Membrane androgen receptor activation triggers pro-apoptotic responses in vitro and in vivo and blocks migration in colon cancer

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Shuchen Gu
aus Guizhou, V.R.China

Tübingen
2011

Tag der mündlichen Qualifikation: 31.08.2011
Dekan: Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter: Prof. Dr. F. Lang
2. Berichterstatter: Prof. Dr. Friedrich Goetz

Acknowledgments

It is with immense pleasure, I record my humble gratitude to my research guide Prof. Dr. Florian Lang, for his expert guidance and critical review throughout the work. My sincere and heart felt thanks for his abundant encouragement.

I am very grateful to my Ph.D supervisor Prof. Dr. Christos Stournaras for his invaluable guidance, endless help, support, thought provoking suggestions and stimulating discussions during the progress of the work. I would also like to thank for his valuable input and suggestions for improving this dissertation.

I would also like to thank Prof. Fritz Götz for evaluating my work and for this great help at my enrollment and in finalizing my thesis. And I would like to thank him for giving me an opportunity to present the dissertation at the Faculty of Biology, Eberhard Karls Universität Tübingen, Germany.

My heartfelt thanks to my colleague Dr. Michael Föller for their help and assistance in organizing the lab work. His friendliness, patience made our lab an exciting place for me to work. My heart felt thanks to my colleague Eva-Maria Gehring, Omaima Nasir, Rexhep Rexhepaj, Hasan Mahmud, Nicole Matzner, Diwakar Bobbala, Syed Qadri for their help, suggestions support and friendship.

I would like to thank all other colleagues of the Institute of Physiology for making the institute, a good place to work.

I dedicate my thesis to my loving parents for being a constant source of inspiration for my work. I am greatly indebted for their support, constructive criticism, prayers and endless love in my life.

Abstract

The classical intracellular androgen receptors (iAR) mediate genomic androgen signals, which take at least more than half an hour. However, the rapid or non-genomic action of androgens takes only seconds to few minutes and involves the activation of androgen membrane binding sites. Although the molecular identity of those membrane binding sites remains still unknown, their expression has been reported in many cell types, including various tumor cells. Activation of membrane androgen receptors (mAR) in prostate and breast cancer cells has been implicated in the regulation of cell growth, motility and apoptosis. Here we analyzed mAR expression and function in colon cancer. Using fluorescent mAR ligands we showed specific membrane staining in mouse colon tumor tissues and in iAR silenced Caco2 cell lines. Stimulation of colon-mAR by testosterone-albumin-conjugates induced rapid actin and tubulin cytoskeleton reorganization and generated apoptotic responses, even in the presence of anti-androgens. We showed that long-term activation of mAR in Caco2 cell lines down-regulated the activity of PI-3K and Akt and induced de-phosphorylation/activation of the pro-apoptotic Bad. Treatment of $APC^{min/+}$ mice significantly decreased the expression of p-AKT and p-Bad levels in tumor tissue. Moreover, mAR activation resulted in a 65% reduction of tumor incidence in chemically induced Balb/c mice colon tumors and an 80% reduction of tumor incidence in $APC^{min/+}$ mice colon tumors. Furthermore, mAR activation strongly inhibited Caco2 cell migration. In accordance with this, vinculin, a protein controlling cell adhesion and actin reorganization, was effectively phosphorylated upon mAR activation. Phosphorylation inhibitors genistein and PP2 inhibited actin reorganization and restored motility. Moreover, blocking actin reorganization by cytochalasin B and silencing vinculin by appropriate siRNA's restored the migration potential. From these results we conclude that mAR activation inhibits the pro-survival signals Akt/Bad *in vitro* and *in vivo,* induces potent proapoptoric responses and blocks migration of colon cancer cells via regulation of vinculin signaling and actin reorganization. Our results point to a central role of mAR in the induction of anti-tumor responses in colon cancer.

Zusammenfassung

Die klassischen intrazellulären Androgenrezeptoren (iAR) vermitteln die genomische Androgenwirkung, die wenigstens 30 Minuten Zeit erfordert. Im Gegensatz hierzu benötigen die schnellen, nichtgenomischen Androgeneffekte nur einige Sekunden bis wenige Minuten durch die Aktivierung von Androgenbindungsstellen in der Zellmembran. Derartige membranständige Androgenbindungsstellen wurden schon in vielen Zelltypen inklusive Tumorzellen nachgewiesen, obwohl die molekulare Identität dieser Bindungsstellen noch immer unbekannt ist. Die Aktivierung dieser membranständigen Androgenrezeptoren (mAR) steht im Zusammenhang mit der Regulation von Zellwachstum, Motilität und Apoptose. Mit der vorliegenden Arbeit wurde die Expression und Funktion von mAR bei Kolonkarzinomen untersucht. Mithilfe fluoreszierender mAR-Liganden konnten membranständige Rezeptoren spezifisch angefärbt werden in Kolonkarzinomgewebe der Maus und in Caco2-Zellen, die nicht über iAR verfügen. Die Stimulierung von mAR in Dickdarmgewebe durch Testosteron-Albumin-Konjugate führte zu einer raschen Reorganisation des Aktin- und Tubulinnetzwerkes und löste Apoptose selbst in Anwesenheit von Antiandrogenen aus. Die längeranhaltende Aktivierung von mAR in Caco2-Zellen führte zu verminderter PI-3- und Akt-Kinaseaktivität und zur Dephosphorylierung und mithin Aktivierung von proapoptotischem Bad. Eine entsprechende Behandlung von APCmin/+-Mäusen verringerte die Expression von p-Akt und von p-Bad signifikant in Tumorgewebe. Darüber hinaus führte die Aktivierung von mAR zu einer Verminderung der Tumorinzidenz um 65% bei chemisch induzierten Dickdarmtumoren von Balb/c-Mäusen und um 80% bei APCmin/+-Mäusen. Weiterhin hemmte die mAR-Aktivierung die Zellmigration von Caco2-Zellen stark. In Übereinstimmung mit diesem Befund war Vinculin, ein Protein, das Zelladhäsion und Aktinreorganisation reguliert, nach mAR-Aktivierung deutlich phosphoryliert. Die Phosphorylierungsinhibitoren Genistein und PP2 hemmten die Aktinreorganisation und reaktivierten die Zellmotilität. Zusätzlich konnte die Blockade der Aktinreorganisation durch Cytochalasin B oder die auf siRNA basierende Herunterregulation von Vinculin das Migrationspotential der Zellen wiederherstellen. Aus diesen Daten kann

geschlossen werden, dass die Aktivierung von mAR die überlebensfördernden Akt/Bad-Signale in vitro und in vivo hemmt, wirkungsvolle proapoptotische Zellantworten induziert und die Migration von Kolontumorzellen über die Regulierung des Vinculin-Signalweges und der Aktinreorganisation blockiert. Diese Erkenntnisse deuten auf eine zentrale Rolle von mAR für die Induktion von Antitumorantworten bei Kolonkarzinom.

Content

Abbreviations

3a-Diol	5a androstane-3a, 17b diol
5aR	5alpha reductase enzyme
AF-1	active activation function
AF-2	ligand-dependent activation function
AKR1C	aldo-keto reductase
Apaf-1	apoptoic protease activating factor-1
APC	adenomatous polyposis coli
AR	androgen receptor
BSA	bovine serum albumin
CAD	caspaseactivated DNase
CAG	encode polyglutamine
CAM	calmodulin
cAMP	cyclic adenosine monophosphate
CIN	chromosomal instability
CYP	cytochromes P450
DBD	DNA-binding domain
DD	death domains
DED	death effector domains
DHT	dihydrotestosterone
EGF	epidermal growth factor
ER	estrogen receptor
ERK	extracellular-signal regulated kinase
FADD	Fas-Associated protein with Death Domain
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
FGF	fibroblast growth factor
GABA	gamma-aminobutyric aci
GDP	guanosine diphosphate
GGC	polyglycine
GP	G-protein
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HNPCC	hereditary nonpolyposis colon cancer
HSD	hydroxysteroid dehydrogenase
IAPs	inhibitor of apoptosis proteins
iAR	intracellular androgen receptor
IBD	inflammatory bowel disease
ICAD	inhibitor of caspase-activated DNase
ICE	interleukin-1β-converting enzyme

IGF1	Insulin-like growth factor 1
IP3	inositol 1,4,5-triphosphate
LBD	C-terminal ligand-binding domain
MAPK	mitogen-activated protein kinase
mAR	membrane associated androgen receptor
MEK	MAPK/ERK kinase
MMR	mismatch repair
MSI	microsatellite instability
PARP	poly ADP-ribose polymerase
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase
ROCKI	Rho-associated coiledcoil forming kinase I
SEER	Surveillance, Epidemiology and End Results
SH2	Src homology domain 2
SH3	Src homology domain 3
SHBG	steroid hormone-binding globulin
SHBGR	steroid hormone-binding globulin receptor
SHC	SH2 Containing Protein
SMAD4	Mothers against decapentaplegic homolog 4
SR	sarcoplasmic reticulum
T	testosterone
TAD	N-terminal transactivation domain
TGFß	transforming growth factor-β
TIF2	Transcription Intermediary Factor-2
TP53	tumor protein P53
TRADD	TNFRSF1A-associated via death domain
VEGF	vascular endothelial growth factor

1. Introduction

1.1 Androgens

Androgens are important male sex steroid hormones. They have many physiological roles leading to the male characteristics and other phenotypes. The major circulating androgen in human tissue is testosterone, which is synthesized mainly by Leydig cells in testis. The effects of testosterone can be classified as virilizing and anabolic. Anabolic effects will make muscle more mass and strength, increase bone density and strength, and stimulate the linear growth and bone maturation. Virilizing effects maturate the sex organs, particularly the penis and the formation of the scrotum in unborn children. And after birth the virilizing effects include a deepening of the voice, growth of the beard and axillary hair. Most of these fall into the category of male secondary sex characteristics. There is another potent androgen, dihydrotestosterone (DHT) in addition to testosterone. It is synthesized mostly in peripheral tissue. The responsibility of DHT results in all of the male secondary sexual characteristics such as deepening of the vocal chords, male hair patterns on the body, hair on the face, oily, and male sexual drive and function. There are other two weak androgens, which are dehydroepiandrosterone and androstenedione. They are mostly synthesized in adrenal glands.

The synthesizing of Androgens is from steroidogenic pathways involving various enzymes and many different intermediates. They are under the control of the stringent regulation through the hypothalamus-pituitary-testis axial. A biosynthetic pathway of androgen starts from cholesterol, which are functions as the precursor. Two androgens, dehydroepiandrosterone and androstenedione are mainly produced in adrenal tissue. Androstenedione is converted into testosterone or estradiol mainly in testis and peripheral tissue. Testosterone is further changed to the more potent 5α-dihydrotestoterone by 5α-reductase, or converted into estradiol by aromatase. Enzymes involved in androgen synthesis pathways are mainly proteins in the cytochromes P450 (CYP) and hydroxysteroid dehydrogenase (HSD) families. [Chang C. 2002] In addition to

the controlled synthesis, androgens are removed from blood through a series of well-orchestrated pathways. They are converted to other active metabolites first, which then followed by sulfation and conjugation with glucuronic acid so as to become more hydrophilic.

The function of androgen is very important. Any change in steps of androgen synthesis will result in diseases. The deficient androgen synthesis often causes male pseudohermaphroditism. Problems in the regulation of androgen synthesis can also lead to male and female pseudohermaphroditism. [Guido M, Uta C.P. 2008] Many genetic diseases due to abnormal androgen secretion have been described. These diseases usually arise due to mutations in the genes involved in steroid metabolism. The cause of hormone-dependent tumors, e.g. prostate cancer, is also due to the change of androgen secretion.

1.1.1. Intracellular Androgen Receptors

The intracellular androgen receptor (iAR) is also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4). It is a type of nuclear receptor which is activated by binding of either the androgenic hormones testosterone or DHT. As other members of the nuclear receptor superfamily, iAR has four major functional regions: an N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD [Mangelsdorf DJ, et al. 1995].

The classic genomic model for steroid hormone action presumes that steroid hormones can freely cross the Plasma Membrane, enter the cytoplasm, and bind to activate specific iAR. The bound steroid receptors act as transcription factors and bind as homodimers or heterodimers to specific DNA response elements in target gene promoters, causing protein synthesis. [Guido M, Uta C.P. 2008] iAR is a kinase substrate and downstream target of receptor-tyrosine kinase (RTK), for example HER-2/neu, and G-protein coupled receptor (GPCR) signalling which can both activate AR independently of androgen. [Nigel C. Bennett, et al. 2010] This genomic-androgen effect typically takes at least more than half an hour. There are also another two pathways activated will

active by iAR. One is binding with the SH3 domain of the tyrosine kinase c-Src to active the MAPK pathway and influence the iAR-mediated transcription via phosphorylation of receptor complexes. The other is binding to steroid hormone-binding globulin (SHBG).The iAR and SHBG bound complexes can activate SHBG receptor and lead to an increase in PKA activity. PKA may influence the iAR-mediated transcription via alteration of phosphorylation status of iAR and iAR coregulators. [Guido M, Uta C.P.2008] (Fig.1)

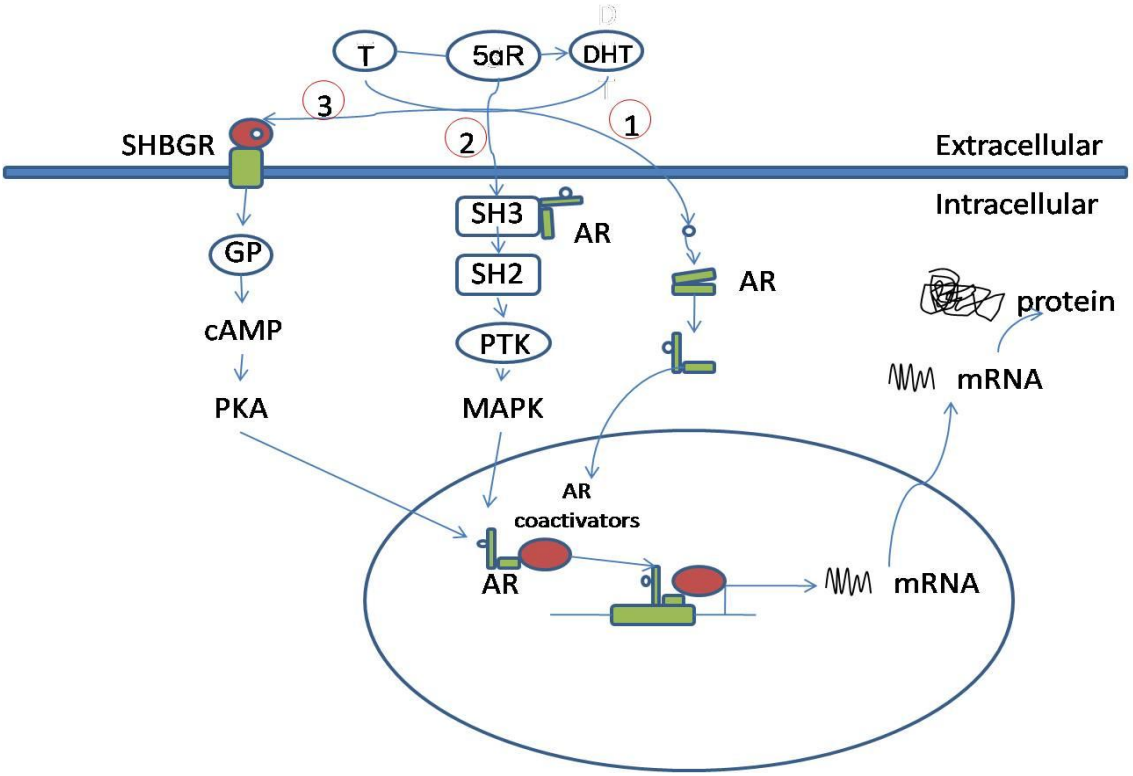


Figure 1: **Androgen actions via intracellular androgen receptor.**

(1) In the classical pathway. (2) Bound with the SH3 domain. (3) Bound to SHBG. *Abbreviations:* T, testosterone; DHT, dihydrotestosterone; 5αR, 5alpha reductase enzyme; AR, androgen receptor; PKA, protein kinase A; GP, G-protein; SH2, Src homology domain 2; SH3, Src homology domain 3; PTK, protein tyrosine kinase; MAPK, mitogen-activated protein kinase; SHBGR, steroid hormone-binding globulin receptor; cAMP, cyclic adenosine monophosphate.

The iAR activated target genes are an array of growth factor genes, e.g. epidermal growth factor (EGF); fibroblast growth factor (FGF); Insulin-like growth factor 1(IGF1); vascular endothelial growth factor (VEGF); transforming growth factor-β (TGFβ). The ability of iAR to cross-talk with key growth factor

signaling events toward the regulation of cell cycle, apoptosis, and differentiation outcomes in prostate cancer cells has been established. IGF, FGF, VEGF, and TGFβ secreted by the prostate stromal cells activate their receptors and interact with iAR signal axis. In prostate epithelial cells, the androgenic signal engages secreted VEGF and TGFβ which affect the prostate tumor microenvironment by inducing angiogenesis, stromal cell growth and differentiation. EGF signaling encounters iAR signal in a tight control of multiple pathways. Growth factor signaling may proceed via iAR signal and regulate the downstream effectors of iAR regulating key cellular processes including proliferation, differentiation, apoptosis, and survival of prostate cancer cells. [Meng-Lei Zhu, Natasha Kyprianou. 2008]

Several studies show that some connections may exist between iAR and the Wnt pathway. The interaction of iAR and the phosphatidylinositol 3-kinase/Akt pathway has been demonstrated in prostate cancer cell lines. The tumor suppressor PTEN, which inhibits the PI3K/Akt pathway, is frequently mutated in prostate cancer. PTEN has been shown to modulate androgen-induced prostate cancer cell growth and iAR-mediated transcription [Li P, et al. 2001; Wen Y et al. 2000]. A study using a synthetic PI3K inhibitor, and re-expression of PTEN in a PTEN-null prostate cancer cell line shows that the involves the Wnt pathway: GSK3β, a downstream effector of PI3K/Akt, also participates in the Wnt pathway; PTEN phosphorylate and inactivate GSK3β, a downstream effector of PI3K/Akt, via PI3K/Akt; GSK3β-dependent inactivation of cytoplasmic β-catenin is was subsequently attenuated; As a result, β-catenin shuttled to the nucleus and augmented ligand-stimulated transcription by iAR. [Sharma M, et al. 2001]

In addition to the transcriptional or genomic mode of activation by steroids, androgens can also exert rapid, nongenomic effects. Similar to the non-genomic action of other steroids, there are certain basic criteria for an androgen induced response to be considered non-genomic in nature. The first criterion is speed. The effect should occur in seconds to minutes. It is not long enough to allow gene transcription or translation. Typically, gene transcription needs several hours after steroid exposure, although the latency for transcription events has been reported to be as short as 7.5 min. It has to take the additional time for

mRNA to be translated into proteins and for those proteins to be processed and induce measurable responses. The cellular responses are changes in free intracellular calcium, and activations of second messenger pathways. The second criterion is membrane mediated. The response should include embedding membrane or associating receptors or binding proteins. The action can be induced even when the steroid is conjugated to molecules which prohibit it from translocation to the nucleus when bound to a receptor. The most common example is the use of testosterone (T) conjugated to large molecules such as bovine serum albumin (BSA). The last is lacking transcription/ translation machinery activation. Experiments using cell lines either lacking the necessary machinery for a genomic response or identifying androgen effects, they are insensitive to inhibitors of transcription and translation. All this demonstrated that certain steroid responses can be elicited in systems where gene transcription or protein synthesis is was unlikely or impossible. [C.D.Foradori, et al. 2008]

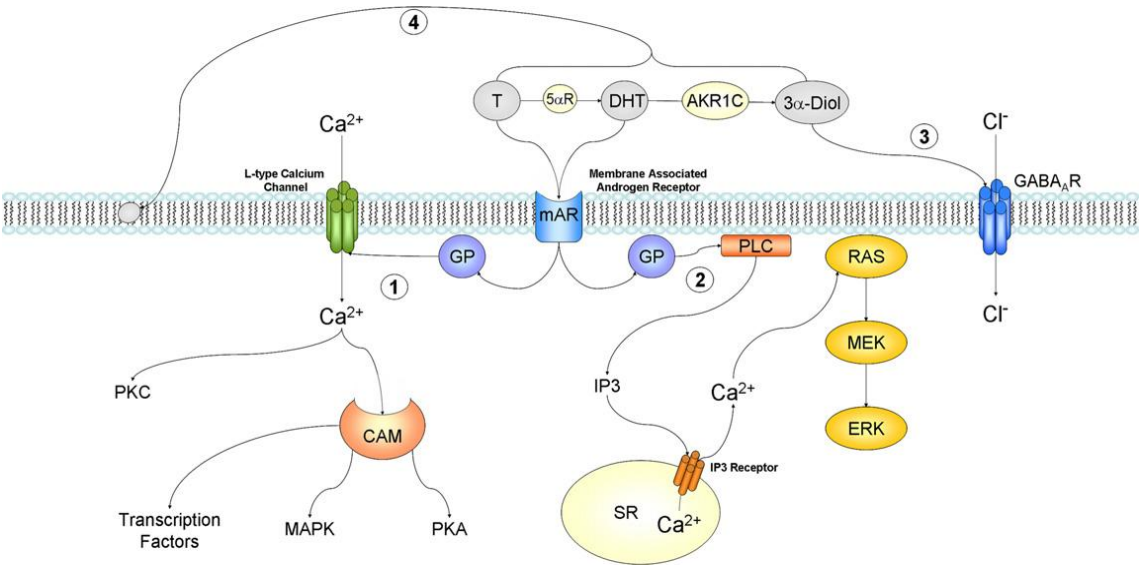


Figure 2: **Non-genomic androgen actions via intracellular ion concentrations and membrane fluidity.**

(1) Androgen interacts with a mAR leading to the activation of L-type calcium channels through an inhibitory G-protein (GP). (2) Androgen interacts with mAR leading to modulation of G-protein activity and subsequent activation of phospholipase C (PLC). (3) DHT's metabolite, 3a-Diol, may interact with the GABAA receptor and lead to increases in intracellular calcium and thus membrane potential. (4) Testosterone and its metabolites can interact with phospholipids in the membrane bilayer to change membrane flexibility and subsequently alter the function of sodium/potassium ATPase and calcium ATPase. Abbreviations: T, testosterone; 5aR, 5alpha

reductase enzyme; AKR1C, aldo-keto reductase; 3a-Diol, 5a androstane-3a, 17b diol; GABA, gamma-aminobutyric acid; GP, G-protein; PKA, protein kinase A; PKC, protein kinase C; CAM, calmodulin; PLC, phospholipase C; IP3, inositol 1,4,5-triphosphate; SR, sarcoplasmic reticulum; MEK, MAPK/ERK kinase; ERK, extracellular-signal regulated kinase. [C.D.Foradori, et al. 2008]

Nongenomic steroid activity involves the rapid induction of conventional second messenger signal transduction cascades. Nongenomic action of androgens can occur through multiple receptors. Androgens activate cAMP and PKA through membrane androgen receptor (mAR). Androgens also induce an elevation in intracellular Ca2+ through mAR to a GPCR (G-Protein Coupled Receptor) by activating an influx through nonvoltage-gated Ca2+ channels. The increasing of intracellular calcium activates signal transduction cascades, which is included PKA (Protein Kinase-A), PKC (Protein Kinase-C), and MAPKs (Mitogen-Activated Protein Kinase). They can modulate the activity of the ARs and other transcription factors. AR can also interact with the intracellular tyrosine kinase c-Src, triggering c-Src activation. One of the targets of c-Src is the adapter protein SHC (SH2 Containing Protein). It is an upstream regulator of the MAPK pathway. The activation of AR are influenced by direct phosphorylation by MAPK [Heinlein CA, Chang C. 2002]. In another side, AR phosphorylation by ERK2 is associated with enhanced AR transcriptional activity and an increased ability to recruit the coactivator ARA70.[Heinlein CA, Chang C. 2002] The SRC family of transcriptional coactivators includes SRC1, SRC3, and TIF2 (Transcription Intermediary Factor-2). They are targets of MAPK phosphorylation and result in an increased ability of these coactivators to recruit additional coactivator complexes to the DNA-bound receptor. The nongenomic, rapid stimulation of second messenger cascades by androgens may ultimately exert biological effects through modulation of the transcriptional activity of AR or other transcription factors. Those modulations may happen by direct phosphorylation of transcriptional activators or their coregulators [Michels G, Hoppe UC. 2008]. In the absence of AR’s cognate ligand the AR can also be activated. Androgen can initiate by various growth factors.

1.1.2. Membrane Androgen Receptor

Scientific evidence accumulated in recent year's points to the existence of membrane androgen receptors (mARs), triggering rapid, non-genomic signals. Although the exact molecular identity of mAR still remains unknown, non-genomic androgen actions manifested within minutes have been reported in various cell types including macrophages and T cells [Benten WP, et al. 1999; Benten WP, et al. 1999], LNCaP [Kampa M, et al. 2002; Wang Z, et al. 2008], T47D [Kampa M, et al. 2005], MCF7 [Kallergi G, et al. 2007], DU145 [Hatzoglou A, et al. 2005; Papadopoulou N, et al. 2008a; Papadopoulou N, et al 2008b], C6 [Gatson JW, et al. 2006], PC12 [Alexaki VI, et al. 2006] or VSMC cells [Somjen D, et al 2004]. These effects are clearly different from those manifested upon activation of the intracellular androgen receptors (iARs) mediating genomic androgen signals resulting in receptor dimerization, nuclear translocation and subsequent activation of androgen-specific target genes.

In prostate cancer, expression of mAR in human tumor cells was initially reported in iAR positive LNCaP cells [Kampa M, et al 2002] and iAR –deficient DU145 cells [Hatzoglou A, et al 2005]. In LNCaP cells study, mAR activation through testosterone-BSA conjugates induced rapid PSA release, fast actin reorganization and additional cell responses like inhibition of cell growth and induction of apoptosis [Hatzoglou A, et al 2005]. The molecular signaling pathway starts from focal adhesion kinase (FAK). Initially, FAK was rapidly phosphorylated and associated with the p85 subunit of the phosphoinositol-3-Kinase (PI-3K). Following this association, the lipid kinase activity of PI-3K and the tyrosine phosphorylation of its p85 regulatory subunit were significantly induced by mAR stimulation. PI-3K activation was accompanied by the downstream upregulation of the Rho small GTPases Cdc42, Rac1, RhoA and RhoB. Rapid activation of these GTPases resulted in actin cytoskeleton reorganization. Yet again, these effects were specific for mAR because three different steroidal and non-steroidal iAR antagonists failed to block the activation of this rapid signaling pathway [Papakonstanti, E. A., et al. 2003]. From these findings it was concluded that mAR activation induced potent apoptotic regression in LNCaP prostate tumor cells controlled by

Rho/ROCK/actin signaling. Interestingly, while LNCaP prostate cancer cells express functional iAR, the DU145 cell line expresses either nonfunctional iAR, or is iAR-deficient. Therefore, DU145 cells fail to respond to iAR-regulated androgen treatment.[Alimirah, F., et al 2006] In this cell model, mAR stimulation by testosterone or T-BSA conjugates induced potent actin reorganization, inhibited cell motility and promoted apoptotic regression. [Papadopoulou N, et al. 2008a] But the signaling pathway is different from LNCaP cells. Specifically, mAR activation bypassed the FAK/ PI-3K signaling pathway, as FAK was shown to be constitutively phosphorylated and mAR stimulation failed to further activate the downstream effectors PI-3K and Rac. An alternative pathway functionally distinct from the FAK/PI-3K/Rac signaling was described. [Papadopoulou N, et al. 2008a] This pathway regulated actin reorganization, the induction of apoptosis and the pro-apoptotic machinery. Indeed, long term down regulation of the pro-survival PI-3K/Akt pathway became evident 12–24 h upon mAR activation as indicated by the significant decrease of the phosphorylation levels of PI-3K and Akt. Furthermore, inhibition of NF-jkB translocation and increased FasL expression were documented, while increased caspase 3 activity was measured [Papadopoulou N, et al 2008b].

A B

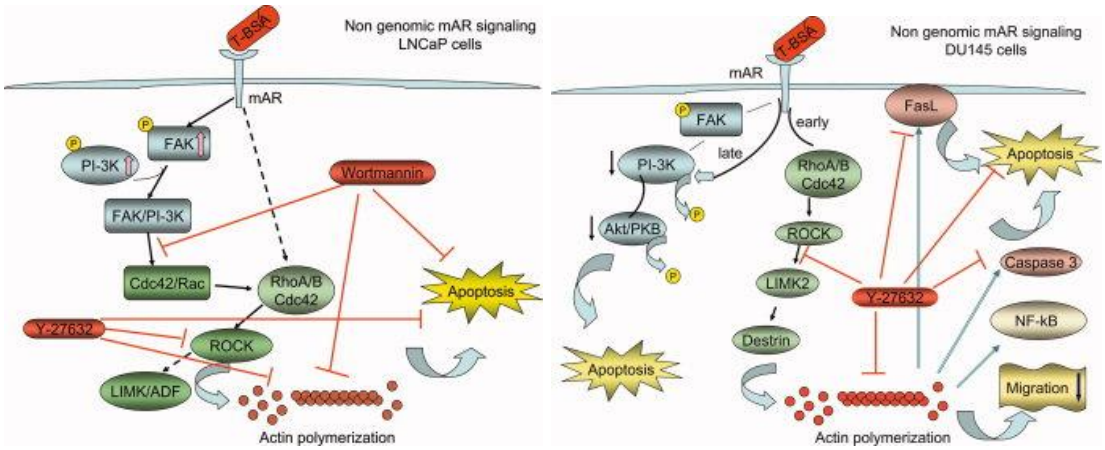


Figure 3: **mAR signaling in human prostate cancer cells**

A) Non-genomic mAR signaling operating in iAR positive LNCaP human prostate cancer cells regulating actin redistribution and apoptosis. Solid arrows indicate events that have been experimentally proven. Dashed arrows indicate unidentified possible links. See text for details.

B) Early and late mAR signaling operating in iAR deficient DU145 human prostate cancer cells regulating actin redistribution, downstream pro-apoptotic signaling, and migration. Solid arrows

indicate events that have been experimentally proven. Dashed arrows indicate unidentified possible links. See text for details. [Papadopoulou N, et al 2009]

In breast cancer, it has been reported that mAR is expressed in T47D and MCF7 human breast epithelial cancer cells. In T47D cells, specific and saturable androgen receptors are present in the membrane and their activation via TBSA conjugates resultes in cell death by apoptosis. [Kampa M, et al 2005] Moreover, pharmacological inhibitors of MEK and p38 kinase were able to block T-BSA induced apoptosis showing a functional implication of these pathways in mAR-dependent apoptosis in T47D cells. However, in MCF7 cells, activation of these receptors by T-BSA conjugates triggered a non-genomic signaling pathway involving FAK and PI-3K phosphorylation and downstream activation of the small GTPase Rac1, ultimately resulting in actin redistribution. Cell migration experiments provided insights in the functional role of mAR stimulation in MCF7 cells. But the activations of mAR did not induce any apoptotic response in this kind of cells. [Kallergi G, et a.l 2007]

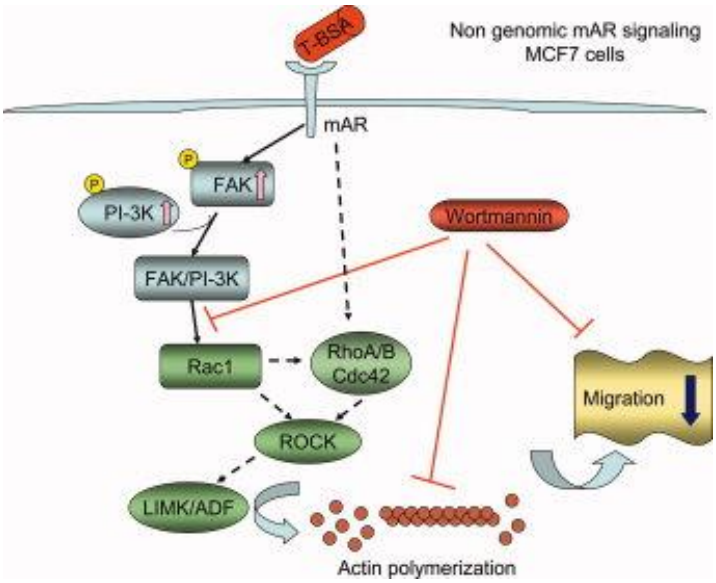


Figure 4: **mAR signaling in breast epithelial cancer cells**
Non-genomic mAR signaling operating in MCF7 breast epithelial cancer cells regulating actin redistribution and cell motility. Solid arrows indicate events that have been experimentally proven. Dashed arrows indicate unidentified possible links. [Papadopoulou N, et al 2009]

Taken together, these studies clearly established that functional mARs trigger strong anti-tumorigenic effects in prostate and breast cancer cells, implying a

potential role of mAR as a novel target for the development of selective cancer treatments [Papadopoulou et al., 2009]. It was shown that mAR activation resulted in actin reorganization regulated by distinct mechanisms involving small GTPases' specific signaling cascades. [Papadopoulou N, et al 2008b] Furthermore, it was shown that mAR activation induced potent apoptotic regression of prostate cancer cells in vitro [Papadopoulou N, et al 2008a] and in mouse xenografts in vivo and suppressed cell growth and motility. [Hatzoglou A, et al 2005, Kampa et al 2006] In breast cancer, activation of mAR in MCF7 breast epithelial cancer cells regulates actin redistribution and cell motility. [Papadopoulou N, et al 2009] However, it remained elusive whether mARs were also expressed in other tumors and whether their activation could result in the induction of anti-tumorigenic effects similar to the ones described in prostate and breast cancer cells.

In my diploma thesis, by using either colon cancer tissues isolated from mice xenograft tumors or two established colon cancer cell lines (Caco2 and HCT116 cells), the expression and function of functional role of mAR has been analyzed. As a result, testosterone binding sites were expressed in the membrane of colon cancer cells and qualify as bona fide membrane androgen receptors as assessed by radioligand binding studies, Scatchard analysis and displacement assays. The activation of those receptors with non permeable testosterone derivatives induced pro-apoptotic responses. [Gu S, et al. 2009]. However, the expression and regulation of pro-survival signals that may compensate mAR induced cell death remained undefined. In addition, the profound anti-tumorigenic mAR action has not been addressed in association with motility and invasiveness. In my PhD work I addressed the anti-apoptotic signaling in mAR expressing colon tumors in vitro and in vivo. Moreover, the migration potential of colon tumor cells upon mAR stimulation was examined and the molecular targets were analyzed in detail, implicated in cell motility regulation.

1.2 Colon cancer

1.2.1. Epidemiology of colorectal cancer

Colon cancer is considered a public health problem worldwide. It is also called colorectal cancer (CRC) or large bowel cancer including cancerous growths in the colon, rectum and appendix. With 655,000 deaths worldwide per year, it is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. The incidence rates are higher in the developed areas, such as Europe, North America and Australia. In Cancer incidence of Europe 2006, it was estimated that 217400 and 195400 new colon cancer cases occurred annually in men and women in Europe, ranking third in the incidence in men and second in women. [Ferlay, J, et al.2007] It's noticeable that the incidence of colon cancer used to be low in some eastern countries, such as Japan and China. However, in recent decades the incidence and mortality of colon cancer in these countries increased sharply, which was probably owing to their adoption of Western life style. The mortality of colon cancer in Japan increased 5.5-folds in the second half of the 20^{th} century [Honda, T., et al. 1999]. The incidence of colon cancer in China doubled in the past 30 years as well [Zhang, Y.Z., et al. 2005]. According to the Surveillance, Epidemiology and End Results (SEER) Program database analysis, 5-year survival rates have risen from 56.5% for patients diagnosed in the early 1980s to as much as 63.2% for those diagnosed in the early 1990s and most recently to 64.9%, a trend due mostly to earlier diagnosis and treatment [Ries LAG, et al. 2008]. One reason for the improving trend is that the prognosis for patients with CRC is highly dependent on stage: 5-year survival rates are over 90% for Dukes A, but only 5% for Dukes D. Unfortunately, only 10% of CRCs are diagnosed early, most patients presenting themselves with the advanced disease [Rockville, MD. 1998].

1.2.2. Etiology of colorectal cancer

The risk factors of CRC include age older than 50, inflammatory bowel disease (IBD), high-fat low-vegetable diet, physical inactivity, smoking, alcohol and

genetic predeposition. [Benson AB 3rd. 2007; Jemal, A., et al 2007] About 20% of CRC are familial, which means gene alterations may mediate the development of CRC [Benson, 2007]. There are two key CRC related hereditary diseases, familial adenomatous polyposis (FAP) and hereditary nonpolyposis colon cancer (HNPCC). FAP is a rare autosomal dominant syndrome caused by an inherited mutation in the APC gene [Strate LL, Syngal S. 2005]. It accounts for approximately 1% to 2% of all CRC cases. HNPCC or termed as Lynch syndrome [Lynch, P.M., Lynch, H.T. 1985], an inherited autosomal dominant syndrome, is caused by inherited mutation in any one of five DNA mismatch repair (MMR) genes and microsatellite instability [Benson AB 3rd. 2007]. HNPCC is predicted to account for 2% or less of all CRC cases [Aaltonen, L.A., et al. 1998].

1.2.3. Genes

There is much progress which has been made in understanding the molecular mechanism of colorectal cancer. A progression from normal mucosa to adenoma to carcinoma was supported by the demonstration of accumulating mutations in genes as K-ras, adenomatous polyposis coli (APC), tumor protein P53 (TP53), and deleted in DCC, all of which are thought to be of significance, but are not able to successfully account for all colorectal cancers. There is heterogeneity in the pathogenetic pathway leading to CRCs, and there are two major tumorigenic pathways. The first is driven by chromosomal instability (CIN), the progress of which involves both oncogenes and tumor-suppressor genes including chromosomes 5q, 17p, and 18q [Fearon, E.R., Vogelstein, B. 1990; Gervaz, P., et al. 2001]. Chromosome 5q genes are responsible for APC, 17p for TP53, and 18q for DCC or Mothers against decapentaplegic homolog 4 (SMAD4). K-ras is the most common oncogene following this pattern. The tumor-suppressor genes APC, TP53, and DCC/SMAD4 play important roles in this sequential adenoma to carcinoma. Another genetic pathway may well be depicted as a consequence of the alteration in mismatch repair (MMR) genes. [Gervaz, P., et al. 2001] When the alteration happens in germinal cells, the hereditary cancer known as hereditary nonpolyposis colorectal cancer (HNPCC) occurs. When somatic cells are affected, microsatellite instability (MSI) would be

unavoidable. MSI is responsible for a subset of sporadic colorectal tumors. [Feng-ying LI, Mao-de LAI 2008]

1.2.4. Colon cancer and steroid receptors

Although colon cancer is not a hormone-dependent tumor, the existence of sex differences in colon cancer incidence was proposed several years ago. The activity of steroid receptor plays a pivotal role in a well-controlled cascade of signals, which maintains the mucosal architecture by the shedding of senescent and apoptotic cells at the surface of the epithelium. The identification of a functional interaction between the Wnt/APC pathway and steroid represents a major goal for several laboratories [Mulholland, et al 2005]. β-Catenin activates a growing number of steroid receptors, resulting in alterations of cell proliferation and tumorigenesis. On the other hand, Wnt signaling appears to be compromised by the action of some steroid receptors. It is also clear that steroid receptors are regionally compartmentalized along the cryptvillus axis, determining the switching on and off of transcription of particular genes with a strong influence on cell fate. The mechanism for the influence of steroid receptors on cell proliferation, differentiation and apoptosis in the gut is complex and still under investigation. Also, the observed phenotypes after steroid receptor activation or inhibition are sometimes contradictory. Steroid receptor effects depend on the amount of agonists, the cell type and the mutational. [Mulholland, et al. 2005] In estrogen receptor, ERβ, dependent or independent of 17β-oestradiol activation has a crucial role in colonic cell homeostasis, including modulation of proliferation and organized cell death. Absence of ERβ results in increased cell turnover in the colonic mucosa. The specific ERβ signaling confers protection against colonic mitogenesis. ERβ modulators might provide options for prevention of colorectal cancer, but variance in signaling of ERβ will complicate the development of treatments. Non-genomic activation of mitogenic cell signaling cascades by oestrogen and clarification of receptor independent coupling mediated by protein kinase C (PKC) δ provide other opportunities for cancer treatment. [Kennelly R, et al. 2008] Colon cancer is also related to androgen receptor. The selective binding between lithocholic acid and iAR supports that diet-related endoluminal substance may play a role in cancer

development model. Moreover molecular alterations such as DNA damage or mutation are the 1st event. [Berta, L., et al 2003] An association between the iAR genotype and colorectal cancer has been observed. Although the expression of iAR, implicated in tumorigenesis has been reported in colon tumors [Slattery ML, et al 2005], the role of functional membrane androgen receptors has not been addressed in colon cancer.

1.3 Apoptosis

Apoptosis is a form of programmed cell death that plays important roles during animal development, immune response, elimination of damaged cells, and maintenance of tissue homeostasis. It is associated with a distinct set of biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane. The name was first introduced by John Kerr [Kerr JFR, et al. 1972] in 1972, refers to the morphological feature of formation of ‘‘apoptotic bodies’’ from a cell. Carl Vogt, however, first described the phenomenon more than 100 years earlier in 1842. Now it has become a major research area in the biomedical sciences.

1.3.1. Morphological features of apoptosis

Apoptosis has a lot of stereotypical morphological changes: The first is the cell shrink which shows deformation and looses contact to its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane, then the cell membrane begins to show blebs and eventually these blebs separate from the dying cell and form "apoptotic bodies". The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. The apoptotic cells also cease to maintain phospholipid asymmetry in the cell membrane, and phosphotidylserine appears on the outer leaflet. The mitochondrial outer membrane also undergoes changes that include loss of its electrochemical gradient, and substances like cytochrome c leak into the cytoplasm. Finally, adjacent cells or macrophages phagocytose apoptotic bodies and the dying cell. Those morphological changes are consequences of

characteristic molecular and biochemical events which occur in an apoptotic cell. Most of them are activated notably by proteolytic enzymes. It finally mediates the cleavage of DNA into oligonucleosomal fragments, in at the same time as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles [Saraste, A; Pulkki, K 2000]. Furthermore, apoptosis is in contrast to the necrotic mode of cell-death. During necrosis, the cellular contents are released uncontrolled into the cell's environment, which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue.

1.3.2. Molecular mechanisms of apoptosis signaling pathways

Apoptosis is executed by intracellular proteases named caspases that are activated during the onset of apoptosis via extrinsic and intrinsic pathways. The intrinsic pathway is triggered by the release of proteins such as cytochrome c from mitochondria to cytosol and the extrinsic pathway is activated by the binding of death-inducing cytokines such as Tumor Necrosis Factor to its receptor at cell surface. Both pathways are regulated at multiple steps to ensure proper apoptosis.

The caspases are of central importance in the apoptotic signaling network which is activated in most cases of apoptotic cell death [Bratton, SB, et al. 2000]. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Actually cell death can only be classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity [Leist, M, Jaattela, M. 2001]. Caspases can be divided into three groups based upon structural differences and substrate preferences, i.e. apoptotic initiators (caspase-2, -8, -9, and -10), apoptotic executioners (caspase-3, -6, and -7), and cytokine processors (caspase-1, -4, -5, -13, murine caspase-11, -12, and -14).

The extrinsic pathway is initiated by ligation of transmembrane death receptors

(Fas, TNF receptor, and TRAIL receptor) with their respective ligands (FasL, TNF, and TRAIL) to activate membrane-proximal caspases (caspase-8 and –10). It in turn cleaves and activates effector caspases such as caspase-3 and –7. Typically the extrinsic pathway involves activating the initiator caspase, caspase-8, which in turn either activates caspase-3 or cleaves the Bcl-2 family member, Bid, leading to the formation of the apoptosome and activation of caspase-9.

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also plays a central role in apoptosis. It regulates the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs [Kaufmann, SH, Earnshaw, WC. 2000; Wang, X. 2001]. This mitochondrial pathway named intrinsic pathways. The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins. It involves members of the Bcl-2 family that regulate cytochrome c released from the mitochondrial intermembrane space to cytoplasm.

1.3.3. Apoptosis responses by AKT pathway

Akt is a good candidate for mediating PI3K-dependent cell-survival responses. An important function of activated PI3K in cells is the inhibition of programmed cell death [Yao and Cooper, 1995]. The first evidence to show that Akt acts as an anti-apoptotic signaling molecule was observed in cerebellar granule neurons after trophic factor withdrawal [Dudek et al., 1997], and in fibroblasts after forced expression of c-Myc [Kauffmann-Zeh et al., 1997]. Subsequent work in many laboratories has established the principle role of Akt in the regulation of cell survival in several cell types, consistent with its ubiquitous expression pattern. Akt has been implicated as an anti-apoptotic in many different cell death paradigms, including withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation and treatment of cells with chemotherapeutic drugs and ischemic shock [Franke et al., 1997; Downward, 1998]. Multiple studies supporting the role of Akt in apoptosis suppression have

connected Akt to cell death regulation either by demonstrating its downregulation following pro-apoptotic insults, or by using gene-transfer experiments that transduce both activated, anti-apoptotic and inactive, pro-apoptotic mutants of Akt.

Taken together, these observations suggest that Akt may play a critical role both in the function of cancer cells and in the pathogenesis of degenerative diseases. By promoting the cell survival of mutated, damaged or transformed cells even under adverse conditions, Akt can promote cancer cell growth by protecting cells from apoptosis, which would otherwise be eliminated by programmed cell death. To experimentally prove the importance of Akt kinases in oncogenic transformation, in a seminal paper, Peter Vogt and colleagues demonstrated that a transformed cellular phenotype could be reverted to normal when using a cell model for PI3K-dependent oncogenesis as long as dominant-negative mutants of Akt were expressed concomitantly [Aoki et al., 1998]. Akt is also likely to play a significant role in degenerative diseases, where excessive or inappropriate cell death occurs possibly because proper trophic factor support is lacking. The relevance of Akt signaling in neurodegenerative disease is supported by studies that examine its activity and function in Alzheimer's disease models in vitro [Hong and Lee, 1997; Weihl et al., 1999]. A role for Akt has also been suggested in other models of human degenerative diseases, including cardiac failure [Matsui et al., 1999] and other cardiovascular diseases where there is increased and chronic loss of cells [Reed and Paternostro, 1999].

1.3.4. Apoptosis responses by Bad

BAD is a distant member of the Bcl-2 family that promotes cell death. Phosphorylation of BAD results in its cytosolic sequestration by the tau form of 14-3-3 proteins and its inactivation, as the phosphorylated form has reduced ability to bind to membrane Bcl-xL. BAD phosphorylation induced by interleukin-3 (IL-3) was inhibited by specific inhibitors of phosphoinositide 3-kinase (PI 3-kinase) [Del Peso L, et al. 1997]. Although signals transduced by the engagement of growth factor receptors, such as cytokines, insulin-like-growth

factor (IGF) and nerve growth factor (NGF), were long known to promote survival, the molecular mechanisms linking their survival-promoting effect to the direct inhibition of apoptosis emerged with the identification of select components of the core apoptotic machinery that are modulated by phosphorylation events downstream of survival signaling [Datta et al., 1999; Amaravadi and Thompson, 2005]. BAD's capacity to bind and neutralize its anti-apoptotic partners, BCL-2, BCL-XL and BCL-W, is inhibited on phosphorylation by survival kinases activated by trophic factors. Various kinases have been shown to phosphorylate BAD. S136 is a preferred substrate for AKT and p70S6 kinases in the PI3K signaling pathway [Datta et al., 1997; Del Peso et al., 1997; Blume-Jensen et al., 1998; Eves et al., 1998; Harada et al., 2001]. S112 and S155 harbor bona fide protein kinase A (PKA) consensus sites that can also be recognized by p90 ribosomal S6 kinase (p90RSK), a kinase activated by the MAPK pathway that shares multiple common substrates with PKA [Datta et al., 2000; Tan et al., 2000; Houslay, 2006]. Modification of BAD by p90RSK is consistent with several studies indicating that the activation of the RAS/RAF/MEK/MAPK pathway modulates BAD phosphorylation [Bonni et al., 1999; Fang et al., 1999; Scheid et al., 1999]. RAF can localize to mitochondria [Wang et al., 1996; Gotz et al., 2005], and its activated forms promote BAD phosphorylation [Fang et al., 1999]. However, RAF modulation of BAD phosphorylation is likely indirect through other kinases such as AKT [Fang et al., 1999; Wiese et al., 2001; von Gise et al., 2001; Gotz et al., 2005]. PIM kinases constitute another class of survival kinases that phosphorylate BAD predominantly on the S112 site [Fox et al., 2003; Yan et al., 2003; Macdonald et al., 2006]. The interrelationship of these phosphorylation events is especially intriguing as it suggests that BAD modification may serve as a node where distinct signaling pathways converge to regulate the core apoptotic machinery. Recent studies have proposed a sequential model of BAD dephosphorylation initiated by pS112 dephosphorylation, which may then expose pS136 and pS155 residues for dephosphorylation [Chiang et al., 2003]. Thus, both phosphorylation and dephosphorylation of BAD at the three serine sites seem to be tiered processes. Although S136 is the apical serine the phosphorylation of which is needed for neutralizing BAD's apoptotic function, pS112 dephosphorylation may be the initial dephosphorylation event required for

promoting the apoptotic activity of BAD. It is also possible that, in addition to directly targeting specific serine sites, BAD phosphatases may inactivate the survival kinases [Andjelkovic et al., 1996; Djouder et al., 2007].

1.3.5. Apoptosis responses by actin polymerasion

Actin organization has been reported to trigger cell death and, potentially, ageing. This function can lie upstream of mitochondrial ROS release [Gourlay CW, Ayscough KR., 2005]. As it has been reported in yeast, in mammalian cells actin stabilization can also induce cell death and this is mediated through changes involving mitochondria [Posey, S. & Bierer, B. 1999, Odaka, C., et al. 2000]. A major regulator of actin that also has a role in apoptosis is gelsolin. This protein is well known to mediate actin reorganization in response to changes in Ca2+ and phosphoinositides *in vivo* and has a role in cell motility. [Kwiatkowski, D. J. 1999] The role of gelsolin in regulating mitochondrial potential might occur through a direct gelsolin binding to mitochondria. [Koya, R. C. et al. 2000]. The key role of gelsolin to enhance actin depolymerization is in protecting from apoptosis [Harms, C. et al. 2004]. The actin regulatory protein cofilin has also been shown to have a key role in the apoptotic process by promoting the depolymerization and severing of actin filaments. On the other hand, changes in actin dynamics might trigger the activation of caspases.

In opossum kidney (OK) cells which express functional characteristics of normal proximal tubular epithelial cells, TNF-α has been reported exerts an apoptotic or antiapoptotic cell response. And TNF-α induces actin cytoskeleton polymerization and their possible role to cell response in OK cells. The signaling mechanism triggered by TNF-α that leads to actin redistribution and to modulation of NF-κB and caspase-3 activity. The signal of TNF-α elicits antiapoptotic effects in OK cells is through the phosphatidylinositol-3 kinase (PI-3 kinase)$\rightarrow$Cdc42$\rightarrow$phospholipase (PLC)-γ1$\rightarrow$ actin cytoskeleton polymerization $\rightarrow$NF-κB nuclear translocation cascade. [Papakonstanti EA, Stournaras C. 2004]

1.4 Cell Migration

Cells migrate in response to multiple situations they encounter during their lives. In pathology, production of abnormal migratory signals may induce the migration of the wrong cell type to the wrong place, which may have catastrophic effects on tissue homeostasis and overall health. Some examples include autoimmune syndromes in which immune cells home to certain locations (joints in rheumatoid arthritis, and the CNS in multiple sclerosis are two examples) and destroy the supporting tissue, causing severe damage; or the process of metastasis, in which tumor cells abandon the primary tumor and migrate to distant tissues where they generate secondary tumors.

There are different modes of cell migration depending on the cell type and the context in which it is migrating. Cells can move as single entities, and the specifics of their motility depend on several factors, adhesion strength and the type of external migratory signals and cues, mechanical pliability, dimensionality, and the organization of the cellular cytoskeleton. The intrinsic properties of the cell interact with the environment to produce a migratory mode or phenotype. Some tumor cells can move by extending membrane blebs, and their actin cytoskeleton is not very organized, either. They have elaborate cytoskeletal structures and adhesions, and their motion is generally slow. It is worth noting that some cell types can switch between these depending on their environment. Cells can also move in groups, including chains of cells and sheet-like layers.

It is generally convenient to parse migration into a useful set of component processes, which are often regulated by the same effectors regardless of the cell type and the mode of migration. These processes include polarization, protrusion and adhesion, translocation of the cell body and retraction of the rear. These processes are coordinated and integrated by extensive transient, signaling networks.

1.4.1. PI3K/Akt signaling pathways in cell migration

Migration is regulated by many gene products and complicated signaling integrated in the concept of focalized adhesion. Main protagonists are protein kinases such as extracellular signal-regulated protein kinase (ERK)1/2, which are the most widely expressed members of the mitogen-activated protein (MAP) kinase family, the phosphatidylinositol 3 kinase (PI3K), the focal adhesion kinase (FAK) and others that can be activated by growth factors, cytokines and ECM [Friedl and Wolf, 2003]. Interestingly, the importance of estrogens in modulating rapid signaling effects that act on these targets has been recently highlighted [Acconcia and Kumar, 2005; Acconcia et al, 2006]. A role for Akt in the control of cell migration, invasion of the extracellular matrix, and ultimately metastasis has been difficult to ascertain. Strikingly, activation of Akt1 has been found to decrease mammary epithelial cell migration, and Akt1 prevents an epithelial-to-mesenchymal transition that resembles events required for metastasis [Irie et al., 2005] and [Yoeli-Lerner et al., 2005]. Two independent mechanisms for this surprising Akt function have been explored. The first found that the inhibitory effect of Akt1 on the in vitro migration and invasion properties of breast cancer cell lines involved a pathway leading to degradation of the nuclear factor of activated T cells (NFAT) transcription factors [Yoeli-Lerner et al., 2005]. However, the molecular mechanism of Akt1-mediated degradation of NFAT is currently unknown. A second group found that siRNA knockdown of Akt1, but not Akt2, led to an increase in the migration of mammary epithelial cells [Irie et al., 2005]. Loss of Akt1, specifically, led to an increase in the activation of Erk1 and Erk2, which was found to be required for the enhanced migration. Again, the mechanism by which Akt1, but not Akt2, inhibits Erk signaling in this system remains unknown. Interestingly, mouse tumor models have also suggested that Akt1 inhibits metastases [Hutchinson et al., 2004], whereas Akt2 promotes metastases [Arboleda et al., 2003]. However, these differential effects of Akt1 and Akt2 on epithelial cell migration may not translate to other cell types. In fact, studies on cell migration using mouse embryonic fibroblasts deficient of specific Akt isoforms have suggested opposite effects on fibroblast migration, with Akt1 promoting migration and with Akt2 inhibiting it

[Zhou et al., 2006]. These studies demonstrate both the importance of crosstalk between the PI3K-Akt pathway and other pathways and the emerging recognition that the three isoforms of Akt can have distinct cellular functions.

1.4.2. Vinculin and cytoskeleton protein Actin in cell migration

Vinculin is a ubiquitously expressed actin-binding protein. It used as a marker for both cell–cell and cell–extracellular matrix (focal adhesion) adherens-type junctions, but its function has remained elusive. A variety of phenotypes of Vinculin-null cells have been shown that the role for vinculin include cell adhesion, cell spreading, focal adhesion stability and strengthening, cell migration and resistance to apoptosis. Vinculin regulate the focal adhesion dynamics, and that transient increases in local phosphoinositide levels. This effect inhibits the vinculin–F-actin interaction, promote focal adhesion turnover and cell motility. Interestingly, the muscle-specific splice variant of vinculin called metavinculin (which contains a 68 amino acid insert in the Vt domain), is localized in dense plaques and costameres, cell–extracellular matrix junctions that are much longer lived than focal adhesions. It could be significant that the Vt/D5 domain of metavinculin interacts less strongly with acidic phospholipids than does the Vt/D5 domain of vinculin [S. Witt et al., 2004]. The association of metavinculin–vinculin heterodimers with F-actin might therefore be relatively resistant to phospholipid competition. That association shows more persistent adhesions. There is much recent interest in the finding that the globular head region of vinculin (Vh) can participate in an intramolecular interaction with the extended vinculin tail (Vt). This interaction masks the binding sites for talin and α-actinin in Vh, F-actin in Vt, and VASP in the proline-rich region between Vh and Vt. All this data has been reported that the cryptic actin-binding site in the vinculin tail is exposed by PIP_2. Interestingly, a talin-related peptide is shown to change the vinculin head–tail interaction, unmasking the actin-binding site. [P.A. Steimle, et al. 1999]. The paxillin-binding site in Vt is apparently not subject to such regulation [A.P. Gilmore and K. Burridge. 1996]. The interaction between Vh and Vt is relieved by acidic phospholipids [P.A. Steimle, et al. 1999, A.P.

Gilmore and K. Burridge. 1996, J. Weekes, et al.1996 and S. Huttelmaier, et al.1998], which bind to Vt [R.P. Johnson, et al.1998], exposing the talin-, α-actinin and VASP-binding sites and a cryptic protein kinase C (PKC) phosphorylation site [J. Weekes, et al.1996, J. Weekes, et al. 1996] in Vt. Whether the actin-binding site(s) in Vt is similarly exposed by acidic phospholipids, as proposed originally [A.P. Gilmore and K. Burridge. 1996 and J. Weekes, et al.1996], is contestable, and some study provides strong evidence that PIP2 inhibits the interaction of Vt with F-actin in vitro [P.A. Steimle, et al. 1999].

Vinculin can be tyrosine phosphorylated and some of the phosphorylation is Src kinases dependent. [Zhiyong Zhang, et al. 2004] The tyrosine phosphorylation sites in vinculin were reported to residues 100, 822and 1065. Tyrosine residue 1065 was phosphorylated by c-Src in vitro, but residue 100 was not phosphorylated by c-Src in vitro, raising the possibility that the phosphorylation of vinculin may be regulated by two, or more, distinct kinases. [Zhiyong Zhang, et al. 2004] Following phosphorylation, vinculin tail showed significantly less binding to the vinculin head domain than the unphosphorylated tail. Some studies have shown that vinculin tail domain modulates the interaction between paxillin and FAK, highlighting one mechanism by which a change in the tail conformation may affect cellular responses. [Subauste MC, et al. 2004] Vinculin phosphorylation residue 822 has been reported to affect interactions with paxillin, which may be a valuable route to change cell motility and survival.

Actin polymerization and adhesion formation are linked. Actin polymerization determines the rate of adhesion assembly and potentially nucleates adhesions that contain activated integrins; conversely, adhesions provide nucleation points that may support actin polymerization. Adhesions and actin are also physically linked and this linkage coordinates adhesion assembly and disassembly and the processes they regulate. Adhesion assembly requires actin polymerization suggesting that the interaction of a subset of adhesion components with actin nucleates the nascent adhesion, which is then stabilized by its association with integrins. The direct interaction of focal adhesion kinase (FAK) and vinculin with the Arp2/3 complex [DeMali et al., 2002; Serrels et al., 2007], the main

nucleator of actin branching and polymerization in lamellipodia, constitutes a possible mechanism for targeting vinculin and FAK to future adhesion sites. The presence of activated integrins in regions of protrusion outside adhesions suggests that they enter the forming adhesion in an activated state [Galbraith et al., 2007; Kiosses et al., 2001]. The other implication is that adhesions might nucleate actin polymerization. This would provide a mechanism for the formation of actin filaments on which adhesions elongate; these appear to elongate from nascent adhesions at the lamellipodium-lamellum interface. This possibility is supported by the observation that purified integrin-adhesion complexes have actin-polymerization activity [Butler et al., 2006]. Although the neutralization of Arp2/3 in β3-integrin-containing adhesion complexes did not impair actin polymerization, targeting of the formin mDia did [Butler et al., 2006].

The organization and dynamics of the actin cytoskeleton are regulated by membrane phosphoinositides at several levels. First, many actin-binding proteins directly interact with phosphoinositides, which regulate the activity and/or subcellular localization of these proteins. Among different PIs, PIP_2 is the best-characterized regulator of the actin cytoskeleton. PIP_2 interacts directly with several actin-binding proteins and regulates their activities [Hilpela P, et al.2004, Sechi AS, Wehland J. 2000, Sheetz MP, et al. 2006, Yamaguchi H, et al. 2009]. Typically, PIP_2 inhibits those actin-binding proteins that promote actin filament disassembly and activates proteins that induce actin filament assembly. Second, phosphoinositides control the subcellular localization of larger scaffolding proteins that are involved in the interplay between the actin cytoskeleton and plasma membrane or intracellular membrane organelles. Finally, proteins controlling the activity of Rho family small GTPases are in many cases regulated by plasma membrane phosphoinositides. The RhoA GTPase has a pronounced role in the formation and regulation of focal adhesion complexes and contractile actomyosin bundles such as stress fibers [Pelham RJ, et al. 1994]. RhoA induces actin polymerization at focal adhesions by activating the Dia1 formin and inhibits actin filament disassembly by initiating a signaling cascade that leads to phosphorylation and subsequent inactivation of the ADF/cofilin family of actin filament severing/depolymerizing proteins through the action of LIM kinases [Hotulainen P, Lappalainen P. 2006, Mahaffy

RE, Pollard TD. 2008, Watanabe N, et al. 1999, Vardouli et al 2005]. Furthermore, RhoA promotes contractility by activating the myosin light-chain kinase through ROCK kinase [Totsukawa G, et al. 2000].

Focal complexes are regulated by signaling via Rac1 or cdc42 small GTPases and are marked by the early recruitment of vinculin [J.V. Small *et al. 2002* and C.D. Nobes and A. Hall. 1995]. Vinculin is a large protein that contains binding domains for multiple cytoskeletal proteins, including actin, α-actinin, talin, paxillin, VASP, ponsin, vinexin and protein kinase C (PKC) [D.R. Critchley. 2000 and B. Geiger *et al. 2001*]. Its head and tail regions physically interact in a resting state to mask most binding sites [D.R. Critchley. 2000]. The open, 'activated', conformation of vinculin is revealed by exposure to PIP2 and exposes all binding sites. Past studies have revealed that vinculin plays a central role in mechanical coupling of integrins to the cytoskeleton, as well as in the control of cytoskeletal mechanics, cell shape, and protrusion amplitude and cell motility. Vinculin binding to the arp2/3 complex might be but one way that the actin-nucleation machinery can be coupled to new sites of adhesion, and testing this hypothesis now presents cell biologists from different fields with a fascinating new challenge.

1.5 Cell and animal model of colorectal cancer

The Caco-2 cell line is an immortalized line of heterogeneous human epithelial colorectal adenocarcinoma cells, developed by the Sloan-Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh. It has been extensively used over the last twenty years as a model of the intestinal barrier. When cultured as a monolayer, Caco-2 cells differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. In many respects, the Caco-2 cell monolayer mimics the human intestinal epithelium. [Richard B van Breemen , Yongmei Li. 2005]

Like Caco2 cell, HCT116 is another kind of human epithelial colorectal adenocarcinoma cells. It established from the primary colon carcinoma of an

adult man; cells were described to carry a RAS mutation in codon 13 and to be tumorgenic in nude mice.

IEC06 cells are derived from the rat small intestine and were produced from a single clone. They were originally described by Quaroni and colleagues as a homogenous population of epithelial-like cells. It has been shown with large, oval nuclei, growing as tight colonies of polygonal, closely opposed cells [Quaroni et al., 1979].

BALB/c mouse is an albino, laboratory-bred strain of the House Mouse. BALB/c mice are useful for research into both cancer and immunology. They are reported as having a low mammary tumor incidence. However, they can develop other types of cancers, most commonly reticular neoplasms, lung tumors, renal tumors and colon cancer.

ApcMin (Min, multiple intestinal neoplasias) is a point mutation in the murine homolog of the APC gene. APC is tumor suppressor gene, which involved in causing colorectal cancer. It is involved in both sporadic and familial forms of colorectal cancer. A mutation in the APC gene is the earliest detectable molecular abnormality in colorectal cancer. APCMin/+ mouse model carries defective APC, which results in the spontaneous development of colorectal tumors.

2. Aims of the studies

From the studies published so far, it became evident that functional mARs trigger potent antitumor effects in prostate and breast cancer cells. These findings imply a potential role of mAR as a novel target for the development of selective cancer treatments (reviewed in Papadopoulou et al., 2009). However, it remained elusive whether mARs are also expressed in other tumors and whether their stimulation could result in the induction of anti-tumor effects similar to the ones described in prostate and breast cancer cells. The aim of the present work was to study the expression of functional mARs in colon cancer tissue. The main goal of this work was to address the role of mAR activation toward major characteristics of tumor cells, namely cell survival and cell migration. Because mAR activation has been shown to promote strong apoptotic regression (Kampa M, et al.2002, Gu S, et al.2009), we analyzed the functionality of the prosurvival PI-3K/Akt pathway. In addition, because this signaling pathway also plays a major role in the invasive potential of tumor cells, we further studied the migration potential upon mAR activation. Our findings indicate that prosurvival signaling prevails in colon tumor cells but is strongly downregulated upon mAR stimulation *in vitro* and in colon tumor tissues isolated from BALB/c and APCMin/+ mice following treatment with mAR agonists. Furthermore, mAR activation blocked migration and invasiveness of colon tumor cells, mainly recruiting the adhesion-and actin cytoskeleton–regulator vinculin. This work provides novel mechanistic insights into the regulation of the proapoptotic and antimigratory mAR effects in colon tumors.

3. Materials and methods

3.1 Materials

3.1.1. Chemical and biological reagents

1,2-dimethylhydrazine (DMH; Sigma-Aldrich, St.Louis, USA)
Annexin V-FITC (BD Biosciences,USA)
anti-actin antibody (Cell signaling, Beverly, US)
anti-tubulin antibody (Cell signaling, Beverly, US)
ApoAlert® Caspase Colorimetric Assay kit (Clontech, USA)
APOPercentage Apoptosis Assay kit (Biocolor,Carrickfergus, UK)
aprotinin (Sigma-Aldrich, St. Louis, US)
APS (Merck, Darmstadt, Germany)
Biomax x-flim (Kodak, Rochester, US)
BSA (Roth, Karlsruhe, Germany)
Developing solution (Kodak, Rochester, US)
Dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, US)
DMEM medium (Gibco, Carlsbad, US)
DRAQ-5 dye (Biostatus, Leicestershire, UK)
ECL detection reagent (Amersham, Louisville, UK)
EGTA (Sigma-Aldrich, St. Louis, US)
estradiol (Sigma-Aldrich, St. Louis, US)
fetal bovine serum (Gibco, Carlsbad, US)
filter paper (Whatman, Maidstone, UK)
FITC-conjugated goat anti-rabbit IgG(Molecular Probes, Eugene, US)
fixing solution (Kodak, Rochester, US)
formaldehyde (Roth, Karlsruhe, Germany)
goat anti rabbit IgG antibody (Amersham, Louisville, UK)
goat serum (Invitrogen, Carlsbad, US)
gold antifade reagent (Invitrogen, Carlsbad, US)
HSA-FITC(Sigma-Aldrich, St. Louis, US)
in *situ* Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland)

methanol (Roth, Karlsruhe, Germany)
NaCl (Roth, Karlsruhe, Germany)
NaF (Sigma-Aldrich, St. Louis, US)
nonfat milk (Roth, Karlsruhe, Germany)
Paraformaldehyde (Roth, Karlsruhe, Germany)
PBS tablet (Gibco, Carlsbad, US)
PCA (Sigma-Aldrich, St. Louis, US)
phalloidin (Sigma-Aldrich, St. Louis, US)
PMSF (Sigma-Aldrich, St. Louis, US)
PVDF blot membrane (Millipore, Billerica, US)
rhodamine-phalloidin (Molecular Probes, Eugene, OR)
SDS loading buffer (Roth, Karlsruhe, Germany)
sodium citrate (Roth, Karlsruhe, Germany)
sodium orthovanadate (Roth, Karlsruhe, Germany)
standard pelleted food (C1310, Altromin, Heidenau, Germany)
sucrose (Sigma-Aldrich, St. Louis, US)
synthetic dextran sulfate sodium (DSS; Wako Pure Chemical Industries, Led. Japan)
TEMED (Roth, Karlsruhe, Germany)
testosterone-HSA ((Sigma-Aldrich, St. Louis, US))
Testosterone-HSA-FITC(Sigma-Aldrich, St. Louis, US)
Tris (Roth, Karlsruhe, Germany)
Tris-HCl (Roth, Karlsruhe, Germany)
Triton X-100 (Roth, Karlsruhe, Germany)
TWEEN-20 (Roth, Karlsruhe, Germany)

3.1.2. Equipment

-20°C refrigerator (Liebherr, Lindau, Germany)
4°C refrigerator (Heraeus, Massachusets, US)
-80°C refrigerator (Sanyo, Osaka, Japan)
Balence (Sartorius, Goetingen, Germany)
Biorad ChemiDoc XRS (Biorad, Hercules, US)
Cell culture hood (Thermo, Waltham, US)
Cell incubator (Heraeus, Massachusets, US)
Centrifuge 22R (Heraeus, Waltham, US)
Centrifuge 5417 R (Eppendorf, Hamburg, Germany)
Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany)
Cyrostat (Thermo, Waltham, US)
Electrophoresis cell (Biorad, Hercules, US)
Electrophoresis power supply (Biorad, Hercules, US)
Electrophoretic transfer cell (Biorad, Hercules, US)
Folie bag sealer (Roth, Karlsruhe, Germany)
Heator (Schutron, Pocklington, UK)
Magnetic stirrer (Roth, Karlsruhe, Germany)
Pippets (Abimed, Langenfeld, Germany)
Shaker (Roth, Karlsruhe, Germany)
Spectronic GENESYS 6 UV-Vis Spectrophotometer (Thermo, Waltham, US)
Vortex (Peqlab,Erlangen, Germany)
Waterbath (Labortechnik, Seelbach, Germany)

3.2 Methods

3.2.1. Cell culture

The Caco2 human colon cancer cell lines and IEC06 non transformed intestinal cells were obtained from the American Type Culture Collection (Manassas, VA) and were studied between passages 60 and 70. CACO2 at 20,000/ml were cultured in DEME medium supplemented with 20% fetal bovine serum in culture flasks in a CO_2 incubator at 37°C. Based on previous titration experiments [Gu et al., 2009] we have used throughout this study a 10-7 M testosterone-HAS concentration for mAR stimulation.

3.2.2. Preparation of steroid solution

Before each experiment testosterone-3- (O-carboxymethyl) oxime-Human Serum Albumin, referred to as testosterone-HSA (or Testo-HSA), DHT and estradiol, were dissolved in serum-free culture medium at a final concentration of 10^{-5} M. This stock solution was incubated for 30 min at room temperature with 0.3% charcoal and 0.03% dextran, centrifuged at 3000 x g and passed through a 0.45 µm filter to remove any potential contamination with free steroid. Testosterone-HSA, estradiol and DHT solutions were used at a final concentration of 10^{-7} M throughout all studies. If not otherwise stated all treatments and incubations with steroids including apoptosis assays were performed in serum-containing medium. Testosterone-HSA-FITC or control HSA-FITC constructs were generated by conjugating Testosterone-HSA or HSA with FITC using standard techniques.

3.2.3. *In vivo* animal experiment

Colon carcinoma was generated as described previously (Wang et al., 2004). In a first series of experiments, 7-week old Balb/c mice (both male and female) were divided into two groups, A (n=5) and B (n=7). Both groups

underwent carcinogenic treatment. At the age of 9 weeks animals were subjected to three cycles of alternating administration of distilled water containing 30 g/L synthetic dextran sulfate sodium (DSS; molecular mass 5000 Da; Wako Pure Chemical Industries, Led. Japan) for 7 days followed by distilled water for subsequent 14 days after intraperitoneal pretreatment with 20 mg/kg 1, 2-dimethylhydrazine (DMH; Sigma-Aldrich Corp. St.Louis.MO.USA). Group B mice received in addition to the carcinogenic treatment 5 mg/kg testosterone-HSA subcutaneously injected three times per week throughout the study period. All mice were sacrificed at the age of 20 weeks. After death, the entire colorectum from the colorectal junction to the anal verge was examined. Fresh specimens were placed in liquid nitrogen and subsequently stored at -80°C for further analysis. Then, the colon was opened longitudinally, washed with PBS, and divided into three portions (proximal, middle and distal). After macroscopic inspection the colon was fixed in a 40% g/L formaldehyde buffer solution (pH.7.4).

In APC mice, animal experiments were carried out in mice of either sex with mutated apc resulting in spontaneous colon tumor development (APCMin/+) obtained from the Jackson Laboratory (USA). The animals were housed under controlled environmental conditions (22-24°C, 50-70% humidity and a 12-h light/dark cycle). Throughout the study the mice had free access to standard pelleted food (C1000, Altromin, Lage, Germany) and tap water. All animal experiments were conducted according to the German law for the care and welfare of animals and were approved by local authorities.

The animals were divided into two groups. Group A, (n = 6) received treatment of 5 mg/kg subcutaneous TAC injection three times per week for 8 weeks. In the control group B (n=4) similar doses of normal saline were given. At the end of 8 weeks all animals were anesthetized with ether and sacrificed. After death, the entire colorectum from the colorectal junction to the anal verge was examined. Then, the colon was opened longitudinally, washed with PBS, and divided into three portions (proximal, middle and distal). Tumors were counted with a dissecting microscope at x3 magnification. After inspection the colon was fixed in a 40% g/L formaldehyde buffer solution

(pH.7.4).

3.2.4. Immunofluorescence analysis and confocal laser scanning microscopy

For testosterone-HSA-FITC staining, 5-µm-thick frozen tissue sections from the Balb/c or APC mouse tumors were fixed with 4% PFA for 15 min and incubated with 5% BSA/1x PBS/0.3% Triton for 1 hour at room temperature. After two washes with PBS 1.5% FBS specimens were exposed to testosterone-HSA-FITC (10-7 M, Sigma) for 1h at room temperature. Nuclei were stained with DRAQ-5 dye (1:1000, Biostatus, Leicestershire, UK) for 10 min at room temperature.

For direct fluorescence microscopy of F-actin, cells were fixed with 3 % paraformaldehyde in PBS for 30 min, permeabilized with 0.5 % Triton X-100 in PBS (10 min) and incubated with rhodamine-phalloidin (Molecular Probes, Eugene, OR, 1:100 dilution) for 40 min in the dark. For indirect immunofluorescence staining, cells were incubated for 2h at room temperature with mouse monoclonal anti-tubulin (Cell signaling, 1: 1000 dilution). Secondary FITC-conjugated rabbit anti-mouse IgG (Invitrogen) was used in a 1: 200 dilution. Nuclei were stained with DRAQ5™ (Biostatus Limited). Slides were mounted using the ProLang® Gold Antifade reagent (Invitrogen).

To quantify the expression of phosphorylated Akt and Bad, 5-µm-thick frozen tissue sections from the APC mice colon tumors were fixed with 4% PFA for 15 min at room temperature. After washing twice with PBS the slides were incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature. Then, the specimens were exposed overnight at 4°C to phospho-Akt (Thr308) (1:800, Cell Signaling, USA) or phospho-Bad (Ser136) (1:100, Santa Cruz Biotechnology, CA). The slides were rinsed three times with PBS and incubated for 1.5 h at room temperature with secondary FITC goat anti-rabbit antibody (1:500, Invitrogen, UK). After three washing steps the

nuclei were stained for 10 min at room temperature with DRAQ-5 dye (1:1000, Biostatus, Leicestershire, UK).

To determine the phosphorylation of Vinculin, cells were cultured on glass cover slips with testosterone-HSA or control without testosterone-HSA for different time periods indicated in the figure legends. After washing twice with PBS, cells were incubated with 4% PFA for 15 min and then incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature. Then, the cells were exposed to anti-vinculin antibodies (1:400, Gene Tex, USA) at 4°C overnight. The cells were rinsed three times with PBS and incubated with secondary FITC goat anti-rabbit antibody (1:500, Invitrogen, UK) or goat anti-mouse antibody (1:500, Invitrogen, UK) for 1.5 h at room temperature. For F-actin staining, cells were incubated with rhodamine-phalloidin (1:100, Molecular Probes, Eugene, OR) for 40 min in the dark. After three washing steps the nuclei were stained with DRAQ-5 dye (1:1000, Biostatus, Leicestershire, UK) for 10 min at room temperature. All the slides and coverslips were mounted with ProLong Gold antifade reagent (Invitrogen).

All Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 40_/1.3 NA DIC. Images were analyzed with the instrument's software.

3.2.5. Immunoprecipitation and Westen blotting

Cells were incubated with 10-7 M testosterone-HSA for the indicated time periods, washed twice with ice cold PBS and suspended in 500 µl ice-cold lysis buffer (50mM Tris/HCl, 1% TritonX-100 pH 7.4, 1% sodium deoxycholate, 0.1% SDS, 0.15% NaCl, 1 mM EDTA, 1 mM sodium orthovanadate) containing protease inhibitor cocktail (Sigma). The protein concentration was determined using the Bradford assay (BioRed). Sixty µg of protein were solubilized in sample buffer at 95ºC for 5 min and resolved by 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a PVDF

membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 1 h. Then, the membrane was incubated with phospho-Akt (Thr308), phospho-PI-3K p85 (Tyr458)/p55 (Tyr199) (1:1000, Cell Signaling, USA), or phospho-Bad (Ser136) (1:100, Santa Cruz Biotechnology, CA) at 4°C overnight. After washing (PBST) and subsequent blocking the blot was incubated with secondary anti rabbit antibody (1:2000, Cell Signaling, USA) or anti-mouse antibody (1:5000, GE Healthy, USA) for 1 h at room temperature. After washing, antibody binding was detected with the ECL detection reagent (Amersham, Germany). For controls the blots were stripped in stripping buffer (Carl Roth, Germany) at 56°C for 30 min. After washing with PBST blots were blocked with TBST + 5% milk for 1 h at room temperature. Then, they were incubated with anti-Akt, or anti-Bad (1:100, Santa Cruz Biotechnology, CA) antibodies at 4°C overnight. After washing with PBST and incubation with anti-rabbit antibody (1:2000, Cell Signaling, USA), antibody-binding was detected and quantified with Quantity One Software (Biorad, Germany).

For immunoprecipitation, equal amounts of protein (500 µg) in the presence or absence of 50µM genistein were subjected to immunoprecipitation with a monoclonal anti-phosphotyrosine antibody (7 µg/500 µg total protein, Santa Cruz Biotechnology, CA). After incubation with the antibody for 1h at 4°C, 50µl of the homogeneous protein A suspension was added into the mixture and incubated overnight at 4°C on a rocking platform. After three washing steps, samples were resuspended in SDS sample buffer, subjected to SDS electrophoresis and transferred to nitrocellulose membrane. Proteins were incubated with vinculin monoclonal antibody (1:100, Santa Cruz Biotechnology, CA) followed by the appropriate anti-mouse antibody (1:5000, GE Healthy, USA). Detection of protein bands was succeeded with ECL kit. Bands were quantified with Quantity One Software (Biorad, Germany).

3.2.6. Measurement of G/total actin ratio by Triton X-100 fractionation

For measurements of the monomeric (Triton soluble) and polymerized (Triton

insoluble) actin, Caco2 cells were incubated for different time point with or without or testosterone-BSA (10.7 M). Then, 500 µl of Triton-extraction buffer (0.3% TritonX-100; 5 mM Tris, pH 7.4; 2 mM EGTA; 300 mM sucrose; 2 µM phalloidin; 1 mM PMSF; 10 µg/ml leupeptin; 20 µg/ml aprotinin; 1 mM sodium orthovanadate; and 50 mM NaF) were added, and the mixture was incubated for 5 min on ice. After removing the buffer, soluble proteins were precipitated with equal volumes of 6% PCA. The Triton-insoluble fraction remaining on the plate was precipitated with 1 ml of 3% PCA. Equal volumes of each fraction were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resulting protein-bands were transferred onto nitrocellulose membrane, and the membrane was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris, pH 7.6; 137 mM NaCl; 0.05% Tween-20) for 1 h at room temperature. Antibody solutions (in TBS-T) were added for 1 h at room temperature rabbit anti-actin first antibody and second horseradish peroxidase-coupled antibody (Chemicon, Temecula, CA)]. Blots were developed by using the ECL system, and the band intensities were quantitated by PC-based image analysis.

3.2.7. Matrigel and transwell assay

Matrigel assays were performed using BD BioCoat™ BD MatrigelTM Invasion Chambers (BD Bioscience, USA). Matrigel was placed in each insert with 8.0 µm pore size in a 24-well plate. The chamber was allowed to polymerize at 37°C for 1 h. The inserts were then washed with serum-free DMEM. 100 µl of complete cell culture medium with 1 × 105cells was then seeded onto the insert. Five hundred µl of complete cell culture medium with 10-7 M testosterone-HSA or testosterone-BSA (Sigma) in the presence or absence of 10-6 M cytochalasin B (Sigma) was added into the well below the insert. In control experiments cells were pre-incubated with 10-7 M testosterone albumin conjugates for 2 h. Then, TAC was washed out with complete cell culture medium and five hundred µl were added into the well below the insert. After a 24 h incubation, the insert was wiped with a wet cotton swab. The lower surface was gently rinsed with PBS, the cells were fixed and stained with DAPI for 10 minutes, rinsed again with sterile water and allowed to dry. After removing the membranes from the inserts, they were mounted with

ProLong Gold antifade reagent (Invitrogen). To determine the total number of migrating cells, the slices were viewed and imaged under the microscope, and the number of cells/field in 10 random fields was counted. Experiments were performed in triplicates.

The transwell assay was performed using transwell inserts (BD Bioscience, USA). The inserts were then washed with serum-free DMEM, 100 μl of complete cell culture medium with 1 × 105 cells were seeded onto the insert. 500 μl of complete cell culture medium with 10-7 M testosterone-HSA or testosterone-BSA (Sigma) in the presence or absence of 10-6 M cytochalasin B (Sigma) 10-6 M anastrozole (Sigma), 10-6 M flutamide (Sigma), 5*10-6 M PP2 (Sigma), or 5*10-6 M genistein (Sigma) was added into the well underside of the insert for 24 h at 37°C and 5% CO2. In control experiments cells were pre-incubated with 10-7 M testosterone albumin conjugates for 2 h. Then, TAC was washed out with complete cell culture medium and five hundred μl were added into the well below the insert. After 24 h incubation, cells were fixed, stained with DAPI for 10 min and microscopically analyzed as described above.

3.2.8. Wound healing assay

For the wound healing assay, confluent cells cultures were scraped with a pipette tip across a 24-well-plate. Following wounding, culture medium was replaced with fresh medium and cells were exposed to 10-7 M testosterone-HSA (Sigma) in the presence or absence of 10-6 M cytochalasin B (Sigma) for the indicated time points. It should be noted that the methodology for the wound healing assay requires that confluent cells are used, in order to provide the necessary cell layer to create wounding by scraping. On the other hand, subconfluent cells were used in matrigel and transwell assays (see below), to ensure correct evaluation of cell motility and invasiveness.

3.2.9. siRNA experiments

Caco2 cells were grown in DMEM medium containing 10% fetal calf serum

under standard culture conditions (37°C, 5% CO2). 4x104 cells were seeded in 24 well plates and cultivated with fresh culture medium for 8 h. The cells were subsequently transfected with validated siRNA for Vinculin (ID# s14764, Ambion, Darmstadt, Germany) or with a negative control siRNA using siPORT Amine (Ambion) transfection agent according to the manufacturer's protocol. The efficiency of silencing was checked by Western blot 72 h after transfection. Upon silencing, 37.6% of the vinculin protein was still detectable in cells treated with siRNA for Vinculin compared to cells treated with a negative control siRNA.

3.2.10. TUNEL assay

The colonic cancer tissue was cut to 8 µm frozen sections and subsequently fixed in 4 % paraformaldehyde for 30 min at room temperature. After rinsing with PBS the samples were permeabilized in a solution of 0.1 % Triton X-100 in sodium citrate for 2 min. Samples, washed with PBS, were then incubated in the TUNEL reaction mix for 1 h at 37oC, according to the manufacturer's instructions (Roche, Germany). Nuclei were stained with DRAQ5™ (Biostatus Limited). Sections were analyzed with a confocal laser scanning microscope (Carl Zeiss).

3.2.11. APOPercentage apoptosis assay

Caco2 cells were cultured in 96-well plates for the APOPercentage apoptosis assay (Biocolor Ltd., Belfast, Ireland). In the presence or absence of 10-6 M anastrozole (Sigma), they were stimulated or not with 10-6 M TAC for 24 hours in serum containing medium. Untreated cells cultured in serum free medium were used as positive control for the apoptotic response.30 mins before the incubation time is reached, add 5 µl APOPercentage Dye to the centre of the well. Incubate for the remaining 30 min of the assay. Then Syringe off the culture medium/dye mixture, and gently wash the cells twice with 200 µl/well PBS. For apoptosis quantitation, the amount of dye within the labeled cells can subsequently be released into solution, and the concentration is measured at a wavelength of 550 nm, using Spectronic

GENESYS 6 UV-Vis Spectrophotometer (Thermo, Waltham, US).

3.2.12. Statistical analysis

Data are provided as means ± SEM; n represents the number of independent experiments. Data were tested for significance using unpaired student's t-test when two-sample means were tested. Differences were considered statistically significant when p-values were < 0.05. All statistical analysis was performed with GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com.

4. RESULTS

4.1 mAR expression in colon cancer cell lines

In my diplom thisis, while analyzing in vivo mAR expression in paraffin blocks generated from xenograft tumor tissues of various origins, we have noticed significant mAR expression in colon cancer xenograft specimens. In line with these findings mAR expression was subsequently detected by confocal laser scanning microscopy using the fluorescent testosterone-HSA-FITC conjugate in cultured HCT116- (Figure5 e,f), or in Caco2-colon cells (Figure 5a,b) while HSA-FITC labeled CaCo2 or HCT116 cells showed no apparent staining (Figures 5 c, d, g, h). These results indicate that mAR in expressed in colon cancer cell lines. Interestingly, mAR staining could not be detected in the non-transformed intestinal cell line IEC06 (Fig. 6A). These staining experiments and the fact that testosterone-HSA-FITC is an impermeable conjugate disclosed mAR expression preferentially in colon cancer cell lines and tumors. In addition, mAR could be also detected in iAR silenced of Caco2 cells by using testosterone-HSA-FITC. These results imply that the molecular identity of mAR is probably not identical with iAR (Fig. 6B)

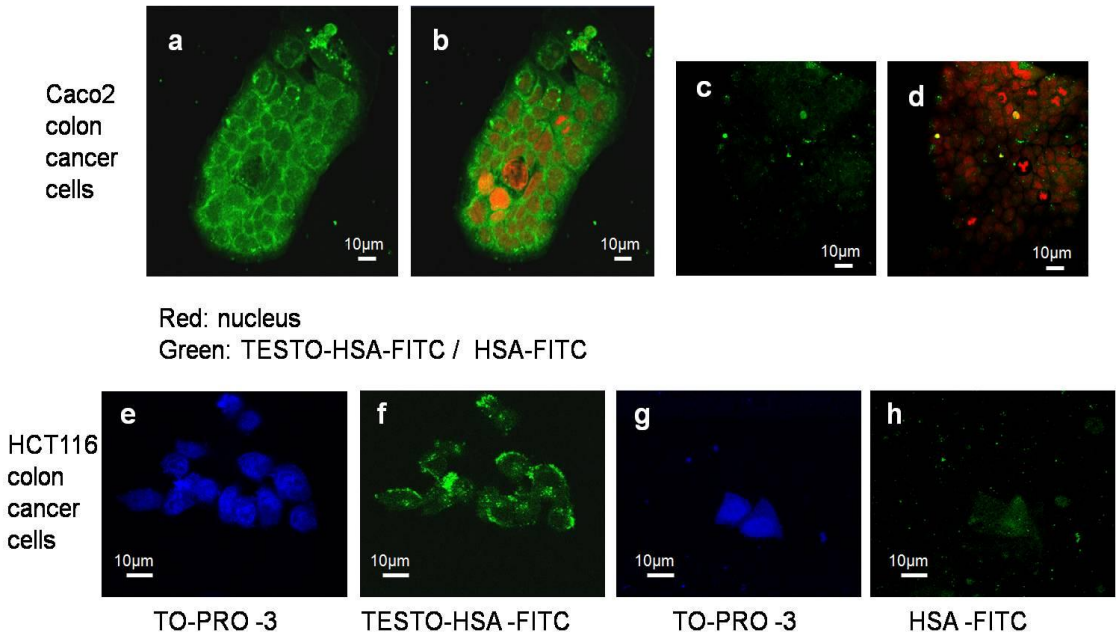
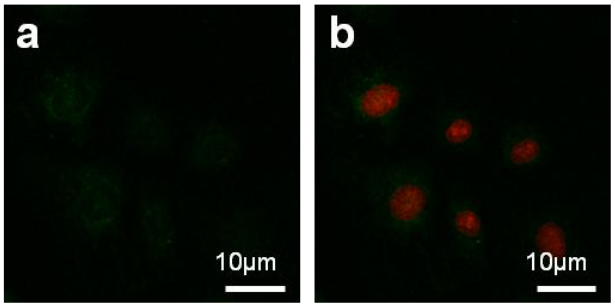


Figure 5 Membrane staining of mAR in Caco2 and HCT 116 colon cancer cells

Confocal laser scanning microscopic analysis of Caco2 cells (a-d) and HCT 116 cells(e-h) stained with testosterone-HSA-FITC, showing specific FITC related fluorescence at the cell membranes, or HSA-FITC, showing no apparent membrane staining. Visualization of nuclei was evident by DRAQ5™ or TO-PRO-3 staining. Magnification, ×100.

A



B

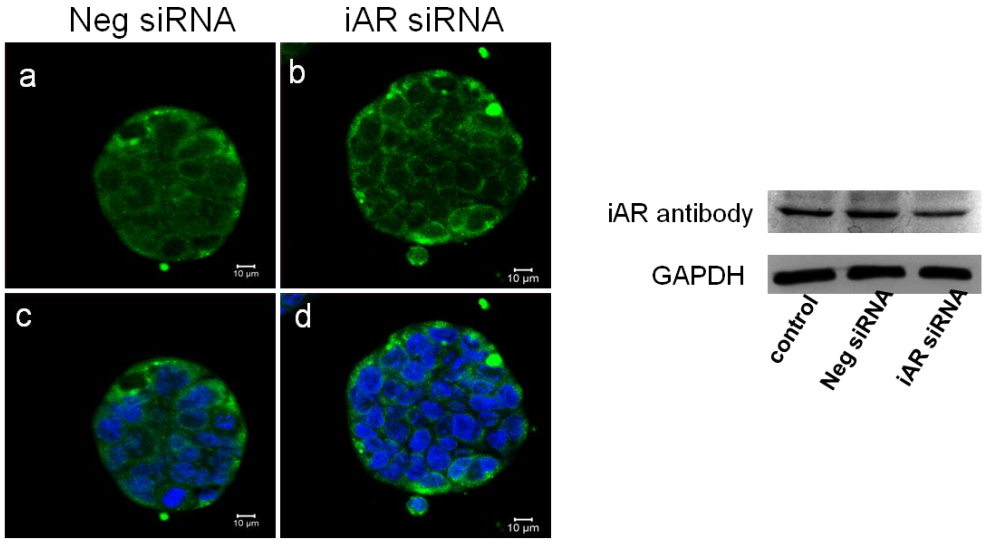


Figure 6 Membrane staining of mAR in IEC 06 cells and iAR silenced Caco2 cells.

A) Confocal laser scanning microscopic analysis of IEC 06 cells (a-b) stained with testosterone-HSA-FITC, showing no apparent membrane fluorescence at the cell membrane. Visualization of nuclei was evident by DRAQ5™ staining.

B) Confocal laser scanning microscopic analysis of iAR silenced cells (a-d) stained with testosterone-HSA-FITC, showing membrane fluorescence at the cell membrane. Visualization of nuclei was evident by DRAQ5™ staining.

4.2 mAR expression in 2 different colon cancer animal models

The findings provided so far indicate that mAR are expressed in colon cancer cell lines Caco2 and HCT-116 *in vitro*. [Gu S, et al. 2009] Thus, we aimed to further evaluate the *in vivo* effects of albumin-conjugated androgens in colon cancer animal models. To this end we first estimated the expression of mAR in colon tumors generated in Balb/c mice and APC mice. As shown in figure 7, using testosterone-HSA-FITC we detected specific, FITC-related fluorescence in membrane specimens of Balb/c mice colon tumors (Fig. 7 A, a, a1), and APC mice colon tumors (Fig.8). No apparent staining could be identified in tissues labeled with HSA-FITC (Fig. 7A, b) and no apparent staining could be found in healthy tissue labeled with testosterone-HSA-FITC (Fig. 7B).

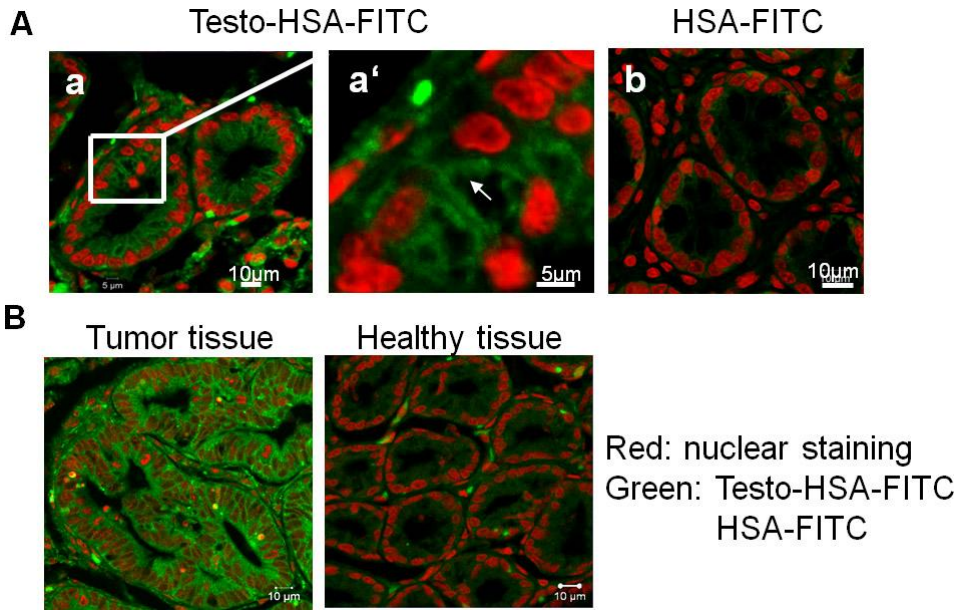


Figure 7: In vivo testosterone-HSA expression in BALB/c mice

A) Confocal laser scanning microscopic analysis of BALB/c colon tumor frozen sections stained with testosterone-HSA-FITC (a, a'), showing specific FITC related fluorescence at the cell membranes. No apparent membrane fluorescence was shown in control samples stained with HSA-FITC (b).

B) Confocal laser scanning microscopic analysis of BALB/c colon tumor and healthy frozen sections stained with testosterone-HSA-FITC, showing specific FITC related fluorescence at the cell membranes of tumor sections.

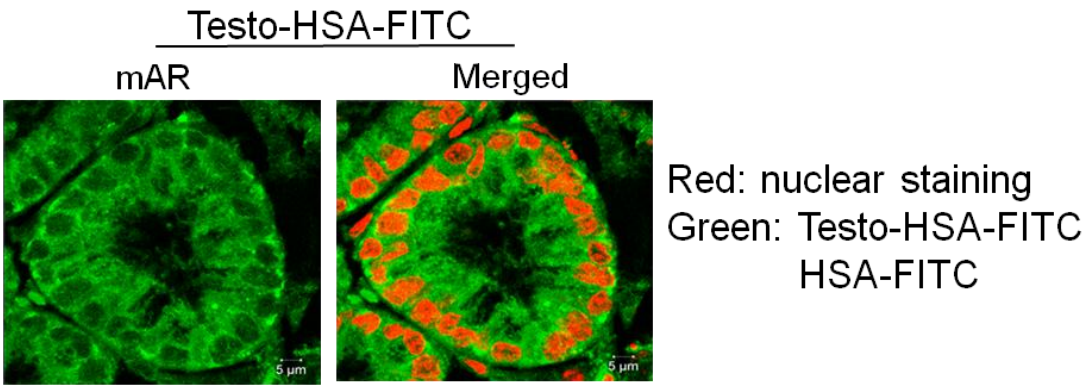


Figure 8: In vivo testosterone-HSA expression in APC mice

Confocal laser scanning microscopic analysis of APCMin/+ colon tumor frozen sections stained with testosterone-HSA-FITC, showing specific FITC-related fluorescence at the cell membranes. Visualization of nuclei was evident by DRAQ5™ staining. Magnification, ×100.

4.3 mAR activation by testosterone-HSA was followed by extensive reduction of tumor incidence *in vivo*

Having a clear indication for mAR-expression, the 12-week tumor incidence of colon tumors generated in Balb/c mice was assessed by chemical carcinogenesis (see Experimental Procedures) in the presence or absence of continuous testosterone-HSA treatment. The animals used for these studies were divided in two groups comprising 5 and 7 animals. One group (7 animals) was treated subcutaneously (3 times/week, for 12 weeks) with 5mg/kg testosterone-HSA, whereas the other group (5 animals) remained untreated. The results (Figure 9, A) show that testosterone-HSA-treatment produced a clear and significant reduction of tumor incidence by 65%. The histological analysis of tumors by Tunel assay (Figure 9, B) confirmed that apoptotic cells were present in significant numbers predominantly at the tumors of animals treated with testosterone-HSA, while they were significantly less in the non-treated animals. These results collectively show that mAR is a functional target that may be used for the selective elimination of colon cancer cells *in vivo*.

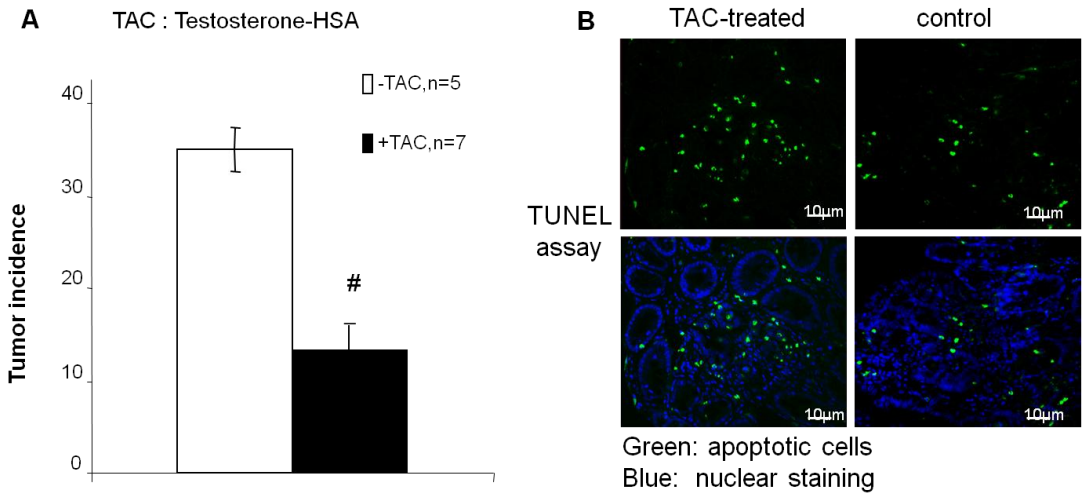


Figure 9: **In vivo testosterone-HSA effects on tumor incidence in BALB/c mice**

A) Arithmetic means ± SEM of colonic tumor incidence in BALB/c mice. Following treatment with the carcinogenic drug 1,2 dimethylhydrozine followed by dextrane sodium sulphate, one group (7 animals) was treated subcutaneously (3 times/week for 12 weeks) with 5mg/kg testosterone-HAS (black bar), whereas the other group (5 animals) remained untreated (white bar). # indicates significant difference between both groups (# P<0.01).

B) After treatment, the colonic cancer tissue was cut to 8 µm frozen sections and fragmented DNA was assessed using TUNEL assay according to the manufacturer's instructions. Confocal laser scanning microscopy analyzed samples. Magnification, ×100.

To further establish the *in vivo* role of mAR activation in mice model, in a second series of experiments APC mice have been used. In these experiments, animals were divided in two groups comprising 6 and 4 animals. One group (6 animals) was treated subcutaneously (3 times/week, for 8 weeks) with 5mg/kg testosterone-HSA, whereas the other group (4 animals) remained untreated. As shown in Fig. 10A, testosterone-HSA treatment resulted in a significant reduction of the tumor incidence by 80%. The histological analysis of tumors by TUNEL assay confirmed that apoptotic cells were present in appreciable numbers predominantly in the tumors of animals treated with testosterone-HSA (Fig. 10B, left panels) whereas they were significantly less abundant in the non-treated animals (Fig. 10B, right panels).

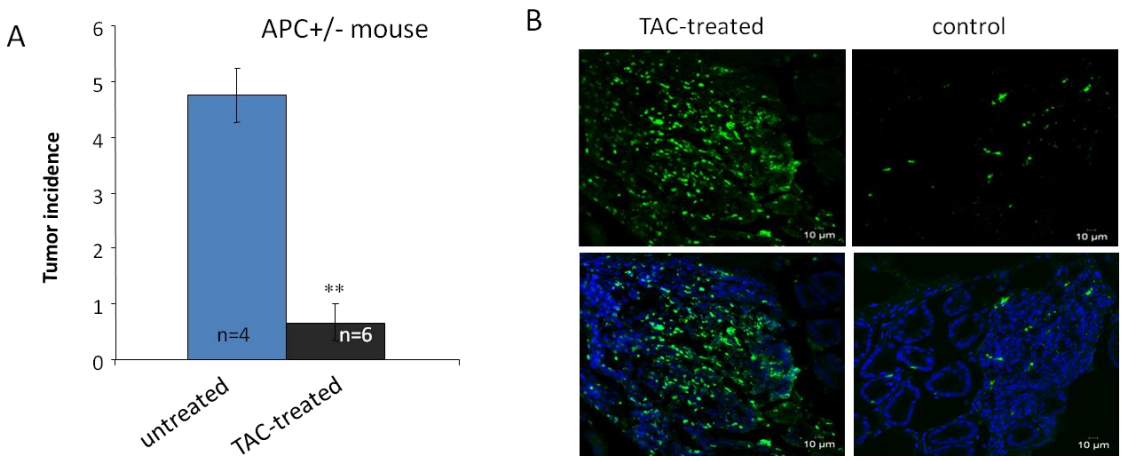


Figure 10: ***In vivo* testosterone-HSA effects on tumor incidence in** $APC^{Min/+}$ **mice**

A) Arithmetic means ± SEM of colonic tumor incidence in APC $^{Min/+}$ mice. The mice were either treated with 5 mg/kg testosterone-HSA subcutaneously 3 times/week for 8 weeks, (n=6 animals black bar) or treated with 5 mg/kg of normal saline subcutaneously 3 times/week for 8 weeks (n=4 animals white bar). # indicates significant difference between both groups (** P<0.01).

B) After treatment, the $APC^{Min/+}$ colonic cancer tissue was cut to 8 µm frozen sections, and fragmented DNA was assessed using TUNEL assay according to the manufacturer's instructions. Confocal laser scanning microscopy analyzed samples. Magnification, ×100.

4.4 p-Akt and p-Bad are downregulated in colon tumor tissues treated by testosterone-HSA

Activated Akt is a pro-survival factor controlling phosphorylation and activity of various pro-apoptotic gene products [Cardone MH, et al. 1998, Datta SR, et al. 1997]. Moreover, the role of this kinase in inactivating the pro-apoptotic function of Bad via phosphorylation is well-documented [Downward J. 1998, Vanhaesebroeck B, et al. 2000]. Finally, downregulation of Akt and Bad has been reported previously in mAR-stimulated prostate cancer cells (Papadopoulou et al Mol Canc 2008) implying that this prosurvival signaling may be down regulated by mAR. Accordingly, the activity of AKT and Bad was further analyzed in colon tumor tissues treated or not with testosterone-HSA. To address this, first I assessed the 8-week incidence of colon tumors

spontaneously developed in APC$^{Min/+}$ mice in the presence or absence of continuous testosterone-HSA treatment. In our experiments, animals were divided in two groups comprising 6 and 4 animals. One group (6 animals) was treated subcutaneously (3 times/week, for 8 weeks) with 5mg/kg testosterone-HSA, whereas the other group (4 animals) remained untreated. Immunohistochemical analysis with either anti p-Akt (Thr308) or anti p-Bad (Ser136) antibodies revealed strong expression of p-Akt and p-Bad in colonic tumor tissue from untreated mice and marked downregulation of both phosphorylated proteins in colon tumor tissues from testosterone-HSA treated animals (Fig. 11). These results clearly prove the anti-tumor potential of testosterone-albumin conjugates in colon cancer and corroborate previously reported data obtained in a chemically induced colon tumor model in Balb/c mice. Furthermore, our results identify Akt/Bad as downstream targets of mAR in colon cancer *in vivo*.

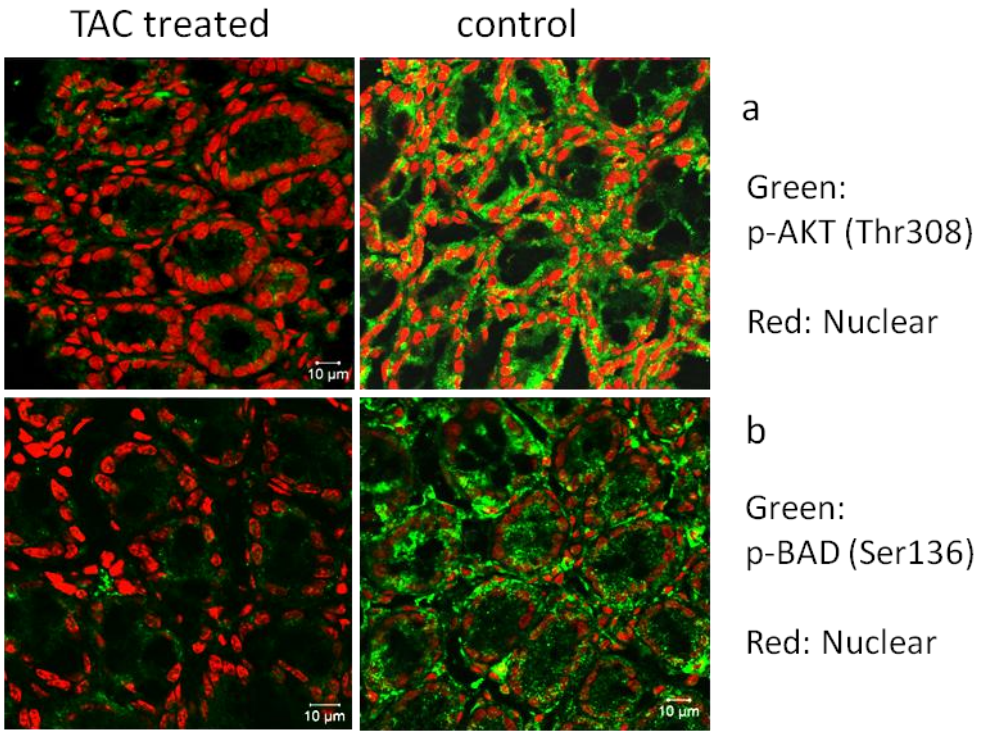


Figure 11: **In vivo testosterone-HSA stimulation inhibits Akt activity and induces Bad de-phosphorylation in APCMin/+ mice**

Confocal laser scanning microscopic analysis of TAC-treated and untreated APCMin/+ frozen colon tumor sections stained with (a) anti-phospho-Akt (Thr308) and (b) anti-phospho-Bad

(Ser136) antibodies. Anti-rabbit-FITC was used as secondary antibody and DRAQ5™ for nuclei staining. Magnification, ×100.

4.5 mAR stimulation inhibits Akt activity and induces Bad de-phosphorylation in Caco2 but not in IEC06 cells

Having established a role of AKT/Bad downstream of mAR activation in colon cancer tissues, we sought to determine the possible inactivation of p-Akt /p-Bad in colon cancer cells treated by testosterone-HSA. Consistent with its well documented oncogenic role in tumor cells, high basal levels of active, phosphorylated Akt (p-Akt) were detected in Caco2 colon tumor cells (Fig 12A). Stimulation of mARs with testosterone-albumin conjugates (TAC) induced a long-term and profound de-phosphorylation of this kinase that became evident 2 hours upon TAC treatment and sustained for at least 12 hours (Fig. 12A). Interestingly, in non transformed IEC06 intestinal cells that do not express mAR [Gu S, et al. 2009], p-Akt levels were very low and remained unchanged during TAC treatment (Figure 10C), indicating mAR specificity for the regulation of this pro-survival factor. Moreover, Figure 1B clearly shows that mAR stimulation by TAC resulted in de-phosphorylation/activation of Bad following kinetics similar to that of Akt, reaching minimum levels of phosphorylated Bad (p-Bad) after 12 h (Fig. 12B). In line with these findings, the Akt-upstream regulator PI-3K was de-phosphorylated upon long term TAC treatment (Fig 12D), implying that the pro-survival PI-3K/Akt signaling is downregulated in mAR activated Caco2 cells.

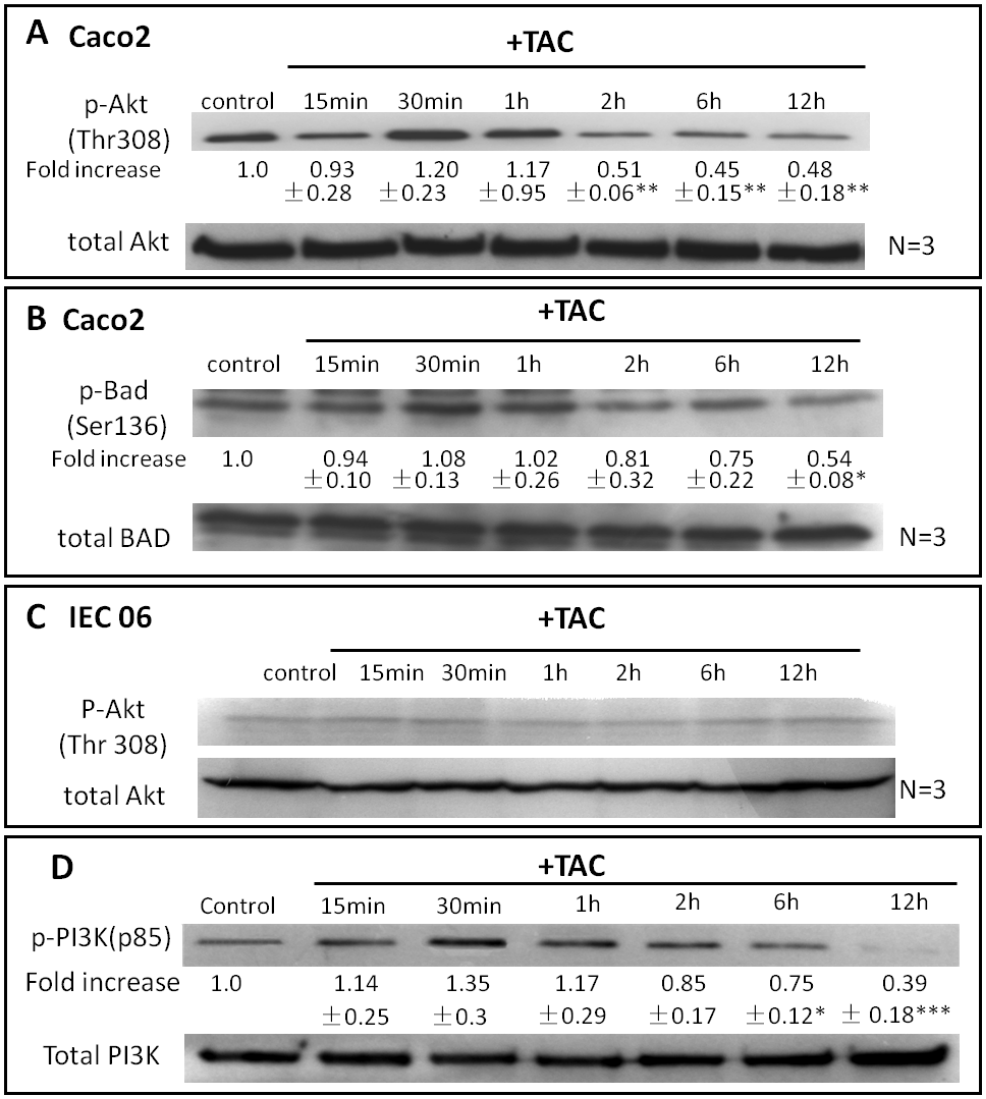


Figure 12**: De-phosphorylation/inactivation of Akt and Bad in Caco2 and IEC 06 cells.**
A) Caco2 cells were exposed to 10^{-7} M testosterone-HSA for the indicated time periods. The ratio of the cellular content of the phosphorylated residues (Thr 308) versus the total isoform of Akt was measured in cell lysates by Western blotting using specific antibodies for each form and was normalized to the corresponding control.

B) Caco2 cells were exposed to 10^{-7} M testosterone-HSA for the indicated time periods. The ratio of the cellular content of the phosphorylated residues (Ser 136) versus the total isoform of Bad was measured in cell lysates by Western blotting using specific antibodies for each form and was normalized to the corresponding control.

C) IEC 06 cells were exposed to 10^{-7} M testosterone-HSA for the indicated time periods. The ratio of the cellular content of the phosphorylated residues (Thr 308) versus the total isoform of Akt was measured in cell lysates by Western blotting using specific antibodies for each form and was normalized to the corresponding control.
D) Caco2 cells were exposed to 10^{-7} M testosterone-HSA for the indicated time periods. The

ratio of the cellular content of the phosphylated PI3K versus the total isoformof PI3K was measured in cell lysates by Western blotting using specific antibodies for each form and was normalized to the corresponding control.
Blots show a representative experiment, the numbers below each lane correspond to the mean values ± SE from three independent experiments (*P<0.05; **P<0.01***P<0.001) indicating the fold-decrease in the phosphorylation level for the indicated time point normalized to the controls.

4.6 mAR activation triggered rapid actin and tubulin reorganization in colon cancer cells

Cytoskeleton reorganization is a prominent early functional response of various cancer cells to steroid hormones targeting membrane binding sites [Koukouritaki et al., 1997, Kampa et al., 2002, Kampa et al., 2006, Papadopoulou et al., 2008a]. Accordingly, to analyze the functional impact of mAR in colon cancer rapid cytoskeleton modifications was investigated in Caco2 cells upon activation of mAR with testosterone-HSA for various time intervals. Cellular actin cytoskeleton dynamics were initially assessed by appropriate quantitative techniques as described in Papakonstanti et al., 2007. As shown in figure 13A, quantitative immunoblot analysis of Triton X-100 insoluble cytoskeletal pellets and corresponding supernatants revealed a significant decrease of the Triton-soluble (monomeric) to total actin ratio in Caco2 cells treated with 10^{-7} M testosterone-HAS, indicating actin polymerization. This effect was evident 15 min upon testosterone-HSA treatment; and returned to nearly control levels after 1-2 hours (Fig. 13A). The quantitative data were fully supported by confocal laser scanning microscopic analysis, showing redistribution of microfilamentous structures and formation of stress fibers and filopodia in testosterone-HSA treated cells (Fig. 13B).

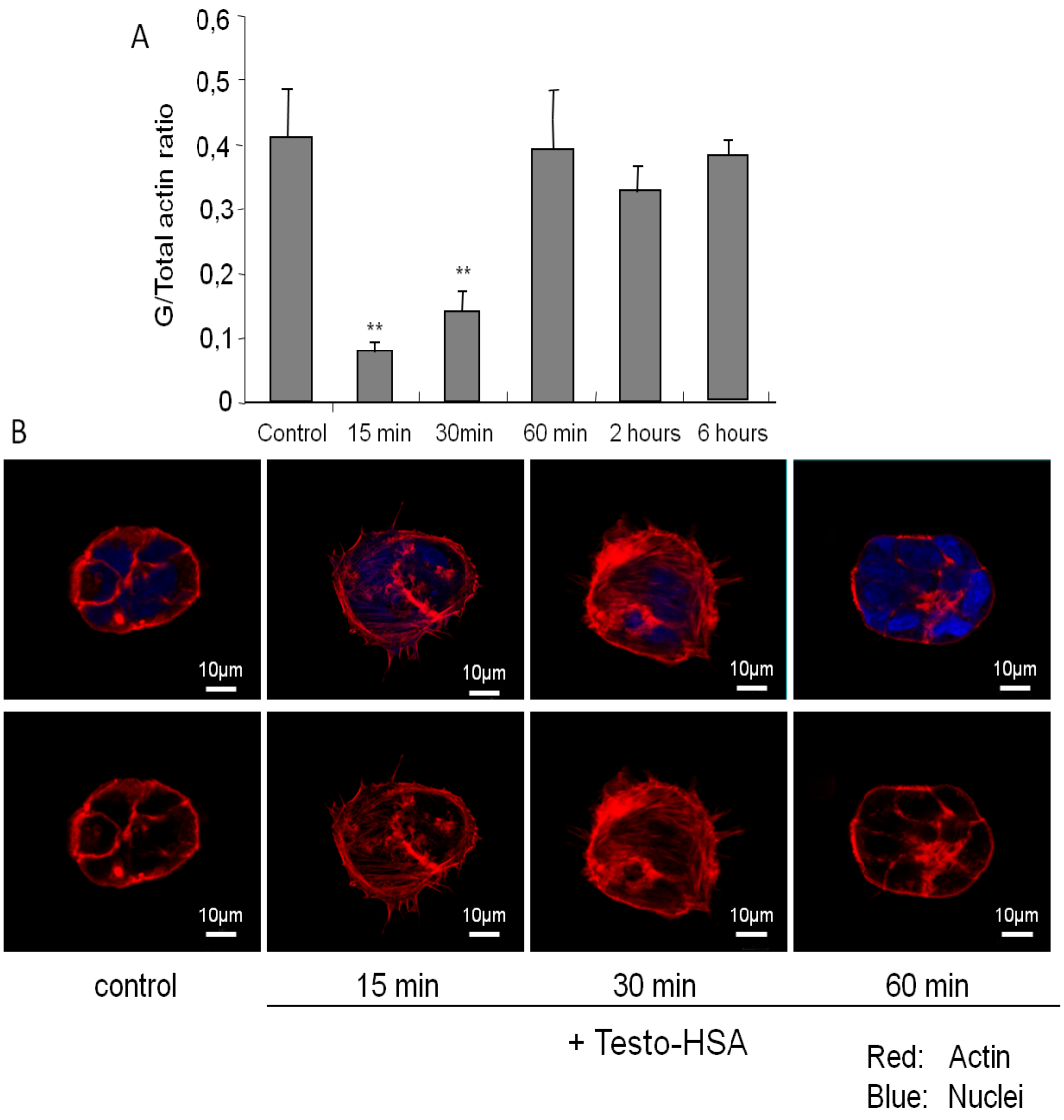


Figure 13: **Modulation of the dynamic equilibrium G- and Total actin in testosterone-HSA stimulated Caco2 cells.**

24h serum starved cells were stimulated with 10^{-7} M androgen conjugate for the indicated time points.
A) Total and G- actin were measured by quantitative immunoblot analysis after Triton X-100 subcellular fractionation. Bars present the G/Total actin mean value ± SE of four independent duplicate experiments (** $P < 0.01$).

B) Cells were stained with rhodamine-phalloidin for filamentous actin and DRAQ5™ for nuclei.

Tubulin cytoskeleton reorganization was further analyzed by confocal laser scanning microscopy. A clear redistribution of the microtubular network became evident in cells treated with 10^{-7} M testosterone-HSA for 15 to 60 minutes (Fig. 14).

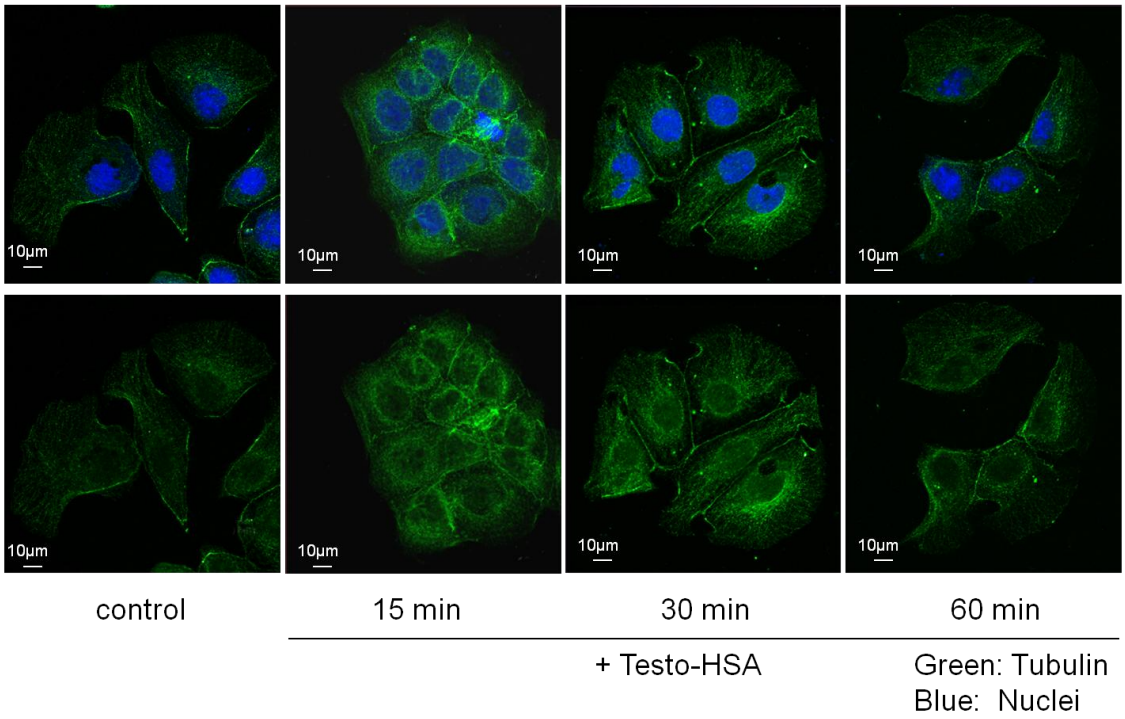
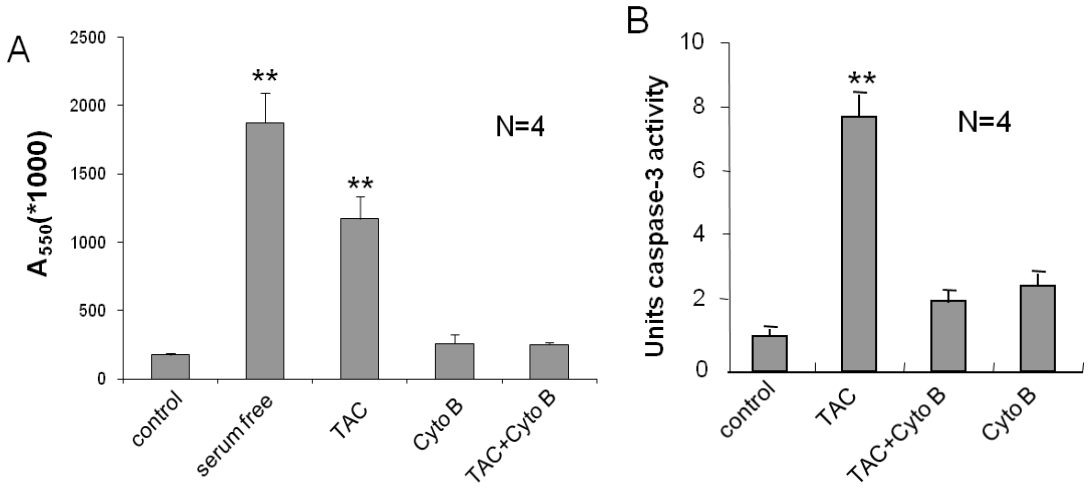


Figure 14: **Modulation of the dynamic equilibrium rapid tubulin reorganization in testosterone-HSA stimulated Caco2 cells.**

Caco2 cells treated or not with 10^{-7} M testosterone-HSA for different time points were cultured in coverslips, fixed and stained with rabbit anti-α-tubulin. Anti-rabbit-FITC was used as secondary antibody and DRAQ5™ for nuclei staining. Confocal laser scanning microscopy analyzed samples. Magnification, ×100.

Previously, it has been reported that activation of mAR with non permeable testosterone derivatives induced pro-apoptotic responses [Gu S Diploma Thesis, Gu S, et al. 2009]. However, the mechanism regulating the mAR-induced apoptotic responces is still unknown. In recent years, the cross-talk between actin cytoskeleton components and apoptotic signaling has attracted specific interest. Indeed, modifications of actin dynamics seem to be crucial for apoptotic responses [Gourlay CW, et al. 2005, Franklin-Tong VE,et al. 2008]. More recently the functional role of actin reorganization in regulating the pro-apoptotic responses induced by mAR was established in prostate cancer cells [Papadopoulou N, et al. 2008,] Based on these results we assessed the mAR-dependent apoptosis and caspase-3 activation in the presence of anti-actin drugs. As shown in Figures 15A,B, in Caco2 cells pre-

treated with cytochalasin B, at a concentration (10-7M) which blocks actin redistribution without exerting toxic effects [Stournaras et al 1996], the mAR-induced apoptotic response (Fig 15A) and caspase-3 activation (Fig 15B) were abolished. These results indicate that actin redistribution is a mandatory step for the apoptotic response of mAR-stimulated colon cancer cells.



TAC: Testosterone-HSA Cyto B: cytochalasin B

Figure 15: **Pro-apoptotic effects of testosterone-HSA, DHT and Estradiol in the absence or presence of inhibitors in Caco2 cells.**

A) Quantitative APOPercentage apoptosis assay of testosterone-HSA stimulated Caco2 cells and similar experiments in the presence of cytochalasin B (Cyto B). Cells were exposed to 10^{-7} M testosterone-HSA for 24 hours and proapoptoric responses were assessed by the APOPercentage apoptosis assay. Equally, cells pre-treated or not with 10^{-7} M Cyto B or flutamide, were exposed to testosterone-HSA for 24 hours. Cells serum starved for comparable periods of time served as a positive control for apoptosis. Bars present the mean OD measured at 550 nm. ** P<0, 01, n=4.

B) Cells were pre-treated or not with Cyto B for 1h and then exposed or not to 10^{-7} M testosterone-HSA for 4h, lysed and incubated with the caspase-3 substrate DEVD conjugated to the chromophore pNA according to the manufacturer's instructions. Caspase-3 activity was measured at 405 nm. ** P<0, 01, n=4.

4.6 mAR activation inhibits cell motility in colon cancer cells

We further examined whether mAR-stimulation regulates motility and invasiveness of colon cancer cells. To this end, we assessed the migration capacity of Caco2 cells treated or not with testosterone-HSA by using the matrigel-, transwell- and wound healing-assays, respectively. As shown in Figure 16, the matrigel assay revealed that cell invasiveness was reduced by 90% following testosterone-HSA-treatment of Caco2 cells. Very similar results were also obtained when cells were treated with testosterone-BSA, indicating that both testosterone albumin conjugates produce similar effects in the inhibition of cell motility. In line with this, cell motility assessed by the wound-healing assay confirmed the inhibition of the migratory capacity of colon cancer cells by mAR stimulation (Fig. 17A). The inhibition of the migratory potential of tumor cells by mAR stimulation was further corroborated by the transwell assay (Fig. 17B). Interestingly, in the presence of non toxic concentrations of cytochalasin B that block the well described mAR-induced actin reorganization the inhibition of cell motility was partially restored in all experimental procedures (Fig 16, 17).

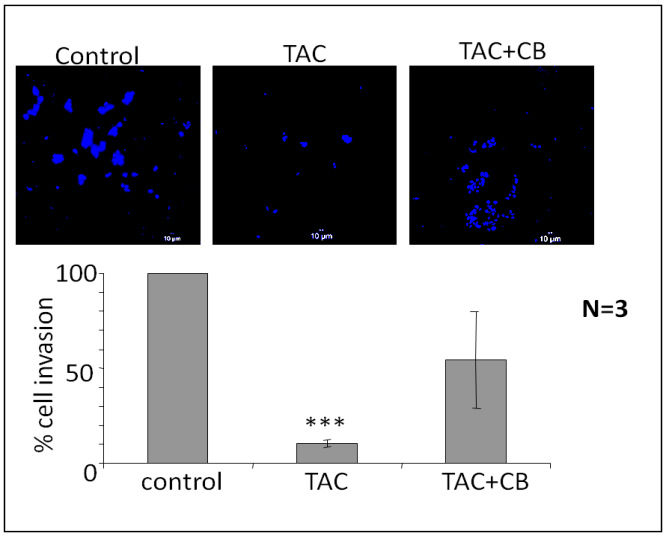


Figure 16: **Effect of testosterone-HSA on Caco2 human colon cancer cell invasion**

Cells were cultured with 10^{-7} M testosterone-HSA in the presence or absence of 10^{-6} M cytochalasin B on the Matrigel-coated upper compartment of Transwell culture chambers,

provided with an 8 μm pore size polycarbonate filter, according to the manufacturer's instructions. 24 h later, Matrigel was removed by scraping, and invaded cells, attached to the lower surface of the filter, were stained with DAPI. The slices were imaged under the microscope and the number of cells in 10 random fields was counted. Bars represent the % of cell invasion in control and treated cells. (n=3).

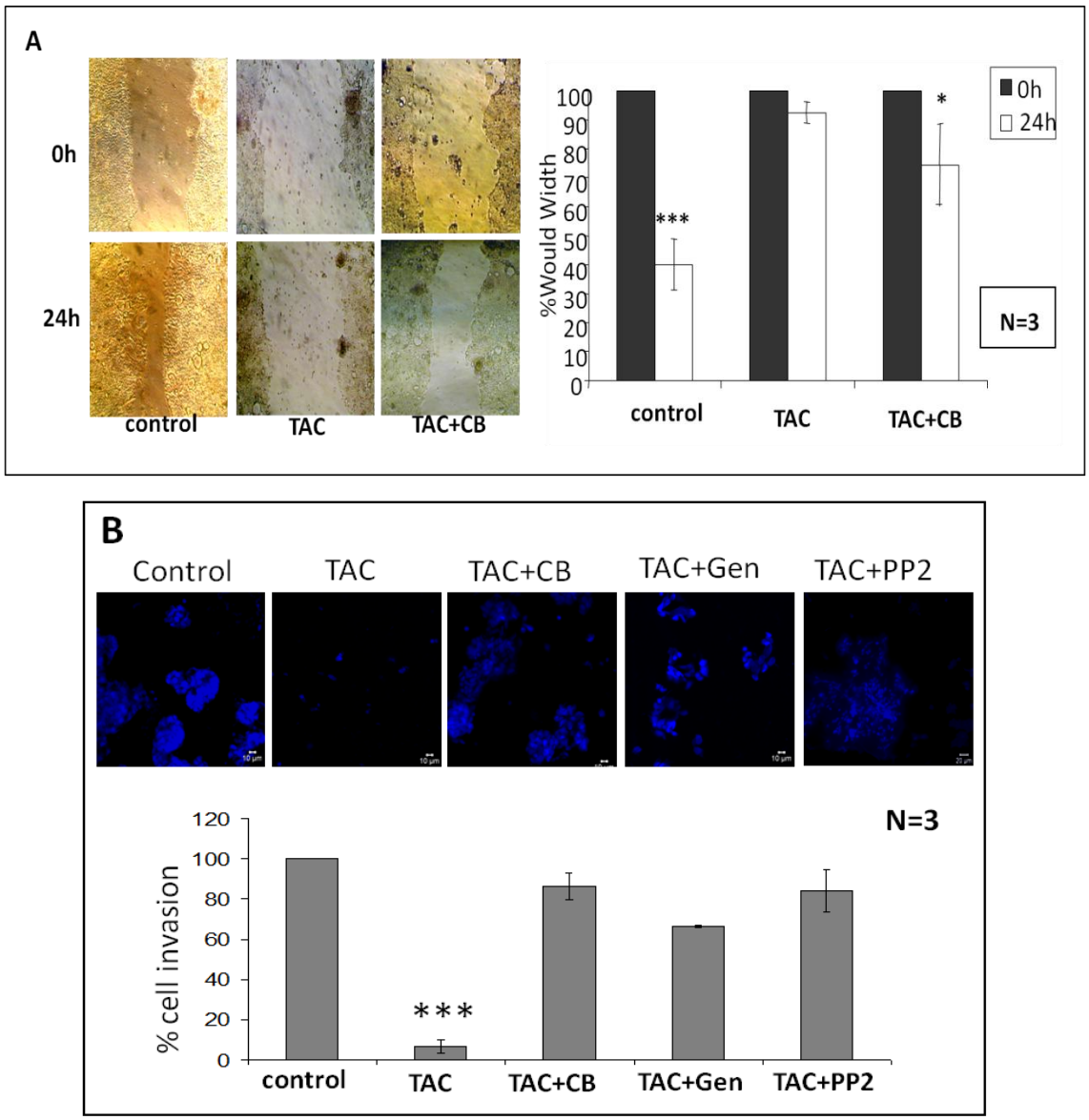


Figure 17: **Effect of testosterone-HSA on Caco2 human colon cancer cell migration**

A) Wound healing assay of Caco2 cells. Following 24 h culture the confluent monolayer was scratched with a pipette tip to create a cell-free area. Testosterone-HSA 10^{-7} M was added, and wound closure was documented by microphotography of the same region after 24 h. Bars represent the width of the wound in control and treated cells.

B) Cells in the presence or not of 50μM genistein and PP2 were cultured with 10^{-7} M

testosterone-HSA in the absence or presence of 10^{-6} M cytochalasin B on the Transwell culture chambers, provided with an 8 µm pore size polycarbonate filter, according to the manufacturer's instructions. 24 hours later, invaded cells, attached to the lower surface of the filter, were stained with DAPI. The slices were imaged under the microscope, and the number of cells in 10 random fields was counted. Bars represent the % of cell invasion in control and treated cells. (n=3), *P<0,05; ***P<0,001

Finally, to further confirm the androgen specificity of the mAR induced effects, we analyzed cell motility and the apoptotic response of TAC treated Caco2 cells in the presence of the aromatase inhibitor anastrozole. As shown in Figure 18 and 19, both, the migration potential and the apoptotic response could not be influenced by this inhibitor.

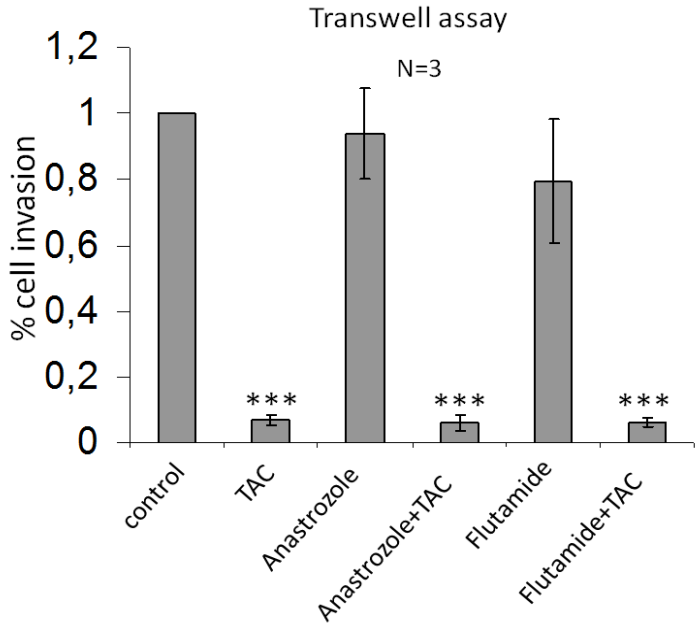


Figure 18: **Motility effects of testosterone-HSA in the absence or presence of inhibitors in Caco2 cells**

Cells in the presence or not of 10^{-6} M anastrozole or 10^{-6} M flutamide were cultured with 10^{-7} M testosterone-HSA on the Transwell culture chambers, provided with an 8 µm pore size polycarbonate filter, according to the manufacturer's instructions. 24 hours later, invaded cells, attached to the lower surface of the filter, were stained with DAPI. The slices were imaged under the microscope, and the number of cells in 10 random fields was counted. Bars represent the % of cell invasion in control and treated cells.

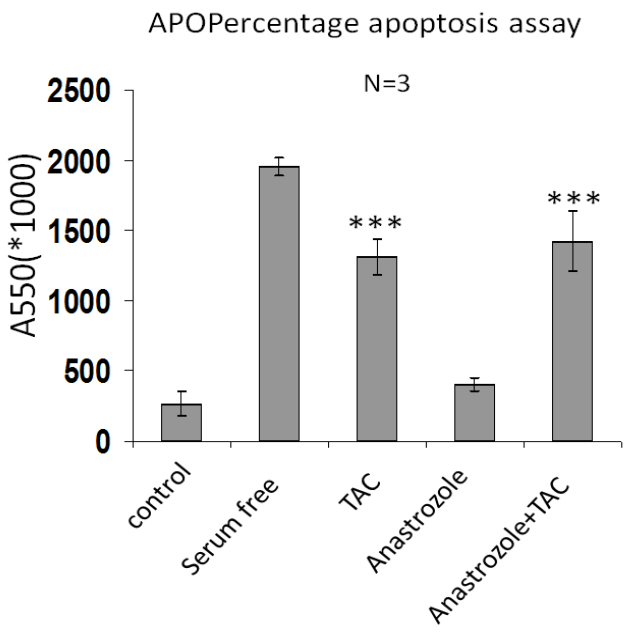


Figure 19: **Pro-apoptotic effects of testosterone-HSA in the absence or presence of inhibitors in Caco2 cells**

Quantitative APOPercentage apoptosis assay of TAC stimulated Caco2 cells and similar experiments in the presence of anastrozole. Cells were exposed or not to 10^{-7} M testosterone-HSA for 24 hours and proapoptoric responses were assessed by the APOPercentage apoptosis assay. Equally, cells pre-treated or not with 10^{-6} M anastrozole was exposed to testosterone-HSA for 24 hours. Cells serum starved for comparable periods of time served as a positive control for apoptosis. Bars present the mean OD measured at 550 nm. *** P<0,001, n=3.

4.7 mAR activation triggers vinculin phosphorylation

The findings presented in Figure 16 and 17 suggested that actin restructuring may control cell migration upon mAR activation in colon cancer cells. As tyrosine phosphorylation of specific proteins is linked to actin cytoskeleton dynamics and cellular motility [Wozniak MA, et al. 2009, Zhao J, et al. 2009], we sought to determine whether mAR effects on cell motility were evident in the presence of genistein and PP2 that represent widely used tyrosine phosphorylation inhibitors. Fig. 17B shows that this was indeed the case, as genistein and PP2 efficiently blocked mAR induced inhibition of cell migration. Notably, both inhibitors had no effect on its own in this assay. We subsequently checked for molecular targets potentially regulating these

effects. We focused on vinculin, an adhesion protein that participates in cell-cell adhesions and that was described to regulate migration and actin organization [Bailly M, et al.2003]. As shown in Fig 20A, mAR stimulation with testosterone-HSA disclosed a substantial increase in the phosphorylation of vinculin within 15 min remaining at least for 12 h. Confocal microscopy analysis fully confirmed the western blotting data. Indeed, immunostaining of testosterone-HSA-treated Caco2 cell preparations with anti-vinculin antibodies (Fig. 20B) revealed gradually increased vinculin spots observed at the focal adhesions. Triple immunostaining with anti-vinculin antibodies, rhodamine-phalloidin for F-actin staining and DRAQ-5 dye nuclear staining clearly documented the formation of actin filaments emanating from the vinculin spots of focal adhesions (Fig. 20B).

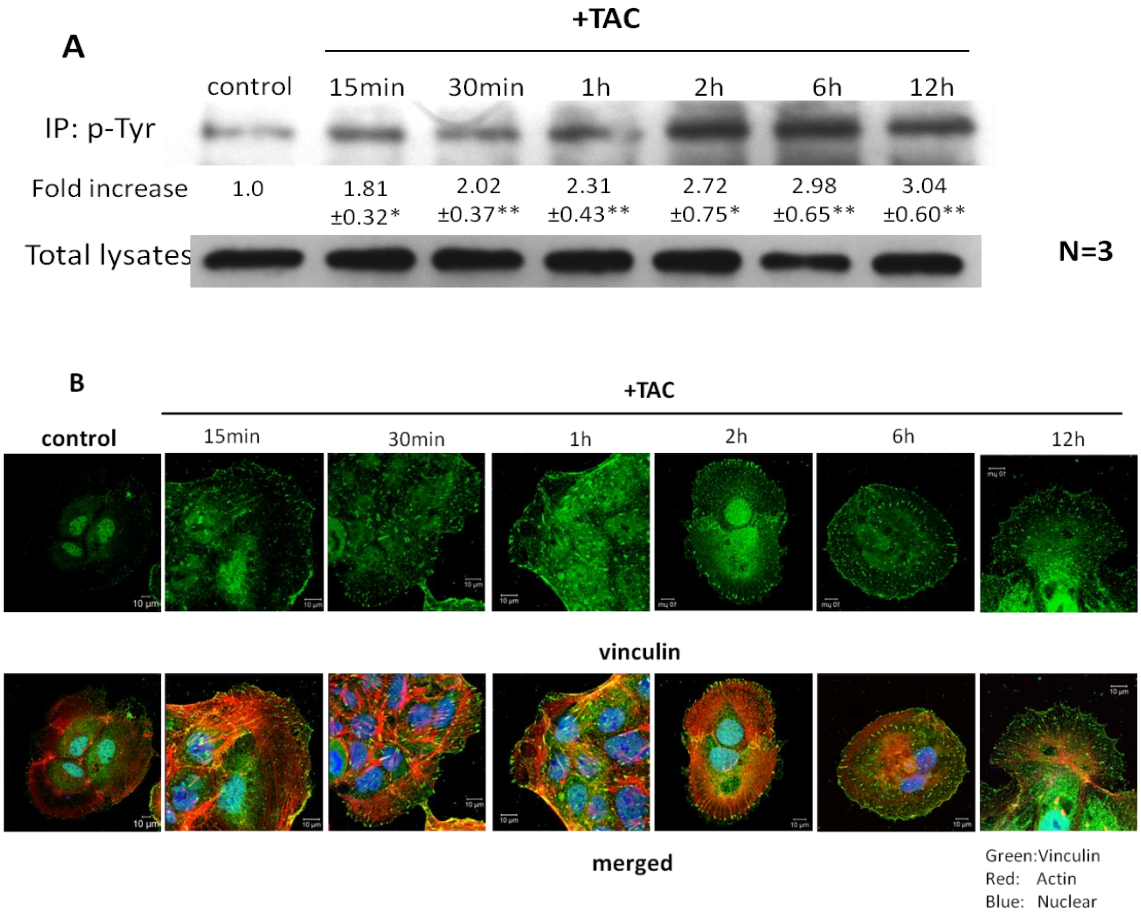


Figure 20: **Effects of testosterone-HSA on vinculin phosphorylation and on Caco2 cell morphology**

A) Caco2 cells were stimulated with 10^{-7} M testosterone-HSA for the indicated time periods. Following cell lysis equal amounts of proteins were immunoprecipitated (IP) with an anti-phosphotyrosine (p Tyr) antibody. The tyrosine-phosphorylated as well as equal amounts of

total lysates were immunoblotted (IB) with a specific antibody against vinculin. The immunoblots were analyzed by densitometry. The intensity of phosphorylated vinculin bands was normalized to the intensity of the corresponding total vinculin bands. Blots show a representative experiment, whereas the relative fold increase (mean values ± SE from three independent experiments) in vinculin phosphorylation with that of untreated cells taken as 1 are indicated. (**P<0, 01)

B) Confocal laser scanning microscopic analysis of vinculin and actin in mAR- activated Caco2 cells. Cells treated or not with 10^{-7} M testosterone-HSA for different time periods were cultured on coverslips, fixed and stained with mouse anti-vinculin, anti-mouse-FITC as secondary antibody, DRAQ5™ for nuclei staining and rhodamine-phalloidin for filamentous actin staining. Magnification, ×100.

Interestingly, in the presence of the tyrosine phosphorylation inhibitor genistein, vinculin phosphorylation was effectively blocked (Figure 21A). Moreover, confocal laser scanning analysis revealed that both the expression of the characteristic vinculin spots and the formation of actin filaments, shown in Fig. 20B, disappeared in mAR-stimulated Caco2 cells in the presence of genistein (Fig. 21B). These results, coupled with the observed blocking effect of genistein in mAR-induced cell motility of CaCo2 cells (Fig. 17B), provide indirect evidence that vinculin phosphorylation may participate in mAR signaling towards actin reorganization and cell motility regulation.

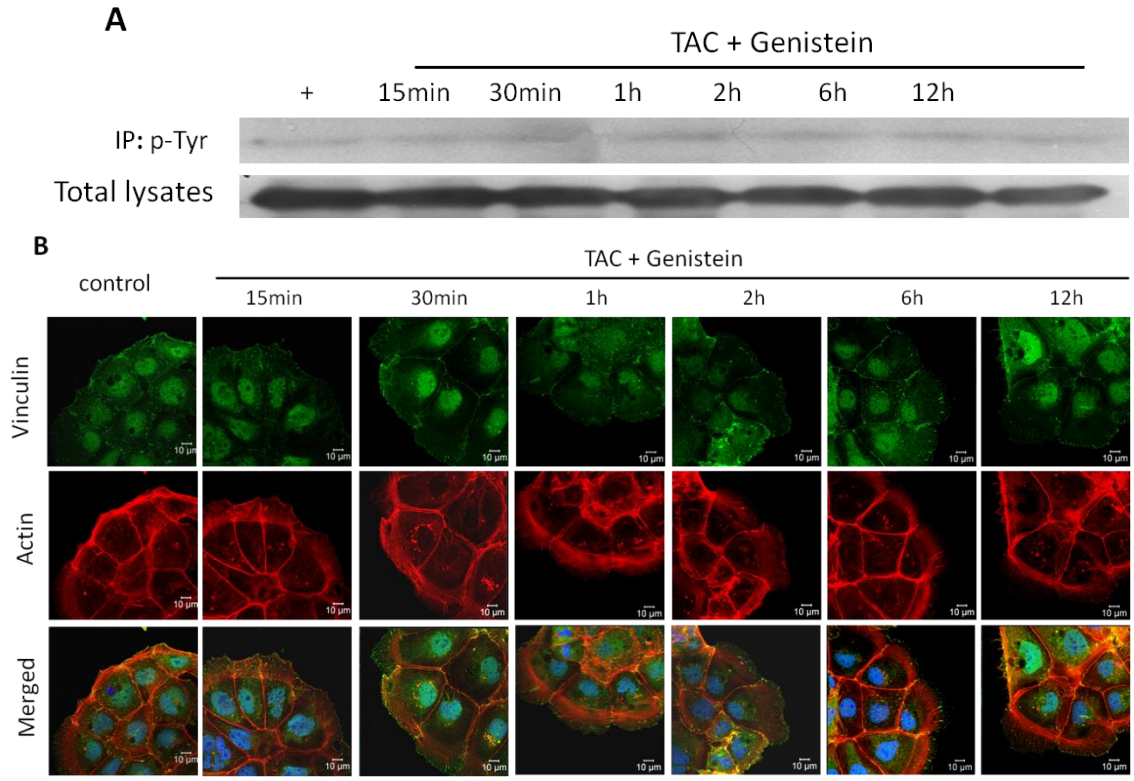


Figure 21: **Phosphorylation of Vinculin in TAC-treated Caco2 cells in the presence of genistein**

A) Caco2 cells in the presence of genistein were stimulated with 10^{-7} M Testosterone-HSA for the indicated time periods. Following cell lysis equal amounts of proteins were immunoprecipitated (IP) with an anti-phosphotyrosine (p-Tyr) antibody. The tyrosine-phosphorylated as well as equal amounts of total lysates were immunoblotted (IB) with a specific antibody against the vinculin. The intensity of phosphorylated vinculin bands was normalized to the intensity of the corresponding total vinculin bands.

B) Confocal laser scanning microscopic analysis of vinculin and actin in mAR-activated Caco2 cells in the presence of genistein. Cells treated with 10^{-7} M testosterone-HSA for different time periods were cultured in coverslips, fixed and stained with mouse anti-vinculin, anti-mouse-FITC as secondary antibody, DRAQ5™ for nuclei staining and rhodamine-phalloidin for filamentous actin staining. Magnification, ×100.

4.8 Vinculin is necessary for actin reorganization and migration of mAR stimulated Caco2 cells

To provide direct and specific experimental evidence for the regulatory action of vinculin in mAR-stimulated Caco2 cells, we designed short interfering

RNAs (siRNAs) that could induce down-regulation of endogenous vinculin. The siRNA was capable of inducing knockdown of endogenous vinculin to almost 70% of the control levels when introduced into Caco2 cells by transient transfection (Fig. 22A). The effect of the siRNA was specific, as an unrelated siRNA had no apparent effect on the protein levels of vinculin (Fig. 22A). Analysis of vinculin and actin cytoskeleton of cells under such conditions of siRNA transient transfection showed a dramatic inhibitory effect on the ability of testosterone-HSA to affect vinculin morphology and actin reorganization (Fig. 22B). In contrast, the control siRNA did not interfere with the actin and vinculin responses to testosterone-HSA.

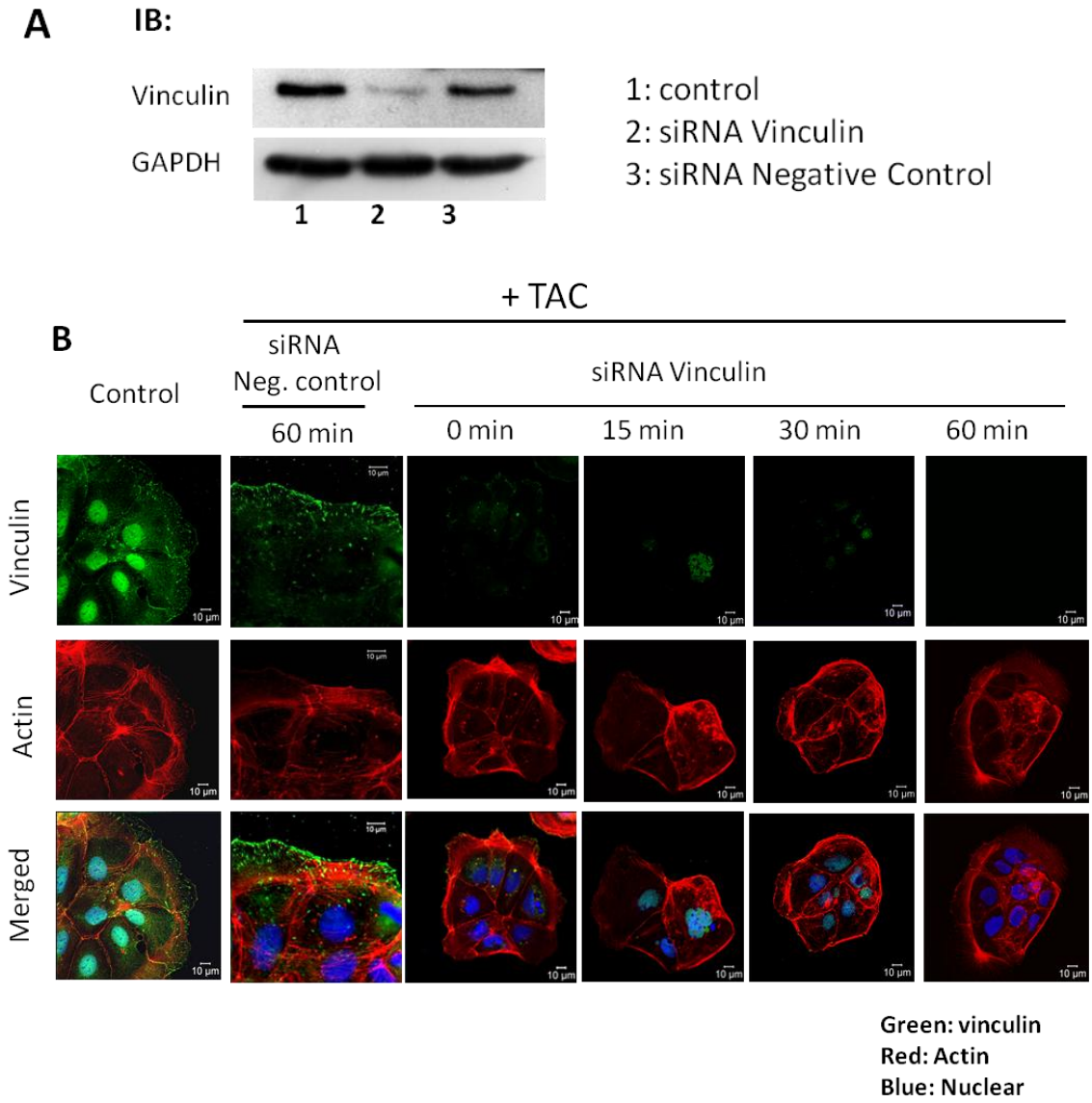


Figure 22: **Effect of testosterone-HSA on Caco2 cells silenced with vinculin siRNA**

A) Caco2 cells transfected with vinculin siRNA or a negative control siRNA were lysed, and

equal amounts of total lysates were immunoblotted (IB) with a specific antibody against vinculin and GAPDH. The immunoblots were analyzed by densitometry.

B) Confocal laser scanning microscopic analysis of vinculin and actin in mAR-activated Caco2 cells silenced either with vinculin siRNA or a negative control siRNA. Transfected cells treated with 10^{-7} M testosterone-HSA for different time periods were cultured in coverslips, fixed and stained with mouse anti-vinculin, anti-mouse-FITC as secondary antibody, DRAQ5™ for nuclei staining and rhodamine-phalloidin for filamentous actin. Magnification, ×100.

Vinculin was shown to be important in regulating adhesion dynamics and cell migration [Huynh N, et al. 2010], and it was postulated that vinculin may connect early adhesion sites to the actin-driven protrusive machinery [Zhao J, et al. 2009]. In addition, the relative decrease of endogenous vinculin levels led to a significant restoration of the migratory capacity of Caco2 cells upon mAR activation, as indicated by both the matrigel- and transwell-assays (Fig 23).

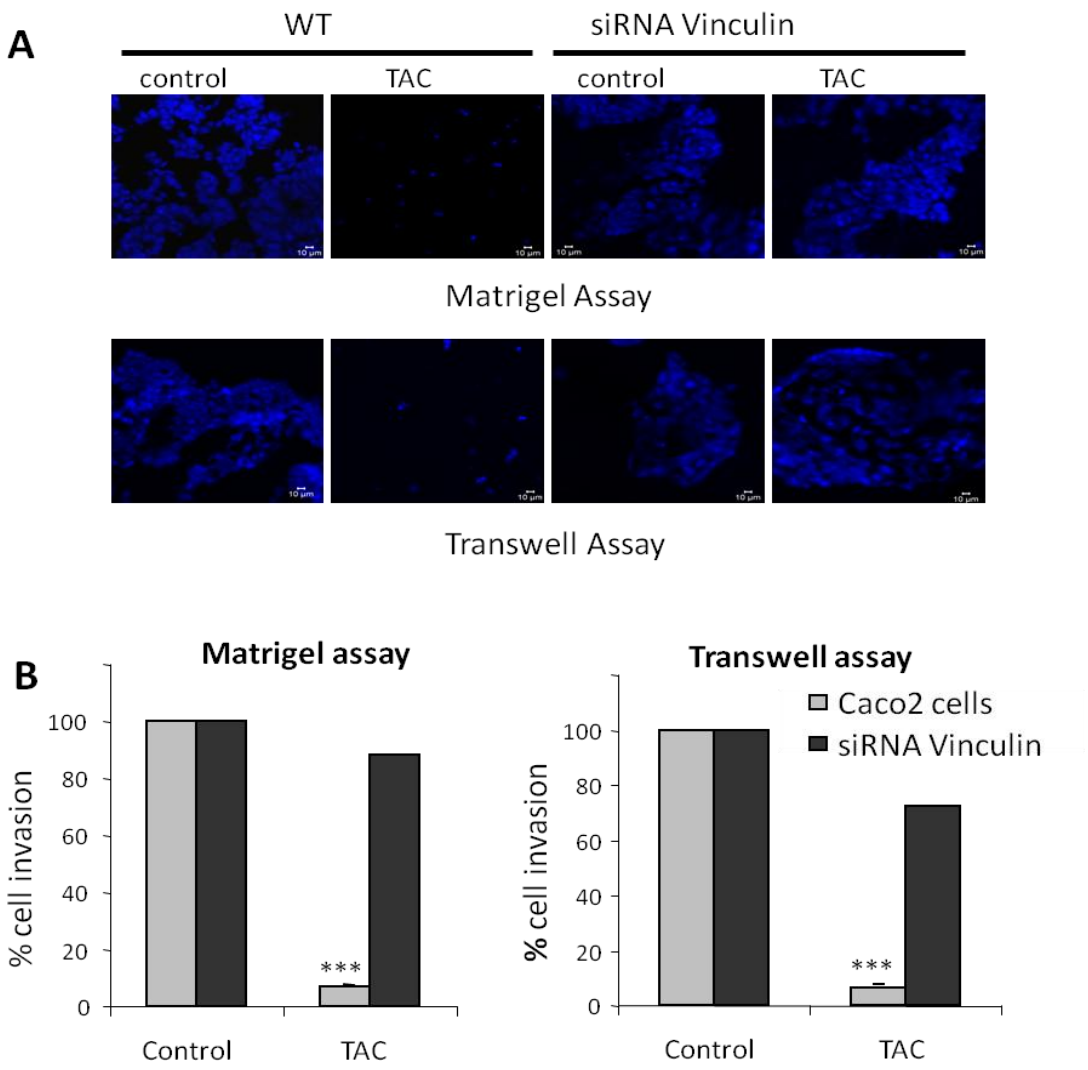


Figure 23: **Effect of testosterone-HSA on Caco2 cells silenced with vinculin siRNA**

A) Representative motility experiment of Caco2 cells transfected or not with vinculin siRNA. Cells were cultured in the presence of 10^{-7} M testosterone-HSA on the Matrigel-coated upper compartment or Transwell culture chambers, provided with an 8 µm pore size polycarbonate filter, according to the manufacturer's instructions. 24 h later, invaded cells, attached to the lower surface of the filter, were stained with DAPI.

b) Quantification of the motility experiments shown in (C) for Caco2 cells silenced with vinculin siRNA and cultured in the presence of 10^{-7} M testosterone-HSA as described in Fig. 14 and 15 B.

Similar results were obtained in cells pre-treated with testosterone-HSA for 2h prior assessing the migration potential by the matrigel- and transwell-assays in the presence or absence of siRNAs against vinculin or the corresponding

negative siRNA control (Fig 24). This experiment also suggests that the observed inhibitory signals on cell migration and adhesion upon testosterone-HSA treatment are triggered very early upon mAR activation when cells are still viable and cannot be attributed to an artificial response of dying cells measured after 24h of TAC treatment (as presented in Fig. 16 and 17). We conclude that vinculin is a critical component downstream of mAR that regulates the responses to actin cytoskeleton reorganization and migration potential in colon tumor cells.

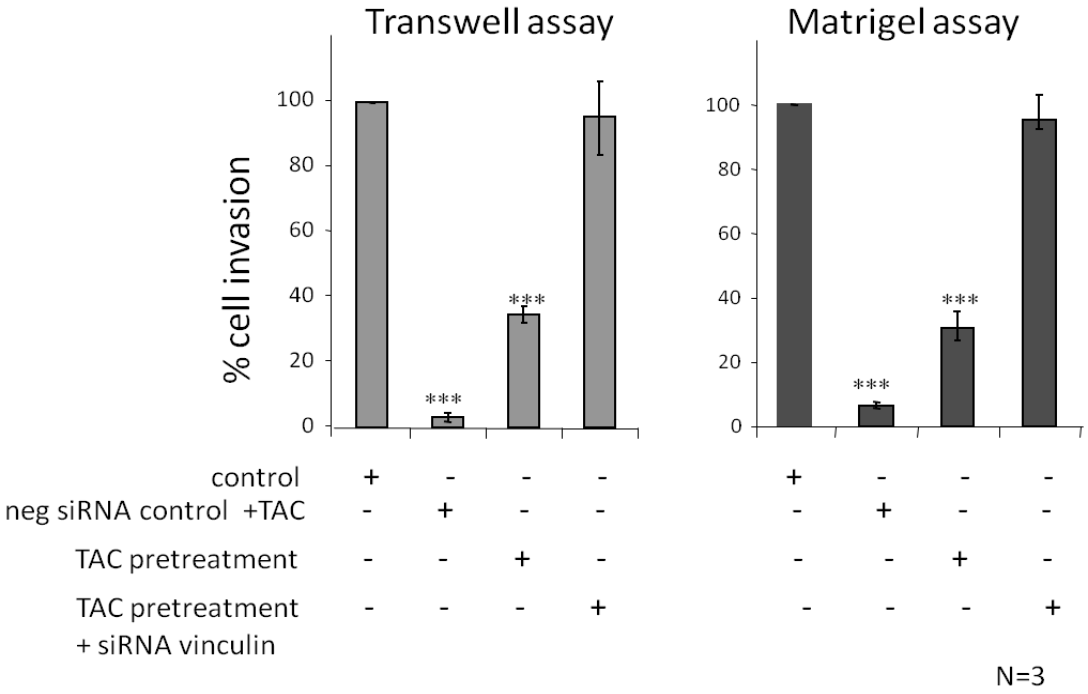


Figure 24: **Effect of testosterone-HSA on Caco2 cells silenced with vinculin siRNA**

Quantification of control matrigel and transwell motility experiments with Caco2 cells pre-treated with 10^{-7} M testosterone-HSA for 2 h and silenced with either negative control siRNA or vinculin siRNA. Bars represent the % of cell invasion in control and treated cells. (n=3), ***P<0.001.

5. Discussion

In recent years a lot of studies introduced the concept of nongenomic steroid hormone actions, which explains the observations related to rapid steroid effects. Nongenomic steroid actions have been reported for most prominent steroids [Losel R, Wehling M 2003, Heinlein CA, and Chang C 2002]. Although the nature of these membrane steroid sites was elusive until recently, the identification of a membrane progesterone receptor [Zhu Y, et al 2003, Falkenstein E, et al 1999, Falkenstein E, et al 1996, Zhu Y, et al 2003] and the isolation of a membrane glucocorticoid-binding protein with homologies with κ-opioid receptors [Evans SJ, et al 2000] show that these proteins might belong to the seven-transmembrane G protein-coupled receptors. This hypothesis is further supported by recent findings showing that the orphan G protein-receptor, GPRC6A can be activated by anabolic steroids, including testosterone. This report suggests that GPRC6A may mediate the non-genomic effects of testosterone and other anabolic steroids [Pi M,et al 2010]. Colorectal cancer is one of the most common cancers and the third leading cause of cancer death. The existence of sex differences in colon cancer incidence was proposed several years ago. The neoplasia occurs more often in men than in women in nearly all countries [Haenszel, W. and Correa, P. 1971]. The colon cancer has been reported to be related to a lot of intracellular steroid receptors, like intracellular estrogen receptor, intracellular thyroid hormone receptor and intracellular androgen receptors [I. D_Errico and A. Moschetta 2008]. The importance of intracellular androgen receptors in colorectal cancer is supported by many avenues of research. They prove that androgen receptors show altered binding characteristics in colon cancer. However, It still remained unknown whether mARs are also expressed in colon cancer and whether their activation could result in the induction of anti-tumorigenic effects similar to those described in other cancer cells.

In the present work we provide experimental evidence that membrane androgen receptors are expressed in colon tumors. Using tissue specimens from colon tumors and established colon tumor cell lines we show here that colon cancer cells express functional mARs. [Gu S, et al. 2009] Moreover,

membrane-impermeable testosterone albumin conjugates induce considerable apoptosis via activation of the pro-apoptotic executor caspase-3. [Gu S, et al. 2009] The observed mAR-activated effects are specific and independent from the classical intracellular androgen receptors (iAR), since they were manifested in the presence of the anti-androgen flutamide. In addition, mAR staining could be also detected in iAR silenced Caco2 cells (Fig 6B) and iAR-deficient DU145 human prostate cancer cells [Hatzoglou A, et al. 2005]. All those imply that the molecular identity of mAR is probably not identical with iAR, targeted to the plasma membrane.

5.1 Membrane androgen receptor activation in colon cancer triggers pro-apoptotic responses in vitro and in vivo

The results from my diplom thesis have show that membrane-impermeable testosterone albumin conjugates induced considerable apoptosis via activation of the pro-apoptotic executor caspase-3. Moreover, the results from other studies indicated as well that membrane androgen receptors are predominantly expressed in tumor cells. Activation of these receptors triggers pro-apoptotic responses. One possible rationalization for the expression of those receptors is that tumor cells may compensate mAR-dependent apoptosis by over-expressing anti-apoptotic proteins or other compensatory mechanisms that collectively protect against mAR-dependent pro-apoptotic effects. Previous reports support this assumption: Indeed, iAR deficient DU145 human prostate cancer cells were shown to over-express the pro-survival PI-3K/Akt pathway, which was down-regulated following long-term mAR activation [Papadopoulou N, et al. 2008A]. In addition, the FAK/PI3K pathway was constitutively activated in DU145 cells and mAR activation was unable to further alter the short-term phosphorylation levels of those kinases [Papadopoulou N, et al. 2008], while long term activation induced significant de-phosphorylation [Papadopoulou N, et al. 2008A]. In these cells, PI-3K was constitutively activated [Papadopoulou N, et al. 2008], whereas long-term mAR stimulation by specific agonists induced dephosphorylation of both, PI-3K and its downstream effector Akt [Papadopoulou N, et al. 2008A].

In this study, pro-survival signals are effectively downregulated in mAR-expressing colon tumors following stimulation by testosterone-albumin conjugates (TAC) both, *in vitro* and *in vivo*. This was concluded by findings showing that phosphorylated kinase Akt, which is constitutively upregulated in colon tumors but not in non-transformed cells (Fig 12 A, C), was significantly downregulated upon long-term mAR activation by TAC. In line with this, PI-3K was de-phosphorylated upon long term TAC treatment (Fig 12 D), implying that the pro-survival PI-3K/Akt signaling is downregulated in mAR activated Caco2 cells. In addition, the pro-apoptotic Bad protein was efficiently dephosphorylated and thus activated by TAC following similar kinetics (Fig. 12B). These results suggest that, in contrast to non transformed intestinal IEC06 cells, mAR-expressing colon tumor cells express activated pro-survival signals that may protect them from apoptotic cell death. mAR activation downregulated the activity of these signals via dephosphorylation, a finding consistent with the strong apoptotic regression upon mAR stimulation reported recently [Gu S, et al. 2009].

Recent studies using mouse xenografts have shown that a testosterone–albumin conjugate (testosterone-BSA) induced potent apoptotic regression of prostate tumors *in vivo* [Hatzoglou A, et al. 2005]. In addition, testosterone-BSA was also reported to potentiate the paclitaxel-mediated cytotoxicity both *in vitro* and *in vivo* [Kampa M, et al. 2006]. These reports are supported by *in vivo* experimental findings presented in this work. Indeed, when Balb/c mice were treated with testosterone-HSA and the 12-week tumor incidence of colon tumors was assessed the chemically induced tumors were reduced by 65% in the testosterone-HSA-treated animals. Most probably this effect was due to the apoptotic regression of tumor cells as indicated by the Tunel assay. These results point out clearly that activation of mAR by testosterone-HSA significantly affects the incidence of colon tumors *in vivo* and they are in line with the previously reported prostate tumor regression in mice [Hatzoglou A, et al. 2005, Kampa M, et al. 2006]. Interestingly, mAR is strongly expressed in tissues derived from p53-deficient xenograft tumors. Since p53 is a frequently inactivated gene in tumors, it is interesting to hypothesize that mAR activation

may result in eradication of p53 tumors *in vivo*. Notwithstanding the above, and despite the fact that additional experiments are required for the detailed evaluation of mAR-dependent biological effects in colon cancer, our data support the recently postulated notion [Papadopoulou N, et al. 2009] that mAR may represent a novel specific tumor target.

These results were also confirmed by *in vivo* experiments in $APC^{Min/+}$ mice. This mouse model is carrying defective adenomatous polyposis coli (APC) resulting in gastrointestinal tumors being developed spontaneously [Gourlay CW, et al. 2005]. Indeed, the colon tumor incidence observed in the TAC-treated animals was significantly reduced by 80% (Fig. 10A). Interestingly, and in line with the findings in colon tumor cells pointing to downregulation of pAKT/pBad (Figure 11) histological immunostaining analysis revealed that both, p-Akt and p-Bad were effectively downregulated in colon tumor specimens isolated from TAC-treated animals (Fig. 13). These findings collectively provide novel mechanistic evidence, pointing to p-Akt/p-Bad signaling, which may control the mAR-induced anti-tumorigenic effects *in vitro* and *in vivo*. It should be noted that $APC^{Min/+}$ mutant mice of either sex were used in this study to avoid conflicting data regarding the sex-related prevalence of colon tumors of $APC^{Min/+}$ mutant mice. It is pointed out that the enhanced susceptibility of male mice to intestinal tumor growth [Smith KJ, et al. 1993, Hinoi T, et al 2007] results rather from the classical androgen receptor, since mAR anti-tumorigenic effects seem to be independent from the classical androgen receptor signaling [Hatzoglou A, et al. 2005, Gu S, et al. 2009, Papakonstanti EA, et al. 2003, Papadopoulou N, et al. 2008].

Taken together, the results presented in prostate and colon tumors imply that mAR expression is associated with active pro-survival pathways thus protecting cells from apoptotic regression. Activation of these receptors triggers specific signaling to restrict this pro-survival machinery. This assumption is fully supported by the *in vitro* and *in vivo* results presented here, arguing that the *in vitro* findings reported previously are not simply a side effect of mAR activation.

5.2 Membrane androgen receptor activation blocks migration

The connection between actin cytoskeleton components and androgen signaling has attracted specific interest in recent years [Ting HJ, et al. 2008]. Actin dynamics seem to be crucial for apoptotic responses [Gourlay CW, et al.2005, Franklin-Tong VE, et al.2008]. The findings in our present work further underscored the key role of actin cytoskeleton rearrangements in regulating apoptosis. Indeed, it was clearly shown that actin (and tubulin) reorganization represent major early events following mAR activation by testosterone-HSA. Moreover, early blockade of actin rearrangement by depolymerizing drugs e.g. cytochalasin B, virtually abrogated the pro-apoptotic responses (Fig. 15A, B). The involvement of the early actin rearrangement in mediating the late apoptotic responses was addressed in earlier studies in prostate cancer cells. In these studies it was shown that inhibition of either up-stream or down-stream signals regulating early actin polymerization blocked the late activation of NFkB and FasL signaling [Papadopoulou N, et al. 2008A]. Although this pro-apoptotic signaling was not addressed in the present study we hypothesise that the actin reorganization is an early functional step in the pro-apoptotic response. These findings, which are in close agreement with similar results reported recently in prostate cancer cells treated with testosterone albumin conjugates [Papadopoulou N, et al. 2008 and 2008A], further emphasize the functional cross-talk between cytoskeleton rearrangements and regulation of apoptosis [Gourlay CW, et al.2005, Franklin-Tong VE, et al.2008].

Akt has been also shown to play a major role in the invasive potential of colon cancer cells in response to a variety of stimuli e.g. hergulin [Yoshioka T, et al. 2010], PAK1 [Huynh N, et al. 2010], Sprouty-2 [Holgren C, et al.2010]. Moreover, inhibition of Akt-dependent pathways has been linked to reduced cell motility in colon cancer [Lai TY, et al. 2010], while mAR stimulation downregulates p-Akt [Papadopoulou N, et al. 2008A and this work] and mAR-dependent activation has been show to block cell motility and invasion on

prostate cancer cells [Hatzoglou A, et al. 2005]. Thus, we sought to determine an effect of mAR activation on cell motility in colon tumor cells. The present observations further reveal that mAR stimulation modulates specific molecular targets controlling cell motility. Activation of mAR by two distinct testosterone albumin conjugates (testosterone-HSA and testosterone-BSA) markedly inhibited cell motility as documented by different assays (Fig 16 and 17). The possibility that testosterone conjugates may be converted to estrogen and influence the overall interpretation of our results was also considered. However, previous binding studies in colon cancer [Gu S, et al. 2009] as well as in prostate cancer cells [Kampa M, et al. 2002] have clearly indicated that estrogens (and progesterone) displaced radiolabeled testosterone with significant lower affinity (10^4 to 10^2-fold). These findings imply that even if such a conversion takes place, it cannot influence the mAR induced effects described so far, because of the high androgen selectivity of these membrane receptors. In addition, control experiments showed that neither migration nor apoptotic responses were influenced by the aromatase inhibitor anastrozole (Figure 18 and 19), further supporting the androgen specificity of the mAR induced effects. Finally, since flutamide did not influence the motility effects nor the apoptotic responses in colon cancer [Gu S, et al. 2009], it is believed that the mAR pool mediating the observed effects is most likely unrelated to a membrane-associated form of the classical, intracellular AR that may be present in the plasma membrane of colon tumor cells. Although the existence of such form of membrane tethered intracellular AR has not been reported in colon cells, experimental data in prostate cells place iAR on the cell membrane [Lind GE, et al. 2004]. In that case, however, and in sharp contrast to what we have observed in our assays in colon tumors, membrane associated iAR induced cell proliferation (instead of apoptosis) which was efficiently blocked by anti-androgens or anti-estrogens [Lind GE, et al. 2004]. In conclusion, our data support the existence of an active, non-AR/ER related membrane receptor bearing anti-cancer action in colon cancer cells. This conclusion is in line with recent findings demonstrating that iAR could not be detected in membrane preparations of CaCo2 cells [Gu S, et al. 2009].

Inhibition of migration is usually correlated with impaired expression/activation

of adhesion molecules and reorganization of focal contact structures, including actin cytoskeleton [Yoshioka T, et al. 2010]. Polymerization of actin filaments against cellular membranes provides the force for a number of cellular processes such as migration, morphogenesis, and endocytosis [Saarikangas J, et al.2010]. Since actin reorganization is a major effect of mAR activation in tumor cells [Kampa M, et al. 2002, Kallergi G, et al. 2007, Gu S, et al. 2009, Papadopoulou N, et al. 2008], we focused on the molecular mechanism underlying the mAR-induced inhibition of cellular motility in Caco2 cells (Fig. 16 and 17). Our results demonstrate that vinculin is a main target of mAR activation that may regulate cell motility. Phosphorylation of vinculin (Fig. 20A) was an early and persistent event leading to significant morphological changes of Caco2 cells. It was clearly correlated with actin restructuring as indicated by the visualization of newly organized actin filaments emanating from the vinculin spots on the cell adhesion contacts (Fig. 20B). Interestingly, vinculin silencing or inhibition of vinculin phosphorylation reversed largely actin reorganization and the inhibition of migration. These findings imply that vinculin phosphorylation/activaton upon mAR stimulation regulates cell adhesion and inhibits the migration potential of Caco2 tumor cells. This conclusion is in line with several reports in the literature. Thus, vinculin was shown to be important in regulating adhesion dynamics and cell migration [Huynh N, et al. 2010], and it was postulated that vinculin may connect early adhesion sites to the actin-driven protrusive machinery [Zhao J, et al. 2009]. According to this model vinculin stabilizes focal adhesions and thereby suppresses cell migration, an effect that is relieved by modifications of inositol phospholipids [Holgren C, et al.2010]. Although the precise role of vinculin in focal adhesions remains to be elucidated, recent experimental evidence suggest that vinculin overexpression reduces cell migration, whereas vinculin downregulation enhances cell motility [Holgren C, et al.2010]. This hypothesis is in line with the results presented in our study, showing inhibition of cell motility ahead of vinculin activation in mAR stimulated Caco2 cells. Notably, this effect was efficiently reversed by silencing of endogenous vinculin utilizing short interfering RNAs. Finally, it is worth noting that the effects of TAC on cell invasion are manifested even after short treatment of cells with this compound (Fig. 23). Moreover, these effects are still silenced by siRNAs against vinculin

(Fig. 23). These results clearly indicate that the inhibitory signals on invasion are activated early upon mAR stimulation, are dependent on vinculin and are present in cells well before they commit to the mAR induced apoptotic program.

6. Conclusions

In conclusion, the results presented here add a clear and significant piece of evidence on the potential anti-tumorigenic role of membrane androgen receptors.

They indicate that

- The functional mAR is expressed not only in hormone-dependent tumors but also in colon tumors.
- mAR conjugates also induced rapid actin and tubulin reorganization.
- The activation through steroid albumin conjugates induces potent pro-apoptotic responses regulated by cytoskeletal rearrangements.
- The long term activity of the pro-survival regulators PI-3K, Akt and Bad is effectively suppressed
- mAR Activation Inhibits Cell Motility in Colon Cancer Cells.
- The specific molecular target for cell adhesion, vinculin is modulated upon mAR activation.

Since these molecules may adapt signaling pathways involved in apoptosis, cell survival and motility [Migliaccio A, et al. 2000, Hanks SK, et al.2003], we hypothesize that they represent key signaling effectors regulating the mAR dependent anti-tumorigenic effects reported in our studies. Further experiments are now needed to address the molecular identity of mAR and to evaluate the potential role of these signaling targets for the development of novel anti-tumorigenic strategies based on specific mAR activation. These receptors may represent specific targets for the development of novel drugs since their activation drastically regress tumor growth and tumor incidence *in vivo*.

Reference

A.P. Gilmore and K. Burridge, (1996). Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. Nature 381, pp. 531–535.

Aaltonen, L.A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomaki, P., Chadwick, R.B., Kääriäinen, H., Eskelinen, M., Järvinen, H., Mecklin, J.P., de la Chapelle, A. (1998). Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med. 338, 1481–7.

Acconcia F, Barnes CJ, Kumar R. (2006). Estrogen and tamoxifen induce cytoskeletal remodelling and migration in endometrial cancer cells. Endocrinology ;147:1203-1212.

Acconcia F, Kumar R. (2005). Signaling regulation of genomic and nongenomic functions of estrogen receptors. Cancer Lett;20:1-14.

Alexaki VI, Charalampopoulos I, Kampa M, Nifli AP, Hatzoglou A, Gravanis A, Castanas E. (2006). Activation of membrane estrogen receptors induce pro-survival kinases. J Steroid Biochem Mol Biol. 98:97-110.

Alimirah, F., Chen, J., Basrawala, Z., Xin, H., and Choubey, D. (2006). DU-145 and PC-3 human prostate cancer cell lines express androgen receptor: implications for the androgen receptor functions and regulation. FEBS. Lett. 580, 2294–2300

Alpert E, Quaroni A, Goldenberg DM. 1979.Alteration in tryptic peptide patterns of ferritins purified from human colon carcinoma. Biochim Biophys Act. Nov 23;581(1):193-7.

Amaravadi R, Thompson CB. (2005). The survival kinases Akt and Pim as potential pharmacological targets. J Clin Invest 115: 2618–2624.

American Joint Committee on Cancer. (2002). AJCC Cancer Staging Handbook. 6th ed. New York: Springer-Verla

Andjelkovic M, Jakubowicz T, Cron P, Ming XF, Han JW, Hemmings BA. (1996). Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. Proc Natl Acad Sci USA 93: 5699–5704.

Aoki M, Batista O, Bellacosa A, Tsichlis P and Vogt PK. The akt kinase: molecular determinants of oncogenicity. (1998). Proc. Natl. Acad. Sci. USA, 95, 14950–14955.

Bailly M. (2003) Connecting cell adhesion to the actin polymerization machinery: vinculin as the missing link? Trends Cell Biol. 13:163-165.

Beato M.(1989). Gene regulation by steroid hormones. Cell 56:335–344

Benson AB 3rd. (2007). Epidemiology, disease progression, and economic burden of colorectal cancer. J Manag Care Pharm. 13, S5-18.

Benten WP, et al. (1999a). Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell10: 3113-3123.

Benten WP, et al.(1999b). Functional testosterone receptors in plasma membranes of T cells. Faseb J, 13:123-133.

Berta, L., Fronticelli, B. C., Fazzari, A., Radice, E., Bargoni, A., Frairia, R. and Gaetini, A. (2003). Sex steroid receptors, secondary bile acids and colorectal cancer. A possible mechanism of interaction. Panminerva Med. 45, 261–266.

Blume-Jensen P, Janknecht R, Hunter T. (1998). The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr Biol 8: 779–782.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. (1999). Cell survival promoted by the Ras–MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286: 1358–1362.

Bratton, SB, MacFarlane, M, Cain, K and Cohen, GM. (2000). "Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis." Exp Cell Res. 256(1): 27-33.

Braun AM, Thomas P. (2004). Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod. 71(1):146-55.

Butler, B., Gao, C., Mersich, A. T. and Blystone, S. D. (2006). Purified integrin adhesion complexes exhibit actin-polymerization activity. Curr. Biol. 16, 242-251.

C.D. Nobes and A. Hall, (1995). Rho, rac, and cdc42 GTPases regulate the

assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, pp. 53–62.

Caiazza F, Galluzzo P, Lorenzetti S, Marino M. (2007). 17beta-Estradiol induces ERbeta up-regulation via p38/MAPK activation in colon cancer cells. Biochem Biophys Res Commun 359: 102–07.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science. 282:1318-1321.

Chang C. (2002). Androgen and Androgen Receptor. Springer : 1-2.

Chiang CW, Kanies C, Kim KW, Fang WB, Parkhurst C, Xie M et al. (2003). Protein phosphatase 2A dephosphorylation of phosphoserine 112 plays the gatekeeper role for BAD-mediated apoptosis. Mol Cell Biol 23: 6350–6362.

Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. (2001). Membraneblebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol. 3:339–345.

Culig Z, Klocker H, Bartsch G, Steiner H, Hobisch A. (2003). Androgen Receptors in Prostate Cancer. J Urol. Oct.170(4):1363-1369.

D.R. Critchley, (2000). Focal adhesions – the cytoskeletal connection. Curr. Opin. Cell Biol. 12, pp. 133–139.

Danial NN. and Korsmeyer SJ. (2004). Cell death: Critical control points. Cell. 116: 205-219.

Datta SR, Brunet A, Greenberg ME. (1999). Cellular survival: a play in three Akts. Genes Dev 13: 2905–2927.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 91:231-241.

Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB et al. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell 6: 41–51.

del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 24;278(5338):687-9.

Delattre, O., Olschwang, S., Law, D.J., Melot, T., Remvikos,Y., Salmon, R.J.,

Sastre, X., Validire, P., Feinberg, A.P.,Thomas, G. (1989). Multiple genetic alterations in distaland proximal colorectal cancer. Lancet. 334(8659):353-356.

DeMali, K. A., Barlow, C. A. and Burridge, K. (2002). Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. J. Cell Biol. 159, 881-891.

Denault, JB and Salvesen, GS. (2002). Caspases: keys in the ignition of cell death. Chem Rev 102(12): 4489-500.

Deveraux QL. andReed JC.. (1999) IAP family proteins—supressors of apoptosis. Genes Dev. 13: 239-252.

Ding, D., Xu, L., Menon, M., Reddy, G. P. and Barrack, E. R. (2004). Effect of a short CAG (glutamine) repeat on human androgen receptor function. Prostate 58, 23–32.

Djouder N, Metzler SC, Schmidt A, Wirbelauer C, Gstaiger M, Aebersold R et al. (2007). S6K1-mediated disassembly of mitochondrial URI/PP1gamma complexes activates a negative feedback program that counters S6K1 survival signaling. Mol Cell 28: 28–40.

Downward J. (1998) Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol. 10:262-267.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR and Greenberg ME. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science, 275, 661–665.

Earnshaw WC., Martins LM., and Kaufmann SH.(1999). Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 68: 383-424.

Evans SJ, Searcy BT, Moore FL. (2000). A subset of opioid ligands bind to the membrane glucocorticoid receptor in an amphibian brain. Endocrinology 141:2294–2300.

Evans, R.M. (1988).The steroid and thyroid hormone receptor superfamily. Science. 240, 889-893.

Eves EM, Xiong W, Bellacosa A, Kennedy SG, Tsichlis PN, Rosner MR et al. (1998). Akt, a target of phosphatidylinositol 3-kinase, inhibits apoptosis in a differentiating neuronal cell line. Mol Cell Biol 18: 2143–2152.

Falkenstein E, Heck M, Gerdes D, Grube D, Christ M, Weigel M, Buddhikot M,

Meizel S, Wehling M. (1999). Specific progesterone binding to a membrane protein and related nongenomic effects on Ca2+-fluxes in sperm. Endocrinology 140:5999–6002.

Falkenstein E, Meyer C, Eisen C, Scriba PC, Wehling M. (1996). Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells. Biochem Biophys Res Commun 229:86–89.

Falkenstein E, Norman AW, Wehling M. (2000). Mannheim classification of nongenomically initiated (rapid) steroid action(s). J Clin Endocrinol Metab 85:2072–2075.

Fang X, Yu S, Eder A, Mao M, Bast Jr RC, Boyd D et al. (1999). Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. Oncogene 18: 6635–6640.

Fearon, E.R., Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell, 61(5):759-76.

Feng-ying LI, Mao-de LAI (2008) Colorectal cancer, one entity or three. J Zhejiang Univ Sci. B 10(3):219-229.

Ferlay, J., Autier, P., Boniol, M., Heanue, M., Colombet, M., Boyle, P. (2007). Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol. 18, 581-92.

Ferro, P., Catalano, M. G., Raineri, M., Reato, G., dell_Eva, R., Risio, M., Foa, R., Fortunati, N. and Pfeffer, U. (2000). Somatic alterations of the androgen receptor CAG repeat in human colon cancer delineate a novel mutation pathway independent of microsatellite instability. Cancer Genet. Cytogenet. 123, 35–40.

Fesik. SW. (2000). Insights into programmed cell death through structural biology. Cell. 103: 273-282.

Foradori C.D., Weiser M.J., Handa R.J.. (2008). Non-genomic actions of androgens. Front Neuroendocrinol. May;29(2):169-8.

Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA, Thompson CB. (2003). The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. Genes Dev 17: 1841–1854.

Franke TF, Kaplan DR and Cantley LC. (1997). PI3K: downstream AKTion blocks apoptosis. Cell, 88, 435–437.

Franklin-Tong VE, Gourlay CW. (2008). A role for actin in regulating apoptosis/programmed cell death: evidence spanning yeast, plants and animals. Biochem J. 413(3):389-404.

Freedman, L.P. (1992). Anatomy of the steroid receptor zinc finger region. Endocr Rev. 13: 129-145.

Freeman MR, Cinar B, Lu ML. (2005). Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer. Trends Endocrinol Metab 16:273-279.

Friedl P, Wolf K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer; 3:362-374.

G.L. Zhou, D.F. Tucker, S.S. Bae, K. Bhatheja, M.J. Birnbaum and J. Field. (2006). Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration, J. Biol. Chem. 281 ,pp. 36443–36453.

Galbraith, C. G., Yamada, K. M. and Galbraith, J. A. (2007). Polymerizing actin fibers position integrins primed to probe for adhesion sites. Science 315, 992-995.

Gatson JW, Kaur P, Singh M. (2006). Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147:2028-2034.

Geiger B, Bershadsky A, Pankov R, Yamada KM. (2001).Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. Nat. Rev. Mol. Cell Biol. 2, pp. 793–805.

Gervaz, P., Bouzourene, H., Cerottini, J.P., Chaubert, P., Benhattar, J., Secic, M., Wexner, S., Givel, J.C., Belin, B. (2001). Dukes B colorectal cancer: distinct genetic categories and clinical outcome based on proximal or distal tumor location. Dis. Colon Rectum. 44(3):364-372.

Giardiello, F.M., Brensinger, J.D., Petersen, G.M., Luce, M.C., Hylind, L.M., Bacon, J.A., Booker, S.V., Parker, R.D., Hamilton, S.R. (1997). The use and interpretation of commercial APC gene testing for familial adenomatous polyposis. N Engl J Med. 336, 823-2.

Gilad LA, Tirosh O, Schwartz B. (2006). Phytoestrogens regulate transcription and translation of vitamin D receptor in colon cancer cells. J Endocrinol 191: 387–98.

Gotz R, Wiese S, Takayama S, Camarero GC, Rossoll W, Schweizer U et al. (2005). Bag1 is essential for differentiation and survival of hematopoietic and neuronal cells. Nat Neurosci 8: 1169–1178.

Gourlay CW, Ayscough KR. (2005).The actin cytoskeleton: a key regulator of apoptosis and ageing? Nat. Rev. Mol. Cell. Biol, 6(7):6583-589.

Goyal. L. (2001). Cell death inhibition: Keeping caspases in check. Cell. 104: 805-808.

Gronemeyer H & Laudet V. (1995).Transcription factors 3: nuclear receptors. Protein Profile 2: 1173-1308.

Gu S, Papadopoulou N, Gehring EM, Nasir O, Dimas K, Bhavsar SK, Foller M, Alevizopoulos K, Lang F, Stournaras C. (2009) Functional membrane androgen receptors in colon tumors trigger pro-apoptotic responses in vitro and reduce drastically tumor incidence in vivo. Mol Cancer. 8:114.

Guido M, Uta C.P. (2008). Raptid action of androgens. Neuroendocrinology 29: 182-198.

Haenszel, W. and Correa, P. (1971). Cancer of the colon andrectum and adenomatous polyps. A review of epidemiologic findings. Cancer 28, 14–24.

Hanks SK, Ryzhova L, Shin NY, Brabek J. (2003) Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. Front Biosci. 8:d982-996.

Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. (2001). p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci USA 98: 9666–9670.

Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C, et al. (2005). Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. J Clin Endocrinol Metab, 90: 893-903.

Heinlein CA, Chang C. (2002). The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol 16:2181–2187.

Harms, C. et al. (2004). Neuronal gelsolin prevents apoptosis by enhancing actin depolymerization. Mol. Cell. Neurosci. 25, 69–82.

Hilpela P, Vartiainen MK, Lappalainen P. (2004). Regulation of the actin cytoskeleton by PI(4,5)P2 and PI(3,4,5)P3. Curr Top Microbiol Immunol 282: 117–163.

Holgren C, Dougherty U, Edwin F, Cerasi D, Taylor I, Fichera A, Joseph L, Bissonnette M, Khare S.(2010) Sprouty-2 controls c-Met expression and metastatic potential of colon cancer cells: sprouty/c-Met upregulation in human colonic adenocarcinomas. Oncogene. 2010.264.

Honda, T., Kai, I., Ohi, G. (1999). Fat and dietary fiber intake and colon cancer mortality: a chronological comparison between Japan and the United States. Nutr Cancer. 33, 95-9.

Hong M and Lee VM. (1997). Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neuronsJ. Biol. Chem. 272, 19547–19553.

Hotulainen P, Lappalainen P. (2006). Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. J Cell Biol 173: 383–394.

Houslay MD. (2006). A RSK(y) relationship with promiscuous PKA. Sci STKE 2006: pe32.

Huang DCS. and A. Strasser. (2000). BH3-only proteins—essential initiators of apoptotic cell death. Cell. 103: 839-842.

Hutchinson, J. Jin, R.D. Cardiff, J.R. Woodgett and W.J. Muller. (2004). Activation of Akt-1 (PKB-alpha) can accelerate ErbB-2-mediated mammary tumorigenesis but suppresses tumor invasion, Cancer Res. 64 (2004), pp. 3171–3178.

Huynh N, Liu KH, Baldwin GS, He H. (2010) P21-activated kinase 1 stimulates colon cancer cell growth and migration/invasion via ERK- and AKT-dependent pathways. Biochim Biophys Acta. Sep; 1803(9):1106-13.

I. Budihardjo, H. Oliver, and M. Lutter, et al. (1999). Biochemical pathway of caspase activation during apoptosis. Annu Rev Cell Dev. 15: 269-290.

I. D_Errico and A. Moschetta. (2008). Nuclear receptors, intestinal architecture and colon cancer: an intriguing link. Cell Mol Life Sci. May; 65(10):1523-43.

Igarashi M, Komiya Y. (1991) Tyrosine phosphorylation and immunodetection

of vinculin in growth cone particle (GCP) fraction and in GCP-cytoskeletal subfractions. J Neurosci Res. 1991 Sep; 30(1):266-74.

J. Weekes, S.T. Barry and D.R. Critchley. (1996). Acidic phospholipids inhibit the intramolecular association between the N- and C-terminal regions of vinculin, exposing actin-binding and protein kinase C phosphorylation sites. Biochem J 314, pp. 827–832.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Thun, MJ. (2007). Cancer statistics, CA Cancer J Clin. 57, 43-66.

Jenster G, Trapman J, Brinkmann AO. (1993). Nuclear import of the human androgen receptor. Biochem J. : 293, 761-768.

Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. (1991). Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol.5 : 1396-1404.

Kallergi G, Agelaki S, Markomanolaki H, Georgoulias V, Stournaras C. (2007). Activation of FAK/PI3K/Rac1 signaling controls actin reorganization and inhibits cell motility in human cancer cells. Cell Physiol Biochem, 20:977-986.

Kampa M, Kogia C, Theodoropoulos PA, Anezinis P, Charalampopoulos I, Papakonstanti EA, Stathopoulos EN, Hatzoglou A, Stournaras C, Gravanis A, Castanas E. (2006) Activation of membrane androgen receptors potentiates the antiproliferative effects of paclitaxel on human prostate cancer cells. Mol Cancer Ther, 5:1342-1351.

Kampa M, Nifli AP, Charalampopoulos I, Alexaki VI, Theodoropoulos PA, Stathopoulos EN, Gravanis A, Castanas E. (2005). Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. Exp Cell Res, 307:41-51.

Kampa M, Papakonstanti EA, Hatzoglou A, Stathopoulos EN, Stournaras C, Castanas E. (2002). The human prostate cancer cell line LNCaP bears functional membrane testosterone receptors that increase PSA secretion and modify actin cytoskeleton. Faseb J, 16:1429-1431.

Kampa M, Pelekanou V, Castanas E (2008) Membrane-initiated steroid action in breast and prostate cancer. Steroids, 73(9-10):953-960.

Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P,

Downward J and Evan G. (1997). Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB.(1997). Nature, 385, 544–548.

Kaufmann, SH and Earnshaw, WC. (2000). Induction of apoptosis by cancer chemotherapy. Exp Cell Res. 256(1): 42-9.

Kennelly R, Kavanagh DO, Hogan AM, Winter DC. (2008). Oestrogen and the colon: potential mechanisms for cancer prevention. Lancet Oncol. Apr;9(4):385-91.

Kerr JFR, Wyllie AH, Currie AR. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer;26: 239–571.

Kiosses, W. B., Shattil, S. J., Pampori, N. and Schwartz, M. A. (2001). Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration. Nat. Cell Biol. 3, 316-320.

Kwiatkowski, D. J. (1999).Functions of gelsolin: motility, signaling, apoptosis, cancer. *Curr. Opin. Cell Biol.* 11, 103–108.

Koya, R. C. et al. (2000).Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. *J. Biol. Chem.* 275, 15343–15349.

Lai TY, Chen LM, Lin JY, Tzang BS, Lin JA, Tsai CH, Lin YM, Huang CY, Liu CJ, Hsu HH (2010) 17beta-estradiol inhibits prostaglandin E2-induced COX-2 expressions and cell migration by suppressing Akt and ERK1/2 signaling pathways in human LoVo colon cancer cells. Mol Cell Biochem Sep; 342(1-2):63-70.

Lawen A. (2003) Apoptosis-an introduction. Bioessays. Sep;25(9):888-96.

Leist, M and Jaattela, M. (2001). Four deaths and a funeral: from caspases to alternative mechanisms. Nat. Rev. Mol. Cell Biol. 2(8): 589-98.

Leverrier Y, Ridley AJ. (2001). Apoptosis: caspases orchestrate the ROCK ‘n’ bleb. Nat Cell Biol. 3:E91–E93.

Li P, Nicosia SV & Ba W. (2001). Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells. J Biol Chem 276: 20444−20450.

Li, B. Monks, Q. Ge and M.J. Birnbaum. (2007) Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1α transcription coactivator, Nature

: 10.1038.

Lieberherr M, Grosse B, (1994). Androgens increase intracellular calcium concentration and inositol 1,4,5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein. J Biol Chem. 269:7217-7223.

Lind GE, Thorstensen L, Løvig T, Meling GI, Hamelin R, Rognum TO, Esteller M, Lothe RA. (2004). A CpG island hypermethylation profile of primary colorectal carcinomas and colon cancer cell lines. Mol Cancer. Oct 11; 3:28.

Losel R, Wehling M (2003). Nongenomic actions of steroid hormones. Nat Rev Mol Cell Biol 4:46–56

Lynch, P.M., Lynch, H.T. (1985). Colon cancer genetics. New York (NY). Van Nostrand Rheinhold.

M.J. Arboleda, J.F. Lyons, F.F. Kabbinavar, M.R. Bray, B.E. Snow, R. Ayala, M. Danino, B.Y. Karlan and D.J. Slamon,. (2003). Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells, Cancer Res. 63 pp. 196–206.

Macdonald A, Campbell DG, Toth R, McLauchlan H, Hastie CJ, Arthur JS. (2006). Pim kinases phosphorylate multiple sites on Bad and promote 14-3-3 binding and dissociation from Bcl-XL. BMC Cell Biol 7: 1.

Mahaffy RE, Pollard TD. (2008). Influence of phalloidin on the formation of actin filament branches by Arp2/3 complex. Biochemistry 47: 6460–6467.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M & Chambon P. (1995). The nuclear receptor superfamily the second decade. Cell 83 : 835-839.

Marino M, Galluzzo P, Leone S, Acconcia F, Ascenzi P. (2006). Nitric oxide impairs the 17beta-estradiol-induced apoptosis in human colon adenocarcinoma cells. Endocr Relat Cancer 13: 559–69.

Matsui T, Li L, del Monte F, Fukui Y, Franke TF, Hajjar RJ and Rosenzweig A. (1999). Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. Circulation, 100, 2373–2379.

Meng-Lei Zhu, Natasha Kyprianou. (2008). Androgen receptor and growth

factor signaling cross-talk in prostate cancer cells. Endocr Relat Cancer. 15 :841-9.

Michels G, Hoppe UC. (2008). Rapid actions of androgens. Front Neuroendocrinol. May;29(2):182-98.

Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F. (2000) Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. EMBO J. Oct 16; 19(20):5406-17

Mulholland, D. J., Dedhar, S., Coetzee, G. A. and Nelson, C. C. (2005). Interaction of nuclear receptors with the Wnt/ beta-catenin/Tcf signaling axis: Wnt you like to know? Endocr. Rev. 26, 898–915.

Nagata S. (2000). Apoptotic DNA fragmentation. Exp Cell Res. 256:12–18

P.A. Steimle, J.D. Hoffert, N.B. Adey and S.W. Craig. (1999). Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. J Biol Chem 274, pp. 18414–18420.

Papadopoulou N, Charalampopoulos I, Alevizopoulos K, Gravanis A, Stournaras C. (2008 a). Rho/ROCK/Actin signaling regualtes membrane androgen receptor induced apoptosis in prostate cancer cells. Exp Cell Res, 314: 3162-3174.

Papadopoulou N, Charalampopoulos I, Anagnostopoulou V, Konstantinidis G, Föller M, Gravanis A, Alevizopoulos K, Lang F, Stournaras C. (2008 b). Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-kappaB activity and induces apoptotic responses via Bad, FasL and caspase-3 in DU-145 prostate cancer cells. Mol Canc. 7:88.

Papadopoulou N, Papakonstanti EA, Kallergi G, Alevizopoulos K, Stournaras C. (2009). Membrane androgen receptor activation in prostate and breast tumor cells: Molecular signaling and clinical impact. IUBMB Life, 61(1): 56-61.

Papakonstanti, E. A., Kampa, M., Castanas, E., and Stournaras, C. (2003). A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. Mol. Endocrinol. 17, 870–881.

Papakonstanti, E. A., Stournaras, C. (2004). Tumor necrosis factor-alpha

promotes survival of opossum kidney cells via Cdc42-induced phospholipase C-gamma1 activation and actin filament redistribution. Mol Biol Cell. 15(3):1273-86. Epub 2003 Dec 29.

Pelham RJ, Chang F. (2002). Actin dynamics in the contractile ring during cytokinesis in fission yeast. Nature 419: 82–86.

Pi M, Parrill AL, Quarles LD. (2010) GPRC6A mediates the non-genomic effects of steroids. J Biol Chem. Dec 17; 285(51):39953-64. Epub 2010 Oct 13.

R.P. Johnson, V. Niggli, P. Durrer and S.W. Craig. (1998). A conserved motif in the tail domain of vinculin mediates association with and insertion into acidic phospholipid bilayers. Biochemistry 37, pp. 10211–10222.

Reed JC and Paternostro G. (1999). Postmitochondrial regulation of apoptosis during heart failure. (Proc. Natl. Acad. Sci. USA, 96, 7614–7616.

Richard B van Breemen , Yongmei Li. (2005). Caco-2 cell permeability assays to measure drug absorption. Expert Opin Drug Metab Toxicol. Aug; 1(2):175-85

Ridley AJ, Hall A. (1994). Signal transduction pathways regulating Rho-mediated stress fibre formation: requirement for a tyrosine kinase. EMBO J 13: 2600–2610.

Ries LAG, Melbert D, Krapcho M, et al. (2008). SEER Cancer Statistics Review. National Cancer Institute: 1975-2005.

Rockville, MD. (1998). Agency for Health Care Policy and Research. AHCPR Publication No. 98-003.

Rory Kennelly, Dara O Kavanagh, Aisling M Hogan, Desmond C Winter. (2008)Oestrogen and the colon: potential mechanisms for cancer prevention Lancet. Oncol. Apr; 9(4):385-91.

Posey, S. & Bierer, B. (1999).Actin stabilization by jasplakinolide enhances apoptosis induced by cytokine deprivation. *J. Biol. Chem.* **274**, 4259–4265.

Odaka, C., Sanders, M. L. & Crews, P. (2000). Jasplakinolide induces apoptosis in various transformed cell lines by a caspase-3-like protease-dependent pathway. *Clin. Diagn. Lab. Immunol.* **7**, 947–952.

S. Huttelmaier, O. Mayboroda, B. Harbeck, T. Jarchau, B.M. Jockusch and M. Rudiger, (1998). The interaction of the cell-contact proteins VASP and

vinculin is regulated by phosphatidylinositol-4, 5-bisphosphate. Curr Biol 8, pp. 479–488.

Saarikangas J, Zhao H, Lappalainen P. (2010). Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. Physiol Rev. Jan;90(1):259-89.

Saraste, A and Pulkki, K (2000). "Morphologic and biochemical hallmarks of apoptosis." Cardiovasc Res 45(3): 528-37.

Scaffidi, C, Fulda, S, Srinivasan, A, Friesen, C, Li, F, Tomaselli, KJ, Debatin, KM, Krammer, PH and Peter, ME. (1998). Two CD95 (APO-1/Fas) signaling pathways. Embo J. 17(6): 1675-87.

Scheid MP, Schubert KM, Duronio V. (1999). Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. J Biol Chem 274: 31108–31113.

Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J, Breard J. (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat Cell Biol. 3:346–352.

Sechi AS, Wehland J. (2000).The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P2 influences cytoskeletal protein activity at the plasma membrane. J Cell Sci 113: 3685–3695.

Serrels, B., Serrels, A., Brunton, V. G., Holt, M., McLean, G. W., Gray, C. H., Jones, G. E. and Frame, M. C. (2007). Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. Nat. Cell Biol. 9, 1046-1056.

Sharma M, Chuang WW & Sun Z. (2002). Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3beta inhibition and nuclear beta-catenin accumulation. J Biol Chem 277: 30935−30941.

Sheetz MP, Sable JE, Dobereiner HG. (2006). Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. Annu Rev Biophys Biomol Struct 35: 417–434.

Small JV, Stradal T, Vignal E, Rottner K. (2002). The lamellipodium: where motility begins. Trends Cell Biol. 12, pp. 112–120.

Smith KJ, Johnson KA, Bryan TM, Hill DE, Markowitz S, Willson JK, Paraskeva C, Petersen GM, Hamilton SR, Vogelstein B, et al.(1993) The APC gene product in normal and tumor cells. Proc Natl Acad Sci U S A.

90:2846-2850.

Slattery ML, Sweeney C, Murtaugh M, Ma KN, Wolff RK, Potter JD, Caan BJ, Samowitz W. (2005) Associations between ERalpha, ERbeta, and AR genotypes and colon and rectal cancer. *Cancer Epidemiol Biomarkers Prev.*; 14(12):2936–2942. doi: 10.1158/1055-9965.EPI-05-0514.

Somjen D, Kohen F, Gayer B, Kulik T, Knoll E, Stern N. (2004). Role of putative membrane receptors in the effect of androgens on human vascular cell growth. J Endocrinol. 180:97-106.

Strasser A., O'Conner L., and VM, Dixit. (2000). Apoptosis signaling. Annu Rev Biochem. 69: 217-245.

Strate LL, Syngal S. (2005). Hereditary colorectal cancer syndromes. Cancer Causes Control. Apr;16(3):201-13.

Subauste MC, Pertz O, Adamson ED, Turner CE, Junger S, Hahn KM. (2004) Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. J Cell Biol. May 10;165(3):371-81.

Sun YH, Gao X, Tang YJ, Xu CL, Wang LH. (2006). Androgens induce increases in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells. J Androl . 27:671-678.

Tan Y, Demeter MR, Ruan H, Comb MJ. (2000). BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. J Biol Chem 275: 25865–25869.

Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F. (2000). Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J Cell Biol 150: 797–806.

Vanhaesebroeck B, Alessi DR. (2000) The PI3K-PDK1 connection: more than just a road to PKB. Biochem J. 346 Pt 3:561-576.

von Gise A, Lorenz P, Wellbrock C, Hemmings B, Berberich-Siebelt F, Rapp UR et al. (2001). Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. Mol Cell Biol 21: 2324–2336.

Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F et al. (1999). Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284: 339–343.

Wang Z, Liu L, Hou J, Wen D, Yan C, Pu J, Ouyang J, Pan H. (2008). Rapid membrane effect of testosterone in LNCaP cells. Urol Int.81(3):353-9

Wang, X. (2001). The expanding role of mitochondria in apoptosis." Genes Dev 15(22): 2922-33.

Watanabe N, Kato T, Fujita A, Ishizaki T, Narumiya S. (1999). Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat Cell Biol 1: 136–143.

Waterhouse NJ, Ricci JE, Green DR. (2002). And all of a sudden it's over: mitochondrial outer-membrane permeabilization in apoptosis. Biochimie. 84:113–121.

Weihl CC, Ghadge GD, Kennedy SG, Hay N, Miller RJ and Roos RP. (1999). Mutant presenilin-1 induces apoptosis and downregulates Akt/PKB. J. Neurosci., 19, 5360–5369.

Wen Y et al. (2000). HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res 60 : 6841−6845.

Wiese S, Pei G, Karch C, Troppmair J, Holtmann B, Rapp UR et al. (2001). Specific function of B-Raf in mediating survival of embryonic motoneurons and sensory neurons. Nat Neurosci 4: 137–142.

Witt S, Zieseniss A, Fock U, Jockusch BM, Illenberger S. (2004). Comparative biochemical analysis suggests that vinculin and metavinculin cooperate in muscular adhesion sites, J. Biol. Chem. 279 pp. 31533–31543.

Wozniak MA, Modzelewska K, Kwong L, Keely PJ.(2004) Focal adhesion regulation of cell behavior. Biochim Biophys Acta. 1692:103-119.

Yamaguchi H, Shiraishi M, Fukami K, Tanabe A, Ikeda-Matsuo Y, Naito Y, Sasaki Y. (2009). MARCKS regulates lamellipodia formation induced by IGF-I via association with PIP2 and beta-actin at membrane microdomains. J Cell Physiol 220: 748–755.

Yan B, Zemskova M, Holder S, Chin V, Kraft A, Koskinen PJ et al. (2003). The PIM-2 kinase phosphorylates BAD on serine 112 and reverses BAD-induced cell death. J Biol Chem 278: 45358–45367.

Yao R and Cooper GM. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor.Science, 267, 2003–2006.

Yoeli-Lerner, G.K. Yiu, I. Rabinovitz, P. Erhardt, S. Jauliac and A. Toker. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT, Mol. Cell 20, pp. 539–550.

Yoshioka T, Nishikawa Y, Ito R, Kawamata M, Doi Y, Yamamoto Y, Yoshida M, Omori Y, Kotanagi H, Masuko T, Enomoto K (2010) Significance of integrin alphavbeta5 and erbB3 in enhanced cell migration and liver metastasis of colon carcinomas stimulated by hepatocyte-derived heregulin. Cancer Sci. Vol.11;No.9:2011-2018.

Zhang, Y.Z., Sheng, J.Q., Li, SR., Wu, ZT. (2005). Hereditary predisposition of colorectal cancer and prevalence of hereditary nonpolyposis colorectal cancer in general population of colorectal cancer patients in China. Zhonghua Yi Xue Za Zhi. 85, 2995-3000.

Zhao J, Guan JL. (2009) Signal transduction by focal adhesion kinase in cancer. Cancer Metastasis Rev. 28:35-49.

Zhiyong Zhang, Gonzalo Izaguirre, Siang-Yo Lin, Hwa Young Lee, Erik Schaefer, and Beatrice Haimovich. (2004) The Phosphorylation of Vinculin on Tyrosine Residues 100 and 1065, Mediated by Src Kinases, Affects Cell Spreading. Mol Biol Cell. Sep; 15(9):4234-47. Epub 2004 Jun 30.

Zhou ZX, Kemppainen JA, Wilson EM. (1995). Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol. Endocrinol. 9: 605-615.

Zhu Y, Bond J, Thomas P. (2003). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci USA 100:2237–2242.

Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. (2003). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci USA* 100:2231–2236.

Curriculum Vitae

Personal Information:

Family Name: Gu

Given Name: Shuchen

Date of Birth: Aug. 02 1982

Nationality: Chinese

Gender: Female

Marital Status: Single

Education:

2008.2~ Now **PhD**

Eberhard Karls University Tuebingen

Major: Biology

2005.10 ~ 2008.2 **M.S.**

Martin-Luther-University Halle-Wittenberg

Hochschule Anhalt University of Applied Science

Major: Biomedical Engineering

2000.9 ~ 2005.7 **B.S.**

medical college ,Tongji University (Shanghai, China)

Major: clinical medicine

Experience:

2004.1~ 12 **Intern**

Shanghai East Hospital

Diagnose and treat common illnesses and receive excellent evaluation from doctors and patients.

2002.10~ 12 **Volunteer**

Tumor Convalescent Center of Shanghai

Help patient with daily care and rehabilitation exercise.

Publications obtained during Ph. D Work

- **Gu S**, Papadopoulou N, Nasir O, Föller M, Alevizopoulos K, Lang F, Stournaras C.Activation of Membrane Androgen Receptors in Colon Cancer Inhibits the Prosurvival Signals Akt/Bad In Vitro and In Vivo and Blocks Migration via Vinculin/Actin Signaling.**Mol Med.** 2011 1-2;17(1-2):48-58. Epub 2010 Oct 15.
- **Gu S**, Papadopoulou N, Gehring EM, Nasir O, Dimas K, Bhavsar SK, Föller M, Alevizopoulos K, Lang F, Stournaras C. Functional membrane androgen receptors in colon tumors trigger pro-apoptotic responses in vitro and reduce drastically tumor incidence in vivo. **Mol Cancer** 2009;8:114.
- Wang K*, **Gu S***, Nasir O, Föller M, Ackermann TF, Klingel K, Kandolf R, Kuhl D, Stournaras C, Lang F. SGK1-dependent intestinal tumor growth in APC-deficient mice. **Cell Physiol Biochem**. 2010;25(2-3):271-8 *The authors contributed equally to the manuscript
- Shumilina E, Xuan NT, Schmid E, Bhavsar SK, Szteyn K, **Gu S**, Götz F, Lang F. Zinc induced apoptotic death of mouse dendritic cells. **Apoptosis.** 2010 Jun 22.
- Rexhepaj R, Rotte A, Pasham V, **Gu S**, Kempe DS, Lang F. PI3 kinase and PDK1 in the regulation of the electrogenic intestinal dipeptide transport. **Cell Physiol Biochem.** 2010;25(6):715-22.
- Föller M, Mahmud H, Qadri SM, **Gu S**, Braun M, Bobbala D, Hocher B, Lang F. Endothelin B receptor stimulation inhibits suicidal erythrocyte death. **FASEB J.** 2010 Sep;24(9):3351-9.
- Xuan NT, Shumilina E, Gulbins E, **Gu S**, Götz F, Lang F. Triggering of dendritic cell apoptosis by xanthohumol. **Mol Nutr Food Res.** 2010 Jul;54
- Bhavsar SK, Föller M, **Gu S**, Vir S, Shah MB, Bhutani KK, Santani DD, Lang F. Involvement of the PI3K/AKT pathway in the hypoglycemic

effects of saponins from Helicteres isora. **J Ethnopharmacol**. 2009;126:386-396.

- Nasir O, Wang K, Föller M, **Gu S**, Bhandaru M, Ackermann TF, Boini KM, Mack A, Klingel K, Amato R, Perrotti N, Kuhl D, Behrens J, Stournaras C, Lang F. Relative resistance of SGK1 knockout mice against chemical carcinogenesis. **IUBMB Life** 2009; 61:768-776.
- Föller M, Mahmud H, **Gu S**, Kucherenko Y, Gehring EM, Shumilina E, Floride E, Sprengel R, Lang F. Modulation of suicidal erythrocyte cation channels by an AMPA antagonist. **J Cell Mol Med** 2009; in press.
- Föller M, Sopjani M, Koka S, **Gu S**, Mahmud H, Wang K, Floride E, Schleicher E, Schulz E, Münzel T, Lang F. Regulation of erythrocyte survival by AMP-activated protein kinase. **FASEB J**. 2009;23:1072-1080.
- Rexhepaj R *, Rotte A *, **Gu S**, Pasham V, Wang K, Kempe DS, Ackermann TF, Föller M, Lang F. Tumor suppressor gene adenomatous polyposis coli downregulates intestinal transport. **Pflugers Arch.** 2011 May;461(5):527-36.
- Sopjani M*, Alesutan I*, Dërmaku-Sopjani M, **Gu S**, Zelenak C, Munoz C, Velic A, Föller M, Rosenblatt K P, Kuro-o M; Lang F. Regulation of the Na+/K+ ATPase by Klotho. FEBS Letters. (in publishing)
- Bhavsar SK, Gu S, Bobbala D, Lang F. Janus Kinase 3 is Expressed in Erythrocytes, Phosphorylated Upon Energy Depletion andInvolved in the Regulation of Suicidal Erythrocyte Death. Cell Physiol Biochem. (in publishing)
- Bhandaru M, Rotte A, **Gu S**, Föller M, Lang F. Calciuria in mice carrying a loss of function mutation of the Adenomatous Polyposis Coli gene. AJP-Regul. (Submitted)
- Qadri SM , Mahmud H, Lang E, **Gu S**, Bobbala D, Zelenak C, Siegfried A, Föller M, Lang F. Enhanced suicidal erythrocyte death in mice carrying a loss of function mutation of the Adenomatous Polyposis Coli gene. (Submitted)

- Rexhepaj R, **Gu S**, Alesutan I, Eichenmüller M, Pathare G, Föller M, Kuhl D, Lang F. SGK1-dependent stimulation of intestinal SGLT1 activity by Vitamin D. Pflugers Archive. (Submitted)