**TITLE PAGE**

## Testosterone Rescues the De-Differentiation of Smooth Muscle Cells through Serum Response Factor/Myocardin

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**Running Title**: Testosterone modulates *Srf/Myocd* in the prostate

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**ABSTRACT**

In the last years it has been suggested that androgens exert differentiating effects on smooth muscle cells (SMCs) but the mechanisms underlining remain unknown. The myodifferentiation is controlled by the SRF/MYOCD complex in cardiac and vascular smooth muscle, with little information in others sites. The aim of the present study was to analyze if SRF/MYOCD is involved in prostatic SMCs (pSMCs) differentiation and to determine the role of testosterone in the modulation of the pSMCs phenotype through the SRF/MYOCD gene program.

Firstly it was demonstrated that testosterone up-regulates *Srf/Myocd*mRNA pSMCs in a dose-dependent manner by stimulating primary cultures of pSMCs with different dose of testosterone (10-12 to 10-5M for 24h). This data was correlated with the increase of the SMC-specific genes *Acta2*, *Cnn1* and*Lmod1*and the decrease in the mesenchymal marker *Vim*. Moreover, the inhibition of *Myocd* or *Srf*  with specific siRNAs avoided the myodifferentiating effects of testosterone.

On the other hand, pSMCs were dedifferentiated by the endotoxin LPS 1ug/ml for 48h. This resulted in a down-regulation of *Srf/Myocd* and lead to a dedifferentiated phenotype (characterized by decreased mRNA and protein levels of *Acta2* and *Cnn1*and increased*Vim*compared to controls). To gain insight into the involvement of *Myocd* in SMC dedifferentiation under this proinflammatory conditions, the HCASM cell line was transduced with adenovirus-Myocd and then treated with LPS or TNF for 48h as dedifferentiator stimuli. The overexpression of *Myocd* resulted in a significant induction in *Acta2, Cnn1,*and *Lmod1* mRNA expression, with no significant differences being found in these markers when these cells were stimulated with LPS or TNF.

Finally, pSMCs were challenged with LPS for 48h and then treated with testosterone for additional 48h. The androgen was able to restore the muscular phenotype by normalizing Srf/*Myocd*mRNA levels. At protein level testosterone reestablished the expression of smooth muscle markers. Electron microscopy corroborated the dedifferentiation after LPS stimulation with the development of a secretory profile; meanwhile, testosterone reverted these changes leading to acquire muscular features. In addition, the androgen restored the proliferation rate and Il6 secretion incremented by LPS.

These results provide novel evidence regarding the differentiating role of testosterone on SMCs by modulating *Srf/Myocd*, highlighting that androgens preserve prostatic SMC phenotype, which is essential to maintain the normal structure and function of the prostate.

**INTRODUCTION**

Smooth muscle cells (SMCs) play a key role in contractility, structure and function of the cardiovascular, pulmonary, digestive, and genitourinary systems. The differentiation of SMCs is controlled by transcriptional activation of distinct sets of genes regulated by Serum Response Factor (SRF), a ubiquitous transcription factor that binds to the DNA consensus sequence CC(A/T)6GG, known as CArG box(1). SRF is not SMC-specific but it is modulated by co-regulators that confer cell-specific gene expression. For example, myocardin *(Myocd)* is an SRF co-activator expressed specifically in SMCs and cardiac muscle cell lineages(2). *Myocd* interacts physically with *Srf,* inducing the promoters of SMC-restricted, CArG-dependent genes (2). Increasing evidence implicates *Myocd* as a major player in the molecular switch for SMCs differentiation program. Indeed, a reduction of *Myocd* levels induces attenuation or diminution of smooth muscle phenotype markers in different cells (3, 4).

It is well-stablished that prostatic SMCs (pSMCs) have a pivotal role in the homeostasis of the prostate gland through stromal-epithelial interactions. To this end, normal pSMCs establish communication with the epithelium by secreting growth factors that regulate proliferation, differentiation, and cell death in both epithelial and stromal compartments (5, 6), with the preservation of a differentiated status being critical for the prostate. Under normal conditions pSMCs are characterized by a low rate of proliferation and the expression of a defined cyto-contractile gene program. However, during the development of prostate pathologies, pSMCs change from a quiescent, contractile to a proliferative and secretory phenotype (7, 8). This is particularly clear in prostate cancer and benign prostatic hyperplasia where the stromal compartment of the gland acquires the form of a “reactive stroma” with myofibroblasts being the most aggressive cell in those scenarios (9-11). Additionally, we demonstrated that pSMCs respond to inflammatory stimuli with myofibroblast-like phenotypic alterations, including a decrease in smooth muscle markers and the development of proteinpoyetic organelles *in vivo* (12) as well as *in vitro* (13). Interestingly, loss of the SMC differentiation has been reported to contribute to the maintenance or aggravation of different inflammatory conditions such as atherosclerosis (14, 15) and asthma (16).

Prostatic homeostasis relies on biological activities sustained by androgens (17). pSMCs express androgen receptors (18, 19), but the effects of androgens on the phenotype and physiology of these cells remain poorly understood, with often contradictory findings (20). Even though the preservation of the SMC differentiation is considered a critical issue in the pathophysiology of the prostate, the expression and the role of SRF and its co-activator MYOCD in pSMCs have not been studied yet. We previously described that testosterone contributes to the preservation of a contractile phenotype in pSMCs by reducing the proinflammatory response to bacterial LPS (21), suggesting that androgens have myodifferentiating effects in the prostate. Therefore, the aim of the present study was to analyze if SRF/MYOCD is involved in pSMCs differentiation and to determine the role of testosterone in the modulation of the pSMCs phenotype through the SRF/MYOCD gene program.

**MATERIALS AND METHODS**

Animals

Twelve week-old adult male rats of Wistar strain, weighing 250-350 g, were housed at the Animal Research Facility of the National University of Córdoba in air-conditioned quarters, under a controlled photoperiod (14-h light/10-h darkness) with free access to commercial rodent food and tap water. Animal care and experiments were conducted following the recommendations of the Guidelines of NHI for the Care of and Use of Laboratory Animals, 1996 and approved by a local Institutional committee.

Primary Prostate Smooth Muscle Cell cultures and treatments

Prostate specimens were obtained from six Wistar rats per culture. The cell dissociation and cell cultures have been previously described elsewhere in detail (13). Briefly, tissues were minced into small fragments and treated for 30 min with a digestion solution containing 200U/ml collagenase type IA (Sigma Aldrich, St. Louis, MO, USA) and 0.05% deoxyribonuclease type I (Sigma) in minimal essential medium SMEM (Sigma). Then, dispersed cells were washed 3 times with SMEM, collected by centrifugation for 2 min at 1000xg and finally resuspended and adjusted to 1 x 106 cell/ml. Then, cells were seeded on 6-well culture plates (Corning, New York, USA) at a density of7.5 x 105 cells/well and cultured in the SMC medium MCDB131 (Sigma), supplemented with 10% heat-inactivated fetal calf serum and 10% horse serum (Gibco, Invitrogen, Carlsbad, CA) in a humidified incubator at 37 ºC supplied with 5% CO2 in air. The culture medium was replaced daily for 6 days; after which, it was replaced by serum free medium supplemented with 5ug/ml insulin, 5ug/ml transferrin, 5ng/ml selenite, and 2ng/ml TGFβ1 (Invitrogen, Carlsbad, CA).

In order to analyze the influence of testosterone on the expression of *Srf/Myocd* and smooth muscle-specific genes, cells were treated with testosterone 0, 10-5, 10-7, or 10-12M (in ethanol at a final concentration of 0.01%) by 24h. On the other hand, pSMCs were incubated with LPS (Sigma) (1µg/ml) for 48h as a dedifferentiator stimulus to determine the *Srf/Myocd* and smooth muscle-specific genes levels. Moreover, in order to evaluate if testosterone reverses the dedifferentiation induced by LPS, pSMCs were treated with testosterone 10-7 or 10-12M for additional 48h after LPS challenge. A control group consisted in cells that were treated with vehicle instead of LPS.

Cell cultures and *srf/myocd* manipulation

Multiple independent isolates of human coronary artery smooth muscle cells (HCASM) were purchased from Invitrogen and maintained in medium 231 with growth supplements as provided by the manufacturer. For adenoviral transduction, HASMC were transduced with 100 moi of adenoviral control or *Myocd* for 24 to 48 h. Viral particle titer was determined by plaque assay. For Small Interfering RNA transfections*,*siRNA to SRF was purchased from Ambion (4392420)Thermo Scientific. Additionally, the ON-TARGET plus SMART pool siRNA to human MYOCD (NM\_153604) was from Dharmacon siRNA Technologies. A scrambled siRNA duplex was used for negative control. Lipofectamine 2000(Invitrogen, Frederick MD USA) was used to deliver siRNA according to manufacturer’s instructions. Following overnight siRNA transfection, cells were re-fed with fresh growth medium for 24 h before treatment as indicated. RNA was extracted to determine knockdown efficiency by qPCR.

RNA extraction, cDNA synthesis and RT-PCR and quantitative RT-PCR analysis.

Total RNA was purified using Direct-zol RNA miniprep kit (Zymo Research) accordingly to manufacture´s instruction. Thereafter, 1 μg of total RNA was used as a template for reverse transcription using EpiScript™ Reverse Transcriptase (Epicentre) using random hexamer primers (Fermentas).

The cDNA obtained was subjected to PCR amplification using GoTaq DNA Polymerase (Promega, Madison, WI) and following PCR protocols was perfomed following manufacture´s instruction. The PCR products were visualized in 2% agarose gels and ethidium bromide staining. To semiquantify and compare cDNA levels, the gels were photographed,

Quantitative PCR analysis was performed on an ABI Prism 7500 detection system (Applied Biosystem) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) as previously reported (22). The gene-specific primer sets were obtained from IDT and described in table 1. Relative changes in gene expression were calculated using the 2-ΔΔCt method normalized against the housekeeping gene *Actb or 18s*. For each pair of primers, a dissociation plot resulted in a single peak, indicating that only one cDNA species was amplified. Amplification efficiency for each pair of primers was calculated using standard curves generated by serial dilutions of cDNA obtained from unstimulated pSMCs cells.

Immunofluorescence

pSMCs attached to coverslips were fixed with 4% formaldehyde, permeabilized with 0.25% Triton X-100 in PBS and incubated for 1 h in 5% PBS-BSA to block non-specific binding. Slides were incubated overnight at 4ºC in a humidified chamber with the following primary antibodies: anti-alpha smooth muscle actin (ACTA2) (1/50 mouse monoclonal, Novocastra, Newcastle, UK), anti-vimentin (1/100 mouse monoclonal, Novocastra), anti-CNN11/500 mouse monoclonal, Thermo Scientific, Rockford, IL). Afterwards, the slides were washed three times with PBS and further incubated with Alexa 488- or Alexa 546-conjugated anti-mouse secondary antibody (1/1000 Invitrogen, Frederick MD USA) for 1h and mounted using fluoromount containing DAPI.

To validate the specificity of the immunostaining, controls were performed by applying the same protocol but replacing the primary antibody with 1 % PBS-BSA. Images were then obtained using an inverted confocal laser scanning microscope FluoView FV 1000 (Olympus; Tokyo, Japan).

Western blotting

The pSMCs cultures were washed with ice-cold PBS and harvested in 120 µl cold PBS containing 1.25% Igepal CA-630, 1mM EDTA, 2mM PMSF, and 10µg/ml leupeptin and 10µg/ml aprotinin. The lysate was centrifuged at 14,000xg for 20 min at 4 ºC to pellet the Igepal CA-630-insoluble material and the supernatant was stored in aliquots frozen at -80 ºC until required. The total protein concentration was measured with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA).Denatured protein samples (30µg/lane) were then separated on 12% SDS polyacrylamide gel and blotted onto a Hybond-C membrane (Amersham Pharmacia, Buckinghamshire, UK) with incubation steps being performed in 5% defatted dry milk in PBS/0.1% Tween 20. Membranes were rinsed and incubated for 3 h 1/200 mouse anti-ACTA2 (Novocastra), 1/300 mouse anti-Vimentin (Novocastra), 1/500 mouse anti-calponin (Thermo Scientific). After washing, the blots were incubated with a peroxidase-conjugated (HRP) goat anti-mouse (Jackson Immunoresearch, West Grove, PA) secondary antibody, and revealed with an Enhanced Chemiluminescence detection system (ECL, Amersham Biosciences, Buckinghamshire, UK) following the manufacturer’s instructions. Emitted light was captured on Hyperfilm (Amersham Pharmacia) and densitometry analysis was performed using ImageJ (NHI, Bethesda, MD). The relative expression was compared among different treatments by taking the control group value as a reference. The expression of ACTB (1/5,000; monoclonal anti-αactin; Sigma) was used as an internal control to confirm equivalent total protein loading.

ELISA

In order to quantify IL6 secretion by pSMCs, 1 ml of medium was collected from the plates, centrifuged at 4 ºC at 1400 rpm for 15 min and stored at -20ºC. The secretion was measured by a commercially available sandwich ELISA kit (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer´s instructions.

Proliferation Assay

The cells attached to the coverslips were fixed with 4 % formaldehyde in PBS for 30 min at room temperature, washed in PBS, and permeabilized with 0.5% Triton X-100 for 10min. Non-specific immunoreactivity was blocked with 1% PBS-BSA for 30 min at RT, and the cells were incubated overnight with a monoclonal antibody against Ki-67 (1/50) (BD-Pharmigen) at 4 °C in a wet chamber. After washing in PBS, the cells were incubated with a biotinylated anti-mouse IgG diluted 1/100 (GE Healthcare; Buenos Aires, Argentina). The coverslips were washed again with PBS, and the pSMCs were incubated with the avidin-biotinperoxidase complex (ABC; Vector; Burlingame, USA). The immunoreactivity was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. A total of 3000 immunoreactive cells were examined by light microscopy in randomly chosen fields of each glass slide, in order to establish the percentage of ki-67 immunoreactive cells. Three slides were analyzed for each group, derived from the same cell preparations. Experiments were replicated at least three times with separate batches of cell preparations.

Statistical analysis

Data from more than two groups were analyzed using analysis of variance (ANOVA) with Fisher as the post-test. Statistical testing and calculation of western blot data were performed using the InStat V2.05 software from GraphPad, Inc.

**RESULTS**

***Srf/Myocd* is expressed in pSMCs and it is modulated by testosterone.**

Considering that SRF/MYOCD is the main system involved in cardiac and smooth muscle differentiation (3), we first aimed to analyze its expression in rat pSMCs. As shown in Fig. 1A, pSMCs expressed *Srf* and *Myocd* mRNAs, although at lower levels than other SMCs from vessels or bladder.

To evaluate the effect of androgens on *Srf/Myocd* expression, pSMCs were treated with different doses of testosterone (10-5, 10-7, and 10-12M) for 24h. The mRNA levels of *Srf* and *Myocd* were significantly increased in cells stimulated with testosterone 10-5 M and10-7 M compared to those treated only with vehicle (Fig. 1B, C). This upregulation in *Srf/Myocd* was dose-dependent since testosterone 10-12 M did not yield significant differences.

Considering that the SRF/MYOCD switch strongly induces the expression of smooth muscle-specific genes, we next analyzed the mRNA levels of*Acta2* and *Cnn1*as parameters of smooth muscle differentiation in testosterone-treated pSMCs. As expected, the increase in *Srf/Myocd* expression was followed by an increment in the levels of*Acta2* and *Cnn1*in response to testosterone treatment (Fig 1D and E), suggesting that testosterone has a myodifferentiator role on pSMCs.

**Inflammatory stimuli induce smooth muscle dedifferentiation by down-regulating *Myocd*.**

We previously reported that pSMCs lose their normal contractile phenotype under an inflammatory environment *in vivo (12)*. A similar dedifferentiation process was observed *in vitro* treating cultured primary pSMCs with the proinflammatory agent LPS (13). Here, we analyzed the expression of *Srf/Myocd* in LPS-dedifferentiated pSMCs. As shown in Fig. 2, primary cultures of pSMCs down-regulated the mRNA of both *Srf* (fig. 2A) and *Myocd* (Fig. 2B) after 48 h of LPS stimulation. The low expression of traditional (*Acta2, Cnn1*) (Fig. D) and novel (*Lmod1*(23)) (Fig. 2C) proposed markers of SMC differentiation, along with the induction of *Vim* confirmed the loss of the contractile phenotype of pSMCs after LPS treatment (Fig. 2D). Complimentary, we carried out immunofluorescence analysis to confirm the loss of smooth muscle phenotype -as indicated by reduced intensity of ACTA2 and CNN1and increase of VIM- in response to LPS treatment (Fig. 2E).

To gain insight into the involvement of *Myocd* in SMC dedifferentiation under proinflammatory conditions, the HCASM smooth muscle cell line was transduced with adenovirus carrying Myocd (Myocd-HCASM) and then treated with LPS or TNF as dedifferentiator stimuli. As expected, adenoviral transduction with *Myocd* resulted in a significant induction of *Myocd* mRNA levels compared to empty virus-transduced cells (Fig. 3A). Moreover, *Myocd* overexpression led to a >10-fold increase in *Acta2* mRNA expression (Fig. 3B) and to a 2-to-3-fold increment of *Cnn1* and *Lmod1*mRNA levels (Fig. 3C and D). Thereafter, we tested whether *Myocd* overexpression may be able to avoid inflammation-induced SMC dedifferentiation. When Myocd-HCASM cells were stimulated with LPS 1g/ml for 48hs, the contractile phenotype was conserved as no significant differences in the expression of *Acta2, Cnn1,* and *Lmod1*was detected (Fig. 3B-D).

Since MYOCD overexpression might affect the production of cytokine responsible for the dedifferentiator effect of LPS, we also tested our hypothesis using TNF as a direct dedifferentiating agent. As shown in Fig. 3B-D, the levels of smooth muscle markers did not decrease after TNF(5ng/ml) stimulation in Myocd-HCASM. In fact, *Acta2* and *Lmod1*expression levels were significantly higher than unstimulated cells (Fig. 3B and D). Additionally, when the mRNA for the proinflammatory cytokines *Tnf* and *Il6* were measured, they exhibited similar levels in both Ad-Empty and Ad-Myocd-transduced cells after LPS or TNF treatment (fig. 3E and F). This data suggests that MYOCD overexpression is able to avoid SMC dedifferentiation induced by an inflammatory environment, even when these MYOCD-HCASM cells are as capable of inducing cytokine mRNA after LPS or TNFasHCASM.

**Testosterone restores prostate smooth muscle phenotype.**

Since the *Srf/Myocd* switch was shown to be regulated by testosterone in normal conditions, we hypothesized that androgens could also restore *Srf/Myocd* expression in LPS-induced dedifferentiated cells. To address this, pSMCs were challenged by LPS for 48h and then treated with different doses of testosterone for additional 48h in the presence of LPS

As previously described, pSMCs stimulated with LPS for 48h exhibited lower mRNA levels of *Srf* (Fig. 4A) and *Myocd* (Fig.4B) compared to controls, and maintained low after 96 h of LPS stimulation. Since mRNA levels in both control cells (at 48 and 96h) were similar, we only show control at 96h in figures 4, 5 and 7. Interestingly, the mRNA levels of both *Srf* and *Myocd* were partially restored after treatment with 10-12M testosterone, and fully restored with 10-7M testosterone (Fig.4 A-B).

Taking into account that testosterone restored LPS-repressed *Myocd/Srf* expression; we analyzed morphological features as well as the expression of *Acta2, Cnn1*, and *Vim* at mRNA and protein level to evaluate whether testosterone-induced upregulation of *Myocd/Srf* complex is associated to a more differentiated contractile profile in pSMC. Testosterone treatment increased the mRNA levels of *Acta2* (Fig. 4C) and *Cnn1* (Fig. 4D) compared to LPS-treated cells in a dose-dependent manner. Conversely, testosterone repressed LPS-increased *Vim* mRNA expression (Fig. 4E).

At protein level, control pSMCs exhibited a well-developed contractile phenotype, characterized by high expression levels of ACTA2 and CNN1 with absence of VIM expression (Fig. 5). Significantly, LPS treatment reduced the expression of both CNN1and ACTA2, and increased the expression of VIM, indicating the induction of a myofibroblastic phenotype (Fig 5 A-C). When these dedifferentiated pSMCs were treated with testosterone 10-12M, they partially recovered the expression of ACTA2 (Fig. 5A) and CNN1 (Fig. 5B). Moreover, a complete reestablishment of the muscular phenotype was reached at the highest dose of testosterone (10-7M).

By immunofluorescence, control pSMCs displayed ACTA2-positive microfilaments, intense signal for CNN1, and were negative staining for VIM (fig. 5D). In contrast, LPS-dedifferentiated cells exhibited weak ACTA2 signal in disorganized filament with only few cells exhibiting slight expression of CNN1. pSMCs also showed a high expression of VIM after LPS stimulation. Testosterone treatment promoted a clear dose-dependent recovery of the muscular phenotype, including an intense signal for ACTA2 and CNN1 and a weak VIM intensity compared to the LPS-vehicle group.

The morphological analysis at ultrastructural level revealed that LPS induces multiple changes compatible with a dedifferentiated and secretory phenotype. In response to LPS treatment, we observed numerous dilated rough endoplasmic reticulum (RER) cisternae with secretory content, well-developed Golgi complex, secretory granules as well as extracellular matrix secretion. Testosterone was able to revert these secretory changes and pSMCs displayed similar contractile features compared to control cells (Fig. 6) consisting of well-developed contractile apparatus and scarce organelles, mainly high electron-dense mitochondria corresponding to a smooth muscle phenotype

Different studies have reported that the secretory profile of pSMCs is associated with the secretion of cytokines and the loss of quiescence, acquiring a proliferative phenotype (24, 25). We observed that LPS induced a secretory myofibroblastic-like phenotype in pSMCs, associated with an increased secretion of the pro-inflammatory cytokine IL6 and a higher cell proliferation as determined by Ki67 staining (Fig. 7). Interestingly, testosterone treatment, in a dose dependent manner, reduced IL6 secretion (Fig. 7A) and cell proliferation (Fig. 7B) in LPS-induced dedifferentiated cells. The normalization of these parameters is in accordance with the reestablishment of a well-differentiated smooth muscle phenotype with a low rate of proliferation after testosterone treatment in LPS-induced dedifferentiated pSMCs.

**Knocking-down *Srf/Myocd* impedes the differentiating effect of testosterone on smooth muscle cells**

Taking in mind that testosterone was able to stimulate smooth muscle-restricted gene expression and to restore the contractile phenotype after an inflammatory stimulus; we evaluated the importance of *Srf/Myocd* in androgen-induced myodifferentiation of smooth muscle cells. HCASM were transfected with siRNAs to knock-down the expression of *Myocd* or si*Srf*, or with scramble RNA as controls, and then stimulated using testosterone 10-7M and 10-5M for 24h. As previously demonstrated in primary cultures of pSMCs, testosterone increased the expression of differentiation markers *Myocd, Acta2, Cnn1, and Lmod1*in cells transfected with controls sc-RNA (Fig. 8 open bars). The expression of the androgen receptor (AR) seems to be required for testosterone-induced myodifferentiation (**SUPPL FIG**).

*Myocd* knock-down resulted in a decreased mRNA expression of *Acta2, Cnn1, and Lmod1*under basal conditions (Fig. 8 filled bars). Moreover, *Myocd* knock-down abolished the stimulatory effect of testosterone on the expression of smooth muscle-specific genes such as *Acta2, Cnn1, and Lmod1*. In accordance, *Srf* knock-down suppressed the induction of *Myocd*, *Acta2, Cnn1, and Lmod1*expression in response to testosterone (Fig. 8 grey bars). Altogether, these results indicate that the presence of a fully-functioning *Srf/Myocd* complex is indispensable for the myodifferentiator effect of testosterone.

**Discussion**

The present study demonstrates that the SRF/MYOCD transcriptional switch, a key component inducting the SMC phenotype (26), mediates the myodifferentiating effects of androgens as well as the dedifferentiating actions of LPS and cytokines. Testosterone up-regulated *Srf/Myocd* mRNA expression in pSMC in a dose-dependent manner, which was correlated with the increase of smooth muscle specific genes. Moreover, the inhibition of *Myocd* or *Srf* avoided this effect, indicating that androgens require SRF/MYOCD to act as differentiator factors on SMC. On the other hand, the LPS-induced dedifferentiation process was associated with a down-regulation of *Srf/Myocd*, with testosterone being able to restore the muscular phenotype by normalizing Srf/*Myocd* mRNA levels.

It has widely been reported that *Srf/Myocd* is the master regulator of smooth muscle-restricted gene expression (4). This myodifferentiator system has a high expression in different SMC-rich tissues such as aorta, bladder, intestine, and lung (27). Even though SMCs constitute the main cell type in the prostate stroma (28), here we provide the first evidence on the expression and regulation of the *Srf/Myocd* complex in these cells. The prostate expressed similar levels of *Srf* and *Myocd* than others SMC-rich tissues suggesting that this transcriptional switch could be relevant for the maintenance of pSMC differentiation. Interestingly, when LPS was used as a dedifferentiator factor for pSMC(13), the levels of *Srf/Myocd* were clearly repressed. The downregulation of *Myocd* has been associated with inflammation-induced phenotypic switching of SMCs from a contractile to a secretory status, leading to the acceleration of atherogenesis in vascular SMCs (29). In the prostate gland, the dedifferentiation of SMCs results in a myofibroblastic cell phenotype which is related to the development and maintenance of several diseases (30, 31). For instance, pSMCs become myofibroblasts in response to pro-inflammatory stimuli (12) and during the reactive stroma supporting prostate cancer growth (32). In this context, *Srf/Myocd* may act as a key guardian of smooth muscle homeostasis, avoiding cellular dedifferentiation and uncontrolled proliferation. In fact, our data indicate that *Myocd* overexpression prevents the phenotypic change of SMC in response to inflammatory injuries.

Although androgens have been established as primary regulators of cell growth, differentiation, and development in skeletal muscles(33), their effects on SMCs are poorly understood. Some reports points out that androgens induce the potentiation of contractile activity in intestinal smooth muscle (34) and exert anti-proliferative and proapoptotic effects on porcine coronary SMC in a dose-dependent manner (35). In the male tract, dihydrotestosterone enhances the expression of SMC-specific proteins in the rat prostate (36, 37). In addition, Zhang *et al.* (38) showed that testosterone positively regulates PDE5 expression while castration not only diminishes prostate size but also reduces pSMCs contractility. Consistently, our data reinforces this concept, by showing that testosterone treatment increased the expression of SMCs markers. The myodifferentiator role of androgens on SMC has also been reported in other male tissues as cavernous SMCs (39) but there is little information about the mechanisms.

Considering the pivotal role of SRF/MYOCD on the SMC differentiation program, we hypothesized that testosterone positively modulates *Srf/Myocd* to maintain high expression levels of SMC markers, thus preventing SMC dedifferentiation. Interestingly, when the members of *Srf/Myocd* complex were knocked-down, the effect of testosterone in the expression of SMC differentiation markers was abrogated, indicating that androgens require the fully SRF/MYOCD system to exert the myodifferentiator effect. Altogether, our results provide novel insights into the molecular basis for the beneficial actions of testosterone in smooth muscle biology.

We previously reported that the presence of testosterone prevents the transformation of SMCs to myofibroblasts in an inflammatory environment (12). In order to extend our study into a preclinical scenario, we used LPS-induced dedifferentiated pSMCs which were then treated with testosterone. The present study confirms that restoring testosterone levels rescues pSMC differentiation, in a process associated with the upregulation of the *Srf/Myocd* switch. To our knowledge, this is the first report with evidence showing that testosterone recovers the smooth muscle phenotype in the prostate. Indeed, not only was the expression of SMC-specific markers (at protein and mRNA levels) re-established, but the proliferation rate and the ultrastructural features were reversed as well. Significantly, our results are in agreement with some reports suggesting that prostatic pathologies might be improved by restoring eugonadism (40-42).

In conclusion, the present study provides novel evidence regarding the differentiating role of testosterone on pSMCs by modulating *Srf/Myocd* expression. These data highlight that testosterone preserves pSMCs phenotype, which is essential to maintain the normal structure and function in the prostate gland. Additionally, our study opens frontiers about the importance of preserving physiological testosterone levels as a new target for the treatment of diseases such as prostate cancer, hyperplasia, and prostatitis where the dedifferentiation of pSMCs play a significant role.

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FIGURE LEGENDS

**Figure 1: Testosterone-induced SRF/Myocd expression in pSMCs.**

The expression of *Srf* and *Myocd* (A) was evaluated by RT-PCR in the rat adult prostate, bladder and artery were employed as positive controls and *Gapdh* was used as reference gene.

pSMC were treated with different doses of testosterone (0, 10-5, 10-7, 10-12M) for 24h and mRNA expression of (B) *Srf*, (C)*Myocd* and the indicated SMC markers (D) Cnn1 and (E) *Acta2* were analyzed by qPCR. Relative mRNA levels reflect fold changes relative to the vehicle control, arbitrarily set to 1 (\*p<0,05 *vs.* to control) (\*\*p<0,01 *vs.* to control) . Data are representative of 3 independent experiments.

**Figure 2: LPS induce smooth muscle dedifferentiation by down-regulating Myocd.**

pSMCs were stimulated with LPS (1ug/ml) for 48h in order to evaluated the expression of SRF/Myocd in dedifferentiated pSMCs. The mRNA level of (A) *Srf* and (B) *Myocd,* and (C) *Lemoid* were analyzed by qPCR. In addition the expression of the smooth muscle markers *Acta 2*, *Cnn1* and the fibroblast marker *Vim* were analyzed by qPCR and at protein level by *inmunofluorescence (D, E).* 4′,6-diamidino-2-phenylindole (DAPI; blue) counterstaining indicates nuclei. The mRNA levels are relative to those of b- actin. The expression level of control group was arbitrarily set to 1. Values are indicated as fold change relative to the mRNA levels of control cells. \*p<0,05 *vs.* control \*\*p<0,01 *vs.* control \*\*\*p<0,001 *vs.* control. Data are representative of 3 independent experiments.

**Figure 3: Myocardin reduces the dedifferentiation induces by inflammatory stimuli.**

Human coronary smooth muscle cells (HCASM) were transduced with adenoviral vectors (Ad) (empty; control) or myocardin (Ad.Myocd) then incubated with LPS (1ug/ml) of TNF (5ng/ml) for 24h. **A-D**, Effect of myocardin on the expression of smooth muscle markers genes indicated by qPCR. E-F mRNA levels of the proinflammatory cytokines (TNFa and Il6) after the inflammatory stimuli. Relative mRNA levels reflect fold changes

relative to the control, arbitrarily set to 1. Data are representative of 3 independent experiments.

**Figure 4: Testosterone recovers *Srf/Myocd* expression in LPS-dedifferentiated pSMCs**

pSMCs were stimulated with LPS (1ug/ml) for 48h and then the cells were treated with testosterone (10-7, 10-12M) for additional 48h. qPCR was performed to quantify (A) *Srf* and (B) *Myocd,* (C) *Acta 2*, (D) *Cnn1* and (D) *Vim* mRNA levels relative to those of b- actin. The expression level of control group was set to 1. Values are indicated as fold change relative to the mRNA levels of control cells. \*\*p<0,01 *vs.* control \*\*\*p<0,001 *vs.* control, #p<0,05 *vs.* to vehicle, ##p<0,01 *vs.* vehicle and ###p<0,001 *vs.* vehicle. Data are representative of 3 independent experiments.

**Figure 5: Testosterone reestablishes the expression of smooth muscle cells markers.**

Dedifferentiated LPS-pSMCs were incubated with testosterone (10-7, 10-12M) for 48h. The expression of smooth muscle phenotypic markers (ACTA2, and calponin) and a fibroblastic marker (vimentin) were evaluated by Western blot (A-C) and (D) immunofluorescence demonstrating that testosterone treatments reestablish the smooth muscle profile. 4′,6-diamidino-2-phenylindole (DAPI; blue) counterstaining indicates nuclei. The western blot values were normalized with ACTB expression and represent the mean±SE of three independent protocols. \**p*<0.05 *vs*. control; \*\*p<0.01 *vs*. control; #p<0,05 *vs*. vehicle, ##p<0,01 *vs.* vehicle ANOVA-Tukey.

**Figure 6: Ultrastructural features of pSMCs.**

The pSMC in both controls (A and B) are thin and contractile filaments (arrowheads). After LPS, cells exhibit numerous organelles as well-developed Golgi complexes (g) and dilated RER cisternae with secretory content, secretory granules (\*) and secretion of extracellular matrix (arrows) (C and E). In the presence of testosterone (10-12M, 10-7), pSMCs display similar morphological pattern to their controls with contractile filaments (arrowheads), mitocondria (m) and less proteinpoyetic organeles and extracellular matrix secretion surrounding the cells (F-J). N: nucleus. Bar:1µm.

**Figure 7: Analysis of Il6 secretion and the proliferative rate**

IL6 secretion was measured by ELISA in supernatants of pSMCs (B). Testosterone treatment diminished the cytokine levels, with significant differences observed with the higher dose of testosterone tested. Proliferation was analyzed by ki-67 labelling (A), testosterone significantly decreased cell proliferation at control level with testosterone 10-7M. Data refer to mean ±SE from three independent experiments. \**p*<0.05 *vs.* control; \*\*\**p*< 0.001 *vs.* control; ##*p*<0.01 vs vehicle; ###*p*<0.001 vs. vehicle ANOVA-Tukey**.**

**Figure 8: Testosterone required *Srf/Myocd* axys to induce expression of smooth muscle genes in HCASM.**

qPCR of indicated genes in HASMC siRNA to *Srf, Myocd* or *Scramble (sr)* after stimulation with different doses of testosterone. The expression level of control group was set to 1. Values are indicated as fold change relative to the mRNA levels of control cells. \*\*p<0,01 *vs.* control \*\*\*p<0,001 *vs.* control. n.s non significant. All data are representative of at least 3 independent experiments.

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**DISCLOSURE STATEMENT**

The authors of this manuscript have nothing to declare.