

Original Paper

# Membrane Androgen Receptor Down-Regulates c-Src-Activity and Beta-Catenin Transcription and Triggers GSK-3beta-Phosphorylation in Colon Tumor Cells

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## Key Words

mARs • c-Src • GSK-3beta • Beta-catenin • Cyclin D1 • Actin • Colon tumors

## Abstract

**Background/Aims:** Functional membrane androgen receptors (mARs) have recently been described in colon tumor cells and tissues. Their activation by specific testosterone albumin conjugates (TAC) down-regulates the PI-3K/Akt pro-survival signaling and triggers potent pro-apoptotic responses both, *in vitro* and *in vivo*. The present study explored the mAR-induced regulation of gene products implicated in the tumorigenic activity of Caco2 colon cancer cells.

**Methods:** In Caco2 human colon cancer cells transcript levels were determined by RT-PCR, protein abundance and phosphorylation by Western blotting and confocal microscopy, as well as cytoskeletal architecture by confocal microscopy. **Results:** We report time dependent significant decrease in Tyr-416 phosphorylation of c-Src upon mAR activation. In line with the reported late down-regulation of the PI-3K/Akt pathway in testosterone-treated colon tumors, GSK-3beta was phosphorylated at Tyr-216 after long term stimulation of the cells with TAC, a finding supporting the role of this kinase to promote apoptosis. PCR analysis revealed significant decrease of beta-catenin and cyclin D1 transcript levels following TAC treatment. Moreover, confocal laser scanning microscopic analysis disclosed co-localization of beta-catenin with actin cytoskeleton. It is thus conceivable that beta-catenin may participate in the reported modulation of cytoskeletal dynamics in mAR stimulated Caco2 cells. **Conclusions:** Our results provide strong evidence that mAR activation regulates late expression and/or activity of the tumorigenic gene products c-Src, GSK-3beta, and beta-catenin thus facilitating the pro-apoptotic response in colon tumor cells.

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## Introduction

Membrane androgen receptors (mARs) have been described in various tumors including prostate tumors [1-5], breast tumors [6, 7] and gliomas [8]. Recently we have reported the expression of functional mARs in colon tumor cells and tissues and have addressed their biological effects [9, 10]. Using non-permeable androgen derivatives (testosterone albumin conjugates, TAC) that do not bind to iAR in colon tumor cells [9-11], it was shown that mAR activation triggered rapid non-genomic signaling that regulated various cellular responses including actin reorganization, apoptosis and migration. In particular, it was shown that rapid activation of the FAK/mTOR/p70S6K/PAK1 cascade governs the early actin cytoskeleton rearrangements [11], while vinculin phosphorylation, in association with morphological alterations of the focal adhesions, accounts for the inhibition of colon tumor cell migration [10]. In line with the observed potent pro-apoptotic responses, late down-regulation of the pro-survival PI-3K/Akt signaling activity was observed in colon tumor cells and tissues treated with TAC [10]. These findings imply that mAR and mAR-initiated downstream key signaling molecules may represent important pharmacological targets in tumors (for recent reviews see [5, 12]).

Various proto-oncogenes, including c-Src, and GSK-3/ beta-catenin/ Wnt signaling have previously been described to be actively implicated in the regulation of colorectal tumors [13-16]. Since a prominent biological response to mAR activation in colon tumors is apoptotic regression [9, 10], we have addressed in the present study the potential influence of membrane-initiated testosterone effects on the activity and/or expression of specific proto-oncogenes. Focussing on the non-receptor protein tyrosine kinase c-Src and the GSK-3/ beta-catenin pathway, we report here that mAR activation significantly down-regulates the activity of c-Src and induces GSK-3beta phosphorylation and down-regulation of beta-catenin and cyclin D1 gene expression. These results provide novel mechanistic insight into testosterone-induced mAR actions regulating expression of genes implicated in colorectal tumor progression.

## Materials and Methods

### *Cell cultures*

The Caco2 human colon cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM high glucose medium (Gibco, USA) containing 10% FBS and 1% penicillin/streptomycin. Based on previous titration experiments [9] we have used a  $10^{-7}$  M testosterone-HSA or testosterone-BSA (TAC) concentration for mAR stimulation throughout this study. Before each experiment TAC was 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 3,000 x g and passed through a 0.45  $\mu$ m filter to remove any potential contamination with free steroid. This is a necessary step to disconnect any possible intracellular testosterone and/or iAR interference with the effects mainly induced by the mAR activation.

### *Western blotting*

Cells were incubated with  $10^{-7}$  M TAC for the indicated time periods, washed twice with ice-cold PBS and suspended in 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 a protease inhibitor cocktail (Roche). The protein concentration was determined using the Bradford assay (BioRad). Sixty  $\mu$ g of protein were solubilized in sample buffer at 95 °C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting, proteins were electro-transferred onto a PVDF membrane and blocked with 5% non-fat milk in TBS-0.10% Tween 20 at room temperature for 1 h. Then, the membrane was incubated with anti-phospho-c-Src (Tyr-416; 1:1000, Cell Signaling, USA), anti-c-Src (1:1000, Cell Signaling, USA), anti-phospho-GSK-3beta (Tyr-216; 1:1000, Cell Signaling), anti-GSK-3beta (1:1000, Cell Signaling, USA), phospho-p38 MAPK (Thr180/Tyr182; 1:1000, Cell Signaling), or p38 MAPK (1:1000; Cell Signaling) at 4 °C overnight. After washing

(TBST) the blot was incubated with secondary anti rabbit antibody (1:2000, Cell Signaling) for 1 h at room temperature. Antibody binding was detected with the ECL detection reagent (Amersham, Germany).

#### RT-PCR analysis

To determine beta-catenin and cyclin D1 transcript levels, total RNA was extracted from Caco2 cells in TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After DNase digestion reverse transcription of total RNA (2,5µg) was performed using random hexamers and GoScript Reverse Transcription System (Promega, USA). Real-time polymerase chain reaction (RT-PCR) of the respective genes were set up in a total volume of 20 µl using 500 nM forward and reverse primer and 2x GoTaq qPCR Master Mix (Promega, USA) according to the manufacturer's protocol. Amplification of the house-keeping gene GAPDH was performed to standardize the amount of sample RNA. Cycling conditions were chosen as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 sec, 59 °C for 15 sec and 72 °C for 30 sec.

For the amplification the following primers were used (5'->3' orientation):

beta-catenin (CTNNB1)

Fw: ACTACTGTGGACCACAAGCAGAG

Rev: AGTCCCAAGGAGACCTTCCATC

GAPDH:

Fw: TGAGTACGTCGTGGAGTCCACTG

Rev: GGTGCTAAGCAGTTGGTGGTG;

cyclin D1 (CCDN1)

Fw: AAGCTCAAGTGGAACCTGGC

Rev: TCTGGAGAGGAAGCGTGTGAG

#### Confocal laser scanning microscopy

For beta-catenin staining, cells were cultured on glass cover slips with TAC or controls without TAC for different time periods, as 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-beta-catenin (1:200, Cell Signaling, 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) 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, USA).

#### Statistical analysis

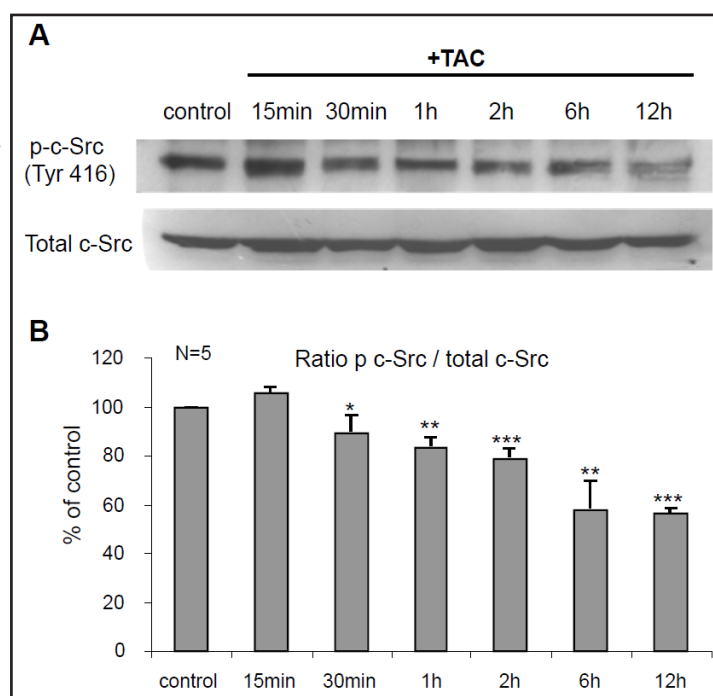
Data are provided as means ± SEM, *n* represents the number of independent experiments. Data were tested for significance using unpaired student's *t*-test or ANOVA as appropriate. Differences were considered statistically significant when *p*-values were < 0.05. Statistical analysis was performed with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

## Results

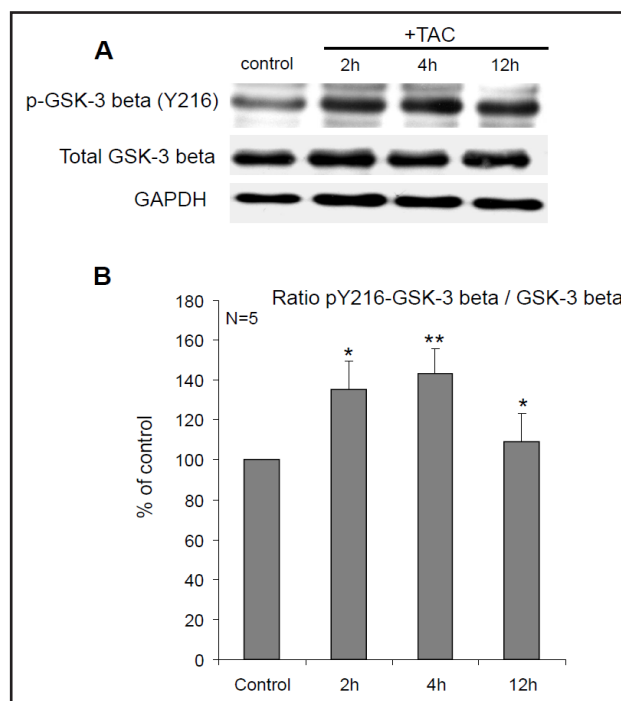
#### TAC down-regulates c-Src phosphorylation

c-Src is a non-receptor protein tyrosine kinase highly expressed in colon tumors [15]. Elevated levels of c-Src activity have been linked to cancer progression and metastasis (for a recent review see [14]). We have addressed the effect of testosterone conjugates-induced mAR stimulation in c-Src activity by analyzing the time dependent Tyr-416 phosphorylation profile of c-Src in Caco2 colon tumor cells. Figure 1 reveals significant, time-dependent down-regulation of c-Src phosphorylation that became evident 30 min upon mAR activation and persisted for at least 12 hours.

**Fig 1.** Effects of TAC on c-Src phosphorylation. Caco2 cells were stimulated with  $10^{-7}$  M TAC for the indicated time periods. Following cell lysis equal amounts of total lysates were immunoblotted with a specific antibody against phospho-c-Src (Tyr-416) and total c-Src. Immunoblots were analyzed by densitometry. A. Original Western blots of phosphorylated c-Src (p-c-Src Tyr416) and total c-Src in CaCo2 cells without (control) or with TAC (100nM) treatment. B. Average  $\pm$  SEM (n=5) of the ratio of p-c-Src to total c-Src in CaCo2 cells without (control) or with TAC (100nM) treatment, untreated cells were taken as 100%. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, (Student's t-test).



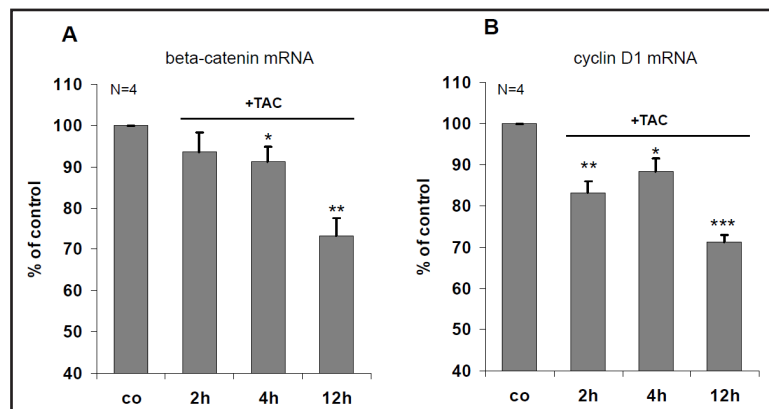
**Fig 2.** Effects of TAC on GSK-3beta phosphorylation. Caco2 cells were stimulated with  $10^{-7}$  M TAC for the indicated time periods. Following cell lysis equal amounts of total lysates were immunoblotted with a specific antibody against phospho-GSK-3beta (Tyr 216) and total GSK-3beta. Immunoblots were analyzed by densitometry. A. Original Western blots of phosphorylated GSK-3beta (p-Tyr-216 GSK-3beta) and total GSK-3beta in CaCo2 cells without (control) or with TAC (100nM) treatment. B. Average  $\pm$  SEM (n=5) of the ratio of p-Tyr-216-GSK-3beta to total GSK-3beta in CaCo2 cells without (control) or with TAC (100nM) treatment, untreated cells were taken as 100%. \*p<0.05, \*\*p<0.01 (Student's t-test).



#### *TAC induces late GSK-3beta phosphorylation*

Glycogen synthase kinase 3 (GSK-3) is a serine / threonine protein kinase shown to participate in a number of apoptotic signaling pathways [17]. Activation of the PKB/Akt pro-survival signaling down-regulates GSK-3 activity [18, 19]. Since mAR activation in colon tumors was shown to induce late down-regulation of PI-3K and Akt activity, both *in vitro* and *in vivo* [10], we sought to define whether GSK-3 phosphorylation is modified after long-term TAC-treatment in colon cancer cells. Figure 2 presents phosphorylation levels of residue Tyr-

**Fig 3.** Time dependent effects of TAC on beta-catenin and cyclin D1 gene transcription. Caco2 cells were stimulated with  $10^{-7}$  M TAC for the indicated time periods. Analysis of beta-catenin, (A) and cyclin D1, (B) mRNA levels by quantitative real-time PCR showing the effects of TAC in Caco2 cells. Bars indicate the mean values of  $2^{-\Delta Ct}$  using GAPDH as housekeeping gene  $\pm$  SEM from n=4 independent experiments, untreated cells were taken as 100%. \*P<0.05, \*\*p<0.01, \*\*\*P<0.001 (Student's t-test).



216 of GSK-3. Interestingly, mAR activation induced statistically significant Tyr-216 GSK-3 phosphorylation after 2 to 12 hours treatment by TAC. This observation points to activation of the kinase that is in line with the reported disruption of PI-3K/Akt activation and the potent pro-apoptotic response of Caco2 cells to TAC treatment.

#### *TAC induces down-regulation of beta-catenin and cyclin D1 gene transcription*

GSK-3 has been shown to phosphorylate  $\beta$ -catenin, thus triggering its degradation by the proteasome. In addition, overexpression of beta-catenin and  $\beta$ -catenin/Wnt signaling is associated with many cancers including colorectal carcinomas [13, 16, 20, 21]. Accordingly, we tested whether mAR activation alters beta-catenin gene expression. As shown in Figure 3A, RT-PCR analysis revealed a significant, late down-regulation of beta-catenin gene transcripts in cells treated with TAC, indicating that mAR activation down-regulates beta-catenin gene expression in colon tumor cells. Since beta-catenin is known to regulate cyclin D1 expression in colon carcinoma cells [22] we further tested cyclin D1 gene transcription in TAC-treated Caco2 cells. Figure 3B reveals significant late down-regulation of cyclin D1 gene transcripts. These findings are in agreement with the anti-tumorigenic action of mAR stimulation in colon cancer cells.

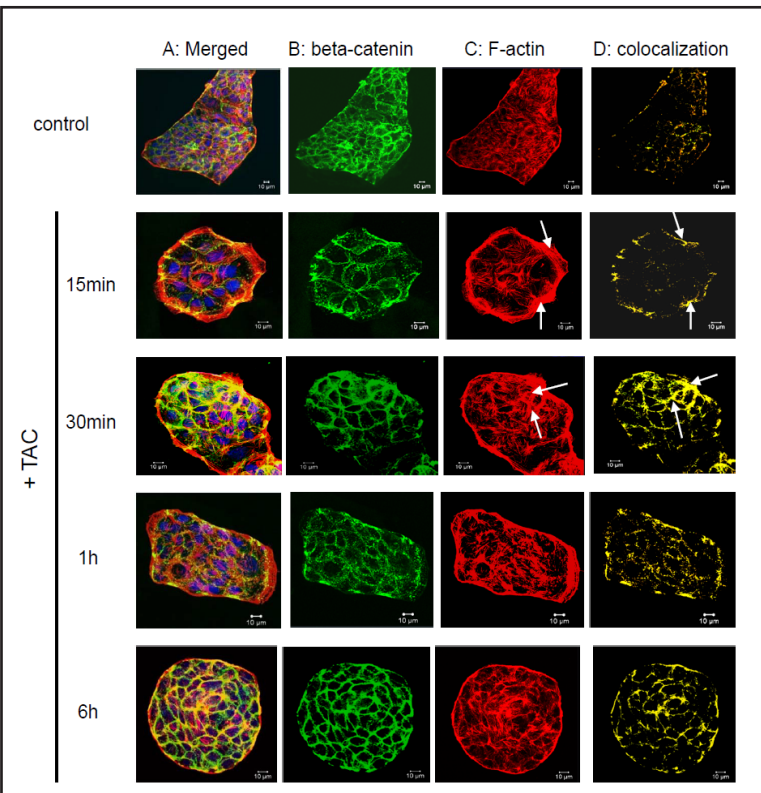
#### *Co-localization of beta-catenin with actin microfilaments upon TAC treatment*

Previous studies have shown that mAR activation generates robust actin cytoskeleton reorganization in colon tumor cells [9]. In addition, beta-catenin has been shown to interact with the cortical actin cytoskeleton in various cell models [23, 24]). Accordingly, using confocal laser scanning microscopy we further analysed whether actin may co-localize with beta-catenin in Caco2 cells. In line with previously reported observations [9, 11], figure 4 demonstrates the profound redistribution of actin structures (actin staining, red) in mAR stimulated cells that became evident as early as 15 minutes after TAC treatment. Interestingly, 30 minutes after TAC treatment a clear co-localization of beta-catenin with actin was observed (Fig. 4); this effect persisted for at least 6 hours. Although the mechanism of mAR-dependent sequestration of beta-catenin to actin is not clear at this stage, this effect most likely reflected formation of an actin meshwork resulting in mAR-dependent reduction of cell motility as reported by others [25].

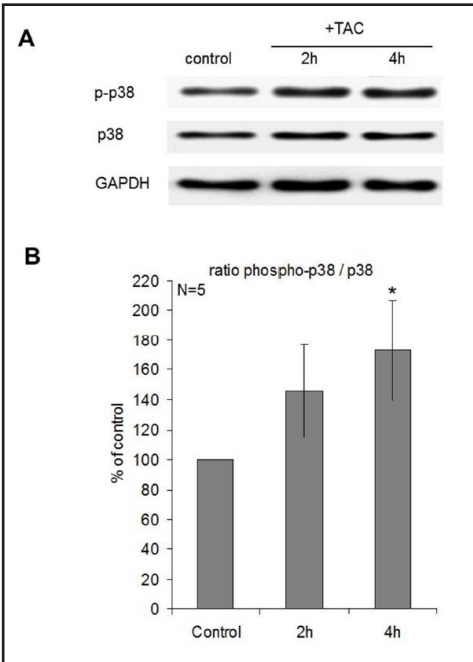
#### *TAC induces significant late p38 MAPK phosphorylation*

p38 MAPK is a mediator of apoptosis in response to a number of cellular signals (for a recent review see [26]). Recently, up-regulated p38 MAPK phosphorylation has been reported in mAR activated breast cancer cells [27]. In line with these reports, our observations indicated that long term (2 and 4 hours) treatment of Caco2 colon cancer cells

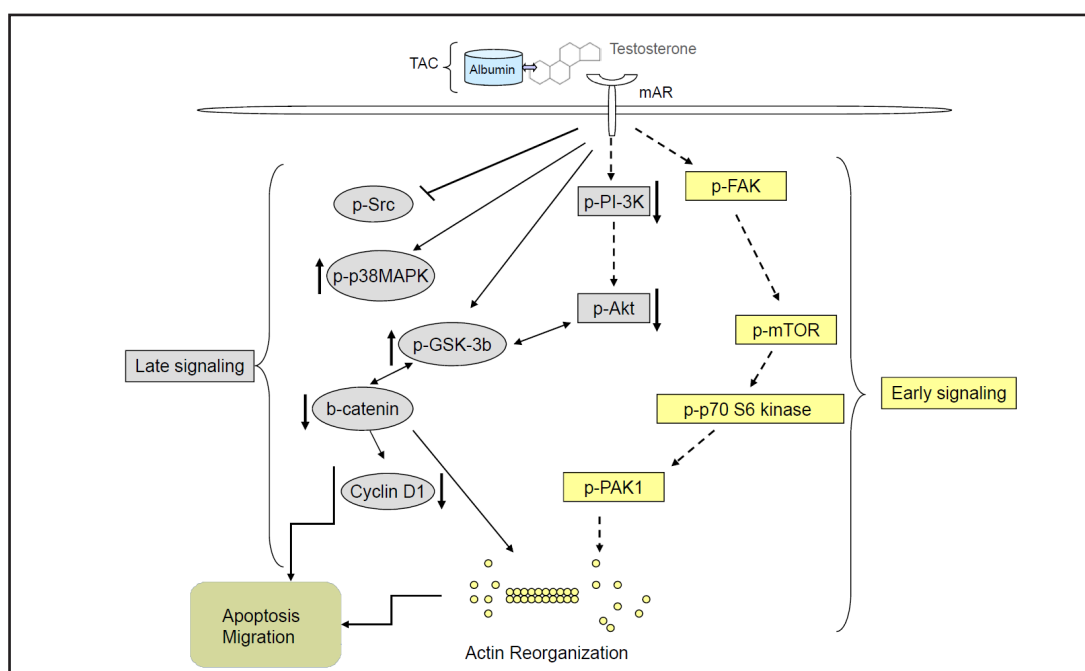
**Fig 4.** Co-localization of beta-catenin with actin microfilaments upon TAC treatment. 24h serum-starved cells were stimulated with  $10^{-7}$  M TAC for the indicated time periods. A. Triple staining micrographs of the cells, stained with anti-beta-catenin (green), rhodamine-phalloidin for filamentous actin (red) and DRAQ5<sup>TM</sup> (blue) for nuclei. Confocal laser scanning microscopy analyzed samples. Magnification  $\times 100$  (Bars: 10 $\mu$ m). B. Cells stained with anti-beta-catenin (green): Confocal laser scanning microscopy analyzed samples. Magnification  $\times 100$  (Bars: 10 $\mu$ m). C. Cells stained with rhodamine-phalloidin for filamentous actin (red). Confocal laser scanning microscopy analyzed samples. Arrows indicate the actin reorganization areas. Magnification  $\times 100$  (Bars: 10 $\mu$ m). D. The co-localization area (yellow) by staining with anti-beta-catenin and rhodamine-phalloidin for filamentous actin. Confocal laser scanning microscopy analyzed samples. Magnification  $\times 100$  (Bars: 10 $\mu$ m).



**Fig 5.** Effects of TAC on p38 MAPK phosphorylation in Caco2 cells. Caco2 cells were stimulated with  $10^{-7}$  M TAC for the indicated time periods. Following cell lysis equal amounts of total lysates were immunoblotted with a specific antibody against phospho-p38 (Thr-180,Tyr-182) and total p38. Immunoblots were analyzed by densitometry. A: Original Western blots of phosphorylated p38 (Thr-180,Tyr-182) and total p38 in CaCo2 cells without (control) or with TAC (100nM) treatment. B: Average  $\pm$  SEM (n=5) of the ratio of phosphor-p38 to total p38 in CaCo2 cells without (control) or with TAC (100nM) treatment, untreated cells were taken as 100% . \*p<0.05 (Student's t-test).



with TAC significantly increased p38 MAPK phosphorylation (Fig. 5). This finding provides strong evidence that p38 MAPK is implicated in mAR-induced effects in colon tumor cells and fully supports previous observations.



**Fig 6.** A model summarizing the current findings on mAR-induced signaling targeting specific pro-survival and tumorigenic gene products in Caco2 cells. Late signaling targets upon mAR stimulation by TAC are presented in grey; yellow signifies early, mAR-stimulated signaling targets. Full arrows signify novel mAR-stimulated signaling events presented in this study. Dotted arrows signify already known mAR-induced early and late signaling events in colon tumors.

## Discussion

In the present study we report that mAR stimulation by TAC significantly decreases c-Src non-receptor tyrosine kinase activity and beta-catenin and cyclin D1 gene transcription in colon tumor cells pointing to down-regulation of these important proto-oncogenes. Moreover, GSK-3 $\beta$  was phosphorylated, while beta-catenin was shown to co-localize with actin structures, implying that beta-catenin may participate in the reported modulation of cytoskeletal dynamics in mAR stimulated cells. These findings, addressing long-term mAR effects (Fig. 6), provide novel experimental evidence further supporting the anti-tumorigenic activity of mARs in colon cancer cells. It should be pointed out that these molecular responses were initiated using the non-permeable androgen derivatives (testosterone albumin conjugates, TAC), which cannot interact with iAR in colon tumor cells, as convincingly shown in previous studies [9-11], and elucidate specific, mAR-triggered effects.

The non-receptor protein tyrosine kinase c-Src is highly expressed in colorectal tumors and actively participates in malignant transformation of those tumors [14, 15, 28]. Elevated levels of c-Src activity foster cancer progression and metastasis by promoting either pro-survival signaling pathways, including PI-3K/Akt, or affecting the activity of tumor suppressor molecules such as PTEN [29]. Moreover, c-Src activity modulates regulators of tumor cell motility, including the FAK/paxillin pathway, or the Rho-GTPases/actin signaling (for recent reviews see [30, 31]). c-Src has also been shown to induce PI-3K-independent Akt activation in various cancers [32]. Our findings showing profound de-phosphorylation of c-Src upon mAR stimulation in colon cancer cells are in line with the previously reported late down-regulation of Akt signaling and the induction of pro-apoptotic responses [10], as well as the inhibition of cell migration [33]. The role of c-Src dependent signalling in the regulation of Tyr-216 phosphorylation/activation of GSK-3 remains to be elucidated.

In line with these findings we further demonstrate mAR-induced down-regulation of the beta-catenin gene expression. Mutations and overexpression of beta-catenin are associated with many tumors, including colon cancer [34]. Recently, physical association of Smad7 and beta-catenin was found to be important for TGFbeta-induced apoptosis of prostate cancer cells [35], while beta-catenin was reported to confer resistance to PI-3K and AKT inhibitors, promoting metastasis in colon cancer [16]. Moreover, we demonstrate the downregulation of cyclin D1 transcription of mAR stimulated colon cancer cells. Cyclin D1 is a major regulator of the cell cycle that controls the progression of cells into the proliferative stage [36] and its transcription has previously been reported to be regulated by beta-catenin in colon carcinomas [22, 37]). Cyclin D1 over-expression is associated with beta-catenin activation in carcinogenesis [38].

We further report the association of beta-catenin with actin in mAR activated colon cancer cells. This association is most likely the result of a mAR-induced reduction in cell motility [33] and has been observed in other epithelial cells in response to actin meshwork activating signaling triggered by Rac1 [25]. Candidate mediators of mAR-induced beta-catenin association to actin could be E-cadherin [25], although interactions via additional cytoskeletal components such as the microfilament-bundling protein fascin [24]) cannot be excluded. Since actin reorganization is a main cellular event in mAR-induced downstream signaling that regulates pro-apoptotic responses [5, 39] the mechanisms linking beta-catenin to TAC induced actin cytoskeleton restructuring are of particular interest. We are currently addressing this issue in our laboratory.

GSK-3beta, being part of the canonical beta-catenin/Wnt pathway, is implicated in mechanisms governing cell proliferation and apoptosis [17, 40, 41]. Through phosphorylation of its target proteins GSK-3beta can promote apoptosis either by activating pro-apoptotic factors such as p53 [42] or by inactivating pro-survival molecules [43]. In various human cancers GSK-3beta may be effective either as tumor suppressor or as tumor promoter (for recent reviews see [44-46]). Our findings showed a significant late phosphorylation of GSK-3beta at Tyr-216 that was reported to enhance the enzymatic activity of this kinase [47]. In line with this, we observed as well a late significant phosphorylation of the Thr-390 residue of GSK-3beta (data not shown), further supporting activation of this kinase. This result, taken together with the down-regulation of beta-catenin transcription, points to a pro-apoptotic activity of GSK-3beta and is in agreement with the reported late down-regulation of Akt phosphorylation and the concomitant apoptotic response demonstrated in TAC-treated colon cancer cells [9, 10]. Indeed, activated Akt phosphorylates serine-9 of GSK-3beta, which inhibits its kinase activity [18, 19, 48]. Moreover, previous studies have revealed that hyperactive GSK-3beta contributes to cell death [49, 50] and that the pro-apoptotic action of GSK-3beta may be partly attributable to the regulation by GSK-3beta of the activities of transcription factors that play prominent roles in the determination of cell fate, including beta-catenin [51]. However, how mAR-induced GSK-3beta phosphorylation may regulate beta-catenin degradation is still unknown. One may consider that GSK-3beta may be involved in E3 ubiquitin ligase activation, which in turn controls cellular beta-catenin levels by ubiquitinylation and proteasomal degradation. Further studies are now required to address this issue.

## Conclusions

Our work provides novel mechanistic insights that may govern the potent pro-apoptotic activity of mAR activation in colon tumor cells. These findings are summarized in the cartoon of Figure 6. Collectively, we demonstrate significant down-regulation of the proto-oncogenes c-Src and beta-catenin, decrease of the cyclin D1 gene transcription, late activation through phosphorylation of GSK-3beta and p38MAPK and finally beta-catenin/actin cytoskeleton interactions. These results indicate that mAR activation is targeting specific pro-survival,

tumorigenic and tumor suppressor gene products and offer potential novel molecular targets for the development of mAR-specific agents.

### Disclosure Statement

The authors declare that they have no competing interests towards any aspect of the work described in this paper.

### Acknowledgements

Authors would like to thank Tanja Loch for the careful preparation of the manuscript. We are also grateful to Dr. E.A. Papakonstanti for noteworthy comments during the preparation of the manuscript draft. This work was supported by the Deutsche Forschungsgemeinschaft (GRK 1302, SFB773, Mercator program) Open Access Publishing Fund of Tuebingen University; University of Crete Research Committee (Grant number KA3452); Deanship of Scientific Research at King Saud University; (Research Group Project No. RGPVPP-018).

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