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Ligand-dependent stabilization of androgen receptor in a novel mouse ST38c Sertoli cell line

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Abstract

Mature Sertoli cells (SC) are critical mediators of androgen regulation of spermatogenesis, *via* the androgen receptor (AR) signaling. Available immortalized SC lines lose AR expression or androgen responsiveness, hampering the study of endogenous AR regulation in SC. We have established and characterized a novel clonal mouse immortalized SC line, ST38c. These cells express some SC specific genes (*sox9*, *wt1*, *tjp1*, *clu*, *abp*, *inhbb*), but not *fshr*, yet more importantly, maintain substantial expression of endogenous AR as determined by PCR, immunocytochemistry, testosterone binding assays and Western blots. Microarrays allowed identification of some (146) but not all (*rhox5*, *spinlwl*), androgen-dependent, SC expressed target genes. Quantitative Real-Time PCR validated regulation of five up-regulated and two down-regulated genes. We show that AR undergoes androgen-dependent transcriptional activation as well as agonist-dependent posttranslational stabilization in ST38c cells. This cell line constitutes a useful experimental tool for future investigations on the molecular and cellular mechanisms of androgen receptor signaling in SC function.

1. Introduction

Sertoli cells (SC) are essential mediators of androgen regulation of spermatogenesis, *via* the androgen receptor (AR)-mediated signaling. In adulthood, SCs maintain a specific environment favorable for the development and proliferation of germ cells (McKinnell et al., 1995; Sharpe et al., 2003) and are involved in the hormonal regulation of spermatogenesis. Indeed, FSH acts directly on SC through FSH receptor, while LH first activates Leydig cells and stimulates testosterone production that acts on SC through binding and activation of AR. AR, a steroid receptor belonging to the superfamily of hormone-activated transcription factors, displays distinct expression profiles in SC during testis development, tightly correlated to the stages of spermatogenesis, as previously reported (Monet-Kuntz et al., 1984; Vornberger et al., 1994; Bremner et al., 1994; Shan et al., 1997; Suarez-Quian et al., 1999; Boukari et al., 2009; Chemes et al., 2008; Rey et al., 2003). Indeed, AR is absent in neonatal SC while its expression becomes maximal at puberty and in adulthood, concomitantly with the progression of spermatogenesis, unlike constant AR expression in Leydig and peritubular myoid cells in both prepubertal and postpubertal testis developmental stages.

Numerous studies in rodents, primates and humans have demonstrated the importance of AR expression in SC for the regulation of spermatogenesis. Several mouse models with total AR invalidation in SC exhibited variable degrees of infertility associated with spermatogenesis impairment (De Gendt et al., 2004; Wang et al., 2009; Verhoeven et al., 2010). In adult human testis, androgens initiate spermatogenesis, a complex cellular differentiation process that does not occur in fetal and newborn testis despite sustained levels of intratesticular androgen production. Thus, the lack of AR in SC in neonatal testis is responsible for a physiological postnatal insensitivity to testosterone (Rey et al., 2003; Chemes et al., 2008; Boukari et al., 2009).

However, the precise molecular mechanisms by which AR regulates physiological functions in SC and supports germ cell development and spermatogenesis are still poorly understood. Although the cell-selective AR deficiency animal models constitute valuable tools to study potential androgen-regulated genes in a testicular environment, it still remains difficult to identify direct androgen-dependent target genes in SC, due to the complexity and entangled paracrine and metabolic pathways existing among the multiple cellular components of the testis.

In order to directly investigate the mechanisms of androgen regulation specific to SC, several attempts to generate immortalized mature SC lines expressing AR have been reported,

resulting in very few postpubertal/mature SC models, such as MSC1 (Rao et al., 2003), S14-1 (Boekelhelde et al., 1993), 42GPA9 (Bourdon et al., 1998) and 15P-1 cells (Paquis-Flucklinger et al., 1993). However, in these cell lines, AR expression was very variable and was rapidly lost after several passages. Moreover, androgen signaling was somehow impaired in these SC models, with very low levels of mRNA *Ar* expression (Rao et al., 2003), as androgens failed to activate endogenous AR, thus necessitating transfection of AR-encoding plasmid in order to study androgen regulation.

The aim of the present study was to establish a novel SC line that maintains a sustained AR expression, allowing investigation of androgen-dependent, SC specific, target genes. Herein, we generated and characterized an original mouse immortalized Sertoli cellular model, named ST38c, harboring substantial expression of endogenous AR that conserves its androgen-dependent transcriptional activation and exhibits agonist-dependent transcriptional and posttranslational regulation. We demonstrated that androgens upregulated AR expression and stabilized AR through posttranscriptional mechanisms. In the present paper, hundreds of androgen-regulated genes have been identified by microarray studies and some of them were validated by real-time quantitative PCR in ST38c cells. This novel cell line thus constitutes a useful experimental system for better understanding the molecular mechanisms of androgen action in SC and may represent a valuable cell-based model for future investigations on the role of SC in spermatogenesis regulation.

2. Materials and methods

2.1. Establishment of the ST38c Sertoli cell line

The ST38c cell line was isolated from the testes of an 8-wk-old transgenic male mouse carrying a transgene in which the SV40 large T Antigen (TAg) was placed under the control of the human vimentin promoter (Schwartz et al., 1991). Mice were bred according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The animal facility was granted approval (N°C94-043-12), given by the Ministère de l'Agriculture, France. All procedures were approved by the local ethic committee CAPSud (N°2012-021). After decapsulation, the testicular tissue was incubated with collagenase (25%) (CLS type I, 146 U/mg from *Clostridium histolyticum*, Worthington Biochemical; Freehold, NJ, USA) for 15 min at 37°C. Cellular debris were removed by sedimentation and the cell-containing supernatant was centrifuged. The cells of the formed pellet were rinsed twice with culture medium and

suspended in 10ml of DMEM/HAM's F12 (1:1) medium containing 20 mM HEPES, pH 7.4, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 20% fetal calf serum (FCS). Cells, cultured in 10 cm² Petri dishes at 37°C in atmosphere containing 5% CO₂, were treated with 20 mM Tris-HCl (pH 7.4) hypotonic solution for 3 min, to remove any residual germ cells. After several serial passages, clonal cells morphologically similar to SC and expressing immunoreactive nuclear SV40 TAg were selected. These cells were expanded and characterized.

2.2. Cell Culture

ST38c cells (passages 6-30) were seeded at 6×10^5 cells in 6cm² Petri dishes with 5 ml of DMEM/HAM's F12 (1:1) medium containing 20 mM HEPES, pH 7.4, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 5% FCS. The medium was replaced by DMEM/HAM's F12 (1:1) with 5% Dextran-Coated Charcoal treated serum (DCC) at least 24 h prior to study androgen signaling in steroid free medium conditions.

2.3. Hormones and Drugs

Dihydrotestosterone (DHT) was purchased from Acros Organics (Noisy Le Grand, France) whereas RU486 (mifepristone), cycloheximide and actinomycin D, were purchased from Sigma (St Louis, MO).

2.4. Quantification of specific androgen-binding sites by Scatchard Analysis

Cells were grown in the DCC medium 24 h before harvesting. After being rinsed twice with cold PBS, pelleted cells were frozen in liquid nitrogen. The frozen cells were ground in a mortar under liquid nitrogen. One volume of powdered cells was homogenized in one volume of TEWG buffer (20 mM Tris-HCl, 1 mM EDTA, 20 mM sodium tungstate, 10% (vol/vol) glycerol, pH 7.4, at 20°C) in a Teflon-glass Potter-Elvehjem (Polylabo, Strasbourg, France) apparatus. Cytosol fractions obtained after centrifugation at 4°C at 15,000 g for 20 min were incubated for 4 h at 4°C with increasing concentrations of [³H]-testosterone (Perkin Elmer GE healthcare). Bound and unbound steroids were separated by the dextran-charcoal technique. Values of binding parameters were determined at equilibrium by Scatchard analysis using computer software Prism 5 (GraphPad Software, San Diego, CA) as previously described (Lombes et al., 1992).

2.5. Gene profiling analysis

RNA extraction was performed using TRIZOL protocol (Invitrogen) and purified with Qiagen column (Rneasy micro) from ST38c cells treated for 24h with either vehicle (EtOH) or 10^{-7} M dihydrotestosterone. The quantity and purity of the extracted RNA was evaluated using a NanoDrop spectrophotometer and its integrity measured using Lab-on-a-chip Bioanalyser 2000 technology (Agilent Technologies, Palo Alto, CA, USA), based on the 28S/18S ribosomal RNAs ratio. Labelling of RNA samples was done according to Agilent oligo Cy5 or Cy3 probes labelling protocol using the Agilent Low Input QuickAmp labelling kit for dye swap strategy. Cy5/Cy3 cRNA mixtures were hybridized to an Agilent mouse genome GeneChip array (G4122F) allowing to independently probe 44K transcripts from whole mouse genome. Scanning of microarrays, data normalization and quality tests were performed as previously described (Khan et al., 2012). Selection of androgen regulated genes was carried out according to fold change (FC) >1.5 , p-value $<10^{-5}$ and intensity >50 cut offs. Among the redundant positive probes matching with the same gene, only one representative response was included in the final list of the 146 androgen-regulated genes. The raw microarray data have been submitted to Array Express database (European Bioinformatics Institute, <http://www.ebi.ac.uk/arrayexpress/>) with the accession number E-MTAB-1732.

2.6. Bioinformatics analysis

Gene ontology and functional clustering was predicted using two bioinformatics resources: the DAVID Bioinformatics Resources (<http://www.david.abcc.ncifcrf.gov>), while further information related to protein families and molecular and biological functions related to the selected genes have been confronted with PANTHER database (www.pantherdb.org).

2.7. Quantitative real time RT-PCR

Total RNA, extracted from cells was processed for RT-PCR, as previously described (Boukari et al., 2009). *Ar* and androgen-regulated target gene candidates identified by microarray analysis were quantified by real-time PCR, with specific primers (primer sequences available upon request). Briefly, 1 μ g of total RNA was treated using the DNase I Amplification Grade procedure (Invitrogen), RNA was reverse-transcribed using the High Capacity cDNA RT kit from Applied Biosystems. Reverse-transcribed samples were diluted 10-fold and used for qRT-PCR using the Power SYBR[®] Green PCR Master Mix (Applied Biosystems). qPCR reactions were carried out on an StepOne Plus Detector (Applied Biosystems) as previously described (Boukari et al., 2007). Ribosomal 18S RNA was used as the internal control for data normalization.

After 24 h treatment with 10^{-7} M dihydrotestosterone, ST38c transcript expression was compared to the basal, not stimulated (ethanol at equivalent concentration) condition. The relative expression level of each gene transcript was normalized with 18S rRNA level and expressed as attomoles of gene per femtomoles of 18S. Results are means \pm SEM of at least three independent experiments.

2.8. Western Blot analysis of AR expression

Total protein extracts, prepared from ST38c cell lysates (20 μ g total protein/lane) were resolved by SDS-PAGE and electrotransferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, UK Limited). Immunoblots were incubated overnight in 5% skim milk-Tris buffer saline/ 0.1% Tween (TBST) before incubation with rabbit anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz) at 1/200 dilution for 1h at room temperature. After several washes, the membranes were incubated with a goat anti-rabbit peroxidase-conjugated second antibody (1:10,000) for 1h at room temperature and proteins were visualized with the ECL⁺ Western blotting analysis system (GE Healthcare). For loading normalization, membranes were incubated with an anti α -tubulin antibody (Sigma). Relative AR protein expression was assessed after normalization with α -tubulin, using the Quantity One Analysis software (Bio-Rad, France). Results are means \pm SEM of at least three independent experiments and represent the relative fold-induction in ligand-stimulated cells compared with basal levels (arbitrarily set at 1).

2.9. Immunofluorescence studies

Cells cultured on Lab-Tek (Nunc, Roskilde, Denmark) were washed in PBS, fixed with 10% formol buffered in PBS (pH 7.3) for 10 min and washed three times in PBS before processing for immunofluorescence assays. Cells were incubated overnight with primary antibodies: rabbit polyclonal antibodies: anti-AR (sc-816, Santa Cruz Biotechnology), anti-WT1 (sc-192, Santa Cruz Biotechnology), anti-SOX9 (Chemicon international) and anti-TJP1 (Invitrogen, Life Technologies), or monoclonal antibodies: anti-SV40 T Antigen (Calbiochem/EMD Millipore) and anti- β -tubulin3 antibody (AbCys, Paris) (Meduri et al., 2002) at 1:50, 1:50, 1:100, 1:50, 1:100, 1:100 dilutions, respectively. Bound immunoglobulins were revealed by goat anti-rabbit Alexa 555 (1:1000) or goat anti-mouse Alexa488 (1:1000) (Invitrogen Cergy-Pontoise) according to the manufacturer's instructions. Negative controls were performed by substituting the primary antibodies with corresponding preimmune immunoglobulins from the same species.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad PRISM version 5 software (GraphPad Software, Inc., La Jolla, USA). Results are expressed as means \pm SEM of at least three independent experiments. Non-parametric Mann-Whitney test was used for comparison between groups.

3. Results

3.1. Morphological and molecular characterization of ST38c Sertoli cells

Sertoli cells (SC) were isolated from testes of an 8-wk-old transgenic male mouse by means of targeted oncogenesis strategy in which the expression of the SV40 large T Antigen was driven by the vimentin promoter (Schwartz et al., 1991). After clonal selection and expansion, the ST38c cells maintained stable morphological aspect during successive passages (P 6-30).

On bright-field microscopic examination, ST38c cells present a homogeneous morphology of SC, as monolayered cells with long cytoplasmic appendages and central ovoid nuclei (Fig.1A). To assess whether ST38c were immortalized, we examined the expression of SV40 large T Antigen by immunofluorescence (Fig. 1B). Positive immunostaining of the SV40 large T Antigen was observed in the nucleus of ST38c cells, thus confirming the immortalized properties of the clonal cell line. To define the precise origin of the ST38c cells, we examined the expression of specific SC markers by immunocytochemistry and RT-PCR (Fig. 1 and Fig. 2A-B). Immunofluorescence studies in ST38c cells revealed the presence of a specific nuclear staining for both SOX9 and WT1 (Fig. 1C-D), two transcription factors expressed in both mature (post-pubertal) and immature (prepubertal) SC. We demonstrated by RT-PCR that ST38c expressed mRNA of other factors known to be involved in SC functions, such as sulphated glycoprotein-2/clusterin (*clu*), transferrin and androgen binding protein (*abp*) (Fig. 2A), consistent with the SC phenotype of ST38c cells. We also examined the expression of β -tubulin3 (*tubb3*), an androgen up-regulated protein, involved in the modulation of the SC cytoskeleton (De Gendt et al., 2011) and found that β -tubulin3 was expressed at both mRNA (Fig. 2A) and protein level as indicated by the cytoplasmic immunostaining (Fig. 1E). As expected for a mature SC line, ST38c cells also express proteins involved in the formation of tight junctions, important for the blood-testis barrier formation, such as zonula occludens1 at both protein (Fig. 1G) and mRNA levels (*tjp1*) (Fig.

2A), while anti-Mullerian Hormone (*amh*) mRNA was not detected in ST38c cells, contrasting with the high AMH expression in newborn testis (Fig. 2B). In order to determine whether ST38c cells are a clonal SC line, we confirmed the absence of Leydig specific cell markers, such as *cyp17a* and LH receptor (*lhr*) mRNA, as illustrated in Fig. 2C. Finally, we also showed that ST38c cells express detectable levels of mRNA for both α and β B subunits of Inhibin B, suggesting that this new cell line might be able to produce Inhibin B (Fig. 2A and 2B). Altogether, these results demonstrate that ST38c cells constitute a homogenous population of mature SC.

3.2. ST38c cells express functional androgen receptors

Since our main objective was to establish a cell line expressing a functional androgen receptor (AR), as opposed to previous SC models, we used different complementary approaches to analyze AR expression in ST38c cells. RT-PCR analysis demonstrated that ST38c cells express AR mRNA (Fig. 3A). Western blot confirmed the presence of AR protein as a ~110 kDa protein, identified in ST38c cell lysates (Fig. 3B), human prostatic LNCaP cells being used as positive control. Immunofluorescence studies further demonstrated that AR is expressed in ST38c cells as a protein with both nuclear and cytoplasm distribution, more pronounced in the nucleus, under the standard experimental conditions (Fig. 3C). We next performed testosterone binding assays to characterize specific sites and their binding parameters for AR. Cytosolic fractions of ST38c cells were incubated with increasing concentrations of [³H]-testosterone for 4h. Scatchard plot analysis revealed that ST38c cells express approximately $5-10 \times 10^3$ specific sites/cell, with an equilibrium dissociation constant (Kd) estimated at ~0.7 nM, consistent with the high affinity of testosterone for AR (Supplemental Fig. S1). Altogether, these data indicate that ST38c cells do express high affinity, high capacity AR.

3.3. Identification of AR-regulated genes in the ST38c cells

To identify androgen-dependent and AR-regulated genes in SC, gene expression patterns were analysed by microarray assays, comparing untreated versus DHT (10^{-7} M) treated ST38c cells. Microarray assay analysis performed as previously described (Khan et al., 2012), allowed identification of a repository of 146 differentially androgen-regulated genes including 72 up-regulated and 74 down-regulated genes, which were selected according to the following criteria: fold change $FC > 1.5$, $p\text{-value} < 10^{-5}$ and intensity > 50 cutoff. A panel of genes selected upon their involvement in the biology of reproduction is presented in Table 1.

Gene ontology was predicted by using DAVID and PANTHER softwares. Among the androgen-up regulated genes in SC, there were several factors involved in testicular development (*ngfr*), androgen mediated transcriptional regulation (*nrip1-rip140*), as well as other candidates required in germ cell differentiation (*pou5f2*, *serpina3m*, *serpina3c*, *serpina3j*, *ctsc*) or genes involved in female gamete generation (*tgfb2*). Among the androgen down-regulated genes in ST38c cells there were several genes involved in the modulation of inflammatory responses (*cxcl5*, *serpina1b*, *cxcl11*), tight junction formation (*cldn19*, but also *cldn1* and *cldn15*) or protein ubiquitination (*uba7*). A large number of genes concerned integrin signaling (*adam11*, *itga8*, *itga11*, *adamts3*, *adamts2*, *itgbl1*), and cell adhesion (*cldn19*, *cdhr1*, *itga11*, *efs*, *cldn15*, *tgfb2*, *itgbl1*, *ncam1*, *vwf*, *omd*, *coll4a1*, *itga8*, *tgfbi*, *cldn1*, *fbln7*, *pstpip1*, *vnn1*, *cntnap2*, *lamc2*, *vcan*, *lmln*, *spon2*, *adam12*, *dpt*). Interestingly, functional annotation clustering analysis (DAVID) revealed that the up-regulated genes preferentially influenced regulation of transcription (*fus*, *klf13*, *sox5*, *rybp*, *tle1*, *tacc1*, *purb*, *qk*, *nrip1*, *prmt1*, *dact1*, *pou5f2*, *rapgef5*, *pdgfc*) and apoptosis (*aldh1a1*, *akt1s1*, *chst11*, *sphk1*, *ngfr*, *tgfb2*). In contrast, the down-regulated genes differentially impacted cytokine (*gm2023*, *amy1*, *cxcl5*, *amy2a4*, *ifnb1*, *tgfbi*, *pf4*, *cxcl11*, *thpo*) and various extracellular signaling pathways (*wnt10a*, *vwf*, *fmod*, *coll4a1*, *adamts16*, *tgfbi*, *spon2*) (for further details, see the Array Express database (European Bioinformatics Institute, <http://www.ebi.ac.uk/arrayexpress/>), where the microarray was registered with the accession number E-MTAB-1732).

In order to confirm the androgen transcriptional regulation in ST38c cells, we next selected several genes including those previously recognized as expressed and/or androgen-regulated in mature SC or in testis. We thus studied their mRNA expression levels by qRT-PCR, comparing basal to androgen (DHT)-stimulated conditions (Fig. 4). Indeed, qRT-PCR analysis validated results of microarray studies. A well-known AR coregulator, *nrip/rip140* mRNA (Heinlein et al., 2002), was significantly induced after androgen exposure by a 2.3 fold factor ($p=0.0022$) (Fig. 4A). Surprisingly, *hsd11b1* mRNA encoding for the 11-beta hydroxysteroid dehydrogenase type 1, exhibited a 5-fold induction upon androgen exposure ($p<0.0001$) (Fig. 4A). We also validated other androgen up-regulated genes such as *gas6* or *aldh1a1* (expressed in testis) (Fig. 4A), as well as androgen down-regulated genes, such as *cldn19* and *gpr126* (Fig. 4B), which presented similar fold changes by RT/qPCR compared to the microarray analysis.

3.4. Androgens stabilize AR protein expression in Sertoli cells

We took advantage of the ST38c cells that do express substantial amounts of functional AR to examine the impact of androgens on AR regulation and turnover in a SC context.

To determine whether androgens could affect *Ar* gene transcription in SC, we measured *Ar* mRNA levels by RT-qPCR in ST38c cells treated or not with 10^{-7} M DHT, a saturating AR concentration. We showed that transcript levels of endogenous *Ar* were induced by androgen exposure in ST38c cells (1.6 fold induction, p-value=0.026), indicating that androgens significantly stimulate *Ar* gene expression in SC (Fig. 4C).

Western blot analysis showed that DHT exposure also induced a strong increase in AR expression in a time-and dose-dependent manner. A two to three fold increase in AR protein expression was observed 6 h after exposure, while a 4.5 fold increase in AR protein was detected at 24 h compared to basal levels (Fig. 5A). When ST38c cells were treated with increasing concentrations of DHT (10^{-10} to 10^{-6} M), AR abundance was clearly augmented as a function of androgen concentration, already visible at a dose as low as 10^{-10} M. A maximal response was obtained with micromolar concentrations, reaching a plateau with higher concentrations (Fig. 5B).

In order to examine whether ligand-dependent AR stability directly involved AR per se, we used RU486, a weak AR antagonist (Fig. 5C). As expected, DHT at 10^{-8} M concentration induced a three-fold increase in relative AR expression. However, the combination of DHT and RU486 decreased AR expression although not significantly, most probably due to the partial agonist activity of RU486 (p-value=0.119) (Fig. 5C).

To further decipher the molecular mechanisms by which androgens stimulated AR abundance in SC, ST38c cells were grown in the presence or absence of actinomycin D, a transcription inhibitor, for various periods of time. As already shown, DHT (10^{-7} M) alone induced a time-dependent increase in AR protein expression, reaching two-fold after 6h exposure (Fig. 5A and B). Actinomycin D (0.4 μ M) alone does not substantially affect AR protein levels over a 6h period despite mRNA synthesis inhibition. Combination of DHT and Actinomycin D resulted in increased steady levels of AR expression during 6 h of exposure, indicating that AR stability is controlled by posttranscriptional events. These findings provide further evidence that androgen-dependent increase in AR abundance in SC was related not only to an enhanced AR messenger expression (Fig. 4C), but also to an increased AR protein stabilization.

To examine whether androgens modify the half-life time of AR protein, ST38c cells were treated either with DHT 10^{-7} M or cycloheximide (5 μ g/ml) or both for 1 h, 3 h or 6 h (Fig. 7). As expected, in the presence of cycloheximide, AR expression rapidly declined and was

almost completely abolished after 6 h cycloheximide treatment. These findings indicate that the $t_{1/2}$ was approximately 3 h in ST38c cells, consistent with previous reports (Syms et al., 1985). On the other hand, as already shown (Fig. 5 and 6), AR protein expression was efficiently induced in the presence of 10^{-7} M DHT. However, the androgen-dependent AR stabilization was also observed in the presence of the protein synthesis inhibitor, cycloheximide (Fig. 7), consistent with an increased half-life time of AR protein. Thus, androgens stabilized AR protein even when protein synthesis was inhibited, suggesting the involvement of direct posttranslational events on AR.

4. Discussion

In the present study, we generated and described a novel immortalized mature Sertoli cell (SC) line, characterized by a substantial and stable expression of endogenous androgen receptor (AR), exhibiting androgen-dependent transcriptional and posttranslational regulation. We established the SC origin and mature phenotype of the ST38c cells, as evidenced by the expression of several specific SC markers and other essential factors implicated in SC functions. The presence of functional AR was unambiguously demonstrated in the ST38c cells making possible investigations on androgen-dependent AR action in a mature SC context.

SC lines have been previously derived from testis of various mammalian species (mouse, rat, sheep), or even of fish (Higaki et al., 2013), using different immortalization strategies, including the targeted oncogenesis method (Rahman et al., 2004). However, these cells either contain a heterogeneous population, such as the MSC1 cell line (Peschon et al., 1992), or display an immature phenotype, such as prepubertal SC-SK11 (Sneddon et al., 2005), TM4 (Mather et al., 1980), or SMAT1 cells (Dutertre et al., 1997), preventing investigation on androgen regulation of SC function given the lack of AR expression in SC during the prepubertal period (Chemes et al., 2008; Boukari et al., 2009; Vija et al., 2013). Previously reported mature immortalized murine SC lines exhibited several limitations or disadvantages, such as the lack of homogeneity for the 15P-1 cell line (Paquis-Flucklinger et al., 1993), or variable degrees of FSH responsiveness owing that 42GPA9 cells expressed FSH-R (Bourdon et al., 1998) while S14-1 cells did not (Boekelheide et al., 1993). In all previously established SC lines, androgen responsiveness and stable AR expression were also an important issue given that immortalized or primary SC presented with rapid loss of AR expression after serial passages such as for SK11 (Sneddon et al., 2005) or SCIT-C8 cells

(Konrad et al., 2005), or expressed very low levels of mRNA *Ar* for the MSC-1 cells (McGuinness et al., 1994).

Herein, we characterized a clonal, mature, immortalized SC line, named ST38c, expressing transcripts of specific SC markers such as *Abp*, transferrin and *clu*, as well as both mRNA and protein of SC markers of maturity (*tjp1/TJP1*). This is the first mature immortalized SC line with relatively high abundance of endogenous AR, which confers androgen-dependent transcriptional responses and exhibits androgen-mediated AR stabilization through posttranslational events. These main functional properties were maintained through several passages (P6-30).

We were interested in studying the mechanisms of AR auto-regulation in the SC context. Regulation of AR levels by androgens has been reported in almost all AR expressing cell types and was found to be both complex and tissue-specific. The androgen-dependent regulation of AR protein and gene expression has been already the subject of numerous studies, mainly in the context of prostatic tissue and prostate cancer, with contradictory results related to the different models used.

We are reporting the androgen regulation and provide possible mechanisms of androgen-mediated AR stabilization in SC. Data in the literature related to androgen-mediated AR regulation in SC are scarce. It was initially reported that androgens increase AR concentration in SC (Verhoeven et al., 1988) while it was briefly mentioned that AR protein expression was stabilized upon androgen treatment, in a primary monkey SC cultures (Chen et al., 2008). In contrast, steady state levels of *Ar* mRNA were found down-regulated after androgen exposure in several cell lines, such as the LNCaP prostatic carcinoma cells, in which Yeap et al showed that reduced mRNA transcription predominated over androgen stabilization of *Ar* mRNA, while in the MDA453 breast cancer cells, transcriptional down-regulation predominated over the reduction of the *Ar* mRNA turnover (Yeap et al, 1999). On the other hand, androgen treatment has been reported to variably up-regulate AR expression in prostate tissue. Takeda suggested a positive control of androgens on AR, given that prostate *Ar* mRNA was greatly reduced following rat castration, while normal AR transcripts were restored upon androgen administration (Takeda et al., 1991). At the protein level, androgen treatment of prostate cells increased AR protein content, as demonstrated by both immunocytochemical and Western blot studies, such an effect suppressed by actinomycin D and cycloheximide, consistent with the involvement of both transcriptional activation and protein neosynthesis (Blanchere et al., 1998). Taken together, owing to the important

variation of AR regulation as a function of cell environment, it was crucial to generate a specific cell model, which could recapitulate AR regulation in a Sertoli cell environment.

Thus, our results significantly differ from those reported in other androgen-responsive tissues, since androgen exposure of ST38c cells resulted in both an increased *Ar* mRNA expression as well as a AR protein stabilization. This androgen-stimulated *Ar* transcription associated with an enhanced AR stability was observed in ST38c cells in a time- and dose-dependent manner. In addition, we showed that AR abundance in the presence of DHT was not completely suppressed by actinomycin D, while AR protein remained stable after combined exposure of DHT and cycloheximide, indicating that both androgen transcriptional up-regulation and androgen posttranslational stabilization contribute equally to AR expression in ST38c cells.

Recently, Hazra et al (Hazra et al., 2013), developed a unique transgenic mouse model, (TgSCAR) in which human AR was specifically overexpressed in SC using the rat androgen-binding protein (*Abpa*) gene promoter. As expected, it was found a strong and premature postnatal expression of AR in SC as early as D2, leading to reduction of SC number and testis weight and associated with upregulation of *rhox5* but also of tight junction transcripts (*cldn11* and *tjp1*). Interestingly, AMH expression rapidly declined to normal levels, however only in postpubertal testis, indicating that differential age-related down-regulation of *amh* might be in part independent of AR expression.

Transcriptomic analysis followed by qPCR validation identified a large series of androgen-regulated genes, some already reported as expressed in testis or androgen regulated. Previous microarrays were mostly performed in AR knockout mouse models, comparing whole testis gene expression of knockout and wild-type animals. Even with sophisticated engineered mouse models of selective ablation of AR in SC, such as SCARKO (Denolet et al., 2006; Abel et al., 2008; Willems et al., 2010) or S-AR^{-y} (Zhang et al., 2012), it is always challenging to unambiguously appreciate whether identified genes are directly or indirectly androgen-dependent and SC specific.

Claudins form a large family of transmembrane proteins involved in tight junction formation with paracellular sealing function (Krause et al., 2008), which have been reported to be androgen regulated during developmental stages in several mouse models. For instance, it was shown that DHT treatment stimulated claudin 11 expression in rat Sertoli cells (Kaitu'u-Lino et al., 2007), as well as mRNA expression and the protein redistribution of claudin 11 in hpg mice testes (McCabe et al., 2012). On the other hand, claudins 3, 11 as well as *tjp1* mRNA and protein were decreased in SCARKO models, associated with defective barrier

formation (Willems et al., 2010). In the ST38c cells, we observed a different pattern of claudin expression, as claudins 19, 15 and 1 were all androgen-downregulated targets, while claudin 3 did not seem to be androgen-regulated and claudins 2, 5 and 11 were barely expressed. These findings are not quite surprising, given that classical and non-classical claudin expression, their regulation and tightness properties, as well as their association to TJP1 seems to be largely dependent upon cell context.

We also identified *rip140* as an androgen up-regulated gene in ST38c cells. *Nrip/rip140* was originally reported as an AR coregulator expressed in epididymal epithelial cells, prostate and testis (Steel et al., 2005). It was shown that *rip140*mRNA steady-state levels were increased by androgens in prostate cancer cells (Carascossa et al., 2006); however, the precise role of *rip140* in SC needs to be further evaluated. We also found that 11 β -hydroxysteroid dehydrogenase type 1 (*hsd11b1*) expression was up-regulated by androgens in ST38c cells. This steroid metabolic enzyme may exert a dual reductase/dehydrogenase enzymatic activity (Chapman et al., 2013; Gathercole et al., 2013) according to the tissue distribution, but most likely regenerating active cortisol or corticosterone in rodents from inactive 11 keto-metabolites (cortisone and 11-dehydrocorticosterone), thus enhancing local glucocorticoid action. *Hsd11b1* was previously shown to be expressed in testis, most notably in Leydig cells (Monder et al., 1994; Gao et al., 1997; Leckie et al., 1998; Gomez-Sanchez et al., 2008; Guo et al., 2012). The role of this glucocorticoid-converting enzyme in SC, the reductase or dehydrogenase activity and its androgen-regulated expression remain to be investigated.

Finally, few androgen-regulated genes previously described as expressed in SCs, such as *rhox5* (Maclean et al., 2005; Hu et al., 2010) presented neither a significant expression, nor androgen regulation in the ST38c cells. Indeed, *rhox5* was absent or very weakly expressed in several other immature or mature Sertoli cell lines (such as 15P1, TM4, MSC1) (Bhardwaj et al., 2008). It has been recently reported that GATA factors (mainly GATA4 and GATA6) are essential for AR regulated *rhox5* transcriptional activation (Bhardwaj et al., 2008). As other Sertoli cell lines (15P1, TM4), our Sertoli cell line, lacked expression of GATA factors, including, GATA4 and GATA6, explaining the lack of *rhox5* transcription.

In sum, our ST38c SC model could certainly complement *in vivo* results obtained with animal models, even though, as most cell-based systems, the ST38c cell line has its advantages as well as its own limitations. For instance, we were unable to efficiently transfect these cells without high cellular mortality, even by means of electroporation. FSH responsiveness was also an important issue. We failed to detect *fshr* mRNA in the ST38c cells under standard experimental conditions. However, in addition to the lack of GATA factors important for *fshr*

transcription, it is known that FSH-R expression highly depends upon cultured conditions, notably timing and duration of gonadotropin exposure. Further studies are needed to reevaluate FSH signaling in SC context.

Anyhow, the ST38c cell model should facilitate not only better understanding of the androgen regulation in SC but also might constitute a suitable system to decipher the molecular mechanisms accounting for the negative correlation between AR and AMH expression in postpubertal Sertoli cells.

Conclusion

To our knowledge, ST38c cells represent the first mature, clonal, immortalized mouse Sertoli cell line, expressing a functional AR. Detailed analysis revealed not only that ST38c express *Ar* RNA messenger and protein, but also that androgens stabilize AR protein turnover in Sertoli cells via post-transcriptional mechanisms. We also investigated androgen induced AR mRNA transcriptional regulation as well as some of the transcriptional androgen-regulated genes, showing that this new cell line might be a valuable model for the study of Sertoli-cell specific gene expression and function.

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Legend to figures:

Figure 1: Morphology and molecular markers expression in ST38c Sertoli cells

Panel **A**: Phase-contrast microphotograph of ST38c shows monolayered cells with long cytoplasmic appendages and central ovoid nucleus. Panels **B-F**: Immunofluorescence and cytochemistry studies on ST38c cells. **B**: Nuclear staining of the SV40 large T antigen (SV40 TAg) confirms the immortalized properties of the clonal cell line. **C**: Nuclear SOX9 immunofluorescence staining; **D**: Nuclear WT1 immunofluorescence staining; **E**: β tubulin 3 cytoplasmic immunofluorescence staining, **F**: zonula occludens 1 protein (TJP1) mostly membrane located staining. Scale bars: 50 μ m.

Figure 2: Characterization of mRNA expression of specific cellular markers in ST38c cells

A: mRNA expression of specific Sertoli cell genes in ST38c cells (WT1: Wilm's Tumour; β tubulin 3; CLU: sulphated glycoproteins-2; ABP: androgen binding protein; Transferrin; TJP1: zonula occludens 1; α IB: inhibin alpha subunit) as determined by RT-PCR.

B: β IB: inhibin beta subunit; AMH: anti-Mullerian hormone. Inhibin beta is expressed in murine ST38c as well as neonate and adult murine testis. AMH is not expressed in ST38c while its expression was very intense in neonate murine testis and barely detected in adult murine testis, consistent with the mature phenotype of the ST38c Sertoli cells.

C: LH receptor and CYP17A genes, specifically expressed in Leydig cells are not detected in ST38c cells, consistent with the Sertoli cell origin of the ST38c cell line as well as its clonality.

Testis from neonate (first day post-natally) and adult mice were included as positive controls. RT- (omission of the reverse transcriptase) and H₂O were included as negative controls.

Figure 3: ST38c cells express the androgen receptor (AR)

A: RT-PCR analysis allowed the detection of a 150bp amplicon demonstrating AR mRNA expression in the Sertoli ST38c cell line. Mouse adult testis was used as positive control. RT- (omission of the reverse transcriptase) and H₂O were negative controls.

B: Western blot analysis of AR expression in ST38c and LNCAP cells, used as a positive control. 20 μ g of total protein lysates from ST38c cells and 4 μ g protein lysates from LNCaP cells were processed for immunoblotting with polyclonal anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins expression was normalized with

α tubulin, blotted using an anti- α tubulin antibody. AR protein expression is revealed as a band of 110 KDa.

C: Immunofluorescence studies reveal the AR mainly nuclear distribution in ST38c cells (left panel). DAPI nuclear staining was used as control (right panel). Scale bar: 50 μ m.

Figure 4: Validation of some androgen-regulated genes in the ST38c cells

Relative expression of *rip140*, *hsd11b1*, *gas6*, *aldh1a1*, *cldn19* and *gpr126* and *Ar* mRNA levels was evaluated by qRT-PCR in ST38c cells treated for 24 h with either vehicle (EtOH) or dihydrotestosterone (DHT) at 10^{-7} M (primer sequences are available upon request).

A: *rip140*, *hsd11b1*, *gas6*, *aldh1a1* were androgen up-regulated.

B: *cldn19* and *gpr126* were androgen down-regulated genes, thus validating the gene expression profile obtained with the microarray studies.

C: Androgens stimulate *Ar* mRNA levels in the ST38c cells. Relative changes in gene expression of the androgen stimulated condition, in a given sample was quantified using the $2^{-(\Delta\Delta C(T))}$ method, to that of another sample represented by the untreated control. Results are expressed as means \pm SEM of at least three independent experiments conducted on six reverse-transcribed samples run in duplicate (A and B), or calculated as attomoles per femtomole of 18S rRNA mRNA relative expression (C). Results were normalized to murine 18S ribosomal RNA. Relative expression in a given sample was for the vehicle (EtOH) treated condition was arbitrarily set at 1 (***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ non parametric Mann Whitney test, GraphPad Prism5).

Figure 5: Androgens up-regulate AR expression in the ST38c cells.

Cell lysates were prepared from ST38c cells and AR expression was analyzed by Western immunoblots using 20 μ g protein lysate per lane. Polyclonal anti-AR antibody (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) detected an AR protein of 110 KDa, as above mentioned. Proteins were normalized by reblotting with anti α -tubulin antibody using the Bio-Rad QuantityOne software. (Bio-Rad, France). Numerized band densities corresponding to AR were normalized to α -tubulin and plotted (right panels) as fold induction relative to AR protein under unstimulated conditions arbitrarily set at 1. Data are means \pm SD of at least three independent determinations experiments.

A: Time-dependent increase in androgen-bound AR protein. ST38c cells were incubated with 10^{-7} M dihydrotestosterone (DHT) for various periods of time 0 h, 6 h, 16 h, 24 h.

B: Dose-dependent increase in androgen-bound AR protein. ST38c cells were stimulated with increasing concentrations of DHT (from 10^{-10} M to 10^{-6} M) for 20 h.

C: ST38c cells were treated for 20 h with either vehicle, DHT at 10^{-8} M alone, with RU486 at 10^{-5} M alone or with both DHT at 10^{-8} M and RU486 at 10^{-5} M.

Figure 6: Posttranscriptional events are involved in androgen-dependent AR stabilization.

A: Western blotting analysis of AR expression in ST38c cells treated either with DHT 10^{-7} M, or with 0.4 μ M actinomycin D or with a combination of both DHT and actinomycin D for various periods of time as indicated.

B: Graphic representation of the relative AR expression in ST38c cells, after 1 h, 3 h or 6 h of treatment with DHT, actinomycin or their association, as previously described in the legend of Figure 6. Relative AR protein expression is expressed as fold induction relative to basal conditions arbitrarily set at 1 except for 6 h incubation where AR expression was normalized at 6h to unstimulated AR expression level at 6 h.

Figure 7: Androgens induce AR upregulation by stabilizing AR protein

AR expression in ST38c cells was studied by Western blotting as described in the Figure legends above.

A: ST38c cells were treated with cycloheximide (5 μ g/ml) for various periods of time as indicated 1h, 3h and 6h. AR expression was progressively reduced as a function of time in the presence of the protein synthesis inhibitor.

B: ST38c cells were treated either with DHT 10^{-7} M or cycloheximide (5 μ g/ml) or a combination of both DHT and cycloheximide for various periods of time as indicated.

C: Graphic representation of relative normalized AR expression in ST38c cells, after treatment with DHT, cycloheximide or their association, as mentioned above. AR expression normalized to α -tubulin was expressed as fold induction relative to basal condition for untreated cells at 0 h, was arbitrarily set at 1.

Supplemental Figure 1: Scatchard Plot analysis for specific androgen-binding sites quantification in ST38c cells.

Cytosolic fractions of ST38c cells, previously maintained in dextran charcoal-coated FBS for 24h, were incubated with increasing concentrations of [3 H]- testosterone (Perkin Elmer) for 4 h at 4°C. Bound (B) and unbound (U) hormone were separated by the charcoal-dextran

technique. U was calculated as the difference between total and bound hormone concentration. The curve was simulated from the best model of interaction. The calculated values of binding parameters were determined at equilibrium. The dissociation constant K_d was estimated at 0.7 nM while the maximum number of specific binding sites was calculated at $5-10 \times 10^3$ sites/cell.