov,[1] Christian Schlensak,[1] Hans Peter Wendel,[1] and Meltem Avci-Adali[1]

[1]Department of Thoracic and Cardiovascular Surgery, University Hospital Tübingen, Calwerstraße 7/1, 72076 Tübingen, Germany; [2]Institute of Medical Genetics and Applied Genomics, University Hospital Tübingen, Calwerstraße 7, 72076 Tübingen, Germany; [3]Department of Conservative Dentistry and Periodontology, Centre of Dentistry, Oral Medicine and Maxillofacial Surgery, University Hospital Tübingen, Osianderstraße 2-8, 72076 Tübingen, Germany

The generation of induced pluripotent stem cells (iPSCs) from patient's somatic cells and the subsequent differentiation into desired cell types opens up numerous possibilities in regenerative medicine and tissue engineering. Adult cardiomyocytes have limited self-renewal capacity; thus, the efficient, safe, and clinically applicable generation of autologous cardiomyocytes is of great interest for the treatment of damaged myocardium. In this study, footprint-free iPSCs were successfully generated from urine-derived renal epithelial cells through a single application of self-replicating RNA (srRNA). The expression of pluripotency markers and the *in vitro* as well as *in vivo* trilineage differentiation were demonstrated. Furthermore, the resulting iPSCs contained no residual srRNA, and the karyotyping analysis demonstrated no detectable anomalies. The cardiac differentiation of these iPSCs resulted in autologous contracting cardiomyocytes after 10 days. We anticipate that the use of urine as a non-invasive cell source to obtain patient cells and the use of srRNA for reprogramming into iPSCs will greatly improve the future production of clinically applicable cardiomyocytes and other cell types. This could allow the regeneration of tissues by generating sufficient quantities of autologous cells without the risk of immune rejection.

INTRODUCTION

Heart failure is currently one of the leading causes of death and affects 23 million people worldwide.[1] In addition to atrial fibrillation and arterial hypertension, myocardial infarction is a common cause of heart failure. The destruction of cardiomyocytes is associated with a reduced pumping capacity of the heart. Because of the extremely low self-renewal capacity of cardiomyocytes with less than 1% per year in adult hearts, a physiologically sufficient regeneration of the myocardium is not feasible.[2] In end-stage heart failure, solely organ transplantation can ensure the survival of patients. To overcome the lack of donor organs for transplantation, there is an explicit need for regenerative therapeutic strategies to treat advanced heart failure.

Previous *in vivo* studies with different species demonstrated that the transplantation of cardiomyocytes can improve the performance of the heart after myocardial infarction.[3,4] However, human implementation is currently failing due to the lack of reliable sources of human cardiomyocytes. To overcome this limitation, various approaches have been developed to generate cardiomyocytes from different cell sources, including bone-marrow-derived stem cells,[5] embryonic stem cells (ESCs),[6,7] and induced pluripotent stem cells (iPSCs).[8,9] In particular, iPSCs are a promising cell source to obtain autologous cells. Since iPSCs are produced from a patient's somatic cells, their generation and application avoid ethical concerns associated with ESCs. Furthermore, rejection reactions are prevented, because the generated cells are autologous. In addition to the application of iPSCs in the field of regenerative medicine, cardiomyocytes derived from iPSCs allow the screening and discovery of drugs, the prediction of cardiotoxicity,[10] and the study of cardiovascular diseases.[11]

Reprogramming of somatic cells into iPSCs was first achieved in murine fibroblasts by Yamanaka and colleagues[12,13] and shortly thereafter in human fibroblasts using retroviral vectors encoding four transcription factors: KLF4, c-MYC, OCT4, and SOX2. Hitherto, human fibroblasts have been the most commonly used cell source for reprogramming studies.[14] Fibroblasts are usually obtained from skin biopsies, which represent an invasive procedure and lead to an injury of healthy tissue. This is associated with pain and additional burden for the patient. Naturally, due to physiological self-renewal of the epithelial tissue in the urinary tract, approximately 2,000 to 7,000 human renal proximal tubule epithelial cells are detached daily and excreted with urine.[15] Thus, the collection and reprogramming of these urine-derived cells represent a promising simple and non-invasive strategy for obtaining patient's own somatic cells.

Received 25 February 2019; accepted 22 July 2019;
https://doi.org/10.1016/j.omtn.2019.07.016.

[4]These authors contributed equally to this work.

Correspondence: Meltem Avci-Adali, PhD, Department of Thoracic and Cardiovascular Surgery, University Hospital Tübingen, Calwerstraße 7/1, 72076 Tübingen, Germany.
E-mail: meltem.avci-adali@uni-tuebingen.de

The genome-integrating viral vectors originally used by Yamanaka et al.[12,13] are carrying a certain risk. The random insertion of retroviral vectors into the genome can lead to mutations and, thereby, to the development of tumors. Furthermore, the retroviral introduction of c-MYC in the cells can lead to increased tumorigenesis in mice.[16,17] Thus, these iPSCs generated by retroviral vectors cannot be clinically used. Meanwhile, several non-integrating approaches have been developed that reduce concerns regarding genetic alterations in iPSCs.[18–22] In particular, synthetic mRNA-based approaches are promising.[23] Thereby, the exogenous delivery of synthetic mRNAs into somatic cells leads to transient expression of desired proteins under physiological conditions by cells' translational machinery. In contrast to plasmid DNA, synthetic mRNAs do not need to enter the cell nucleus. This results in an immediate translation of delivered mRNA in the cytosol and eliminates the risk of genomic integration and insertional mutagenesis.[24] The incorporation of modified nucleotides during the *in vitro* transcription (IVT) or codon optimization[25] can significantly improve the stability of synthetic mRNAs, drastically reduce immune responses triggered by recognition of foreign RNAs, and increase the recruitment and recycling of ribosomes and, thereby, result in enhanced translation efficiency.[26–29]

Due to the transient nature, synthetic mRNAs can be used to express reprogramming factors to obtain footprint-free iPSCs.[22] However, the transient presence of the exogenously delivered mRNAs also necessitates the daily transfection of cells with the synthetic mRNAs during the reprogramming process, which is cost intensive, time consuming, and associated with increased cellular stress. To overcome these hurdles, self-replicating RNAs (srRNAs) can be used to express the reprogramming factors for an extended period of time,[30] without the need for repeated transfections. The srRNA contains the coding sequences of four transcription factors-OCT4, KLF4, SOX2, and c-MYC-and four non-structural proteins (nsP1–nsP4), which encode the RNA replication complex of Venezuelan equine encephalitis (VEE) virus.[30,31]

In this study, using srRNA, footprint-free iPSCs were generated from adult human urine-derived epithelial cells, and the successful generation of beating autologous cardiomyocytes from these iPSCs was demonstrated.

RESULTS

Reprogramming of Urine-Derived RECs into iPSCs Using OCT4, KLF4, SOX2, IRES-cMyc (OKSiM)-GFP srRNA

Cells were collected from urine of 4 different donors. The renal epithelial origin of the isolated and expanded cells at passage 2 or 3 was confirmed using flow cytometry. The analysis showed that 95.8% ± 1.3% of the cells positively express epithelial marker β-catenin and that 99.8% ± 0.2% of the cells express the renal proximal tubular marker CD13 (Figure 1A).

To control the transfection and translation of the srRNA in cells, an IRES-GFP encoding sequence was cloned into the T7-VEE-OKSiM plasmid for the synthesis of OKSiM-GFP srRNA (Figure 1B). For reprogramming, 5×10^4 renal epithelial cells (RECs) were seeded on gelatin-coated wells. Using the established reprogramming protocol (Figure 1C), 21 to 30 days post-transfection, 3 to 25 primary iPSC colonies were obtained per well. After 24 h and reaching approximately 50% confluency (Figure 1D), a single transfection with 0.5 μg OKSiM-GFP srRNA was performed for 4 h. Subsequently, cells were cultivated in B18R-containing medium. Using flow cytometry, a strong GFP reporter protein expression was detected in 7.2% ± 2.8% of the cells 2 days post-transfection (n = 4). On the third day after transfection, 0.8 μg/mL puromycin was added to the medium to select srRNA-transfected cells. After 24 h, only a partial antibiotic-mediated, selective cell death was detected. On day 7 after transfection, a significant reduction in cell numbers was observed, and GFP^+ cells carrying the reprogramming srRNAs survived and grew in colonies. On day 7, the cells underwent severe morphological changes, and small round cells with large nuclei appeared (Figure 1D). Afterward, the medium was changed to E8 stem cell medium containing B18R. In the following days, when the cells reached 90% confluency during the reprogramming process, the cells were split at a 1:2 or 1:4 ratio onto vitronectin-coated plates. On day 13, an increase in colony size was detected. After 26 days, iPSC colonies were identified using live-cell staining (antibody against SSEA-4). Thereafter, B18R was withdrawn from the medium to eliminate the reprogramming srRNA from the cells, and iPSCs were either immediately picked or further cultivated for about 4–7 days until primary iPSC colonies increased in size (D33). Manually picked iPSC colonies were transferred onto the vitronectin-coated wells of a 12-well plate.

Characterization of iPSCs Derived from RECs

Expression of Pluripotency Markers

After the reprogramming of adult somatic RECs into iPSCs, the expression of pluripotency markers was analyzed in iPSCs at passage 4 by immunostaining with specific antibodies. Fluorescence microscopy analyses revealed a strong expression of NANOG, OCT4, SOX2, SSEA-4, TRA-1-60, and LIN28 within the entire iPSC colonies (Figure 2A). Furthermore, flow cytometry analyses demonstrated that 85% ± 6% of iPSCs (passages 5–7) were expressing NANOG and that 91% ± 4% of cells were positive for TRA-1-60 (Figure 2B). Further cultivation and expansion of iPSCs until passage 25, corresponding to 4–5 months in culture, had no effect on TRA-1-60 and NANOG expression (Figure S1). Additionally, gene expression of SOX2, OCT4, LIN28, NANOG, and E-cadherin was quantified using qRT-PCR. The obtained REC-iPSCs (passages 3–6) showed—like the commercially available positive control iPSC cell line WT02, which was generated using a Sendai virus vector from dermal fibroblasts—a significantly high expression of SOX2 (1,510-fold), OCT4 (173-fold), LIN28 (4,376-fold), NANOG (733-fold), and E-cadherin (4,230-fold), compared to the initial RECs (Figure 2C).

Trilineage Differentiation of iPSCs

The differentiation potential of the obtained iPSCs (passages 3–7) into all three germ layers was evaluated *in vitro* using a directed 7-day differentiation protocol (StemMACS Trilineage Differentiation Kit from Miltenyi Biotec) followed by specific antibody staining and

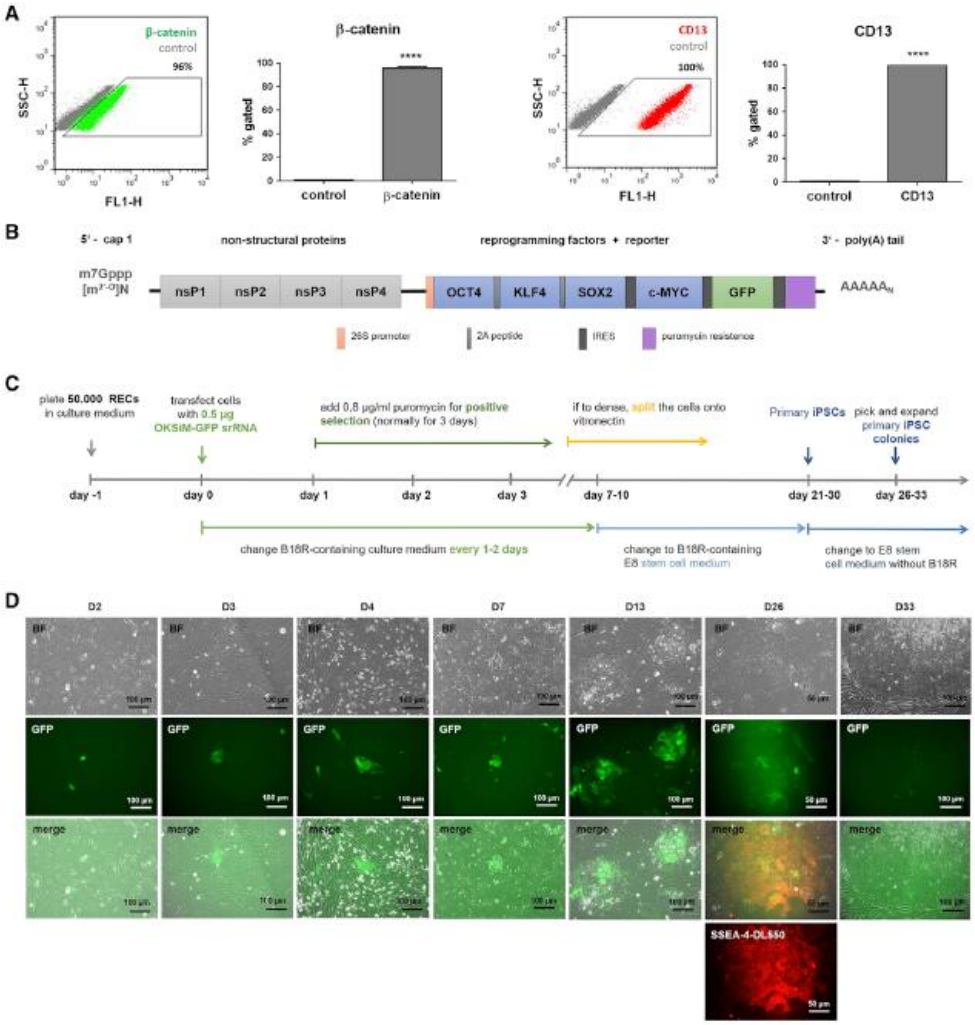

Figure 1. Reprogramming of Urine-Derived RECs into iPSCs Using OKSiM-GFP srRNA

(A) Flow cytometry analysis of RECs after staining with antibodies against epithelial marker β-catenin and renal epithelial marker CD13. Results are shown as mean + SD (n = 4). Statistical differences were determined using paired t test (****$p < 0.0001$). (B) Schematic overview of srRNA construct. The synthetized srRNA contains the coding sequences for the non-structural proteins (nsP1–nsP4) of VEE to allow self-replication of srRNA, the reprogramming factors (OCT4, KLF4, SOX2, and c-MYC), and the reporter protein GFP. The internal ribosome entry sites (IRES) sequences (linked to c-MYC and GFP) control ribosome entry and, therefore, protein production. The 5′ end was modified with a cap1 structure, and a poly(A) tail was added at the 3′ end. For positive selection, the srRNA also encodes a puromycin resistance. (C) Timeline for the generation of iPSCs from RECs using srRNA. (D) Microscopic images of RECs after the transfection with 0.5 µg srRNA. A few cells expressing the reporter protein GFP were detected 2 days after the transfection (D2). At day 3, puromycin was added for 3 days to eliminate cells, which are not transfected (D3). In the following days, GFP-positive cells proliferated and generated colonies (D4). At day 7, mainly GFP-positive cells had survived, and non-transfected cells were dying (D7). At day 13, an increased size of colonies was observed (D13). After 26 days, iPSC colonies were stained with DL550-labeled live antibody against SSEA-4 (D26). Depending on the colony size, primary iPSCs were picked the next day or further cultivated up to 7 days (D33) until colonies increased in size. The detached iPSCs were transferred into vitronectin-coated wells.

quantification using flow cytometry. The mesoderm induction resulted in the generation of elongated endothelial-like cells as well as multilayer accumulations of smooth muscle-like cells (Figure 3). The endoderm induction led to the detection of a dense cell layer similar to that of early hepatocyte-like cells. The ectoderm induction resulted in cells arranged in neural rosettes. The successful

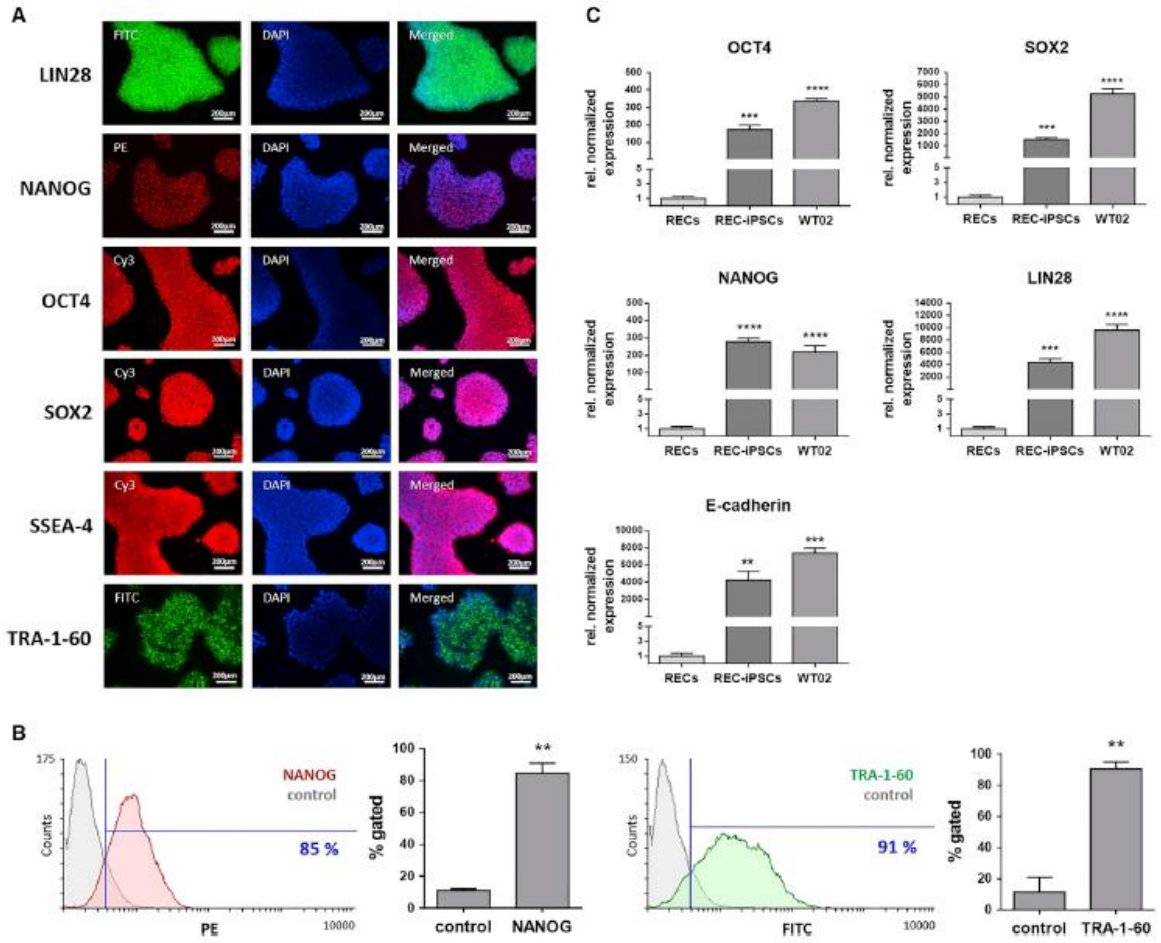

Figure 2. Analysis of Pluripotency Marker Expression in iPSCs Generated from RECs

(A) Representative immunofluorescence microscopy images of iPSCs (passage 4) stained with LIN28-, NANOG-, OCT4-, SOX2-, SSEA-4-, and TRA-1-60-specific antibodies. (B) Flow cytometry analysis of iPSCs (passages 5–7) stained with antibodies specific for NANOG and TRA-1-60. Results are shown as mean + SD (n = 3). (C) Expression analysis of SOX2, OCT4, LIN28, NANOG, and E-cadherin transcripts using qRT-PCR. mRNA levels were normalized to GAPDH mRNA levels, and the results are presented relative to the expression levels in RECs. Results are shown as mean + SEM (n = 4). Statistical differences were determined using paired t test (**p < 0.01; ***p < 0.001, ****p<0.0001).

mesodermal commitment was shown by detecting 48% ± 17% CD31-positive and 74% ± 17% SMA-positive cells. The endoderm differentiation resulted in 95% ± 3% AFP-positive and 97% ± 4% CXCR4-positive cells. The ectodermal distinction was demonstrated by the detection of 96% ± 2% paired box gene 6 (PAX6)-positive and 98% ± 1% class III β-tubulin (TUBB3)-positive cells.

Teratoma Formation of iPSCs

The *in vivo* differentiation capacity of the generated iPSCs into tissue types of all three germ layers was assessed using chorioallantoic membrane (CAM) assay. Therefore, 2×10^6 iPSCs (passages 8–11) were suspended in Matrigel and applied onto the CAM. After 10 days of incubation, the formed cell mass was excised together with the CAM (Figure 4A). Histological analysis revealed mesodermal, endodermal, and ectodermal differentiation of applied iPSCs within the teratoma tissue mass (Figure 4B). The differentiation into mesodermal tissue was demonstrated by the presence of bone-like and connective tissue structures in teratoma sections. The differentiation toward endodermal tissue was shown by the detection of gland- and gut-like epithelial tissues, and the generation of ectodermal tissue was demonstrated by the presence of squamous epithelial tissue and neural epithelium starting to form rosette-like structures.

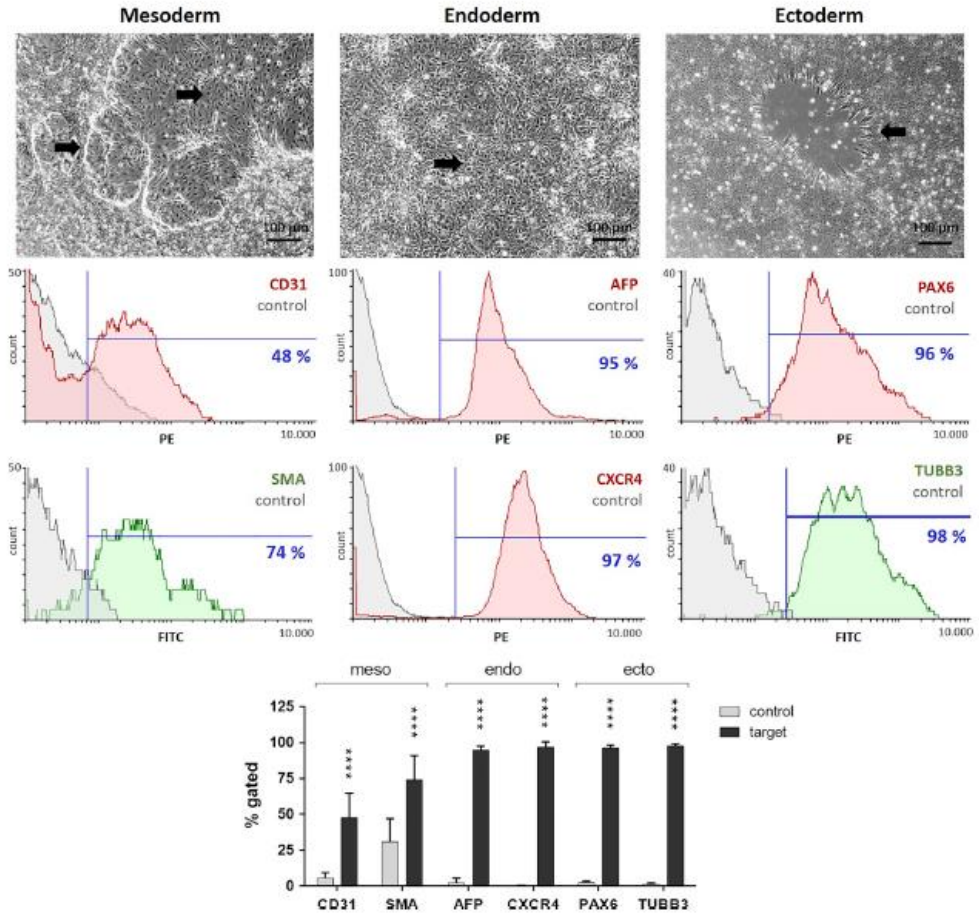

Figure 3. *In Vitro* Differentiation Potential of iPSCs Derived from RECs into the Three Germ Layers: Mesoderm, Endoderm, and Ectoderm

After 7 days of trilineage differentiation, cells with changed phenotypes (indicated with arrows) were observed. Flow cytometry analysis confirmed the differentiation of iPSCs (passages 3–7) into all three germ layers by detection of mesodermal (CD31 and SMA), definitive-endodermal (AFP and CXCR4), and neuroectodermal (PAX6 and TUBB3) cells. Compared to controls, differentiated cells showed significantly increased expression of mesodermal, endodermal, and ectodermal markers. Scale bars of phase contrast microscopic pictures represent 100 μm. Results are shown as mean + SD (n = 4). Statistical differences were determined using one-way ANOVA followed by Bonferroni's multiple comparison test (****p < 0.0001).

Downregulation of Oncogenic Transcription Factors and Elimination of srRNA after the Reprogramming of RECs into iPSCs and Analysis of Genomic Stability

Since a permanently elevated expression of KLF4 and c-MYC is associated with an increased tumorigenesis,[16] the expression levels of these transcription factors were determined in obtained iPSCs (passage 3) using qRT-PCR. The analyses revealed that the expression of KLF4 and c-MYC in iPSCs was not significantly different from the expression levels of the initial RECs (Figure 5A). To eliminate the exogenously delivered srRNA in the reprogrammed cells, B18R protein treatment was discontinued between day 21 and day 30 of reprogramming. The elimination of srRNA was analyzed in iPSCs at passage 3 using qRT-PCR. Thereby, the srRNA-specific nsP2 and nsP4 coding regions were detected. RECs transfected with srRNA (RECs+) and cultivated for 2 days demonstrated approximately 2×10^6-fold higher numbers of srRNA transcripts compared to untransfected RECs (Figure 5B). In contrast, no residual srRNA expression was detected in iPSCs. Subsequently, the obtained amplicons were analyzed using agarose gel electrophoresis. As shown in Figure 5C, solely RECs+ showed transcripts with the expected product length of 192 bp for nsP2 and 238 bp for nsP4. To analyze the genomic stability of iPSCs, karyotyping of chromosomes was performed with the initial urine-derived RECs as well as with the generated iPSCs (at passages 4–6). The results revealed no changes

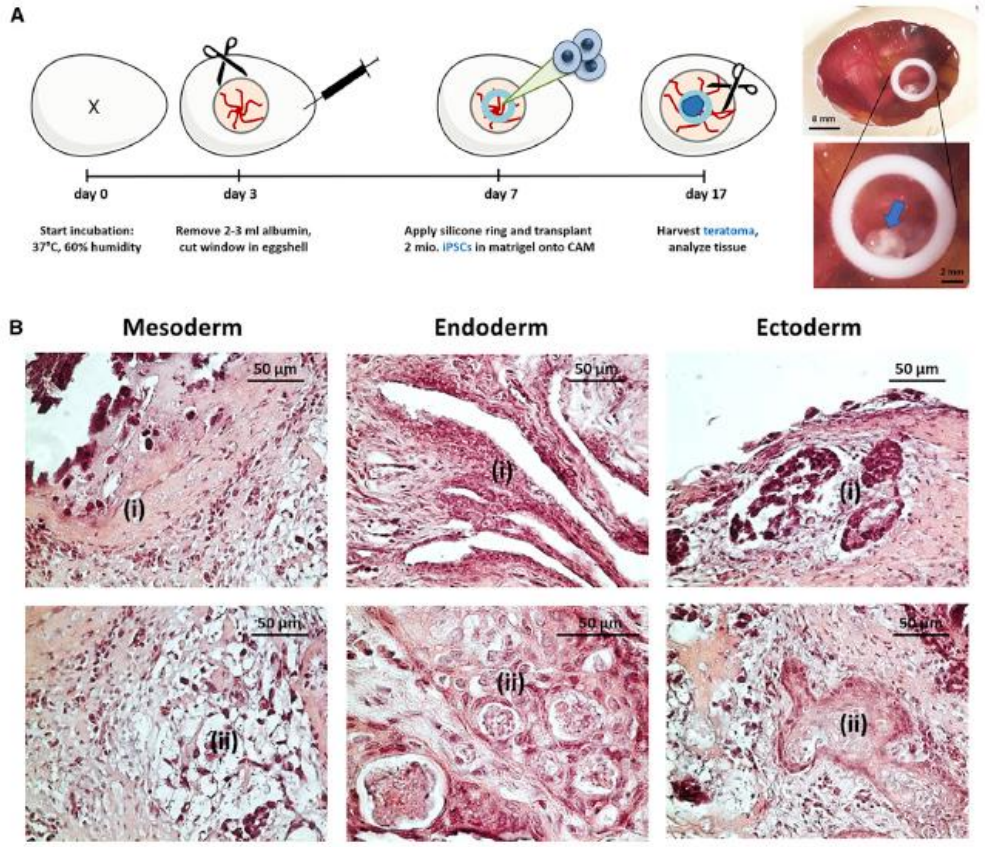

Figure 4. *In Vivo* Analysis of Teratoma Formation by Application of iPSCs onto CAM

(A) Schematic representation of the implementation of CAM assay for the analysis of teratoma formation. 2×10^6 iPSCs (passages 8–11) were applied into the inner area of a silicone ring (Ø 8 mm), which was placed onto the CAM on day 7 of incubation. After 10 days of incubation, teratomas (indicated with an arrow) were excised, and histological analyses were performed. (B) A representative microscopic image of H&E-stained teratoma sections showing iPSC-derived tissues of all three germ layers: mesoderm (i, bone-like tissue; ii, connective tissue), endoderm (i, gut-like epithelium; ii, gland-like epithelium), and ectoderm (i, primitive neural rosettes; ii, squamous epithelium).

in number and appearance of chromosomes, including their length, banding pattern, and centromere position after the reprogramming procedure (Figure 5D).

Characterization of Generated Cardiomyocytes from iPSCs

iPSCs (passages 4–12) generated from 4 different donors were differentiated within 10–14 days into beating cardiomyocytes (passage 0). Using flow cytometry, the yield of cardiac troponin T (cTNT)-positive cardiomyocytes was determined after the enrichment procedure and resulted in 89.2% ± 1.7% cTNT-positive cells (Figure 6A). The fluorescence microscopic overview of cTNT and α-actinin (ACTN2) double-stained cells confirmed the high amount of cardiac cells compared to a few solely DAPI-stained nuclei (Figure 6B). The differentiated cells also showed the typical elongated rod-like shape of cardiomyocytes. More detailed confocal laser scanning micrographs of the cells in passage 1 demonstrated the structural arrangement of the expressed cardiomyocyte-specific proteins, cTNT, cardiac myosin heavy chain (MYH6), or the muscle-specific marker alpha smooth muscle actin (ACTA2) (Figure 6C). The staining of cells with cTNT-specific antibody revealed the cardiomyocyte-specific sarcomeric structures within the cells. Moreover, using qRT-PCR, the expression of cardiomyocyte-specific markers, atrial natriuretic peptide (ANP), cTNT, α-actin cardiac muscle 1 (ACTC1), and MYH6 was analyzed. The obtained cells (passage 0) expressed 5.4×10^4-fold increased levels of ANP, 2.9×10^4-fold higher cTNT levels, 5.5×10^3-fold higher ACTC1 levels, and 2.2×10^4-fold higher MYH6 levels compared to those in the initial RECs (Figure 6D). Furthermore, the presence of cardiac troponin I (TNNI3) was analyzed in cell culture supernatants 13 to 16 days after starting the iPSC differentiation into cardiomyocytes (Figure 6E). A significantly higher concentration of TNNI3 (0.26 ± 0.11 µg/L) was detected in cardiomyocyte cell culture supernatants compared to iPSC culture

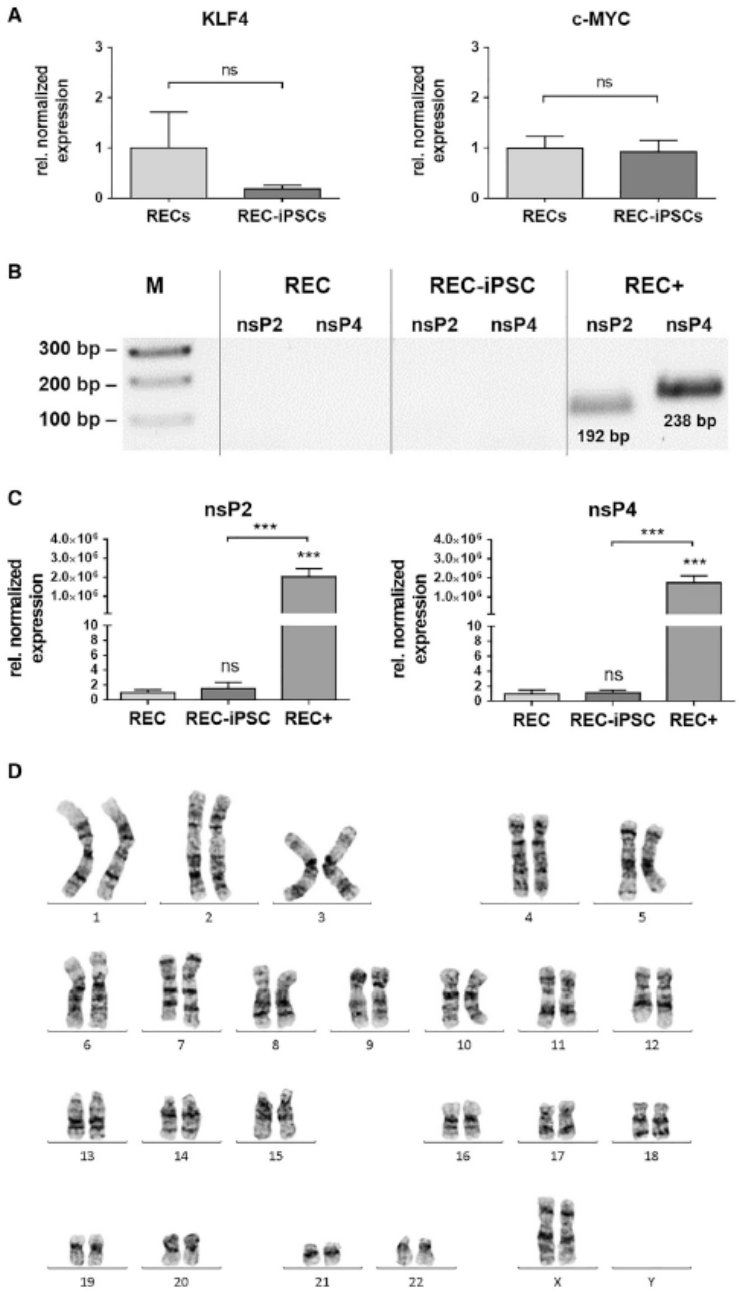

Figure 5. Detection of Oncogenic Transcription Factors and srRNA after the Reprogramming of RECs into iPSCs and Analysis of the Genomic Stability of REC-Derived iPSCs

(A) Analysis of KLF4 and c-MYC expression in iPSCs (passage 3) generated by a single transfection with srRNA. The qRT-PCR results are presented relative to the expression levels in the initial RECs. Results are shown as mean + SEM (n = 4). (B) Quantification of srRNA in RECs 2 days after the srRNA transfection (REC+) and in iPSCs (passage 3) using qRT-PCR. Thereby, nsP2- and nsP4-encoding regions of the srRNA were detected using specific primers. The results are presented relative to the expression levels of initial RECs without srRNA transfection. Results are shown as mean + SEM (n = 4). Statistical differences were determined using paired t test (***p < 0.001). (C) Detection of nsP2 and nsP4 specific amplicons in qRT-PCR products using 1% agarose gel electrophoresis. (D) Representative karyotype image of REC-iPSCs (passage 4) obtained from a female subject showing a normal 46XX karyotype by G-banding (n = 3).

medium supernatants (<0.03 μg/L, which represents the lower TNNI3 detection limit). Thereby, the successful differentiation of iPSCs into cardiomyocytes was demonstrated.

Characterization of Contracting Cardiomyocytes

The rhythmic beating is a typical characteristic of fetal and immature stem-cell-derived cardiomyocytes.[32] Thus, to show and analyze the contraction of obtained cardiomyocytes (passage 0), short video recordings were made for 30 s, with 7 pictures per second. Using MATLAB application Motion GUI, the motion directions were calculated and indicated by arrows in Figure 7A (Video S1). Thereby, wide ranges of motion and directional synchronous contractions were detected. Furthermore, the areas with the highest movement rate are shown in the heatmap (Figure 7B). Beating rates were calculated by the time shift between contractions. Cardiomyocytes produced from the iPSCs of 4 different REC donors showed uniform motion patterns during the recording time, with a mean beating rate of 25.24 ± 3.44 beats per minute, which corresponds to a beating frequency of 0.42 Hz ± 0.06 Hz (Figure 7C). Fluorescence microscopic recordings of Ca^{2+} transients in the cardiomyocyte cultures showed Ca^{2+} oscillations (Figure 7D; Video S2), which proved the electromechanical coupling of obtained cardiomyocytes. These findings revealed that the generated cardiomyocytes were functional in regulating intracellular Ca^{2+} signaling. In addition, the beating rate was determined in the presence of Ca^{2+} antagonist nifedipine or β-adrenoceptor agonist isoproterenol (Figure 7E). The treatment of cardiomyocytes with 0.1, 1, or 10 μM nifedipine led to the complete inhibition of cardiomyocyte

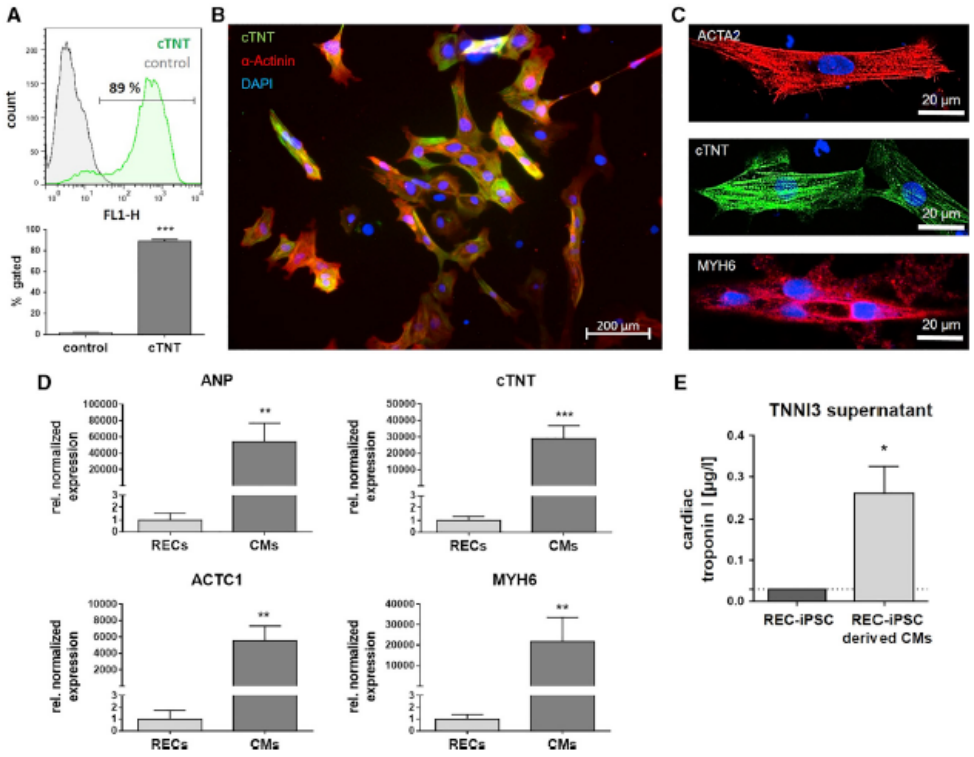

Figure 6. Analysis of Cells Obtained after Cardiac Differentiation of REC-Derived iPSCs

(A) Flow cytometry analysis of cTNT-expressing cells after cardiac differentiation and enrichment. (B) Fluorescence microscopic overview of cTNT- and α-actinin (ACTN2)-positive cells in passage 1 after the cardiac differentiation. Counterstaining of nuclei was performed with DAPI. (C) Confocal-laser-scanning microscopic analysis of cardiomyocyte-specific ACTA2, cTNT, and MYH6 expression. Nuclei were stained with SYTO9. (D) qRT-PCR gene expression analysis of cardiac-specific proteins ANP, cTNT, ACTC1, and MYH6 in passage 1 after the differentiation. (E) Detection of TNNI3 concentration in cardiomyocyte culture supernatants and in iPSC culture supernatants as control. Results are shown as mean + SEM (n = 3). Statistical differences were determined using paired t test (*p < 0.05; **p < 0.01; ***p < 0.001).

contractions. In contrast, the treatment of cardiomyocytes with 0.01 µM isoproterenol increased the beating rate from 32.42 ± 2.09 beats per minute to 40.09 ± 7.71 beats per minute. At an isoproterenol concentration of 0.1 µM, the beating rate reached 50.61 ± 6.52 beats per minute. After the removal of nifedipine and isoproterenol and cultivation of cells for a few hours in cardiomyocyte maintenance medium (CMM), the cells recovered and displayed initial beat rates.

DISCUSSION

In recent years, the ability to generate iPSCs from somatic cells has led to considerable progress in regenerative medicine, and the reprogramming has become a powerful tool in the field of tissue engineering. In this study, we established a method to generate footprint-free cardiomyocytes by using the autologous cell material from the patient's urine and srRNA. First, urine-derived human RECs were reprogrammed using srRNA into iPSCs and then differentiated into beating autologous cardiomyocytes. The obtained iPSCs showed self-renewability and the expression of pluripotency-specific markers, while no residual srRNA and genomic abnormalities were detected. The trilineage differentiation potential of REC-derived iPSCs was demonstrated *in vitro* as well as *in vivo*. Moreover, the cardiac differentiation of these cells resulted in the generation of contractile cardiomyocytes. Thus, the results clearly demonstrated that autologous footprint-free iPSCs as well as cardiomyocytes can be obtained by using the patient's urine as a source for somatic cells. Thereby, invasive biopsies for isolation of somatic cells is not required. Since the obtained cells are footprint-free and autologous, they might have a high potential to be used for the regeneration of injured myocardium.

Urine is a naturally excreted material; therefore, the sampling of patients' urine offers an easy, non-invasive, low-cost, and pain-free method for the collection of sufficient numbers of human somatic cells for reprogramming. Due to natural physiological self-renewal of the epithelial tissue in the urinary tract, approximately 2,000 to 7,000 cells are daily detached and excreted with the urine.[15] In urine,

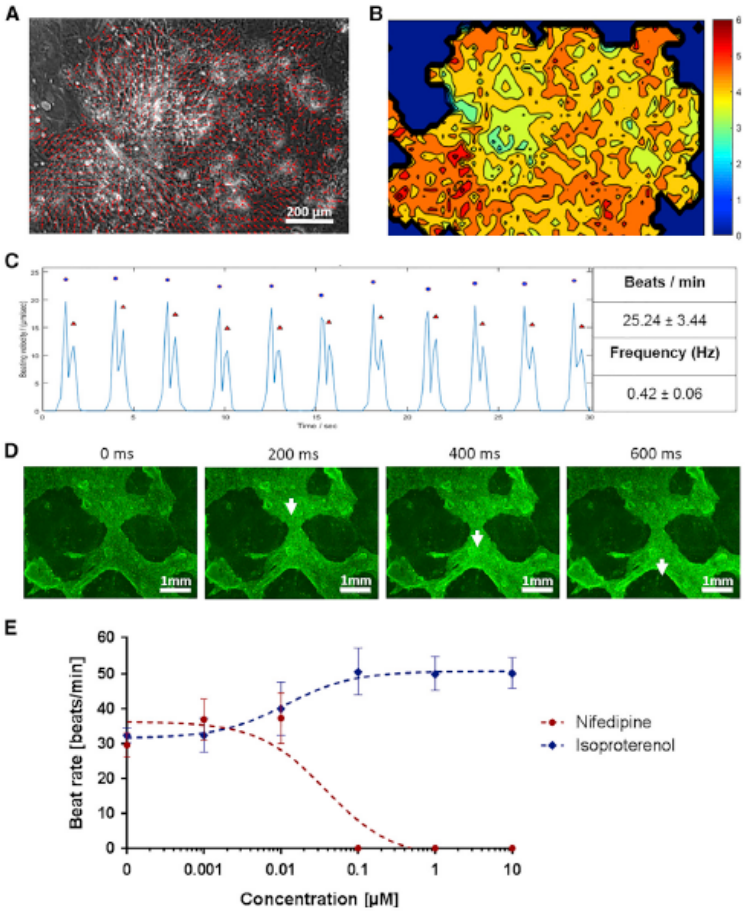

Figure 7. Characterization of Contracting Cardiomyocytes

(A) Arrow-based display of the directed contractions of cardiomyocytes (passage 0). (B) Heatmap depicting the time-averaged magnitude of motion in the x and y directions. An increase of movement is indicated by colors from blue to red. (C) Representative display of beating velocity (micrometers per second) during the recorded time of 30 s. Blue dots indicate contraction, and red triangles indicate the relaxation. Beat rate and frequency (mean ± SD) of iPSC-derived cardiomyocytes were calculated via motion tracking (n = 4). (D) Ca^{2+} imaging of cardiomyocytes. Representative images of every 200 ms are shown. White arrows indicate the position of Ca^{2+} transients. (E) Dose-response curves of isoproterenol- and nifedipine-treated cardiomyocytes (mean ± SD; n = 3).

3 types of epithelial cells can be found: renal, transitional, and squamous.[33] RECs line the nephron as a single layer. An increased number of these cells in the urine can indicate an infection or kidney disease. Transitional epithelial cells are a multilayer of epithelial cells that line the urinary bladder. A few transitional cells are present in the urine of healthy persons, and increased numbers are associated with infection or transitional cell carcinoma. Most often, these cells are also found in the urine after urethral or ureteral catheterization. Squamous epithelial cells line the urethra and vagina, and this type of epithelial cells is most often found in female urine. Large numbers of squamous cells in female urine generally indicates vaginal contamination. However, during a culturing time of 3 to 5 days in renal epithelial proliferation medium, the squamous epithelial cells do not adhere to the cell culture plate and are removed after the medium change. Typically, 3 to 6 small colonies of RECs appear and grow steadily.

In our study, 100–200 mL urine was enough to obtain a sufficient amount of cells for the reprogramming procedure. However, by using an increased amount of urine, more cells could be obtained; thereby, the expansion time of the cells could be reduced. Furthermore, the proliferation rate of the cells can vary between different donors and ages and might influence the reprogramming efficiency. In this study, the isolated cells from urine samples were positive for the epithelial marker β-catenin and the renal proximal tubular marker CD13. In contrast to urine sampling, the commonly used somatic cells are obtained from skin samples (fibroblasts)[14] or blood cells[34] by invasive procedures. Cutting out healthy skin pieces is, of course, a painful procedure and is associated with the risk of infection. Furthermore, the reprogramming of blood cells is difficult, and the efficiency is known to be relatively low.[35] It has also been shown that iPSCs derived from different cell sources maintain a distinct molecular pattern of epigenetic markers, which is linked to the donor tissue.[36] This might result in the improved differentiation potential of iPSCs into the initial somatic cell type. In this study, the successful differentiation of urine-derived REC-iPSCs into cells of all three germline directions was demonstrated.

The reprogramming was performed by a single transfection of RECs with a synthetic srRNA that encodes four reprogramming factors—OCT4, KLF4, SOX2, and c-MYC—as well as the fluorescent reporter protein GFP and contains an open reading frame for puromycin resistance. The puromycin resistance enables the positive selection of srRNA-containing cells, which improves the reprogramming efficiency. In comparison to viral-vector-based methods, the use of srRNA prevents random integration of reprogramming factors into the genome and enables the generation of footprint-free iPSCs. Furthermore, compared to the application of synthetic mRNA for the generation of iPSCs,[22] which requires a daily transfection of 5 different mRNAs into the same target cell, a single transfection of

cells with srRNA is sufficient for the complete reprogramming period.

The addition of B18R protein into the reprogramming medium suppresses the type-I-interferon-associated immune responses[37] to the srRNA; thereby, the premature degradation of the srRNA in the cells can be prevented. After the reprogramming, the termination of B18R addition leads to the degradation of srRNA.[30] Furthermore, the incorporation of IRES-GFP into the srRNA allowed the control of successful transfection and translation of srRNA during the reprogramming process. Additionally, in the absence of the immunosuppressive protein B18R, the decrease of the fluorescence intensity indicated the degradation of the srRNA in the cells. Moreover, the implementation of qRT-PCR using specific primers for the nsP2 and nsP4 regions of the srRNA provided the evidence for the srRNA degradation, since, in obtained iPSCs (passage 3), no srRNA could be detected. The proto-oncogenes c-MYC and KLF4 are expressed in different types of cancer,[38,39] and the permanent overexpression of these genes is associated with an increased tumorigenesis.[16,40] Thus, although the expression of these proteins is required during the reprogramming, after the reprogramming, they should be downregulated. This was also demonstrated in our obtained iPSCs; the expression of c-MYC and KLF4 was not significantly different from the expression in precursor RECs. Furthermore, the decrease of c-MYC and KLF4 expression also indicates the degradation of the srRNA construct in the iPSCs.

In addition to expressing certain proteins responsible for maintaining the pluripotency and the self-renewal capacity of iPSCs, another key feature of iPSCs is the ability to differentiate into each of the three germ layers: mesoderm, endoderm, and ectoderm. We successfully demonstrated the ability of the obtained iPSCs to form each of the germ layers after directed differentiation *in vitro* as well as *in vivo* after the application of iPSCs on CAM. Compared to the teratoma formation in mice, which is incubated for about 4 weeks, the application of 2×10^6 iPSCs on CAM and the incubation time of 10 days were sufficient to form all three germ layers. The cardiac differentiation of renal-epithelial-cell-derived iPSCs resulted already after 7 days in beating cardiomyocytes with a beating rate of approximately 25 beats per minute. The treatment of these cells with 0.1 μM nifidipine resulted in complete inhibition of beating. In contrast, the pharmacological modulation of REC-iPSC-derived cardiomyocytes with isoproterenol led to an increased beating rate.

The future application of these cells for the repair of damaged heart tissues requires the production of pure cardiomyocyte cultures in a large-scale format and the selection of the appropriate subtype-specific cardiomyocytes—nodal, atrial, or ventricular cardiomyocytes.[41–43] Furthermore, the complete differentiation of iPSCs into mature cardiomyocytes should be ensured.[44,45] Dubois et al.[46] demonstrated by using an anti-SIRPA antibody and fluorescence-activated cell sorting (FACS) that cardiomyocytes can be enriched from human pluripotent stem cells. In our study, we used the lactate-based method to enrich cardiomyocytes.[47] Due to differences in energy substrate metabolism, compared to other mammalian cells, cardiomyocytes are able to produce energy also from lactate or fatty acids instead of glucose.[48] Thus, the cultivation of cells in glucose-depleted and lactate-supplemented medium leads to the survival of cardiomyocytes and the elimination of undifferentiated cells. Using this method, 89.2% ± 1.7% cTNT-positive cells were obtained.

Heart failure and myocardial infarction mainly affect the ventricles of the heart; thus, to prevent arrhythmias after transplantation of the cells into cardiac ventricles, the ventricular subtype of cardiomyocytes should be applied. Therefore, cell sorting can be performed to obtain pure cardiomyocyte subtypes for different applications. The commonly used marker specific for ventricular cardiomyocytes is the myosin light chain 2v (MLC-2v), and the myosin light chain 2a (MLC-2a) is considered as a specific marker for atrial cardiomyocytes.

Conclusions

The non-invasive collection of somatic cells from urine and the one-off application of srRNA could allow the easy and efficient generation of sufficient and unlimited numbers of patient-specific iPSCs, which can then be differentiated besides cardiomyocytes also into other desired cell types without any genomic integration. Thereby, personalized cell therapy of different diseases can be enabled, which prevents rejection reactions and the use of immunosuppressive drugs with their long-term complications. Thereby, the outcome of various cell therapy approaches can be greatly improved, and the generated cells can also serve as cell models for studying specific genetic diseases and treatment methods.

MATERIALS AND METHODS

Production of Synthetic srRNA

The T7-VEE-OKSiM plasmid[30] containing the VEE non-structural protein coding sequences (nsP1 to nsP4) to enable the RNA replication and the coding sequences of OCT4, KLF4, SOX2, and c-MYC was purchased from Addgene (LGC Standards, Teddington, UK). To monitor the transfection and reprogramming efficiency, an additional sequence encoding an IRES (internal ribosome entry site) and the reporter protein GFP were cloned by Aldevron (Fargo, ND, USA) into the plasmid, which is then called OKSiM-GFP plasmid.

To amplify the OKSiM-GFP plasmid, *E. coli* competent cells (α-Select Chemically Competent Cells, Bioline, Luckenwalde, Germany) were transformed with 100 ng plasmid and cultivated in lysogeny broth (LB) medium containing 50 μg/mL ampicillin. Plasmid isolation was performed using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). Afterward, 36 μg OKSiM-GFP plasmid was linearized using 5 μL FastDigest MluI restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA) and 20 μL 1× reaction buffer in a total volume of 200 μL for 3 h at 37°C. The linearized DNA was purified using the Isolate II PCR and Gel Kit (Bioline) and analyzed using 1% agarose gel electrophoresis.

For the synthesis of srRNA, IVT was performed for 2 h at 37°C using the RiboMAX Large Scale Production System—T7 Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The IVT reaction mixture contained 10 μg linearized OKSiM-GFP

plasmid DNA and 40 U RiboLock RNase Inhibitor (Thermo Fisher Scientific) in 100 µL. Afterward, DNA templates were removed by adding 1 µL TURBO DNase for 15 min at 37°C. Next, 5′-end capping was performed using the ScriptCap Cap1 Capping System followed by 3′-end polyadenylation with the A-Plus Poly(A) Polymerase Tailing Kit (both from Cellscript, Madison, WI, USA) according to manufacturer's instructions. Following each reaction step, the srRNA was purified using the RNeasy Kit (QIAGEN) and eluted in nuclease-free water. The purity and specific length of generated srRNA products were analyzed using 1% agarose gel containing 2.2 M formaldehyde and 1× GelRed (Biotium, Fremont, CA, USA) in 1× MOPS (3-(N-morpholino)propanesulfonic acid) buffer. Electrophoresis was performed at 100 V for 60 min in 1× MOPS buffer.

Isolation and Cultivation of RECs from Urine

RECs were isolated from 100–200 mL urine from healthy donors (men and women between 25 and 35 years of age) by centrifugation at 400 × *g* for 10 min. Afterward, cells were washed with 50 µg/mL gentamicin and 250 µg/mL amphotericin B (Sigma-Aldrich) containing Dulbecco's PBS (DPBS; Thermo Fisher Scientific) and centrifuged at 200 × *g* for 10 min. Then, cells were suspended in 1 mL primary medium consisting of DMEM/F12 high glucose supplemented with 10% fetal bovine serum (FBS), REGM Renal Epithelial Growth Medium SingleQuots Kit (Lonza, Basel, Switzerland), 50 µg/mL gentamicin, and 250 µg/mL amphotericin B and plated in one well of a 0.1% gelatin-coated 12-well plate. For the next 3 days, 1 mL primary medium was added each day. The obtained cells were then cultivated in proliferation medium (REMC) consisting of 50% renal epithelial (RE) basal medium with REGM Bullet Kit supplements (Lonza, Basel, Switzerland) and 50% mesenchymal cell (MC) proliferation medium (DMEM high glucose supplemented with 10% FBS, 1× GlutaMax, 1× MEM (minimum essential medium) non-essential amino acids (NEAA), 50 µg/mL gentamicin, and 250 µg/mL amphotericin B, 5 ng/mL basic fibroblast growth factor (bFGF), 5 ng/mL platelet-derived growth factor (PDGF)-AB, and 5 ng/mL epidermal growth factor (EGF). Cell culture reagents were obtained from Thermo Fisher Scientific and recombinant human growth factors were obtained from Peprotech (Hamburg, Germany). Cells were cultivated at 37°C with 5% CO_2, and the medium was changed every 2–3 days. After reaching 80% confluency, RECs were detached using 0.04% trypsin/0.03% EDTA, and the reaction was stopped using trypsin-neutralizing solution (TNS; 0.05% trypsin inhibitor in 0.1% BSA, PromoCell, Heidelberg, Germany). Afterward, cells were centrifuged for 5 min at 300 × *g* and seeded on 0.1% gelatin (Sigma-Aldrich Chemie, Steinheim, Germany)-coated cell culture plates. The characterization of RECs was performed by flow cytometry using antibodies specific for epithelial marker β-catenin and renal proximal tubular marker CD13 (Miltenyi Biotec, Bergisch Gladbach, Germany).

Reprogramming of RECs into iPSCs Using srRNA

To perform reprogramming, 5 × 10^4 RECs (from 4 different donors, passages 2–3) were seeded per well of a 12-well plate coated with 0.1% gelatin and incubated overnight at 37°C in proliferation medium. Next day, the cells were incubated for 45–60 min with proliferation medium containing 200 ng/mL B18R interferon inhibitor (Thermo Fisher Scientific) at hypoxia (37°C, 5% CO_2, 5% O_2). For the transfection, lipoplexes were generated by the incubation of 0.5 µg OKSiM-GFP srRNA and 1.5 µL Lipofectamine Messenger Max (Thermo Fisher Scientific) in 0.5 mL Opti-MEM I reduced serum medium (Opti-MEM, Thermo Fisher Scientific) for 15 min at room temperature (RT). RECs were washed with DPBS, and lipoplexes were added. After 4 h of incubation, lipoplexes were discarded, and 1 mL fresh proliferation medium containing 200 ng/mL B18R was added for further incubation at 37°C with 5% CO_2 and 5% O_2 for 24h. Until day 3, the medium was replaced daily, and at day 3, 0.8 µg/mL puromycin (Sigma-Aldrich) was added to the proliferation medium to select srRNA-containing transfected cells. After 2–3 days of incubation, untransfected cells were eliminated. Thereafter, B18R-containing proliferation medium was changed every day. At day 7 of reprogramming, medium was changed to E8 stem cell medium (Essential 8, Thermo Fisher Scientific) supplemented with 200 ng/mL B18R. After the first appearance of iPSC colonies (beginning on day 21), B18R was withdrawn from the medium. iPSC colonies, which were positively stained with DyLight 550-labeled mouse anti-human StainAlive SSEA-4 antibody (Stemgent) were picked manually and seeded on 0.5 µg/cm^2 vitronectin (Thermo Fisher Scientific)-coated tissue culture plates and expanded in E8 medium.

Cultivation of iPSCs Derived from RECs

After reaching confluence, iPSCs were washed once with DPBS and incubated for 5–10 min at RT with DPBS containing 0.5 mM EDTA (Sigma-Aldrich). After the detachment, cells were suspended in E8 medium containing 10 µg/mL ROCK inhibitor Y-27632 (Enzo Life Sciences, Lausen, Switzerland) and passaged at a split ratio of 1:10 or 5 × 10^5 cells per vitronectin-coated well of a 6-well plate or in T25 culture flasks. iPSCs were cultivated at 37°C and 5% CO_2, and E8 medium was changed daily.

Flow Cytometry

Cells were washed with 1 mL DPBS and detached using 0.04% trypsin/0.03% EDTA, and the reaction was stopped by adding TNS (PromoCell). The cells were then centrifuged (5 min at 400 × *g*), washed with DPBS, and fixed for 10 min at RT in 0.5 mL fixation solution (R&D Systems). After washing with DPBS, cells were suspended in washing buffer (Permeabilization/Wash Buffer I, R&D Systems), and 5 µL fluorescently labeled antibody was added and incubated for 45 min at RT. Afterward, cells were washed with 0.5 mL washing buffer, suspended in 200 µL 1× BD CellFIX solution (Becton Dickinson, Heidelberg, Germany), and measured using a BD FACScan flow cytometer (Becton Dickinson) and Flowing Software (Turku Centre for Biotechnology, Turku, Finland).

qRT-PCR

To perform qRT-PCR analysis, 300 ng RNA was reverse transcribed into complementa DNA (cDNA) using the iScript Kit (Bio-Rad). The primers used for the specific amplification of transcripts are listed in Table S1, and they were ordered from Ella Biotech (Martinsried, Germany) and used at a final concentration of 300 nM. Real-time qRT-PCR reactions were performed in a CFX Connect Real-Time PCR

Detection System (Bio-Rad) using IQ SYBR Green Supermix (Bio-Rad). Expression of constitutively expressed gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control for the amount of RNA input. Primers were designed by using the Primer-Blast tool from NCBI.[49] Melting temperatures and self-complementarities were checked using the Oligonucleotide Properties Calculator from Northwestern University Medical School.[50]

The qRT-PCR amplification of cDNA was performed under the following conditions: 3 min at 95°C for one cycle, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 10 s. After 40 cycles, melt curve analysis was performed to ensure the specificity of the products. The qRT-PCR reactions were run in triplicate with a total volume of 15 µL per well. Levels of mRNA for each gene were normalized to GAPDH, and the results are shown relative to control mRNA levels.

Characterization of iPSCs Derived from RECs

Detection of Pluripotency Markers

Immunocytochemistry of iPSCs. 5×10^5 iPSCs (passages 4–5) were seeded on vitronectin-coated glass slides in 12-well plates and cultured for 2–3 days in cell culture medium until reaching 50%–70% confluency. Cells were washed 2× with 1 mL DPBS and fixed for 10 min at RT with 0.5 mL fixation solution (R&D Systems, Minneapolis, MN, USA). After washing with 0.5 mL washing buffer, the cells were incubated for 1.5 h at RT in washing buffer containing 5% BSA. Then, cells were incubated 3 h at RT with fluorescently labeled antibodies in washing buffer containing 1% BSA or overnight at 4°C with primary antibodies. After washing 3× with 0.5 mL washing buffer, the staining of the cells with fluorescently labeled secondary antibodies was performed for 1 h at RT in washing buffer containing 1% BSA. Afterward, the cells were washed 3× with washing buffer, DPBS, and then water. Subsequently, the coverslips were mounted using Fluoroshield mounting medium with DAPI (Abcam, Cambridge, UK). Rabbit anti-human POU5F1 (OCT4) (Sigma-Aldrich Chemie), rabbit anti-human SOX2 (Stemgent, Cambridge, MA, USA), and mouse anti-human LIN28A (6D1F9) (Thermo Fisher Scientific) antibodies were used as primary antibodies. Fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse immunoglobulin G (IgG) (whole molecule; Sigma-Aldrich) and Cy3-labeled goat anti-rabbit IgG cross-adsorbed secondary antibody (Thermo Fisher Scientific) were used according to the manufacturer's instructions. Furthermore, phycoerythrin (PE)-labeled mouse anti-human NANOG antibody (BD, Franklin Lakes, NJ, USA), DyLight 488-labeled mouse anti-human StainAlive TRA-1-60 antibody (Stemgent), and DyLight 550-labeled mouse anti-human StainAlive SSEA-4 antibody (Stemgent) were used. Fluorescence images were taken using an Axiovert 135 microscope and AxioVision 4.8.2 software (Carl Zeiss, Oberkochen, Germany).

Gene Expression Analysis of iPSCs. RNA from 1×10^6 iPSCs (passages 3–6) was isolated using the Aurum Total RNA Mini Kit (Bio-Rad, Munich, Germany) according to manufacturer's instructions, and qRT-PCR analysis was performed to detect the expression of OCT4, SOX2, NANOG, LIN28, E-cadherin, KLF4, and c-MYC. Levels of mRNA for each gene were normalized to that of GAPDH, and the results are shown relative to control mRNA levels in RECs. The commercially available iPSC line WTSli020-A (referred to as WT02 and generated from dermal fibroblasts using Sendai virus vector by the delivery of OCT4, SOX2, KLF4, and c-MYC; European Bank for induced pluripotent Stem Cells; Babraham, UK]) was used as iPSC control.

Trilineage Differentiation of iPSCs

To analyze the ability of the obtained iPSCs to differentiate into the three embryonal germ layers the directed differentiation of iPSCs (at passages 3–7) into meso-, endo-, and ectoderm was tested using the human StemMACS Trilineage Differentiation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according the manufacturer's instructions. Therefore, optimized cell numbers were seeded per well of a vitronectin-coated 12-well plate: mesoderm differentiation (1×10^5 iPSCs), endoderm differentiation (2×10^5 iPSCs), and ectoderm differentiation (1.5×10^5 iPSCs). At day 7, the differentiated cells were analyzed using flow cytometry.

The mesodermal differentiation capacity was analyzed by the formation of endothelial cells, using PE-labeled mouse anti-human CD31 antibody (BD Biosciences, Franklin Lakes, NJ, USA), and the formation of smooth muscle cells, using Alexa Fluor 488-labeled anti-human α-smooth muscle actin (SMA) antibody (R&D Systems). The endodermal differentiation capacity is characterized by the presence of definitive endoderm cells using PE-labeled anti-human α-fetoprotein (AFP) antibody (R&D Systems) and PE-labeled anti-human C-X-C chemokine receptor type 4 (CXCR4) antibody (R&D Systems). Ectodermal differentiation potential was assessed through the presence of neuroectoderm cells using PE-labeled anti-PAX6 antibody (Miltenyi Biotec) and Alexa Fluor 488-labeled anti-human neuron-specific TUBB3 antibody (BD Biosciences).

Teratoma Formation of iPSCs Using Chicken Embryo CAM Assay

To confirm the trilineage differentiation potential of iPSCs, the *in vivo* formation of teratomas was analyzed using CAM assay. Fresh fertilized chicken eggs of the Lohmann White × White Rock breed chicken variety were obtained from the breeding facility Matthias Sittig (Buchholz, Germany). The eggs were incubated for 3 days at 37°C and 60% relative humidity in an egg incubator (Heka-Brutgeräte, Rietberg-Varensell, Germany) and completely rotated twice a day. At day 3 of incubation, 2–3 mL albumin was aspirated by inserting an 18G needle at the tip of the egg without harming the yolk. Subsequently, a semi-permeable adhesive tape, Suprasorb F (Lohmann & Rauscher, Rengsdorf, Germany), was stuck to the eggshell. A circular window (Ø 1–1.5 cm) was cut into the shell. Unfertilized eggs showing no vasculature or heart beating were removed. Then, using the adhesive tape, the window was sealed to prevent dehydration and to minimize the risk of infection. Afterward, the eggs were incubated without rotation. At day 7, 2×10^6 iPSCs (passages 8–11) were suspended in 50 µL cell culture medium and mixed with 50 µL

Matrigel (hECS qualified, Corning). A silicone ring with an inner diameter of 0.8 cm (neoLab, Leonberg, Germany) was carefully placed onto the CAM, and 100 µL Matrigel-containing cells was applied into the inner circle of the ring. The eggs were then sealed and further incubated. At day 17, the CAMs were excised around the application area and fixed overnight at 4°C with 4% paraformaldehyde (Merck, Darmstadt, Germany). After washing with DPBS, the specimens were dehydrated using ascending ethanol series and embedded in paraffin for sectioning. Sections were cut at 8-µm thickness and stained with H&E (Morphisto, Frankfurt, Germany).

Genomic Stability of iPSCs

The genomic stability of iPSCs was analyzed by karyotyping at the Institute of Medical Genetics and Applied Genomics, University of Tübingen, Tübingen, Germany. Therefore, RECs and RE-derived iPSCs (at passages 4–7) were treated for 1 h with Colcemid (Biochrom), incubated with 0.075 M KCl for 30 min at 37°C, and fixed with 1:3 acidic acid:methanol. Karyotyping was performed on G-banded metaphase chromosomes (banding quality of 400–500 bp) using standard cytogenetic procedures. An intact genome was demonstrated by karyotyping (numerical analysis of 15 mitoses and structural analysis of at least 5 mitoses).

Detection of srRNA Elimination after the Reprogramming of Cells

RNA from 1×10^6 iPSCs (passage 3) was isolated using the Aurum Total RNA Mini Kit according to manufacturer's instructions. Using nsP2- and nsP4-specific primers (Table S1) and qRT-PCR, the presence of srRNA in generated iPSCs was analyzed. RNA levels were normalized to GAPDH, and the results are shown relative to control RNA levels in RECs. In addition, to obtain a positive control, cells were transfected with 1 µg srRNA, and the RNA was isolated after 2 days of cultivation.

Differentiation of iPSCs into Cardiomyocytes

The differentiation of iPSCs from 4 different donors into cardiomyocytes was performed using the PSC Cardiomyocyte Differentiation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Therefore, 2×10^5 iPSCs (passages 4–12) were seeded on Geltrex LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific) (1:100 diluted in CMM) or on 0.5 µg/cm^2 vitronectin-coated wells of a 6-well plate. After 3–4 days cultivation in E8 medium, the differentiation protocol started. The cells were incubated for 2 days with cardiomyocyte differentiation medium A and then for 2 days with cardiomyocyte differentiation medium B. On the fifth day of differentiation, CMM was added to the cells for the following days of differentiation.

After 7–12 days, when contracting cardiomyocytes were observed (passage 0), the medium was changed to cardiomyocyte enrichment medium (CEM): RPMI 1640 medium without glucose (Thermo Fisher) containing 0.25% BSA (Fraction V, Sigma-Aldrich), 4 mM sodium lactate (Fisher Chemicals), 4 mM HEPES (Thermo Fisher), and 6.5 µM ascorbic acid (Acros Organics, Geel, Belgium). After 4–6 days, cardiomyocytes were passaged using 1 mL TrypLE solution (Thermo Fisher), and the reaction was stopped by adding an equal volume of TNS (PromoCell). Cell suspension was filtered through a 100-µm cell strainer (Greiner Bio-One, Frickenhausen, Germany) and centrifuged at 200 × *g* for 5 min. The cells were suspended in CMM, seeded onto Geltrex- or vitronectin-coated cell culture plates with a density of 1×10^5 cells per square centimeter (passage 1), and cultivated in a humidified atmosphere at 37°C and 5% CO_2. After 4–6 days of enrichment procedure, the yield of differentiated cardiomyocytes was determined by flow cytometry using FITC-labeled mouse anti-human cTNT antibody from Miltenyi Biotec.

Detection of Cardiomyocyte Markers

Immunocytochemistry of Cardiomyocytes Derived from iPSCs

1×10^6 iPSC-derived cardiomyocytes (passage 1) were plated on Geltrex-coated glass slides in 12-well plates and cultivated for 2 days in CMM. Rabbit anti-human cTNT antibody, mouse anti-human ACTA2 (both from R&D Systems), and mouse anti-human MYH6 antibody (GeneTex, Irvine, CA, USA) were used as cardiomyocyte-specific primary antibodies. NL637-conjugated donkey anti-mouse IgG (R&D Systems) and Cy3-labeled goat anti-rabbit IgG (Thermo Fisher Scientific) were used as secondary antibodies. Primary and secondary antibodies were applied as recommended by the manufacturer. Nuclei were stained using a final concentration of 5 µM SYTO 9 Green Fluorescent Nucleic Acid Stain (Thermo Fisher Scientific) in DPBS. Fluorescence images were taken after washing with DBPS using a Leica TCS SP5 confocal laser scanning microscope and the Leica Application Suite Advanced Fluorescence (2.7.3.9723) software (Leica, Wetzlar, Germany). To obtain fluorescence microscopic overview images of cardiomyocytes derived from iPSCs, cells were stained using FITC-labeled anti-human cTNT and PE-labeled anti-human (sarcomeric) α-actinin (ACTN2) antibodies from Miltenyi Biotech and Fluoroshield Mounting Medium with DAPI (Abcam). Fluorescence images were taken using an Axiovert 135 microscope and AxioVision 4.8.2 software (Carl Zeiss).

Gene Expression Analysis of Cardiomyocytes Derived from iPSCs

RNA from 1×10^6 iPSC-derived cardiomyocytes (12–16 days after starting the differentiation; passage 0) was isolated using the Aurum Total RNA Mini Kit according to manufacturer's instructions, and qRT-PCR analysis was performed to detect the expression of ANP, cTNT, MYH6, and α-actinin, cardiac muscle 1 (ACTC1). Levels of mRNA for each gene were normalized to GAPDH, and the results are shown relative to control mRNA levels in RECs.

Detection of Cardiac Troponin I

1×10^6 iPSCs (passages 4–8) were seeded per well on Geltrex-coated 6-well plates and differentiated for 12–16 days into cardiomyocytes. The supernatants (CMM) of beating cardiomyocyte cultures (passage 0) were analyzed to measure the TNNI3 content using ADVIA Centaur XPT (TnI-Ultra chemiluminescent immunoassay, Siemens Healthcare Diagnostics, Eschborn, Germany) according to the manufacturer's instructions. Additionally, the TNNI3 concentration was

measured in cell culture supernatants of RECs (REMC medium) and REC-derived iPSCs (E8 medium) as well as in fresh CMM.

Characterization of Contracting Cardiomyocytes

Video Microscopy

Video recordings of beating cells were performed to analyze the mechanical beating behavior of obtained cardiomyocytes from 4 different donors. Therefore, 7 images per second were taken for 30 s using an Axiovert 135 microscope and AxioVision 4.8.2 software (Carl Zeiss). The beating center(s) and the beat rate of obtained cardiomyocytes were determined using the MATLAB application Motion GUI.[51]

Analysis of Electromechanical Coupling Using Ca^{2+} Imaging

To analyze the electromechanical coupling of the obtained cardiomyocytes, calcium imaging was performed. Ca^{2+} oscillations are an indication of a fully differentiated cardiac phenotype and key regulator in controlling cardiomyocyte relaxation and contraction. To evaluate the intracellular Ca^{2+} behavior, Ca^{2+} transients were measured from spontaneously contracting cardiomyocytes (passage 0, 4 donors) using the Fluo-4 Direct Calcium Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Beating cardiomyocytes were obtained 12 days after the differentiation of iPSCs on Geltrex-coated wells of a 6-well plate. The obtained cells were incubated for 30 min at 37°C with 1 mL 1× Fluo-4 Direct Calcium Assay Reagent Solution and 1 mL CMM. Using a fluorescence microscope (Axiovert 135), Ca^{2+} transients were recorded within the next 30 min at RT with 5 pictures per second and a 30 ms exposure time at 494 nm excitation wavelength.

Response of Cardiomyocytes to Pharmacological Modulation

To analyze the reaction of the generated REC-iPSC derived cardiomyocytes on pharmaceutical drugs, the cells were treated with 0.0001, 0.001, 0.01, 0.1, 1.0, or 10 μM Ca^{2+} channel blocker nifedipine or β-adrenoceptor agonist isoproterenol. Stock solutions of 100 mM nifedipine or 100 mM isoproterenol (both from Sigma Aldrich) were prepared in DMSO and diluted in CMM. Cardiomyocyte culture medium was replaced 11 or 13 days after starting the differentiation process (passage 0) by preheated (37°C) drug dilutions in CMM and incubated for 5 min at 37°C. Video recordings of 20 s each were performed using AxioCam and an Axiovert 135 microscope, and the beat rate was analyzed using Motion GUI. After recordings, the medium was changed to CMM.

Statistical Analysis

Data are shown as mean ± SD or SEM. Paired t test or one-way ANOVA for repeated measurements followed by Bonferroni's multiple comparison test was performed to compare the means. Two-tailed statistical analyses were performed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA). Differences of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.07.016.

AUTHOR CONTRIBUTIONS

H.S. and M.A.-A. conceived and designed the experiments. H.S. and M.W. performed the experiments with support from A.B., A.-F.P., H.P.W., and M.A.-A., and analyzed the data. U.M.-H. performed karytotyping analysis and interpretation. C.v.O. performed CLSM investigations. H.P.W. and C.S. contributed reagents, materials, and analysis tools. H.S., M.W., and M.A.-A. wrote the paper. M.A.-A. supervised the project.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

For TNNI3 detection in supernatants, the authors would like to thank Dr. Ingo Rettig at the Division of Endocrinology, Diabetology, Vascular Medicine, Nephrology and Clinical Chemistry, Department of Internal Medicine IV, University of Tübingen, Germany. We would also like to thank Jeannette Schöne and Elisa Kächele for excellent cytogenetic technical assistance and Annika Hechler for assistance with the collection of CLSM data. This study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG) through AV 133/7-1.

REFERENCES

1. Roger, V.L. (2013). Epidemiology of heart failure. Circ. Res. *113*, 646–659.
2. Bergmann, O., Zdunek, S., Felker, A., Salehpour, M., Alkass, K., Bernard, S., Sjostrom, S.L., Szewczykowska, M., Jackowska, T., Dos Remedios, C., et al. (2015). Dynamics of cell generation and turnover in the human heart. Cell *161*, 1566–1575.
3. Li, R.K., Jia, Z.Q., Weisel, R.D., Mickle, D.A.G., Zhang, J., Mohabeer, M.K., Rao, V., and Ivanov, J. (1996). Cardiomyocyte transplantation improves heart function. Ann. Thorac. Surg. *62*, 654–660.
4. Watanabe, E., Smith, D.M., Jr., Delcarpio, J.B., Sun, J., Smart, F.W., Van Meter, C.H., Jr., and Claycomb, W.C. (1998). Cardiomyocyte transplantation in a porcine myocardial infarction model. Cell Transplant. *7*, 239–246.
5. Valarmathi, M.T., Fuseler, J.W., Goodwin, R.L., Davis, J.M., and Potts, J.D. (2011). The mechanical coupling of adult marrow stromal stem cells during cardiac regeneration assessed in a 2-D co-culture model. Biomaterials *32*, 2834–2850.
6. Caspi, O., Itzhaki, I., Kehat, I., Gepstein, A., Arbel, G., Huber, I., Satin, J., and Gepstein, L. (2009). In vitro electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. Stem Cells Dev. *18*, 161–172.
7. Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A.B., et al. (2003). Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation *107*, 2733–2740.
8. Zhang, J., Wilson, G.F., Soerens, A.G., Koonce, C.H., Yu, J., Palecek, S.P., Thomson, J.A., and Kamp, T.J. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ. Res. *104*, e30–e41.
9. Burridge, P.W., and Zambidis, E.T. (2013). Highly efficient directed differentiation of human induced pluripotent stem cells into cardiomyocytes. Methods Mol. Biol. *997*, 149–161.
10. Mathur, A., Loskill, P., Shao, K., Huebsch, N., Hong, S., Marcus, S.G., Marks, N., Mandegar, M., Conklin, B.R., Lee, L.P., and Healy, K.E. (2015). Human iPSC-based cardiac microphysiological system for drug screening applications. Sci. Rep. *5*, 8883.
11. Moretti, A., Bellin, M., Welling, A., Jung, C.B., Lam, J.T., Bott-Flügel, L., Dorn, T., Goedel, A., Höhnke, C., Hofmann, F., et al. (2010). Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N. Engl. J. Med. *363*, 1397–1409.
12. Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

13. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

14. Raab, S., Klingenstein, M., Liebau, S., and Linta, L. (2014). A comparative view on human somatic cell sources for iPSC generation. Stem Cells Int. *2014*, 768391.

15. Ingelfinger, J.R. (2002). Nephrogenic adenomas as renal tubular outposts. N. Engl. J. Med. *347*, 684–686.

16. Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313–317.

17. Wernig, M., Meissner, A., Cassady, J.P., and Jaenisch, R. (2008). c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell *2*, 10–12.

18. Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008). Induced pluripotent stem cells generated without viral integration. Science *322*, 945–949.

19. Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature *458*, 771–775.

20. Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I., and Thomson, J.A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. Science *324*, 797–801.

21. Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., et al. (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell *4*, 381–384.

22. Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., et al. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell *7*, 618–630.

23. Steinle, H., Behring, A., Schlensak, C., Wendel, H.P., and Avci-Adali, M. (2017). Concise review: application of in vitro transcribed messenger RNA for cellular engineering and reprogramming: progress and challenges. Stem Cells *35*, 68–79.

24. Rabinovich, P.M., and Weissman, S.M. (2013). Cell engineering with synthetic messenger RNA. Methods Mol. Biol. *969*, 3–28.

25. Thess, A., Grund, S., Mui, B.L., Hope, M.J., Baumhof, P., Fotin-Mleczek, M., and Schlake, T. (2015). Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. Mol. Ther. *23*, 1456–1464.

26. Anderson, B.R., Muramatsu, H., Nallagatla, S.R., Bevilacqua, P.C., Sansing, L.H., Weissman, D., and Karikó, K. (2010). Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. Nucleic Acids Res. *38*, 5884–5892.

27. Karikó, K., Buckstein, M., Ni, H., and Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity *23*, 165–175.

28. Karikó, K., Muramatsu, H., Welsh, F.A., Ludwig, J., Kato, H., Akira, S., and Weissman, D. (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol. Ther. *16*, 1833–1840.

29. Svitkin, Y.V., Cheng, Y.M., Chakraborty, T., Presnyak, V., John, M., and Sonenberg, N. (2017). N1-methyl-pseudouridine in mRNA enhances translation through eIF2α-dependent and independent mechanisms by increasing ribosome density. Nucleic Acids Res. *45*, 6023–6036.

30. Yoshioka, N., Gros, E., Li, H.R., Kumar, S., Deacon, D.C., Maron, C., Muotri, A.R., Chi, N.C., Fu, X.D., Yu, B.D., and Dowdy, S.F. (2013). Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell *13*, 246–254.

31. Petrakova, O., Volkova, E., Gorchakov, R., Paessler, S., Kinney, R.M., and Frolov, I. (2005). Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in Mammalian cells. J. Virol. *79*, 7597–7608.

32. Mummery, C.L., Zhang, J., Ng, E.S., Elliott, D.A., Elefanty, A.G., and Kamp, T.J. (2012). Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. Circ. Res. *111*, 344–358.

33. Ringsrud, K.M. (2001). Cells in the urine sediment. Lab. Med. *32*, 153–155.

34. Loh, Y.H., Agarwal, S., Park, I.H., Urbach, A., Huo, H., Heffner, G.C., Kim, K., Miller, J.D., Ng, K., and Daley, G.Q. (2009). Generation of induced pluripotent stem cells from human blood. Blood *113*, 5476–5479.

35. Kim, Y., Rim, Y.A., Yi, H., Park, N., Park, S.H., and Ju, J.H. (2016). The generation of human induced pluripotent stem cells from blood cells: an efficient protocol using serial plating of reprogrammed cells by centrifugation. Stem Cells Int. *2016*, 1329459.

36. Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M.J., Ji, H., Ehrlich, L.I., et al. (2010). Epigenetic memory in induced pluripotent stem cells. Nature *467*, 285–290.

37. Kim, Y.G., Baltabekova, A.Z., Zhiyenbay, E.E., Aksambayeva, A.S., Shagyrova, Z.S., Khannanov, R., Ramanculov, E.M., and Shustov, A.V. (2017). Recombinant Vaccinia virus-coded interferon inhibitor B18R: Expression, refolding and a use in a mammalian expression system with a RNA-vector. PLoS ONE *12*, e0189308.

38. Foster, K.W., Ren, S., Louro, I.D., Lobo-Ruppert, S.M., McKie-Bell, P., Grizzle, W., Hayes, M.R., Broker, T.R., Chow, L.T., and Ruppert, J.M. (1999). Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. Cell Growth Differ. *10*, 423–434.

39. Foster, K.W., Frost, A.R., McKie-Bell, P., Lin, C.Y., Engler, J.A., Grizzle, W.E., and Ruppert, J.M. (2000). Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Res. *60*, 6488–6495.

40. Yu, F., Li, J., Chen, H., Fu, J., Ray, S., Huang, S., Zheng, H., and Ai, W. (2011). Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene *30*, 2161–2172.

41. Später, D., Hansson, E.M., Zangi, L., and Chien, K.R. (2014). How to make a cardiomyocyte. Development *141*, 4418–4431.

42. Cyganek, L., Tiburcy, M., Sekeres, K., Gerstenberg, K., Bohnenberger, H., Lenz, C., Henze, S., Stauske, M., Salinas, G., Zimmermann, W.H., et al. (2018). Deep phenotyping of human induced pluripotent stem cell-derived atrial and ventricular cardiomyocytes. JCI Insight *3*, 99941.

43. Schweizer, P.A., Darche, F.F., Ullrich, N.D., Geschwill, P., Greber, B., Rivinius, R., Seyler, C., Müller-Decker, K., Draguhn, A., Utikal, J., et al. (2017). Subtype-specific differentiation of cardiac pacemaker cell clusters from human induced pluripotent stem cells. Stem Cell Res. Ther. *8*, 229.

44. Besser, R.R., Ishahak, M., Mayo, V., Carbonero, D., Claure, I., and Agarwal, A. (2018). Engineered microenvironments for maturation of stem cell derived cardiac myocytes. Theranostics *8*, 124–140.

45. Di Baldassarre, A., Cimetta, E., Bollini, S., Gaggi, G., and Ghinassi, B. (2018). Human-induced pluripotent stem cell technology and cardiomyocyte generation: progress and clinical applications. Cells *7*, E48.

46. Dubois, N.C., Craft, A.M., Sharma, P., Elliott, D.A., Stanley, E.G., Elefanty, A.G., Gramolini, A., and Keller, G. (2011). SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. Nat. Biotechnol. *29*, 1011–1018.

47. Tohyama, S., Hattori, F., Sano, M., Hishiki, T., Nagahata, Y., Matsuura, T., Hashimoto, H., Suzuki, T., Yamashita, H., Satoh, Y., et al. (2013). Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. Cell Stem Cell *12*, 127–137.

48. Lopaschuk, G.D., and Jaswal, J.S. (2010). Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. J. Cardiovasc. Pharmacol. *56*, 130–140.

49. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T.L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics *13*, 134.

50. Kibbe, W.A. (2007). OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. *35*, W43–W46.

51. Huebsch, N., Loskill, P., Mandegar, M.A., Marks, N.C., Sheehan, A.S., Ma, Z., Mathur, A., Nguyen, T.N., Yoo, J.C., Judge, L.M., et al. (2015). Automated video-based analysis of contractility and calcium flux in human-induced pluripotent stem cell-derived cardiomyocytes cultured over different spatial scales. Tissue Eng. Part C Methods *21*, 467–479.

8.5. PUBLIKATION V

Generation of iPSCs from jaw periosteal cells using self-replicating RNA

International Journal of
Molecular Sciences

MDPI

Article

Generation of iPSCs from Jaw Periosteal Cells Using Self-Replicating RNA

Felix Umrath [1,†], Heidrun Steinle [2,†], Marbod Weber [2], Hans-Peter Wendel [2], Siegmar Reinert [1], Dorothea Alexander [1,†] and Meltem Avci-Adali [2,*,†]

1 Department of Oral and Maxillofacial Surgery, University Hospital Tübingen, 72076 Tübingen, Germany; felix.umrath@med.uni-tuebingen.de (F.U.); siegmar.reinert@med.uni-tuebingen.de (S.R.); Dorothea.Alexander@med.uni-tuebingen.de (D.A.)
2 Department of Thoracic and Cardiovascular Surgery, University Hospital Tübingen, 72076 Tübingen, Germany; heidrun.steinle@uni-tuebingen.de (H.S.); marbod.weber@uni-tuebingen.de (M.W.); hans-peter.wendel@med.uni-tuebingen.de (H.-P.W.)
* Correspondence: meltem.avci-adali@uni-tuebingen.de; Tel.: +49-7071-29-83334
† These authors contributed equally to this work.

Received: 8 March 2019; Accepted: 30 March 2019; Published: 3 April 2019

Abstract: Jaw periosteal cells (JPCs) represent a suitable stem cell source for bone tissue engineering (BTE) applications. However, challenges associated with limited cell numbers, stressful cell sorting, or the occurrence of cell senescence during in vitro passaging and the associated insufficient osteogenic potential in vitro of JPCs and other mesenchymal stem/stromal cells (MSCs) are main hurdles and still need to be solved. In this study, for the first time, induced pluripotent stem cells (iPSCs) were generated from human JPCs to open up a new source of stem cells for BTE. For this purpose, a non-integrating self-replicating RNA (srRNA) encoding reprogramming factors and green fluorescent protein (GFP) as a reporter was used to obtain JPC-iPSCs with a feeder- and xeno-free reprogramming protocol to meet the highest safety standards for future clinical applications. Furthermore, to analyze the potential of these iPSCs as a source of osteogenic progenitor cells, JPC-iPSCs were differentiated into iPSC-derived mesenchymal stem/stromal like cells (iMSCs) and further differentiated to the osteogenic lineage under xeno-free conditions. The produced iMSCs displayed MSC marker expression and morphology as well as strong mineralization during osteogenic differentiation.

Keywords: induced pluripotent stem cells (iPSCs); jaw periosteal cells (JPCs); self-replicating RNA; reprogramming; iPSC-derived mesenchymal stem/stromal like cells (iMSCs); bone-tissue engineering

1. Introduction

Depending on the diagnosis, the perpetual challenge for clinicians in oral and maxillofacial surgery is to reconstruct and regenerate small as well as large bone defects. In previous studies, we and others demonstrated that human jaw periosteal cells (JPCs) can be used for the generation of bone tissue engineering (BTE) constructs [1–3]. For the regeneration of small defects, the use of the patient's own jaw periosteal cells can be appropriate and the isolated cell numbers sufficient. The reconstruction of large bone resections with BTE products, e.g., for tumor patients, requires extremely high cell numbers. Unfortunately, the production of adequate cell yields is hampered by the occurrence of cell senescence and a decreased osteogenic potential of periosteal stem cells at higher passages. Additionally, primary JPCs, as well as mesenchymal stem/stromal cells (MSCs) from other tissues exhibit donor variability concerning their differentiation potential. This variability can be caused by different factors, such as varying stem cell numbers in heterogeneous starting materials, the tissue of origin, or the isolation

and maintenance techniques [4,5]. Although surface markers have been identified, which allow the enrichment of osteogenic progenitor cells from heterogeneous cell populations via fluorescence or magnetic activated cell sorting (FACS, MACS) [6–8], cell yields are often unsatisfactory because of low sorting efficiencies and high cell mortality.

The use of patient-specific induced pluripotent stem cells (iPSCs) could solve the above-mentioned problems. iPSCs can differentiate into every cell type of the body and their self-renewal capacity allows the expansion of desired cells to extremely high cell numbers [9]. They can be differentiated into mesenchymal stem/stromal like cells (iMSCs), which can serve as an alternative stem cell source for BTE [10]. Additionally, the directed differentiation of iPSCs to iMSCs could result in more homogeneous populations of osteogenic progenitor cells and therefore might help to standardize the starting material for clinical BTE products [11].

For the safe clinical applicability of iPSC-derived cells, the use of reprogramming methods excluding insertional mutagenesis and xeno-contaminations is of crucial importance [12]. Insertional mutagenesis can be prevented by reprogramming cells using synthetic mRNA, which is a safe and efficient reprogramming technique [13]. However, the transient nature of synthetic mRNAs requires daily transfections, which decrease the cell viability and make the process laborious and time-consuming. Yoshioka et al. [14] improved this approach by developing a synthetic polycistronic self-replicating RNA (srRNA) consisting of the OSKM reprogramming factors (OCT4, SOX2, KLF4, cMYC) and the non-structural proteins (nsP1 to nsP4) encoding sequences of the Venezuelan equine encephalitis virus (VEE), which enabled the RNA to replicate inside the transfected cells as long as their innate immune response was suppressed by the use of the interferon inhibitor B18R [14]. Using this srRNA, a single transfection can be sufficient to reprogram cells, and the removal of B18R leads to the degradation of the srRNA and results in the generation of footprint-free iPSCs.

In the present study, we used an optimized srRNA construct containing a green fluorescent protein (GFP) encoding sequence to enable the live monitoring of transfected cells and the adaptation of the reprogramming protocol to the cell types used. For the production of clinically applicable BTE constructs, animal-derived components have to be excluded from every part of the generation procedure. Thus, in this study, we established for the first time a continuous xeno-free workflow from the initial primary JPC isolation to iPSC generation and differentiation into iMSCs until the final stage of mineralized tissue.

2. Results

2.1. srRNA Transfection Efficiency

To generate iPSCs, JPCs were seeded into 12-well plates at a density of 2.5×10^4 cells/well and transfected with 0.25 ng of srRNA. After 24 h, transfected cells expressing GFP were detected by fluorescence microscopy (Figure 1a–c). An average transfection efficiency of $33 \pm 2\%$ was determined by the measurement of GFP-positive cells via flow cytometry (Figure 1d). Significantly higher (1.67-fold) median fluorescence index (MFI) values were detected in srRNA transfected JPCs compared to untransfected JPCs (Figure 1e).

2.2. JPC Reprogramming

The reprogramming was performed with JPCs from three different patients according to Figure 2a. To select the srRNA containing cells 24 h after the transfection, 1 µg/mL puromycin was added to the medium. Furthermore, to enhance reprogramming efficiency, 250 µM sodium butyrate (NaB), which is a histone deacetylase inhibitor, was added to the medium. Puromycin selection was continued until day five, when all cells in the untransfected control wells were dead (Figure 2b). In comparison, Figure 2c shows the surviving cells transfected with srRNA at day five. After puromycin selection, cells were passaged and seeded in 1:5 ratio into vitronectin coated wells of 12-well plates. The first iPSC colonies emerged between day 12 and 15 (Figure 2d,e). Single iPSC colonies were picked and

transferred into vitronectin coated 12-well plates containing E8 medium with 10 µM Y27632 ROCK inhibitor and were maintained in E8 medium without Y27632 from the next day onward.

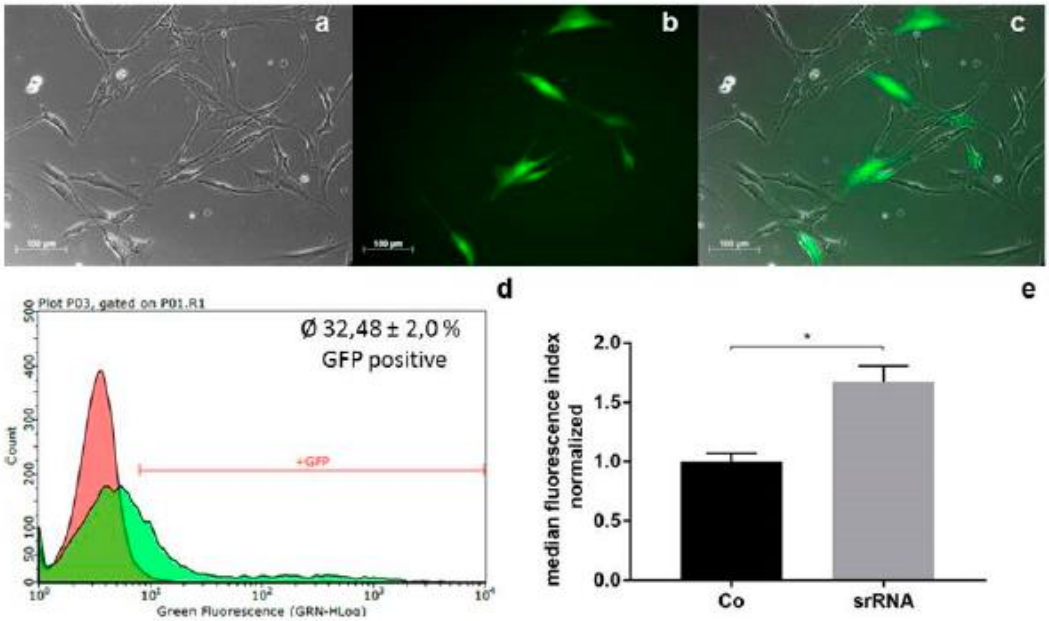

Figure 1. Transfection of jaw periosteal cells (JPCs) with self-replicating RNA (srRNA). (**a**–**c**) Representative (**a**) brightfield, (**b**) green fluorescent protein (GFP), and (**c**) merged images of JPCs 24 h after srRNA transfection. (**d**) Representative histogram of flow cytometry measurements of untransfected (red) and srRNA transfected (green) JPCs 24 h after transfection. (**e**) Average normalized median fluorescence index (MFI) values + standard deviation (SD) of srRNA transfected and untransfected (Co) JPCs relative to MFI values of untransfected (Co) samples were calculated and compared using Student's *t*-test ($n = 3$, * $p < 0.05$).

2.3. Characterization of iPSCs

2.3.1. Pluripotency Marker Expression

To characterize the generated iPSC clones, the expression of pluripotency markers was analyzed via immunostaining and flow cytometry. Figure 3a shows the strong fluorescence staining of Oct4, Sox2, Lin28, Nanog, TRA-1-60 and SSEA4 across all cells of the stained iPSC colonies. Furthermore, the surface marker expression of the generated iPSCs was compared to the initial JPCs using flow cytometry (Figure 3b). In the obtained iPSCs, the expression of MSC markers CD73 and CD105 was significantly downregulated, while pluripotent stem cell markers SSEA-4, TRA-1-60, and TRA-1-81 were significantly upregulated. JPCs and iPSCs were both positive for CD90 and negative for SSEA-1, as expected. Furthermore, the expression of MSC specific (CD73, CD44) and pluripotent stem cell specific (OCT4, NANOG, ALP, TERT) transcripts were analyzed by qRT-PCR. A significantly increased expression of pluripotent stem cell markers OCT4, NANOG, ALP, and TERT was detected in iPSCs compared to JPCs, while the expression of MSC specific transcripts CD73 and CD44 was significantly downregulated (Figure 3c).

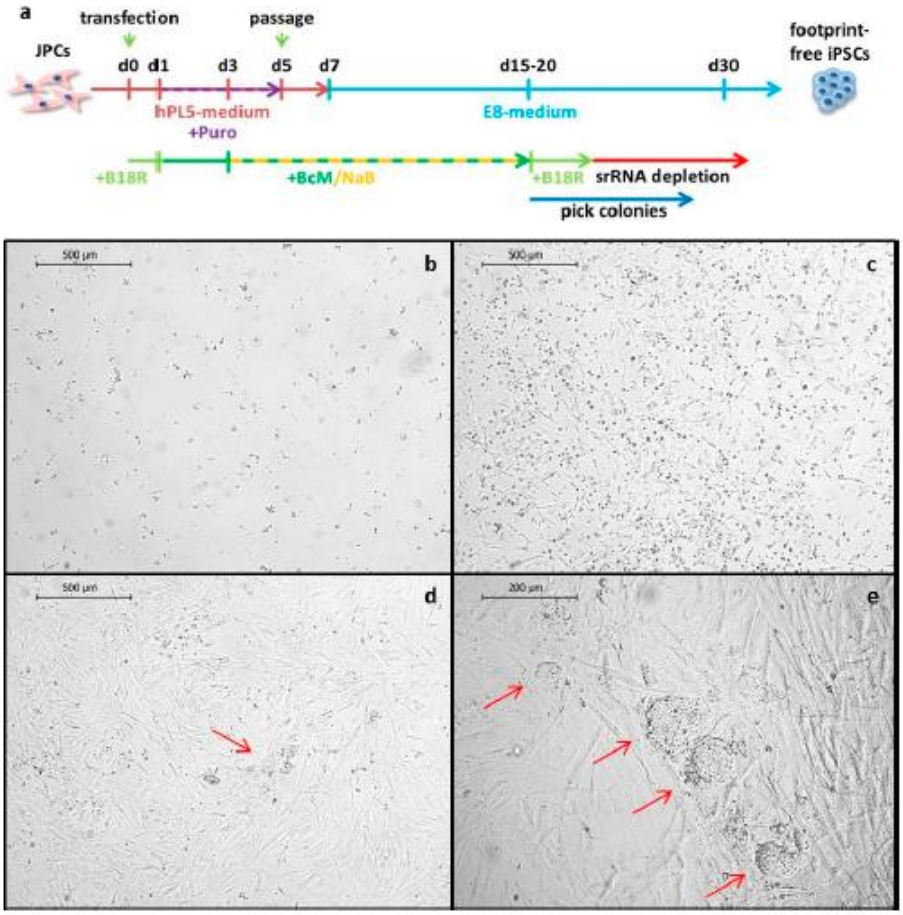

Figure 2. Reprogramming of JPCs. (**a**) Timeline of JPC reprogramming. JPCs were transfected (d0) in hPL5-medium containing 0.2 µg/mL B18R protein. On day one, the medium was changed to hPL5 containing 25% conditioned medium containing B18R (BcM) and 1.0 µg/mL puromycin (Puro). Puromycin selection was continued until day five (purple arrow). Cells were passaged on day five, and on day seven, the medium was changed to Essential 8 (E8) containing 25% BcM. Sodium butyrate (NaB) was added to the medium from day three to 15. When the first induced pluripotent stem cell (iPSC) colonies emerged, the medium was changed to E8 containing 0.2 µg/mL B18R protein. iPSC colonies were picked at day 15 or later. (**b–e**) Representative bright field images of JPCs during srRNA-based reprogramming. (**b**) Untransfected JPCs treated with puromycin at day five. (**c**) srRNA transfected JPCs treated with puromycin at day five. (**d**) srRNA transfected JPCs at day 12 with the first iPSC-colonies (indicated by a red arrow). (**e**) srRNA transfected cells at day 15 with iPSC-colonies (indicated by red arrows).

2.3.2. Differentiation Potential of iPSCs into the Three Germ Layers In Vitro and In Vivo

The differentiation capacity of the generated iPSCs to form all tissues of the three germ layers is characteristic for pluripotent stem cells and was first assessed in vitro by a 7-day trilineage differentiation protocol. As shown in Figure 4a, endothelial, hepatocyte-like and neural-like cells were obtained after the differentiation of iPSCs. The mesoderm, endoderm, and ectoderm differentiation potential was confirmed by tissue specific antibody staining and quantification using flow cytometry. Mesodermal differentiation resulted in 59 ± 23% CD31-positive and 89 ± 11% SMA-positive cells. Endodermal induction yielded 96 ± 2% AFP-positive and 99 ± 1% CXCR4-positive cells. Ectodermal differentiation was demonstrated by the detection of 94 ± 3% Pax6-positive and 89 ± 6% Tuj1-positive cells.

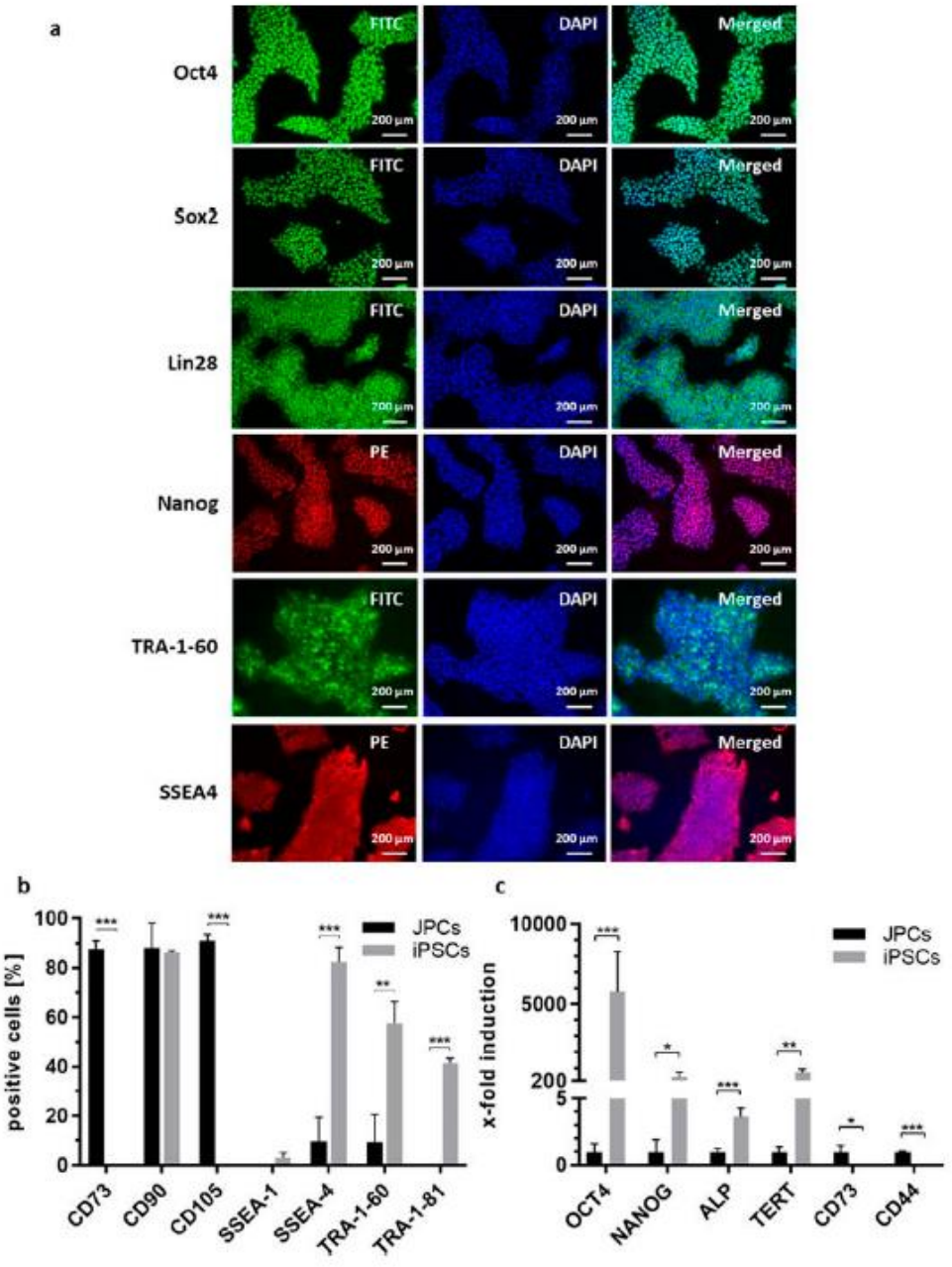

Figure 3. Pluripotency marker expression of JPC-derived iPSCs. (**a**) Oct4, Sox2, Lin28, Nanog, TRA-1-60 and SSEA4 immunostaining of iPSCs. (**b**) Surface marker expression of JPCs and JPC-derived iPSCs analyzed by flow cytometry and compared using Student's *t*-test ($n = 3$, ** $p < 0.01$, *** $p < 0.001$). (**c**) Gene expression analysis of JPCs and JPC-derived iPSCs by qRT-PCR. Gene expression levels were normalized to levels of GAPDH. Mean values + SD of iPSC and JPC gene expression were displayed relative to those of JPCs. Statistical significance was calculated using Student's *t*-test. ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Using a chicken embryo chorioallantoic membrane (CAM) assay, the in vivo differentiation of iPSCs was analyzed. 10 days after the application of iPSCs onto the CAM, teratoma formation could be observed. Subsequently, teratomas were sectioned and stained with hematoxylin & eosin (H&E) and tissue types of the mesodermal (bone-like tissue), endodermal (gut-like tissue) and ectodermal (squamous epithelium) lineage could be identified (Figure 4b).

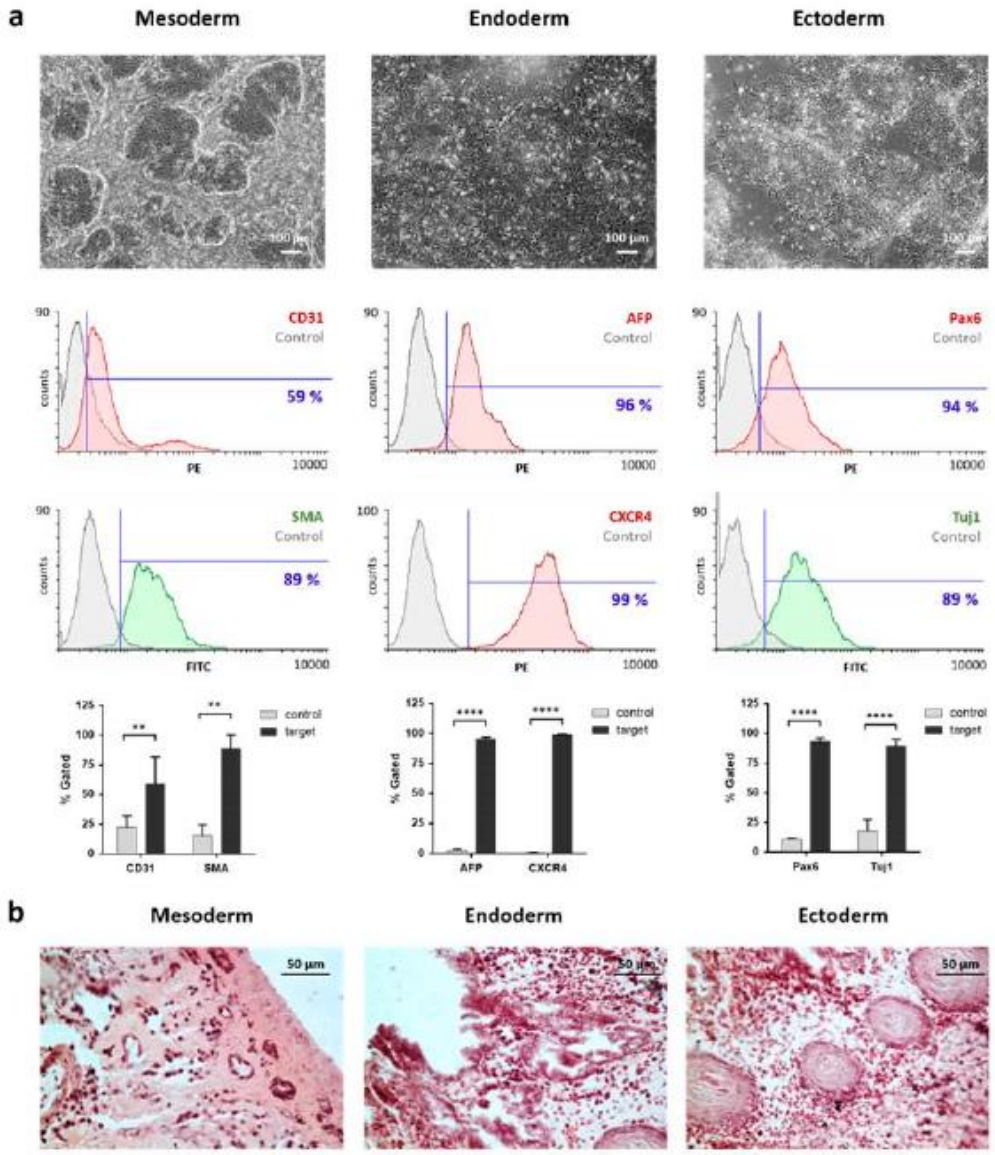

Figure 4. Trilineage differentiation potential of JPC-derived iPSCs. (**a**) Microscopic pictures of mesodermal, endodermal and ectodermal in vitro differentiation showing different morphologies after six days of germ layer specific differentiation. Flow cytometry analysis of differentiated cells was performed after staining with specific antibodies compared to untreated controls. Data are shown as mean + SD. Differences were compared using one-way ANOVA ($n = 3$, ** $p < 0.01$, **** $p < 0.0001$). (**b**) Microscopic images of in vivo teratoma formation of iPSCs using a chorioallantoic membrane (CAM) assay. H&E stained sections showed mesodermal (bone-like), endodermal (gut-like) and ectodermal (squamous epithelium) tissue.

2.3.3. Detection of srRNA in iPSCs and Karyotyping of iPSCs

After the successful generation of iPSCs, the presence of srRNA in the cells was analyzed. Therefore, qRT-PCR was performed with RNA isolated from JPCs and JPC-derived iPSCs and primer pairs specific to the nsP2 and nsP4 sequences. JPCs transfected with srRNA and cultivated for 48 h, served as positive control (JPC+). These samples showed high amounts of nsP2 and nsP4 compared to untransfected JPCs and iPSCs (Figure 5a). In contrast, the nsP2 and nsP4 amount measured in iPSCs was similar to that of untransfected JPCs, which demonstrates the absence of srRNA in the

reprogrammed cells. Karyotyping of JPC-derived iPSCs resulted in normal karyograms without chromosomal aberrations (Figure 5b).

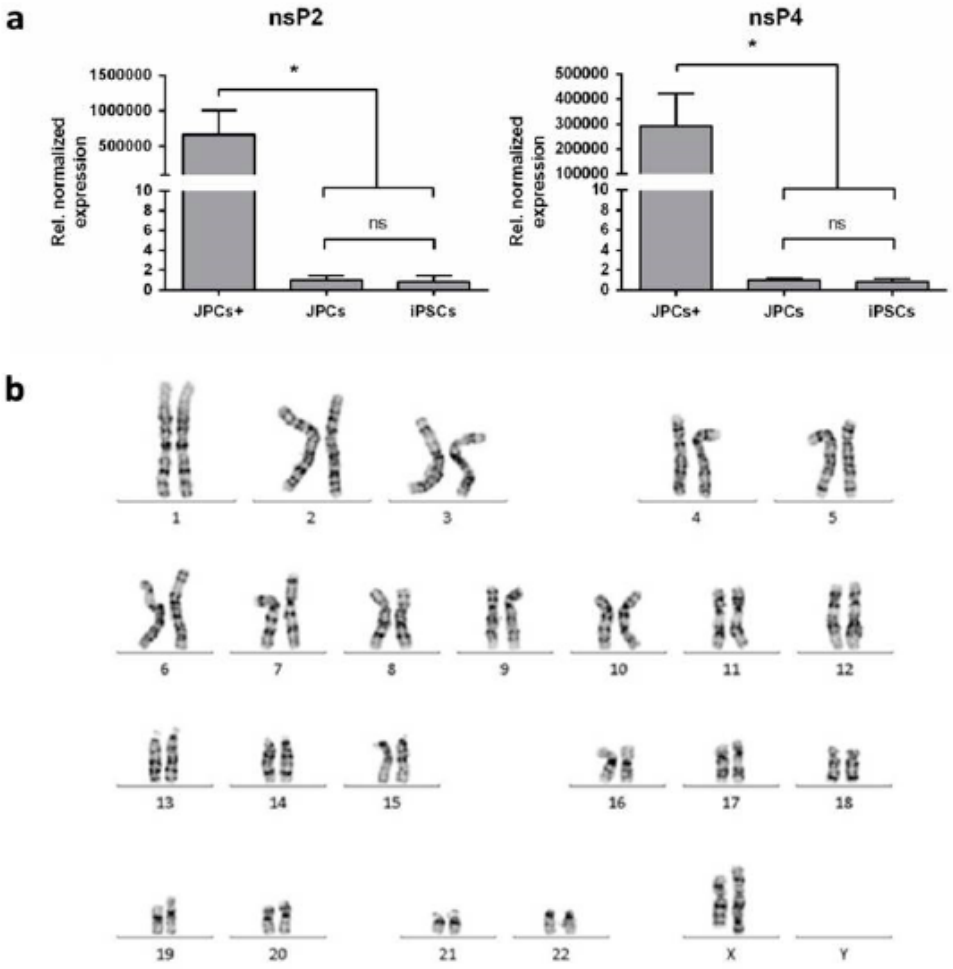

Figure 5. Elimination of srRNA after iPSC generation and karyotyping of JPC-derived iPSCs. (**a**) qRT-PCR analysis of nsP2 and nsP4 transcripts in iPSCs (passage 3), untreated JPCs and srRNA containing JPCs (JPCs+) 48 h post-transfection. Data are shown as mean + SEM. Differences were compared using one-way ANOVA ($n = 3$, * $p < 0.05$, ns = not significant) (**b**) Representative karyogram of JPC-derived iPSCs showing a normal karyotype (46, XX).

2.3.4. Differentiation of iPSCs into iMSCs and Their Characterization

To be able to use iPSCs for BTE, the differentiation potential of iPSCs into the osteogenic lineage has to be demonstrated. Therefore, iPSCs were first differentiated into iMSCs (Figure 6a). For this purpose, iPSCs (Figure 6b) were cultivated without passaging for 10 days to stimulate spontaneous differentiation. Subsequently, cells were passaged as single cells and incubated in hPL5 medium with ascorbic acid until their morphology changed to a spindle shaped MSC-like appearance (3–5 passages, Figure 6c,d). Cells exhibiting MSC morphology (Figure 6e) were expanded in hPL5 medium before osteogenic differentiation and characterization.

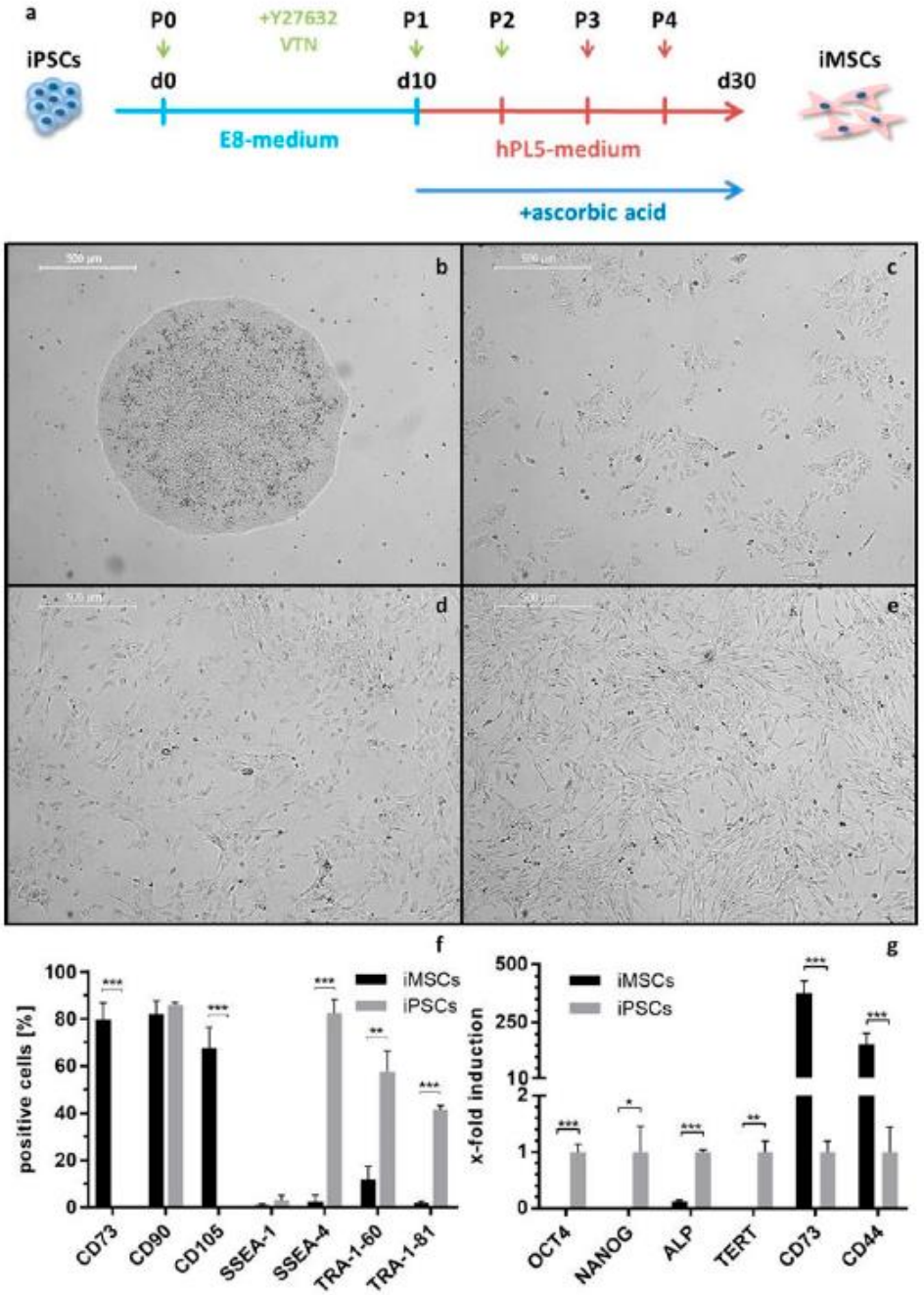

Figure 6. Differentiation of iPSCs into iPSC-derived mesenchymal stem/stromal like cells (iMSCs). (**a**) Timeline: iPSCs were seeded onto vitronectin coated plates in the presence of the ROCK inhibitor Y27632 and cultivated for 10 days in E8 medium (light blue line). Thereafter, human platelet lysate hPL5 medium containing 150 µM ascorbic acid was added to the cells (red line). Cells were passaged 3–5 times until they showed homogeneous MSC-like morphology after approximately 30 days. (**b–e**) Change of morphology during the differentiation of iPSCs into iMSCs. (**b**) iPSC colony before differentiation. (**c**) Cells after single cell plating in passage 0. (**d**) Differentiating iMSCs in passage two and (**e**) iMSCs in passage four (scale bars represent 500 µm). (**f**) Surface marker expression of iMSCs compared to iPSCs detected by flow cytometry. (**g**) Gene expression levels of iMSCs were normalized to levels of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as x-fold induction relative to iPSCs (set to 1). Differences in surface marker, and gene expression were compared using Student's *t*-test. ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Using flow cytometry, the surface marker expression of obtained iMSCs was analyzed (Figure 6f). The expression of MSC markers (CD73 and CD105) was significantly upregulated in iMSCs, while iPSC markers (SSEA-4, TRA-1-60, and TRA-1-81) were significantly downregulated compared to iPSCs. iMSCs and iPSCs were both positive for CD90 and negative for SSEA-1 (Figure 6f). In addition, gene expression of MSC markers (CD73, CD44) and iPSC markers (OCT4, NANOG, ALP, and TERT) was quantified by qRT-PCR (Figure 6g). iPSC markers were significantly downregulated in iMSCs compared to iPSCs, while MSC markers were significantly upregulated.

2.3.5. Osteogenic Differentiation of iMSCs

To demonstrate the functionality of iMSCs and their potential to be used for BTE applications, iMSCs were subjected to osteogenic differentiation. To differentiate the iMSCs under xeno-free conditions, cells were treated with osteogenic medium supplemented with human platelet lysate (hPL) instead of fetal bovine serum (FBS). After 15–20 days of differentiation, cells were fixed, and calcium phosphate precipitation was stained with alizarin red. As displayed in Figure 7, all iMSCs were able to produce strong mineral deposits.

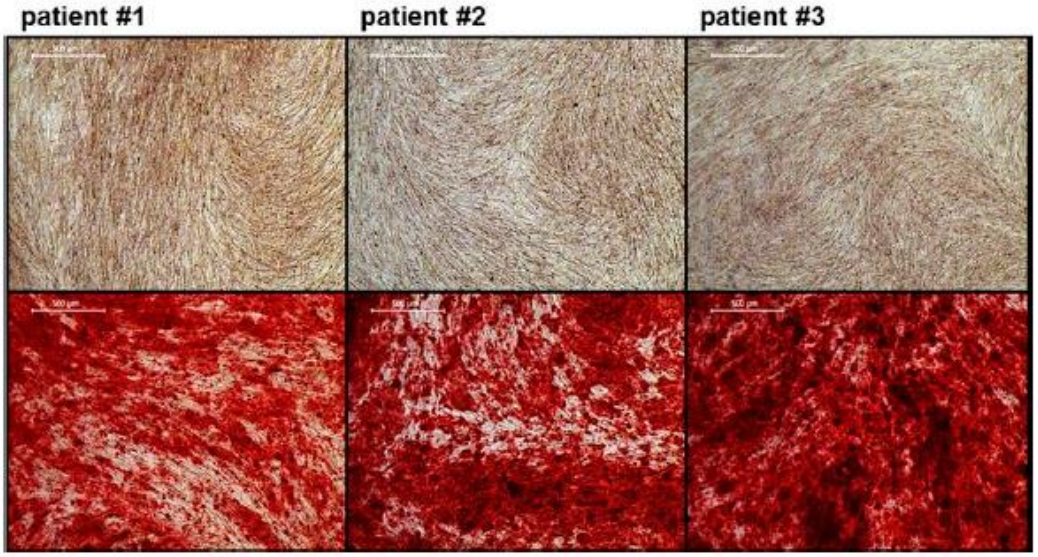

Figure 7. Osteogenic differentiation potential of iMSCs. iMSCs incubated without osteogenic stimuli (control, upper panel) and osteogenic medium (lower panel) for 15–20 days, stained with alizarin red (scale bars represent 500 µm).

3. Discussion

Jaw periosteum is an excellent, but limited source of osteogenic progenitor cells. To establish stem cell based regenerative therapies for large bone defects in oral and maxillofacial surgeries, appropriate numbers of good quality stem cells are needed. However, it is difficult to obtain sufficient cell numbers from cell cultures expanded from primary cells. The occurrence of cell senescence and the loss of differentiation potential are problems to be solved. Thus, iPSCs are a promising alternative cell source which might help to solve these problems. Unfortunately, the use of iPSC derived cells still has some safety issues concerning insertional mutagenesis caused by reprogramming vectors, teratoma formation, and infections or immune responses caused by xenogenic media supplements. In our study, we established protocols to overcome these issues and to bring therapeutic applications of iPSC-derived osteogenic progenitor cells closer to the clinic. To generate footprint-free iPSCs and to address the problem of insertional mutagenesis, srRNA was used, which combines the safety of synthetic mRNA-based reprogramming and the convenience of a single transfection.

The complete removal of srRNA after B18R withdrawal was demonstrated in the generated iPSCs at passage three, as also shown by Yoshioka et al. [14]. The additional presence of a GFP encoding sequence in the srRNA allowed convenient monitoring of srRNA translation and depletion following termination of immunosuppression by B18R interferon inhibitor. Thus, it allowed the adaptation of reprogramming/transfection protocols for JPCs. The reprogramming protocol established in this study worked reliably for JPCs from all tested patients and could also be successfully applied to human gingival fibroblasts (data not shown).

The osteogenic differentiation of iPSCs can be performed with different protocols, however recently a two-step approach was described, where iPSCs were first differentiated iMSCs, and in a second step subjected to osteogenic differentiation [10,15]. The differentiation of iPSCs into iMSCs has several advantages, e.g., the exclusion of teratoma formation risk [16] and the possibility to use cell culture and differentiation protocols standardized for MSCs. Usually, the differentiation of iPSCs to iMSCs can either be performed via embryoid body formation or by the incubation of iPSCs (when growing as colonies or plated as single cells) with differentiation media, followed by several passaging steps [17].

In this study, we modified a protocol from Luzzani et al. (2015), who also used hPL containing media for the differentiation of iPSCs into iMSCs [18]. To improve the yield of iMSCs, iPSC colonies were cultivated for extended periods without passaging prior to single cell plating. The resulting high cell densities probably primed the cells towards mesenchymal differentiation.

As shown by flow cytometry and gene expression analysis, the surface marker and gene expression profile of the obtained iMSCs were similar to that of typical MSCs [4]. Subsequent osteogenic differentiation resulted in strong mineral deposition, which demonstrated the promising potential of these cells for BTE applications.

In the present work, an important step towards clinical application was made by removing all xenogenic compounds from the protocols throughout the process. This was possible by replacing FBS with hPL for medium supplementation. Our attempts to generate iPSCs from JPCs using commercially available defined MSC-media failed due to low cell viability after the srRNA transfection or puromycin selection.

The translation of iPSCs from basic research to clinical application has made substantial progress in the past years. The first clinical trial using iPSCs to treat macular degeneration has been launched in Japan in 2014, and was able to demonstrate the safety of iPSC-derived regenerative therapies [19]. Further, a clinical trial using iPSC-derived MSCs for the treatment of steroid-resistant acute graft versus host disease (GvHD) has been started in Australia in March 2017 [20]. Preliminary results of this trial also proved the safety of the iPSC-derived MSCs and showed an improvement in severity of GvHD in 14 out of 15 patients.

These trials raise hopes for other iPSC applications to reach the clinical level in the near future even though it is still a long way for iPSCs to make their way into clinical routine.

4. Materials and Methods

4.1. Xeno-Free Isolation and Culture of JPCs

JPCs derived from three patients were included in this study in accordance with the local ethical committee (approval number 074/2016BO2, 17.05.2016) and after obtaining written informed consent. Jaw periosteal tissue was cut in small pieces with a scalpel and incubated in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% hPL (ZKT Tübingen gemeinnützige GmbH), 100 U/mL penicillin-streptomycin (Lonza, Basel, Switzerland), 2.5 µg/mL amphotericin B (Biochrom, Berlin, Germany), 50 µg/mL gentamicin (Lonza), and 10 µg/mL ciprofloxacin (Sigma-Aldrich, St. Louis, USA) for 1–2 weeks. Outgrowing cells were passaged using TrypLE Express (Thermo Fisher Scientific) and expanded and frozen in passage one using Cryo SFM freezing medium (Promocell, Heidelberg,

Germany). From passage two onward, JPCs were grown in hPL5-medium (DMEM/F12 containing 5% hPL, 100 U/mL penicillin-streptomycin, and 2.5 µg/mL amphotericin B).

4.2. Production of srRNA

The T7-VEE-OKS-iM plasmid, a gift from Steven Dowdy (Addgene plasmid # 58972), containing sequences encoding the non-structural proteins (nsP1 to nsP4) for self-replication, the reprogramming factors Oct4, Klf4, Sox2, and cMyc [14] and an additionally added internal ribosome entry site (IRES)-GFP was amplified in *E.coli* and plasmids were isolated using QIAPrep (Qiagen, Hilden, Germany). After the linearization with MluI restriction enzyme (Thermo Fisher Scientific), 10 µg template DNA was transcribed in vitro using RiboMAX large-scale production system T7 Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Afterwards, 2 U TURBO DNase was added for 15 min at 37 °C. For 5′-end capping, ScriptCap Cap1 Capping System was used followed by 3′-end polyadenylation with A-Plus Poly(A) Polymerase Tailing Kit (both from Cellscript, Madison, WI, USA) according to the manufacturer's instructions. Following each reaction step, srRNA was purified using RNeasy Kit (Qiagen). The specific lengths of the generated DNA and srRNA products were analyzed using 1% agarose gel electrophoresis.

4.3. Production of B18R-mRNA

The coding sequence for B18R was inserted by Aldevron (Fargo, ND, USA) into the pcDNA 3.3 plasmid. Pseudoruridine-5′-triphosphate (Ψ-UTP) and 5-methylcytidine-5′-triphosphate (m5CTP) modified B18R mRNA was generated by in vitro transcription (IVT) as previously described in our studies [21].

4.4. Generation of Integration-Free iPSCs from JPCs Using srRNA

4.4.1. Preparation of Conditioned Medium Containing B18R (BcM)

JPCs were expanded in hPL5 medium and passaged into a T75 flask to reach approx. 80% confluency at the next day. To perform B18R-mRNA transfection, the medium was aspirated and replaced by 6.5 mL Opti-MEM (Thermo Fisher Scientific). The transfection cocktail was prepared according to the manufacturer's instructions (500 µL Opti-MEM, 7.5 µg of B18R-mRNA, 15 µL Lipofectamine 3000 (Thermo Fisher Scientific)), added to the medium and incubated for 4 h at 37 °C. Subsequently, medium was changed to 15 mL hPL5 medium. After 24 h, medium was collected and replaced with 15 mL fresh hPL5. The collected BcM was stored at −20 °C. Medium collection was repeated until day three and the collected BcM was pooled, sterile filtered with a 0.2 µm filter, aliquoted and stored at −20 °C.

4.4.2. Transfection of JPCs with srRNA and Reprogramming

JPCs (2.5–5 × 10^4 cells) were seeded into 12-well plates to reach 30–50% confluency the next day. Medium was changed to 0.5 mL hPL5 supplemented with 0.2 µg/mL recombinant B18R protein (eBioscience, San Diego, CA, USA) 30 min prior to transfection. The transfection cocktail (25 µL Opti-MEM, 0.25 µg srRNA, 0.5 µL TransIT mRNA Boost Reagent (Mirus Bio LLC, Madison, WI, USA), and 0.5 µl TransIT mRNA Reagent (Mirus Bio LLC)) was prepared according to the manufacturer's instructions and added to the wells.

24 h after transfection, the medium was replaced with hPL5 containing 25% BcM and 1 µg/mL puromycin (Thermo Fisher Scientific) and changed every other day. To determine the transfection efficiency, cells were harvested after 24 h using TrypLE Express, resuspended in BD Cytofix/Cytoperm Solution (BD Bioscience, Franklin Lakes, NJ, USA) and GFP expression was measured by flow cytometry using the Guava EasyCyte 6HT-2L instrument (Merck Millipore, Billerica, MA, USA). From day three until day 15, 250 µM sodium butyrate (NaB, Selleck Chemicals LLC, Houston, TX, USA) was added to the medium. On day five, after successful puromycin selection, srRNA containing

cells were passaged using TrypLE Express and split in 1:5 ratio into 12-well plates coated with 0.5 mL of a 5 µg/mL vitronectin solution (Thermo Fisher Scientific). Untransfected cells died within the five days of puromycin treatment. Two days after passaging (day seven), the medium was changed to Essential 8 medium (E8, Thermo Fisher Scientific) containing 25% BcM and replaced by fresh medium every day. When first iPSC colonies emerged, medium was changed to E8-medium supplemented with 0.2 µg/mL recombinant B18R protein. Single iPSC colonies were picked and transferred into vitronectin coated 12-well plates containing E8 medium supplemented with 10 µM Y27632 ROCK inhibitor (Selleck Chemicals LLC). iPSCs were maintained in E8 medium with daily medium changes and passaged every 4–6 days.

4.5. Characterization of JPC-Derived iPSCs

4.5.1. Immunostaining for Detection of Pluripotency

On vitronectin coated glass slides, 5×10^5 iPSCs were cultivated for 2–3 days and fixed using fixation solution (R&D Systems, Minneapolis, MN, USA) for 10 min. Afterwards, cells were washed with wash buffer (Permeabilization/Wash Buffer I, R&D Systems) and blocked with 5% BSA in wash buffer for 1–2 h at RT. Antibody staining was performed according to the manufacturer's instructions in wash buffer containing 1% BSA. Cells were incubated for 3 h at RT with fluorescently labelled antibodies (PE-labelled mouse anti-human Nanog antibody (BD Bioscience), DyLight™ 488 labelled mouse anti-human StainAlive™ TRA-1-60 antibody (Stemgent, Cambridge, MA, USA), and DyLight™ 550 labelled mouse anti-human StainAlive™ SSEA-4 antibody (Stemgent)). The incubation with primary antibodies (rabbit anti-human POU5F1 (Oct4) (Sigma-Aldrich), rabbit anti-human Sox2 (Stemgent), and mouse anti-human LIN28A (6D1F9) (Thermo Fisher Scientific)) were performed overnight at 4°C. After washing with wash buffer, cells were incubated for 1 h in wash buffer containing 1% BSA with fluorescently labeled secondary antibodies (FITC-labelled sheep anti-mouse IgG (Sigma-Aldrich) or Cy3-labelled goat anti-rabbit IgG (Thermo Fisher Scientific)). Finally, the cells were washed and stained using Fluoroshield mounting medium with DAPI (Abcam, Cambridge, UK). Fluorescence microscopic images were taken using Axiovert135 microscope and AxioVision 4.8.2 software (Carl Zeiss, Oberkochen, Germany).

4.5.2. Tri-Lineage Differentiation of iPSCs In Vitro

The ability of iPSCs to differentiate into the three germ layers was analyzed using the human StemMACS™ Trilineage Differentiation Kit (Miltenyi, Bergisch Gladbach, Germany) according the manufacturer's instructions. 1×10^5 iPSCs were seeded per well of a 12-well plate for mesoderm differentiation, 2×10^5 cells for endoderm differentiation, and 1.5×10^5 iPSCs for ectoderm differentiation. The cells were analyzed after seven days of differentiation using flow cytometry.

Therefore, cells were detached, fixed for 10 min at RT in fixation solution (R&D Systems) and washed with DPBS. Then, the cells were incubated for 45 min at RT with wash buffer (Wash Buffer I, R&D Systems) containing 5 µl of each of the following antibodies: Mesoderm differentiation: Alexa Fluor 488 labelled anti-human α-smooth muscle actin (α-SMA) antibody (R&D Systems) and PE labelled mouse anti-human CD31 antibody (BD Biosciences), Endoderm differentiation: PE-labelled anti-human C-X-C chemokine receptor type 4 (CXCR4) antibody and PE-labelled anti-human α-fetoprotein (AFP) antibody (both from R&D Systems, Minneapolis, USA), Ectoderm differentiation: Alexa Fluor 488 labelled anti-human neuron-specific class III β-tubulin (Tuj1) antibody (BD Biosciences) and PE-labelled anti-human paired box gene 6 (Pax6) antibody (Miltenyi). After washing with wash buffer, cells were fixed using CellFIX solution (BD Biosciences) and analyzed using FACScan flow cytometer (BD Biosciences) and Flowing Software (Turku Centre for Biotechnology, Turku, Finland).

4.5.3. Teratoma Formation on Chicken Embryo Chorioallantoic Membrane (CAM)

To confirm the differentiation potential of iPSCs into the three germ layers, an in vivo teratoma formation was performed using CAM assay. After three days of incubation at 37 °C, 2-3 mL albumen was aspirated from fertilized chicken eggs (Lohmann White x White Rock) and a window was cut into the shell and sealed with semi-permeable adhesive tape. After seven days of incubation at 37 °C, a silicone ring was placed onto the CAM and 2×10^6 iPSCs mixed with 50 µL E8 medium and 50 µL Matrigel (Corning, New York, NY, USA) were transferred into the inner circle of the silicone ring. The eggs were then sealed again and further incubated at 37 °C. After 10 days (day 17 of incubation), the teratoma cell mass with the surrounding CAM was excised and fixed at 4 °C with 4% paraformaldehyde (Merck, Darmstadt, Germany) overnight. After washing with DPBS and ethanol dehydration, the samples were embedded in paraffin for sectioning and H&E (Morphisto, Frankfurt, Germany) staining.

4.5.4. Detection of Residual srRNA in the Reprogrammed iPSCs

The presence of srRNA in the generated iPSCs was analyzed by qRT-PCR using primers specific for sequences encoding the non-structural proteins nsP2 and nsP4: nsP2 (fw: 5′-TCC ACA AAA GCA TCT CTC GCC G-3′, rev: 5′-TTT GCA ACT GCT TCA CCC ACC C-3′) and nsP4 (fw: 5′-TTT TCA AGC CCC AAG GTC GCA G-3′, rev: 5′-TGT TCT GGA TCG CTG AAG GCA C-3′). For RNA isolation, 1×10^6 iPSCs at passage three were used (Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) and 300 ng RNA was reverse transcribed into complementary DNA (cDNA) using iScript Kit (Bio-Rad). qRT-PCR reactions with 40 cycles were performed in CFX Connect Real-Time PCR Detection System (Bio-Rad) using IQ™ SYBR® Green Supermix (Bio-Rad) and 300 nM primer with following conditions: 3 min at 95 °C (1 cycle); 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 10 s. The mRNA expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are shown relative to the initial JPCs.

4.5.5. Karyotyping of iPSCs

iPSCs were grown to 60–80% confluency in a 6-well plate and incubated for 90 min with 0.15 µg/mL KaryoMAX colcemid solution (Thermo Fisher Scientific). Then, cells were detached using Accutase (Thermo Fisher Scientific) and inactivated with DMEM/F12 containing 10% FBS. The cell suspension was centrifuged (300× *g* for 5 min), the supernatant was discarded and 1.5 mL of 0.075 M KaryoMAX KCl solution (Thermo Fisher Scientific) was added and incubated at 37 °C. After 30 min, 100 µL fixative (3:1 methanol/acetic acid) was added and incubated for 10 min. After centrifugation, the cells were resuspended in fixative, incubated for 1 h at RT, and then stored over night at 20 °C. Chromosome analysis was performed by the Institute of Medical Genetics and Applied Genomics of the University Hospital Tübingen.

4.6. Differention of iPSCs into iMSCs

Very low concentrations of iPSCs (≤ 10% confluency) were seeded into vitronectin coated (0.5 mL of 5 µg/mL solution) 12-well plates and cultivated without passaging for 10 days to stimulate spontaneous differentiation. After this period, the cells were detached using Accutase and transferred into vitronectin coated 6-well plates containing E8-medium and 10 µM ROCK inhibitor Y27632 (passage 1). The next day, the medium was changed to hPL5 supplemented with 150 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich) and medium was replaced every other day. After reaching 80% confluency, cells were passaged into vitronectin coated 6-well plates (split ratio 1:3) using Accutase and ROCK inhibitor was added to the medium. For following cell passages TrypLE Express was used and no further ROCK inhibitor or vitronectin was used. Cells were passaged until the morphology of the cells had changed to a spindle shaped MSC-like appearance (3–5 passages). Cells exhibiting

MSC morphology were expanded in hPL5 medium before using the cells for osteogenic differentiation and characterization.

4.7. Osteogenic Differentiation of iMSCs

For osteogenic differentiation, iMSCs were cultivated in osteogenic medium (DMEM/F12 containing 10% hPL, 100 U/mL penicillin-streptomycin (Lonza), 2.5 µg/mL amphotericin B, 0.1 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), β-glycerophosphate (AppliChem, Darmstadt, Germany), and 4 µM dexamethasone (Sigma-Aldrich)) with medium changes every 2–3 days. After 15–25 days, cells were fixed with 4% formalin and stained for 20 min with 1 mL of 40 mM Alizarin red solution (pH 4.2, Sigma-Aldrich). Unbound dye was removed by washing with deionized water and images were taken using an inverted microscope (Leica, Wetzlar, Germany).

4.8. Flow Cytometric Analysis of JPCs, iPSCs, and iMSCs

The expression of pluripotency markers (SSEA-1, SSEA-4, TRA-1-60, TRA-1-80) and MSC-markers (CD73, CD90, CD105) was analyzed by flow cytometry. Cells were detached using TrypLE Express and 1×10^5 cells per sample were incubated on ice for 15 min in 20 µL blocking buffer (PBS, 0.1% BSA, 1 mg/mL sodium azide (Sigma-Aldrich), and 10% Gamunex (human immune globulin solution, Talecris Biotherapeutics GmbH, Frankfurt, Germany)). Then, 50 µL FACS buffer (PBS, 0.1% BSA, 1 mg/mL sodium azide) as well as phycoerythrin (PE) and allophycocyanin (APC) conjugated antibodies (for individual volumes see Table 1) were added and incubated on ice for 20 min. After two washing steps with 200 µL FACS buffer, flow cytometry measurements were performed using the Guava EasyCyte 6HT-2L (Merck Millipore, Billerica, MA, USA).

Table 1. List of antibodies used for flow cytometry.

Human Antigen	Volume per Sample (µL)	Isotype	Conjugate	Company
SSEA1	5	human recombinant antibody (REA)	PE	Miltenyi, Bergisch Gladbach, Germany
SSEA4	5		PE	
TRA-1-60	5		PE	
TRA-1-81	5		PE	
REA-Isotype	5		PE	
CD73	5	mouse IgG1	PE	BD Biosciences, Franklin Lakes, NJ, USA
CD90	1		PE	
CD105	10		APC	BioLegend, San Diego, CA, USA
IgG1-Isotype	10		APC	
IgG1-Isotype	5		PE	R&D Systems, Minneapolis, MN, USA

4.9. Gene expression Analysis of JPCs, iPSCs, and iMSCs by qRT-PCR

RNA isolation from JPCs, iPSCs and iMSCs (1×10^5 cells per sample) was carried out using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The amount of isolated RNA was quantified with a Qubit 3.0 fluorometer and the corresponding RNA BR Assay Kit (Thermo Fisher Scientific). The first-strand cDNA synthesis was performed with 0.5 µg of RNA using the SuperScript Vilo Kit (Thermo Fisher Scientific). The quantification of mRNA expression levels was performed using the real-time LightCycler System (Roche Diagnostics, Mannheim, Germany). For the PCR reactions, commercial OCT4, NANOG, ALP, CD44, and CD73 primer kits (Search LC, Heidelberg, Germany) and DNA Master SYBR Green I kit (Roche, Basel, Switzerland) were used. The amplification was performed with a touchdown PCR protocol of 40 cycles (annealing temperature between 68–58 °C), following the manufacturer's instructions. GOI (gene of interest) transcript levels of each sample were normalized to those of the housekeeping gene GAPDH, divided by the corresponding control samples and displayed as x-fold induction indices.

4.10. Statistical Analysis

For the statistical evaluation of data, means + standard deviation (SD) or standard error of mean (SEM) were calculated. Student's *t*-test or one-way analysis of variance (ANOVA) for repeated measurements followed by Bonferroni's multiple comparison test was used. All statistical analyses were performed double-tailed using GraphPad Prism 6.01. A p-value < 0.05 was considered significant.

5. Conclusions

In this study, we generated for the first time, footprint- and xeno-free iPSCs from JPCs by the transfection of srRNA encoding the reprogramming factors. We conclude that JPCs can function as starting material for the generation of clinical grade autologous iPSCs. The differentiation of JPC-iPSCs to iMSCs leads to the generation of cells with a high osteogenic potential, which are a promising source of osteogenic progenitor cells for BTE. Using cGMP grade hPL as a medium supplement, clinically applicable osteogenic progenitor cells can be obtained.

Author Contributions: Methodology, validation, investigation, data curation, writing original draft preparation, F.U. and H.S.; validation, investigation, data curation, M.W.; conceptualization, funding acquisition, project administration, supervision, writing-review and editing, D.A. and M.A.-A.; resources, writing-review and editing, H.-P.W and S.R.

Funding: This research was funded by German Research Foundation, grant number AL 1486/6-1/AV 133/7-1.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ANOVA	analysis of variance
APC	allophycocyanin
BcM	conditioned medium containing B18R
BSA	bovine serum albumin
BTE	bone tissue engineering
CAM	chorioallantoic membrane
cGMP	current good manufacturing practice
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
GOI	gene of interest
H&E	haematoxylin and eosin
hPL	human platelet lysate
IgG	immunoglobulin G
iMSC	iPSC-derived mesenchymal stem/stromal like cell
iPSC	induced pluripotent stem cell
IRES	internal ribosome entry site
JPC	jaw periosteal cell
m5CTP	5-methylcytidine-5′-triphosphate
MACS	magnetic-activated cell sorting
MFI	median fluorescence index
mRNA	messenger RNA
MSC	mesenchymal stem/stromal cell
NaB	sodium butyrate
OSKM	OKT4, SOX2, KLF4, cMYC

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Puro	puromycin
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
ROCK	rho-associated protein kinase
SD	standard deviation
SEM	standard error of mean
srRNA	self-replicating ribonucleic acid
Ψ-UTP	pseudoruridine-5′-triphosphate

References

1. Alexander, D.; Hoffmann, J.; Munz, A.; Friedrich, B.; Geis-Gerstorfer, J.; Reinert, S. Analysis of OPLA scaffolds for bone engineering constructs using human jaw periosteal cells. *J. Mater. Sci. Mater. Med.* **2008**, *19*, 965–974. [CrossRef] [PubMed]
2. Ferretti, C.; Mattioli-Belmonte, M. Periosteum derived stem cells for regenerative medicine proposals: Boosting current knowledge. *World J. Stem Cells* **2014**, *6*, 266–277. [CrossRef] [PubMed]
3. Roberts, S.J.; van Gastel, N.; Carmeliet, G.; Luyten, F.P. Uncovering the periosteum for skeletal regeneration: The stem cell that lies beneath. *Bone* **2015**, *70*, 10–18. [CrossRef] [PubMed]
4. Dominici, M.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.; Krause, D.; Deans, R.; Keating, A.; Prockop, D.; Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **2006**, *8*, 315–317. [CrossRef] [PubMed]
5. McLeod, C.M.; Mauck, R.L. On the origin and impact of mesenchymal stem cell heterogeneity: New insights and emerging tools for single cell analysis. *Eur. Cells Mater.* **2017**, *34*, 217–231. [CrossRef] [PubMed]
6. Alexander, D.; Schafer, F.; Olbrich, M.; Friedrich, B.; Buhring, H.J.; Hoffmann, J.; Reinert, S. MSCA-1/TNAP Selection of Human Jaw Periosteal Cells Improves their Mineralization Capacity. *Cell. Physiol. Biochem.* **2010**, *26*, 1073–1080. [CrossRef] [PubMed]
7. Olbrich, M.; Rieger, M.; Reinert, S.; Alexander, D. Isolation of osteoprogenitors from human jaw periosteal cells: A comparison of two magnetic separation methods. *PLoS ONE* **2012**, *7*, e47176. [CrossRef] [PubMed]
8. Umrath, F.; Thomalla, C.; Poschel, S.; Schenke-Layland, K.; Reinert, S.; Alexander, D. Comparative Study of MSCA-1 and CD146 Isolated Periosteal Cell Subpopulations. *Cell. Physiol. Biochem.* **2018**, *51*, 1193–1206. [CrossRef] [PubMed]
9. Yu, J.; Thomson, J.A. Induced Pluripotent Stem Cells. In *Principles of Tissue Engineering*; Lanza, R., Langer, R., Vacanti, J., Eds.; Academic Press: Boston, MA, USA, 2014; pp. 581–594.
10. Luzzani, C.D.; Miriuka, S.G. Pluripotent Stem Cells as a Robust Source of Mesenchymal Stem Cells. *Stem Cell Rev. Rep.* **2017**, *13*, 68–78. [CrossRef] [PubMed]
11. Frobel, J.; Hemeda, H.; Lenz, M.; Abagnale, G.; Joussen, S.; Denecke, B.; Saric, T.; Zenke, M.; Wagner, W. Epigenetic rejuvenation of mesenchymal stromal cells derived from induced pluripotent stem cells. *Stem Cell Rep.* **2014**, *3*, 414–422. [CrossRef] [PubMed]
12. Seki, T.; Fukuda, K. Methods of induced pluripotent stem cells for clinical application. *World J. Stem Cells* **2015**, *7*, 116–125. [CrossRef] [PubMed]
13. Warren, L.; Manos, P.D.; Ahfeldt, T.; Loh, Y.H.; Li, H.; Lau, F.; Ebina, W.; Mandal, P.K.; Smith, Z.D.; Meissner, A.; et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **2010**, *7*, 618–630. [CrossRef] [PubMed]
14. Yoshioka, N.; Gros, E.; Li, H.R.; Kumar, S.; Deacon, D.C.; Maron, C.; Muotri, A.R.; Chi, N.C.; Fu, X.D.; Yu, B.D.; et al. Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell* **2013**, *13*, 246–254. [CrossRef] [PubMed]
15. Wu, Q.; Yang, B.; Hu, K.; Cao, C.; Man, Y.; Wang, P. Deriving Osteogenic Cells from Induced Pluripotent Stem Cells for Bone Tissue Engineering. *Tissue Eng. Part B Rev.* **2017**, *23*, 1–8. [CrossRef] [PubMed]

16. Zhao, Q.; Gregory, C.A.; Lee, R.H.; Reger, R.L.; Qin, L.; Hai, B.; Park, M.S.; Yoon, N.; Clough, B.; McNeill, E.; et al. MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 530–535. [CrossRef] [PubMed]
17. Steens, J.; Klein, D. Current Strategies to Generate Human Mesenchymal Stem Cells In Vitro. *Stem Cells Int.* **2018**, *2018*, 6726185. [CrossRef] [PubMed]
18. Luzzani, C.; Neiman, G.; Garate, X.; Questa, M.; Solari, C.; Fernandez Espinosa, D.; Garcia, M.; Errecalde, A.L.; Guberman, A.; Scassa, M.E.; et al. A therapy-grade protocol for differentiation of pluripotent stem cells into mesenchymal stem cells using platelet lysate as supplement. *Stem Cell Res. Ther.* **2015**, *6*, 6. [CrossRef] [PubMed]
19. Takahashi, M. Retinal Cell Therapy Using iPS Cells. *Nippon Ganka Gakkai Zasshi* **2016**, *120*, 210–224; discussion 225. [PubMed]
20. Cynata Therapeutics Limited. *A Study of CYP-001 for the Treatment of Steroid-Resistant Acute Graft versus Host Disease*; Cynata Therapeutics Limited: Carlton, Australia, 2017.
21. Avci-Adali, M.; Behring, A.; Steinle, H.; Keller, T.; Krajeweski, S.; Schlensak, C.; Wendel, H.P. In vitro synthesis of modified mRNA for induction of protein expression in human cells. *J. Vis. Exp. JoVE* **2014**. [CrossRef] [PubMed]

DANKSAGUNG

Mein besonderer Dank richtet sich an Prof. Dr. Hans-Peter Wendel für seine freundliche und stetig ermutigende Unterstützung und für Möglichkeit meine Doktorarbeit im Klinischen Forschunslabor der Thorax-, Herz- und Gefäßchirurgie am Universitätsklinikum Tübingen durchzuführen. Ebenfalls möchte ich meinen Dank an Prof. Dr. Stevanovic für die Betreuung und die Begutachtung der Dissertation aussprechen.

Ganz besonders danken möchte ich Prof. Dr. Meltem Avci-Adali, die mich als Bereuerin der Arbeit mit viel Geduld, außerordentlichen Ideen und Ratschlägen, sowie konstruktiver Kritik motivierend unterstützt und gefördert hat. Insbesondere danken möchte ich auch für das Engagement mit dem Sie mir in allen Lebenslagen zur Seite gestanden ist und den Begriff „Doktor-Mutter“ für mich maßgebend geprägt hat.

Einen großen Dank möchte ich auch allen derzeitigen und ehemaligen Mitarbeitern des THG-Labor-Teams ausprechen, die mich über die Jahre hinweg unterstützt haben und mir mit Rat und Tat zur Seite standen. Die großartige Zusammenarbeit in diesem äußerst kollegialen Betriebsklima hat mir sehr geholfen Probleme zu lösen und mit viel Freude an der Arbeit zu bleiben. Besonders viel Freude bereitet und Unterstützung und Nachsicht entgegegen gebracht haben mir auch meine Büro-Kollegen, Bernd, Sonia, Marbod, Katharina, Andi, Kathi und Jochen, ohne die mein Alltag nicht so angenehm gewesen wäre; ich werde unsere unzähligen Späße vermissen.

Zu guter Letzt möchte ich meine tiefe Dankbarkeit genüber meiner Familie und Freunden aussprechen, die mich während der ganzen Zeit motiviert und unterstützt haben, und mir viel Geduld und Verständnis entgegengebracht haben.