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FULL TITLE:

Androgen receptor expression during C2C12 skeletal muscle cell line differentiation.

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SUMMARY

The Androgen Receptor (AR) pathway is involved in the development of skeletal muscle but the molecular basis of androgen-related myogenic enhancement are still unclear. We have investigated AR expression and localization during myoblasts–myotubes differentiation in skeletal muscle cell line C2C12. AR expression increases during proliferation and commitment phase and its levels remain elevated in myotubes. In proliferating and committed cells in the absence of testosterone, AR protein localizes in the nuclei whereas it is almost totally localized in the cytoplasm in myotubes. Low testosterone doses shift the receptor in the nuclei without increasing the amount of total protein. High doses of T induce a significant increase of AR expression during proliferation and differentiation. Few information are available on AR targets that drive myogenic process. In our study, testosterone induces myogenin, myosin heavy chains (MyHC) and GRIP-1 expression, suggesting that AR and its coregulatory proteins are pivotal factors in skeletal muscle differentiation.

Key words: androgen receptor, testosterone, skeletal muscle

INTRODUCTION

The androgen-androgen receptor (AR) signaling pathway is crucial for the determination and differentiation of reproductive organs in men. The activation of this pathway induces important effects in the development of accessory tissues such as brain and skeletal muscle (Herbst et al., 2004) . Strong evidence indicate that testosterone (T), the main circulating androgen secreted by testicular Leydig cells under pulsatile luteinizing hormone stimulation, increases muscle mass and strength, both under physiological and pathological conditions. Physiologic T replacement therapy in healthy young hypogonadal men is associated with significant gains in fat free mass, muscle size and maximal voluntary strength (Bhasin et al., 2000; Snyder et al., 2000; Wang et al., 2000) Furthermore, the effects of androgens in male body composition is established by the use of T supplementation in older men with low levels of circulating hormone, being associated with a significant increase in lean body mass and a reduction in fat mass (Ferrando et al., 1998; Moreley et al., 1993; Sih et al., 2006; Snyder et al., 1999; Tenover et al., 1992) . The improvement of maximal voluntary strength in men with low T concentrations, is also dependent by T replacement therapy (Bhasin et al., 1997) . It has been shown that T supplementation increases muscle mass primarily inducing fiber hypertrophy (Bhasin et al., 1996; Mooradian et al., 1987) due to an increase of protein synthesis (Ferrando et al., 1998; Rooyackers et al., 1997; Sheffield-Moore et al., 1999; Urban et al., 1995) and a decrease of muscle protein breakdown (Ferrando et al., 2002). Evidences in ARKO mice, which have deletion of the genomic actions of the androgen receptor, demonstrate that androgens play a physiological role in achieving peak muscle mass in males but not in females (MacLean et al., 2008)

Emerging data show that T-AR complex activates genes involved in myogenesis. AR is a member of the superfamily of ligand-activated transcription factors encoded by a gene located at Xq 11-12, has three functional domains respectively responsible for

transactivation, ligand binding and binding to DNA (Gobinet et al., 2002; Kemppainen et al., 1992; Roy et al., 2001) . Most of the unliganded AR is localized in the cytoplasmic compartment of target cells, in which it is stored as a multiprotein complex with heat-shock protein and immunophilins. During ligand binding, the receptor dissociates from the multiprotein complex, recruits coactivators and translocates to the nucleus. Subsequently, in concert with co-activators and co-repressors, it binds the promoter region and modulates the expression of androgen-responsive genes that regulate cell fate determination (Gobinet et al., 2002) . AR expression has been detected in skeletal muscle cells (Chang et al., 1995; Doumit et al., 1996), in fibroblasts, and in vascular endothelial cells, smooth muscle and mast cells (Sinha-Hikim et al., 2003) . Animal studies have shown that fiber hypertrophy is associated with increased expression of AR (Hickson et al., 1983; Hickson et al., 1985); accordingly, clinical reports have indicated that T induces muscle hypertrophy through the expression of its receptor (Bhasin et al., 2001; Sheffield-Moore 2000) . AR has been shown also in satellite cells and in CD34+ stem cells of interstitium (Sinha-Hikim et al., 2004). In young men testosterone-induced fiber hypertrophy is associated with an increase in number and dimension of satellite cells, the major source of myogenic precursors involved in mammalian muscle regeneration (Wozniak et al., 2005), suggesting an intriguing potential role for T in reactivation of the myogenic program (Sinha-Hikim et al., 2003). Moreover, it has been demonstrated that androgens can promote the commitment of mesenchymal pluripotent C3H10T1/2 cells into myogenic lineage, through an androgen receptor-mediated pathway (Singh et al., 2003).

In this study we focused our attention on the expression and the localization of AR during skeletal muscle cells differentiation with or without exogenous T. Using C2C12 cells line we show that AR expression is increased during proliferation and commitment of these

cells and that it remains at high level in myotubes. Interestingly, when the cells became differentiated, AR is stored in the cytoplasm and activated only after T treatment.

RESULTS

Androgen receptor expression during C2C12 differentiation.

In order to analyze the AR and related genes expression during skeletal muscle differentiation, we used mouse myoblasts C2C12 cells developing a classical differentiation protocol. We plated cells at low density in a growing medium (GM), leaving them to proliferate for 72 hours until reaching the committed status. Then we incubated cells in a low serum medium in order to induce terminal differentiation. We checked gene expression 24, 48 and 72 hours after seeding in GM and at two terminally differentiated conditions (2 and 4 days after switch in DM). (Figure 1A). In order to study AR expression during C2C12 differentiation in the absence or in the presence of exogenous T, we used both complete and serum-deprived complemented medium, to get rid of possible interfering effect due to serum.

We showed no differences between the two different conditions on AR, myogenin and myosin heavy chains mRNA expression, even if cells growth in DCS supplemented medium express a lower amount of myogenic marker (i.e. myogenin) (data not shown). Since we wanted to evaluate our model in a more physiological context, we performed all the following experiments in complete serum supplemented media. We checked the transition from a proliferating to a mature muscle phenotype, concomitant with terminal cell cycle exit, by analyzing some classical cell cycle marker. In muscle cells, cell cycle exit is characterized by the activation of myogenin, a transcription factor strictly expressed in terminal differentiated myotubes (Hasty et al., 1993), the repression of cyclin D1 (Guo et al., 1997) and the activation of cell cycle inhibitor p21 (Halevy et al., 1995). QPCR showed a significant increase of myogenin ($p < 0.001$) and p21 ($p < 0.001$) mRNA expression. Also

cyclin D1 mRNA levels were down regulated ($p < 0,001$) in accordance with our experimental model (Fig 1B).

We initially investigated AR mRNA and protein expression during the conversion of myoblasts into multinucleated myotubes, in absence of exogenous T. QPCR showed that AR mRNA expression was significantly increased during proliferation until the committed phase (2,65 fold from 24 hours to 48 hours and 5 folds from 48 hours to D0, $p < 0,01$) and it persisted high in terminally differentiated myotubes (Figure 2A). In accordance with mRNA, AR protein followed the same pattern of expression (4 folds induction from 24 hours to 48 hours and 3 folds from 48 hours to committed phase, $p < 0.001$) (Figure 2B). These results reveal a central role of AR in earlier phases of myogenesis.

AR localization during muscle differentiation

The inactivated AR is localized in the cytoplasmic compartment as a multiprotein complex with heat-shock proteins and immunophilins. Ligand activation is followed by the dissociation from the multiprotein complex, recruitment of coactivators and translocation in the nuclei. (Gobinet et al., 2002).

We investigated the intracellular localization of AR during muscle differentiation. As already shown in Fig. 2B, under basal condition AR total protein was up regulated until D0 and its levels remained unchanged in D2 and D4. The subcellular pattern of localization turned out to be interesting: whereas the cytoplasmic expression profile resumed total protein distribution, in the nuclear compartment AR protein levels increased during proliferation and turned down to basal level in differentiated myotubes (Fig 3A, upper panel).

Effect of testosterone treatment

A. AR localization

When cells were treated with 10nM testosterone, a significant increase in total and nuclear AR protein levels was observed during the proliferation and the differentiation process

(Fig. 3A, lower panel; Fig. 3B, C, D). Interestingly when lower concentrations (0.1 and 1nM) of T were used, a significant increase of AR protein was found only in the nuclear compartment whereas its levels remained roughly unchanged when total protein content was examined (showed only most relevant data at D2 and D4, Fig 4). All data were confirmed by immunofluorescence (Figure 5). In differentiated myoblasts AR was detected predominantly in the cytoplasm whereas a strong staining was found in the nuclear compartment of T-induced cells, as expected after AR nuclear translocation. All these results suggest an important shift of pre-existent AR from the cytoplasm into the nucleus, since the total protein level was not increased.

B. AR expression

Emerging data show that T exerts its effect in muscle cells by an androgen receptor (AR) dependent pathway. Since C2C12 cells express AR under basal condition, we investigated the effect of different doses of T on its expression levels, throughout the whole differentiation process.

Fig. 6A shows a clear dose dependent effect of T on AR mRNA levels in undifferentiated myoblasts (24h and 48h), whereas treatment with only 10nM testosterone was able to significantly induce AR expression at all studied time points (8.25, 19.74, 22.98, 30.21 and 43.15 folds respectively at 24h, 48h, D0, D2 and D4; $p \leq 0.01$) suggesting that skeletal muscle cells increase their responsiveness to T during proliferation phase.

The described effects of T were specifically dependent on AR, since treatment with 100-fold molar excess of the AR antagonist bicalutamide was able to revert AR mRNA induction (Fig 6B); antagonists alone had no effect on AR messenger expression.

C. Myogenin expression

As described yet, myogenin is a transcription factor that strictly marks terminal differentiated myotubes (Hasty et al., 1993). Previous report showed that myogenin expression is enhanced in AR-stably transfected myoblasts by T treatment (Lee 2002). In

our experimental model, myogenin expression started at D0 reaching maximal level at D2 and D4 (Fig 1B); T exposure determined a significant increase of myogenin expression. Interestingly, T-induced increase in myogenin mRNA expression appeared at D0; notably, at this stage a dose-response effect was observed on myogenin mRNA levels (Fig 7A). Such effect was evident also at a protein level, when nuclear protein was analyzed (Fig 7B). Myogenin mRNA increase was antagonized by the addition of 100-molar excess of bicalutamide (Fig 7C) which completely abolished T effects. These data strongly suggest that T induced myogenin induction is AR dependent. Similar data were obtained in steroid deprived condition (data not shown).

D. Myosin heavy chains expression

The Myosin heavy chains (MyHC) are terminally differentiated muscle cells markers. In normal adult skeletal muscle fibres, four MyHC isoforms may be expressed: one slow, the MyHC-1 and three fast, MyHC-IIa, MyHC-IIb and MyHC-IIx/d (Schiaffino et al., 1996)

In the AR knockout (ARKO) mice, it has been demonstrated that androgen-AR signalling pathway may have important roles in determining the ratios of skeletal muscle fiber types in muscle by regulating protein levels of skeletal muscle fiber-specific proteins (Altuwaijri et al., 2004). In our experimental model, testosterone (T) upregulated the expression of MyHC-IIb, MyHC-IIx/d and MyHC-I mRNAs (Fig 8); MyHC-IIa was not expressed (not shown). The effect of Testosterone (T) induction became visible at D0, confirming our hypothesis that T may accelerate differentiation process in skeletal muscle cells. 100 fold molar excess of bicalutamide (Fig 8) were able to inhibit the Testosterone-mediated MyHCs upregulation, demonstrating that it is involved an AR-mediated pathway. Moreover, the effect of androgens were most marked on MyHC-I mRNAs, in accordance with previous observation in ARKO mice muscle where AR-signaling pathway decreases slow-twitch fibers expression (Altuwaijri et al., 2004).

E. GRIP-1 expression

The transcriptional activity of AR is affected by coregulators that influence a number of functional properties of AR, including ligand selectivity and DNA binding capacity (Heinlein et al., 2002). An elegant study shows that GRIP-1, a steroid receptor coactivator, interacts with MEF-2C mediated transcription and formation myotubes, indicating that steroid receptors coactivators may play important role in skeletal muscle differentiation (Chen et al., 2000). We investigated the regulation of GRIP-1 during cells differentiation upon T treatment. QPCR clearly indicated that in untreated cells GRIP-1 mRNA was up regulated during the differentiation process ($p < 0.001$). Interestingly T significantly increased GRIP-1 mRNA expression, 2 folds at D0 and about 3 folds at D2 and D4 ($p < 0.01$) (Fig 9), consistent with the hypothesis that GRIP-1 could interact with AR during skeletal muscle differentiation.

DISCUSSION

In the present study we show that testosterone (T) and androgen receptor are critical factors in earlier phases of myogenesis. AR is maintained at high levels in the cytoplasm of terminally differentiated myotubes and shift into the nucleus under the effect of T even at low doses. Myogenin and GRIP-1 expression, two important factors involved in the transcriptional machinery of skeletal muscle cells, are also induced by T. Several *in vitro* and *in vivo* reports demonstrate that the androgen-AR signaling pathway is required for skeletal muscle development and for sustaining muscle mass, strength and protein synthesis (Herbst et al., 2004). Our data provide original information on the expression and localization of AR during C2C12 skeletal muscle cells differentiation, in the presence or in the absence of T stimuli. In reproductive tissue T is converted to dihydrotestosterone (DHT) by the action of the enzymes 5α -reductase type 1 and 2. Considering that the levels of these enzymes in skeletal muscle are very low (Bhasin et al., 2003), T represents the

main ligand for AR to induce muscle differentiation. C2C12 cells have been widely used as a model of skeletal muscle differentiation *in vitro* (Galbiati et al., 1999) due to their capacity to proliferate or differentiate under appropriate experimental conditions. In previous reports about the AR involvement in C2C12 cells differentiation, no detectable AR protein levels were found (Altuwaijri et al., 2004; Lee 2002); consequently in these studies all experiments were performed on AR stably transfected cell line. In our study, QPCR, Western Blot and Immunofluorescence showed that our cellular model clearly express AR. To our knowledge this is the first report on the expression and localization of endogenous AR during C2C12 cells differentiation. Altuwaijri et al (2004) showed that transfected AR protein was up regulated in myotubes and that transcription of AR gene is increased during the muscle differentiation. We investigated in detail the natural timing of endogenous (not transfected) AR expression confirming that AR is up regulated in myotubes. Furthermore we demonstrated that a significant increase of AR mRNA and protein occurs just during the proliferation phase. Interestingly, in terminally differentiated myotubes AR mRNA and protein levels remained stably elevated until the differentiation process was complete. The increase of AR expression in the absence of ligand during the phases leading to the terminal differentiation may suggest that muscle cells acquire high responsiveness to androgens. In myotubes AR mRNA and protein levels reached and retained the highest AR degree, probably required to maintain the differentiated status; therefore high AR levels could be considered as a marker of terminally differentiated myotubes. A similar expression pattern of AR and other nuclear receptors has been described in mouse 3T3-L1 preadipocytes during adipose differentiation, suggesting that nuclear receptors may serve as biomarkers and potential therapeutic targets to interfere with cellular differentiation (Fu et al., 2005). The treatment of C2C12 and C3H 10T1/2 pluripotent mesenchymal cells with testosterone has been shown to induce its own receptor expression (Lee 2002; Singh et al., 2003). We demonstrated that all the used

doses (0.1, 1 and 10nM) of T were able to induce a dose-response increase in AR mRNA and protein expression during myoblasts proliferation, suggesting that in this phase the skeletal muscle cells should be able to enhance their responsiveness to androgen also in presence of low hormone concentration. Bicalutamide, a specific non-steroidal AR antagonist, is able to revert the T-induced effects. This demonstrates for the first time that testosterone modulates C2C12 cells androgen responsiveness affecting the expression of its own receptor by a specific AR-dependent pathway. In agreement with Bhasin et al, who have demonstrated that androgens stimulate AR expression, inducing myogenic differentiation and inhibiting adipogenesis in C3H 10T1/2 pluripotent mesenchymal cells through an AR-mediated pathway (Singh et al., 2003), we show that androgens exert their biological action on skeletal muscle differentiation by a ligand-dependent positive feedback on AR expression that increase the system responsiveness.

Since we confirmed the role of AR in muscle differentiation, we have further investigated in detail its subcellular localization. It is well known that ligand-induced nuclear translocation is necessary for the AR transcriptional activity (Gobinet et al., 2002). We observed that endogenous AR protein is up regulated during the course of C2C12 myogenic differentiation. Cytoplasmic receptor fraction showed a similar expression profile of total protein. Surprisingly we detected an increase in nuclear receptor only during proliferation phase; in differentiated myotubes the high levels of AR are almost totally localized in the cytoplasm. 10nM T treatment increased total protein expression and was able to shift AR in the nuclear compartment at all differentiation time points. Interestingly, in our experimental model low doses of T are able to shift, and then to activate, receptor in the nuclei in differentiated myotubes also in absence of total protein expression improvement. These results, together with the demonstration of high cytoplasmic levels of AR in myotubes, could explain a fine capacity of mature skeletal muscle tissue to rapidly respond to T also in presence of very low (i.e. 0.1nM) circulating T levels variations.

Recent reports demonstrated that androgens are able to induce the expression of some myogenic markers (Lee 2002; Singh et al., 2003). In particular we studied the androgen-induced myogenin expression in C2C12 skeletal muscle cells demonstrating that T is able to up regulate myogenin mRNA and protein synthesis by an endogenous AR-dependent pathway. Furthermore we showed that the myogenin up regulation appears into committed cells that are still growing in high serum medium. Our data are slightly different from those of Lee (Lee 2002) who observed that T stimulated myogenin induction only in C2C12 cells seeded in low serum medium. We consider that this inconsistency is related to the different experimental model used: we analyzed the ligand-induced effect on an endogenous (not transfected) receptor in a different time course. We also showed that T induces MyHCs mRNA expression via AR pathway. Interestingly, bicalutamide alone was able to down regulate MyHC IIb and MyHC slow mRNA also in the absence of the ligand. This evidence can be explained by the presence of interfering steroids in complete serum that could constitutively activate AR. Such effect has already been described in other experimental settings where mineralocorticoid receptor (MR) antagonists were able to basally down regulate MR target genes in 3T3-L1 preadipocytes (Caprio et al, 2007). Transcriptional activity of nuclear receptors is modulated by coregulatory proteins that interact with nuclear receptors to enhance or reduce transactivation of target genes (Heinlein et al., 2002). GRIP-1 (also known as TIF-2) is an AR coactivator expressed in proliferating and differentiated C2C12 cells that potentiates skeletal muscle differentiation by acting as a critical coactivator for MEF-2C-mediated transcription (Chen et al., 2000). Here we report that GRIP-1 mRNA is up regulated during C2C12 differentiation and that T induces GRIP-1 mRNA expression. To our knowledge, this is the first demonstration of T-induced GRIP-1 expression in skeletal muscle cells suggesting a relationship between the skeletal muscle transcriptional machinery and AR-mediated cofactor regulation. Since T effects on AR target genes were obtained after a prolonged incubation time, we can

hypothesize that AR activation by T may regulate the expression or the activity of other transcription factors that may subsequently control cell differentiation. Further studies are necessary to elucidate this aspect.

Testosterone and related androgens are largely used for doping purposes (Brown et al., 2006; Saudan et al., 2006) and represent the substances most frequently responsible for adverse analytical findings among drugs prohibited by the world Anti-Doping Agency (www.wada-ama.org). Our data show that testosterone influences the differentiation of C2C12 cells (that may be considered as activated satellite cells) to mature myotubes, by modulating the expression and localization of its receptor and by influencing myogenic program. On the other hand, the effects of low doses of testosterone on mature muscle fibers allow us to speculate that *in vivo* even physiological (probably routinely undetectable) amounts may exert relevant effects on “primed” muscle cells.

Finally, our study may stimulate further investigations on the changes induced by factors modulating muscle mass regeneration in order to better understand the role of androgens on muscle differentiation, organization and repair.

MATERIALS AND METHODS

Cell culture

C2C12 mouse myoblasts were a kind gift of Dr. Antonio Musarò, Department of Histology and Medical Embryology, University of Rome La Sapienza, Italy. Cells were maintained at 37°C, 5% CO₂ in a humidified incubator in Dulbecco's modified Eagle's medium without phenol red supplemented with 15% fetal calf serum, 2mM L-Glutamine, 100U/ml of penicillin G sodium, 100 µg/ml of streptomycin sulphate (Growth Medium, GM). Cells used in all experiments were at passage 4. All media and serum were purchased from Invitrogen.

Myoblasts differentiation and androgens treatment

For cell differentiation experiments, C2C12 myoblasts were plated at a density of 2×10^4 cells/ml in GM, supplemented with normal or dextran-charcoal stripped serum. Differentiation into myotubes was induced after 72 hours by switching 80 – 90% confluent cells to Dulbecco's modified Eagle's medium without phenol red supplemented with 2% horse serum, 2mM L-Glutamine, 100U/ml of penicillin G sodium, 100 µg/ml of streptomycin sulphate (Differentiation Medium, DM).

Cells were harvested for molecular analysis at 24, 48 (proliferating myoblasts) and 72 hours (D0, committed myoblasts) after seeding in GM and at 2 days and 4 days (D2 and D4) after switch in DM (differentiated myotubes). Testosterone (Sigma), Bicalutamide (Astrazeneca) or vehicle (ethanol), diluted as indicated in the results in GM or DM, were added to the medium at the start of experiments and at D0 when medium was changed to GM into DM.

RNA extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (QPCR)

Cells were washed twice with 1X Phosphate Buffer Saline (PBS, Invitrogen), collected with scraper and immediately lysated in 1 ml of TRIzol™ Reagent (Invitrogen). Total RNA was extracted following manufacturer's indications. The purity, integrity and yield of RNA was monitored by micro-capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies) using the RNA 6000 LabChip kit. 1 µg of total RNA was treated with DNase I Amplification Grade (Invitrogen) and reverse-transcribed using the SuperScript™ III (Invitrogen). Quantitative PCR was performed in Mx3000P™ light cycler (Stratagene) using iTaqSupermix with rox (Biorad) as indicated by manufacturer's. All primers were optimized for real-time RT-PCR amplification checking the generation of a single amplicon in a melting curve assay and the efficiency in a standard curve amplification (> 98% for each couple of primers). Quantitative RT-PCR sample value was normalized for the expression

of cyclophilin mRNA. The relative expression of investigated genes was calculated by Mx3000P™ software version 2.0 (Stratagene) and is reported as arbitrary units. In all experiments each sample was analyzed in duplicate. Sequences of primers are given in table 1.

Protein extraction and Western Blot analysis

Cells were washed twice with 1X PBS, collected with scraper, centrifuged 10' at 1200rpm at 4°C and immediately lysed for protein extraction. To extract total proteins, pellet was resuspended in lysis buffer (50mM Tris-HCl pH7.6, 0,01% NP40, 0,1% SDS, 150mM NaCl, 5mM EDTA, 100mM NaF, 10mM NaPPi, 2mM Na₃VO₄, 2mM PMSF and 0.01 mg/ml of Aprotinin, Leupeptin and Pepstatin). After 20' in ice, sample was centrifuged 20' at 14000rpm at 4°C and supernatant (total proteins) was collected and stored at -80°C until use. NE-PER Nuclear and cytoplasmic extraction Reagents (Pierce) was used to extract nuclear and cytosolic fraction of cell proteins, following manufacturer's protocol. Proteins concentrations was determined using the Micro BCA Protein assay Reagent (Pierce).

Protein samples (50µg for nuclear and cytoplasmic extracts; 100µg for total extracts) were separated in a SDS-polyacrilamide gel and transferred to a nitrocellulose membrane. Transfer was verified by Ponceau S staining. The membrane was blocked 60 min at RT with 10% nonfat dry milk in Tris Buffered Saline (T-TBS, 50mM Tris Base, 0,9% NaCl, Tween 20 0.001%, pH 7,4). Anti-Androgen Receptor (N-20, Santa Cruz), anti-Myogenin (M-225, Santa Cruz), anti-Nucleolin (H-250, Santa Cruz) and anti-α-Tubulin (TU-02, Santa Cruz) antibodies were added followed by incubation overnight at 4°. After a 30 min incubation at room temperature, the membrane was washed three times with T-TBS and incubated for 60 min at RT with HRP-labeled anti-rabbit or anti-mouse (Jackson ImmunoResearch) in 5% non fat dry milk. The membrane was then washed three times and the antibodies were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) with Typhoon 9200 (Amersham Biosciences). Quantitative analysis was

performed using Imagequant TL Image analysis software (Amersham Biosciences); sample value was normalized for housekeeping gene (nucleolin or α -tubulin as indicated in the text) and is reported as arbitrary units.

Immunofluorescence (IF) analysis

Cells were seeded on polyisinated slides and grown as described in “Myoblasts differentiation and androgens treatment” section of Materials and Methods. For IF analysis, cells were rinsed twice with cold 1X PBS, fixed with 4% paraformaldehyde 10' at room temperature (RT), rinsed three times with 1X PBS, permeabilized by incubation in 0.2% Triton in 0.1M Tris-HCl pH 7,6 and rinsed accurately in 1X PBS. Cells were incubated in blocking solution (3% bovine serum albumin in 1X PBS) for 30' at RT and further incubated with 1:200 in 1X PBS anti-Androgen Receptor primary antibody (N-20, Santa Cruz Biotechnologies) 1 hour at RT. After three washes in 1X PBS, cells were incubated with 1:2000 anti-rabbit Alexa Fluor (Molecular Probes) in the dark 30' at RT. Cells were rinsed three times with 1X PBS and slides mounted with 70% glicerol in 1X PBS. Images were acquired by Nikon TE2000-U microscope and Nikon CCD camera at 60X magnification.

Statistical analysis

Statistical analysis was performed using Student's *t* test (Jandel Scientific). Data are presented as mean \pm standard deviation and are the average of at least four different independent experiments using independent cell cultures in each experimental settings. P values of <0.05 were considered significant.

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FIGURES LEGENDS

Figure 1: Validation of C2C12 cell differentiation model.

A: microscopic analysis of cell differentiation. C2C12 cells were cultured at low confluence in the growing medium (GM) until committed status, then shifted in differentiating medium (DM). 24h and 48h are representative points of proliferating myoblasts; D0 is representative of the committed myoblast status, reached 72h after seeding. D2 and D4 are differentiated myoblasts cultured respectively 2 and 4 days in DM (magnification 60X). B: QPCR analysis of cell differentiation molecular markers (B: myogenin; C: cyclin D1; D: p21) at different time points during differentiation. The results shown represent the mean of three independent experiments using independent cells culture in each experimental setting. ** $p < 0.001$; statistical significance was analysed between each time point versus previous.

Figure 2: AR expression during cells differentiation.

A: Time course of AR mRNA expression during cells differentiation analysed by QPCR. * $p < 0.01$; statistical significance was analysed between each time point versus previous. The results shown represent the mean of three independent experiments using independent cells culture in each experimental setting. B: Western blotting analysis of AR protein expression at the same time points studied in QPCR. Upper panel shows representative immunoblot showing AR expression; α -tubulin (lower panel) was used as loading control. Lanes: 1: 24h; 2: 48h; 3: D0; 4: D2; 5: D4. Histograms show the mean \pm standard deviation of four different western blot quantifications. ** $p < 0.001$.

Figure 3: Testosterone effects on AR protein expression and localization

A: Western Blot analysis of total, cytosolic and nuclear AR protein. Upper panel: untreated cells; middle panel: 10nM T treated cells; lower panel: loading control, normalizer gene (α -

tubulin for total and cytosolic fraction and nucleolin for nuclear fraction). Lanes: 1: 24h; 2: 48h; 3: D0; 4:D2; 5: D4. B,C,D: Graph of quantified data from Western Blot experiments; B: total AR; C: cytosolic AR; D: nuclear AR. Bars represent mean \pm standard deviation of five different independent experiments; statistical significance is indicated between untreated (white bars) versus 10nM T treated cells (grey bars) in each time point analysed. ** $p < 0.001$.

Figure 4: Dose-dependent effect of testosterone upon AR protein expression and localization

A: Western blot analysis of AR protein expression under different doses of T treatment. Upper panel: nuclear AR; middle panel: total AR; lower panel: nucleolin (loading control). Lanes 1 – 4: protein extracts at D2; lanes 5 – 8: protein extracts at D4. Lanes: 1, 5: untreated cells; 2, 6: 0.1nM treated ; 3, 7: 1nM treated cells; 4, 8: 10nM treated cells. Bars (B,C) show quantification of four different independent experiments. Statistical analysis was performed between treated versus untreated cells in each time point. * $p < 0.01$; ** $p < 0.001$.

Figure 5: Testosterone effects on AR protein expression and localization

Immunofluorescence analysis of AR localization in C2C12 cells in absence (left pictures) or in presence (right pictures) of 10 nM T. One of three similar studies is shown. Magnification 60X.

Figure 6: Dose-dependent effect of testosterone on AR mRNA expression

A: Time course of AR mRNA expression in C2C12 cells grown in presence of 0.1, 1 or 10nM T treatment or in presence of vehicle alone (untreated), by QPCR. Bars represent mean \pm standard deviation of five different independent experiments; statistical analysis was performed between treated versus untreated cells in each time point. * $p < 0.01$; ** $p < 0.001$. B: Bicalutamide reverts T-induced up-regulation of AR mRNA at all studied time points. QPCR analysis of AR mRNA expression in C2C12 cells grown in presence of

10nM T (+T), 100 fold molar excess bicalutamide (B) alone (+B), 10nM T and 100 fold molar excess B (+T +B) or vehicle alone (untreated). Bars show quantification of four different independent experiments. Statistical analysis was performed between T treated versus T + B treated cells in each time point. * $p < 0.01$; ** $p < 0.001$.

Figure 7: Testosterone induces myogenin mRNA and protein expression through a specific activation of AR

A: QPCR analysis of myogenin mRNA expression in C2C12 cells at D0, D2 and D4, grown in presence of 0.1, 1 or 10nM T concentrations, or in presence of vehicle alone (untreated). Bars represent mean \pm standard deviation of five different independent experiments; statistical analysis was performed between treated versus untreated cells in each time point. ** $p < 0.001$. B: Western blot analysis of myogenin protein expression under 10nM T treatment. Upper panel: untreated cells; middle panel: 10nM T treated cells; lower panel: nucleolin (loading control). Lanes: 1: 24h; 2: 48h; 3: D0; 4: D2; 5: D4. Bars show quantification of four different independent experiments. Statistical analysis was performed between treated versus untreated cells in each time point. ** $p < 0.001$. C: QPCR analysis of myogenin mRNA expression in C2C12 cells grown in presence of 10nM T (+T), 100 fold molar excess B alone (+B), 10nM T and 100 fold molar excess B (+T +B) or vehicle alone (untreated). Bicalutamide reverts T-induced up-regulation of myogenin mRNA expression at all studied time points. Bars show quantification of four different independent experiments. Statistical analysis was performed between T treated versus T + B treated cells in each time point. ** $p < 0.001$.

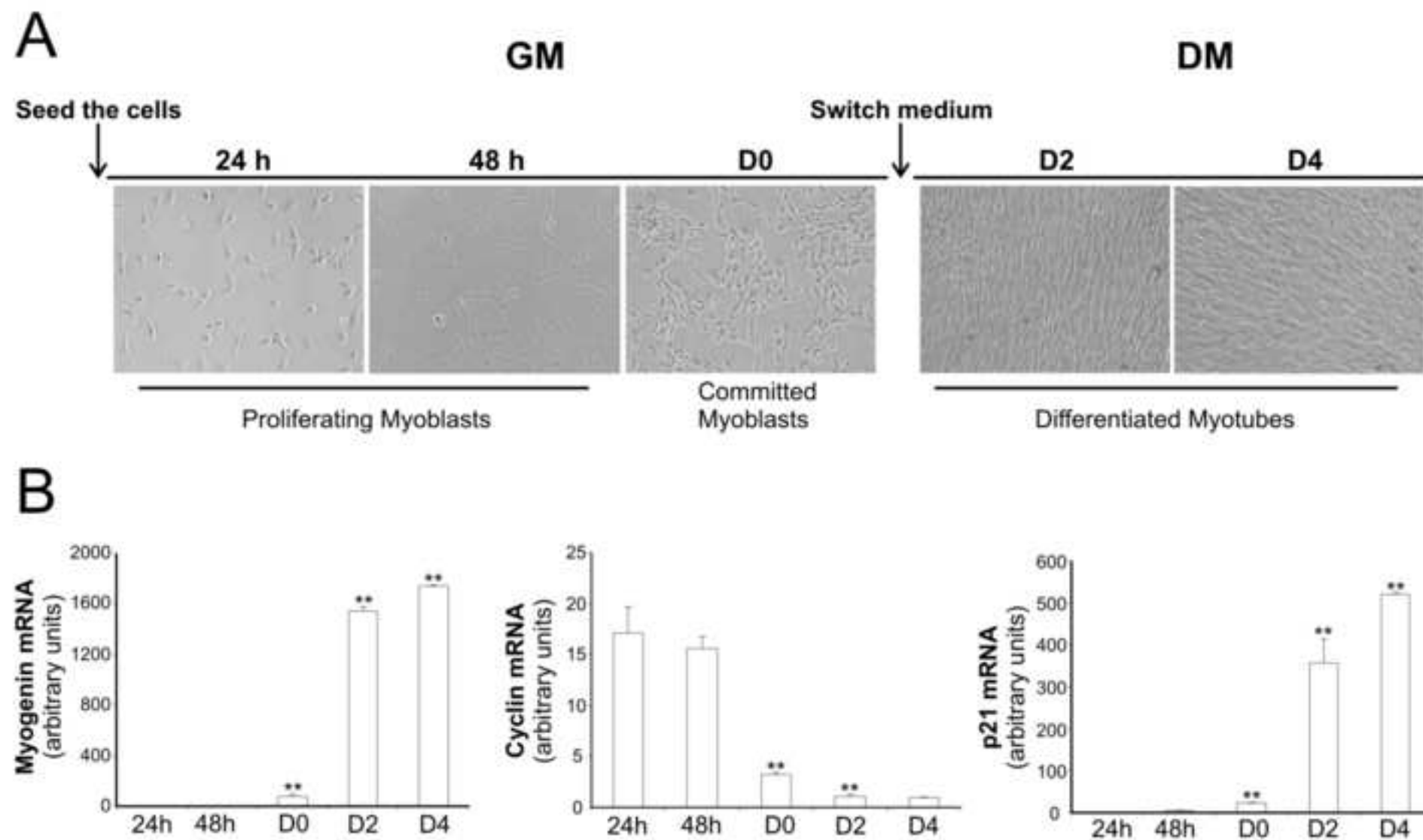
Figure 8: Testosterone induces myosin heavy chains (MyHCs) mRNA expression through a specific activation of AR

QPCR analysis of MyHCs mRNA expression in C2C12 cells grown in presence of 10nM T (+T), 100 fold molar excess bicalutamide (B) alone (+B), 10nM T and 100 fold molar excess B (+T +B) or vehicle alone (untreated). A: MyHC IIb fast; B: MyHC IIx/d fast; C:

MyHc slow. Bars represent mean \pm standard deviation of four different independent experiments; statistical analysis was performed between testosterone treated versus untreated cells in each time point. ** $p < 0.001$.

Figure 9: Testosterone induces GRIP-1 mRNA expression in a time-dependent manner
QPCR analysis of GRIP-1 mRNA expression in C2C12 cells grown in presence of 10nM T or of vehicle alone (untreated). Bars represent mean \pm standard deviation of five different independent experiments; T is able to significantly up-regulate GRIP-1 expression only after the committed status. Statistical analysis was performed between treated versus untreated cells in each time point. ** $p < 0.001$.

Figure 1



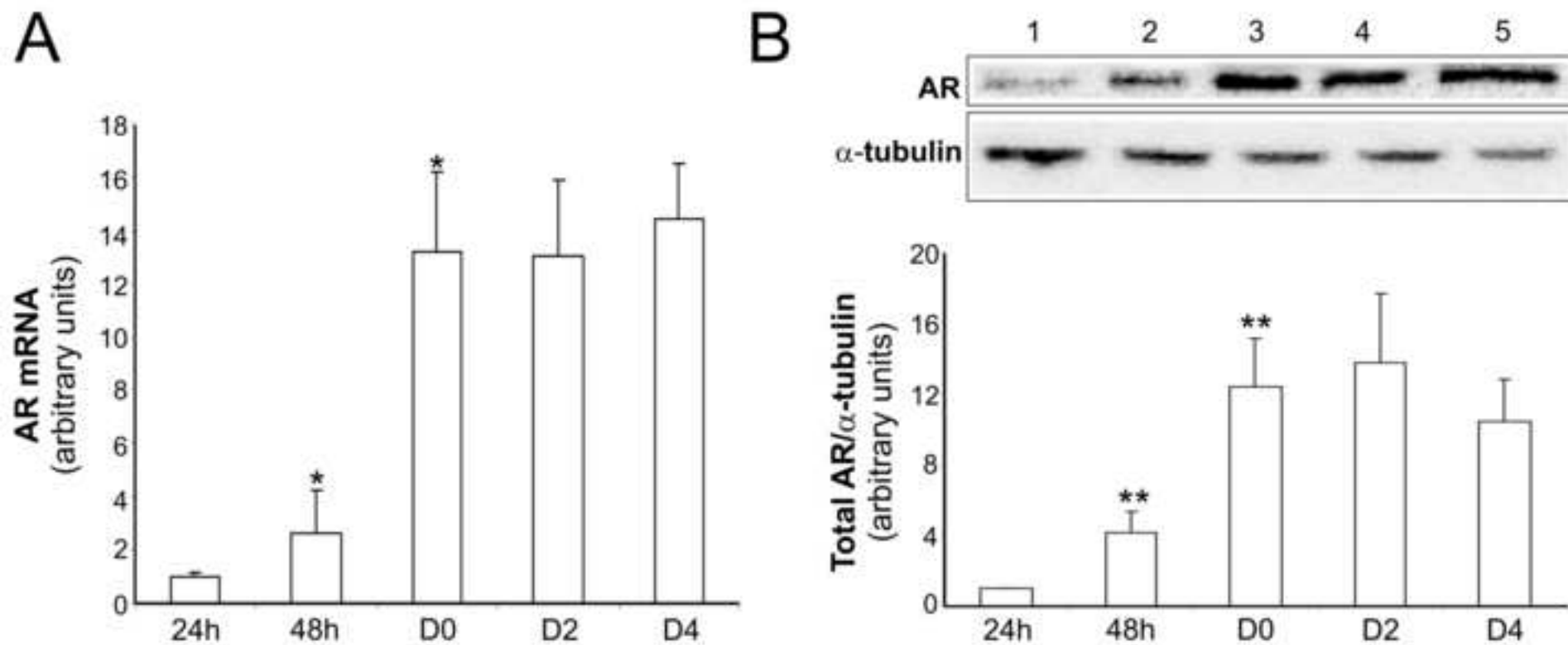


Figure 3

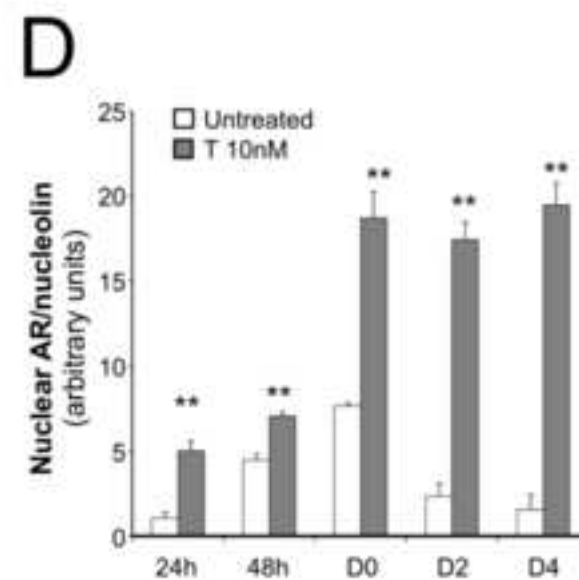
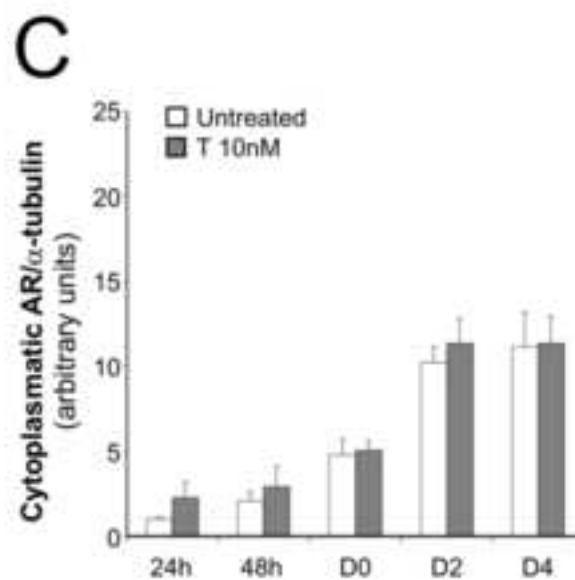
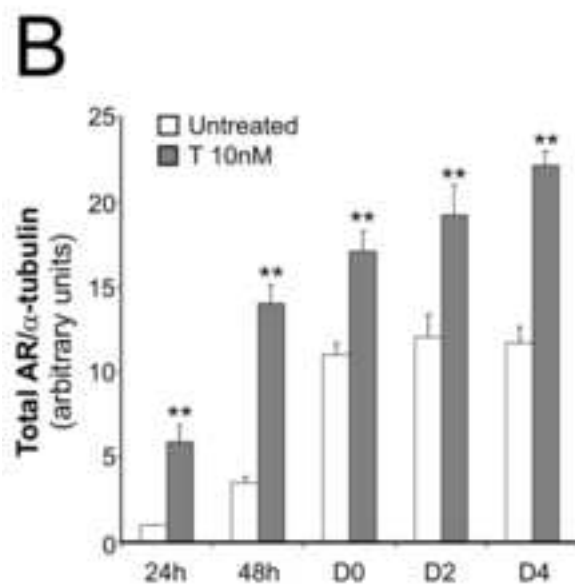
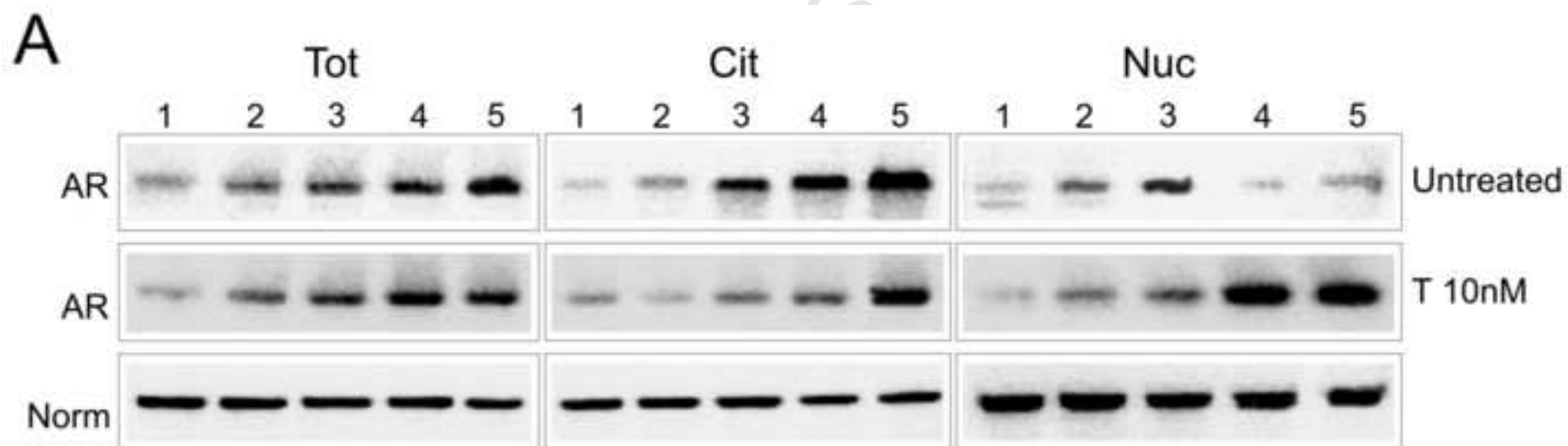
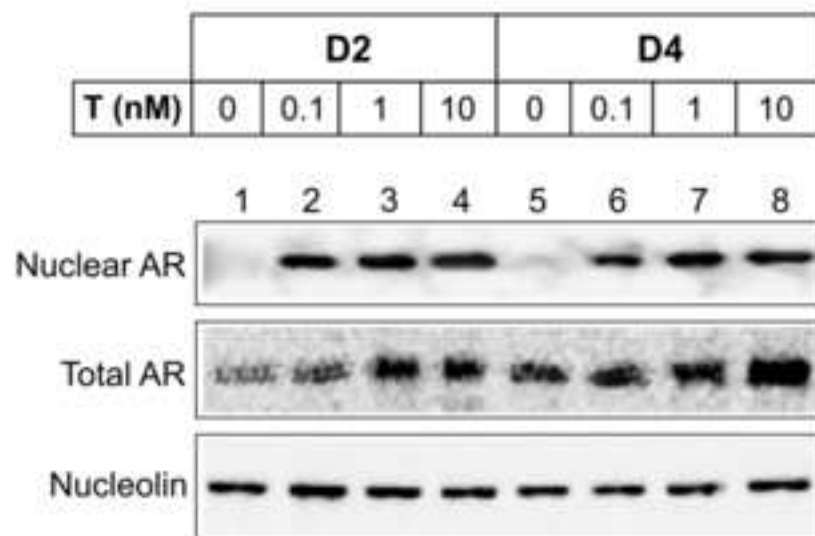
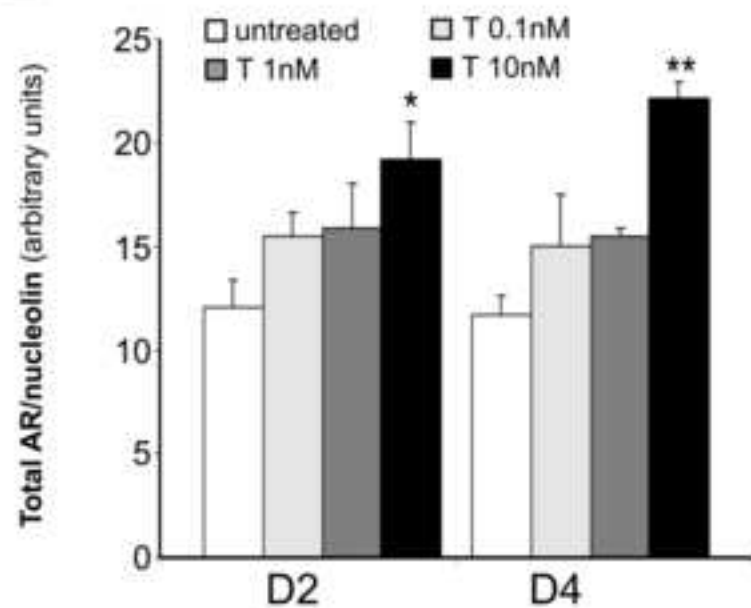


Figure 4

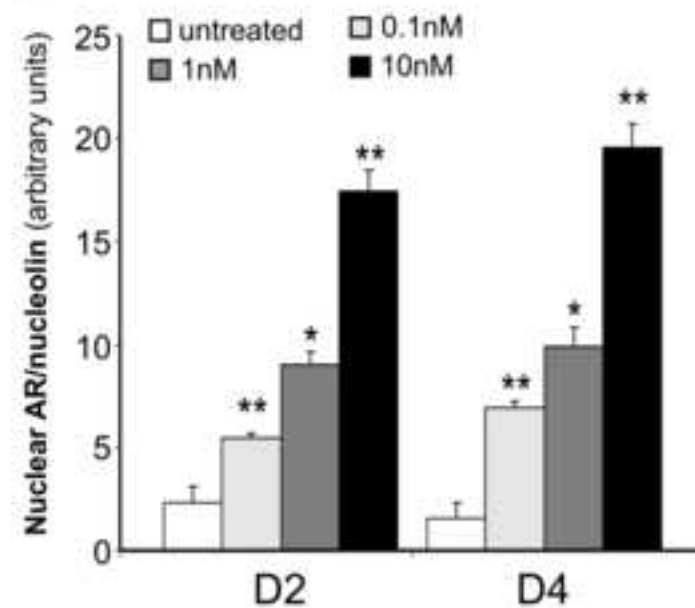
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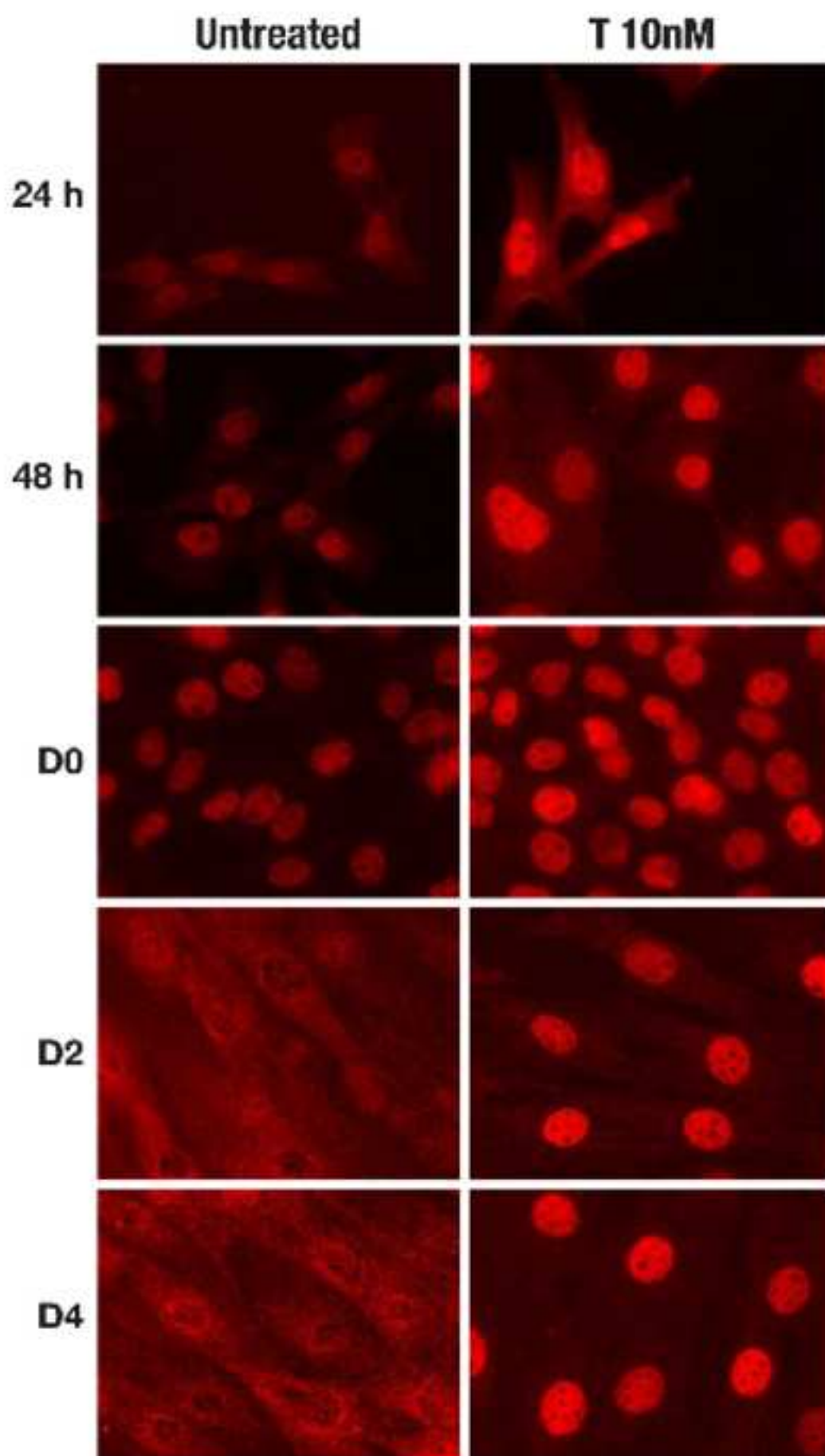


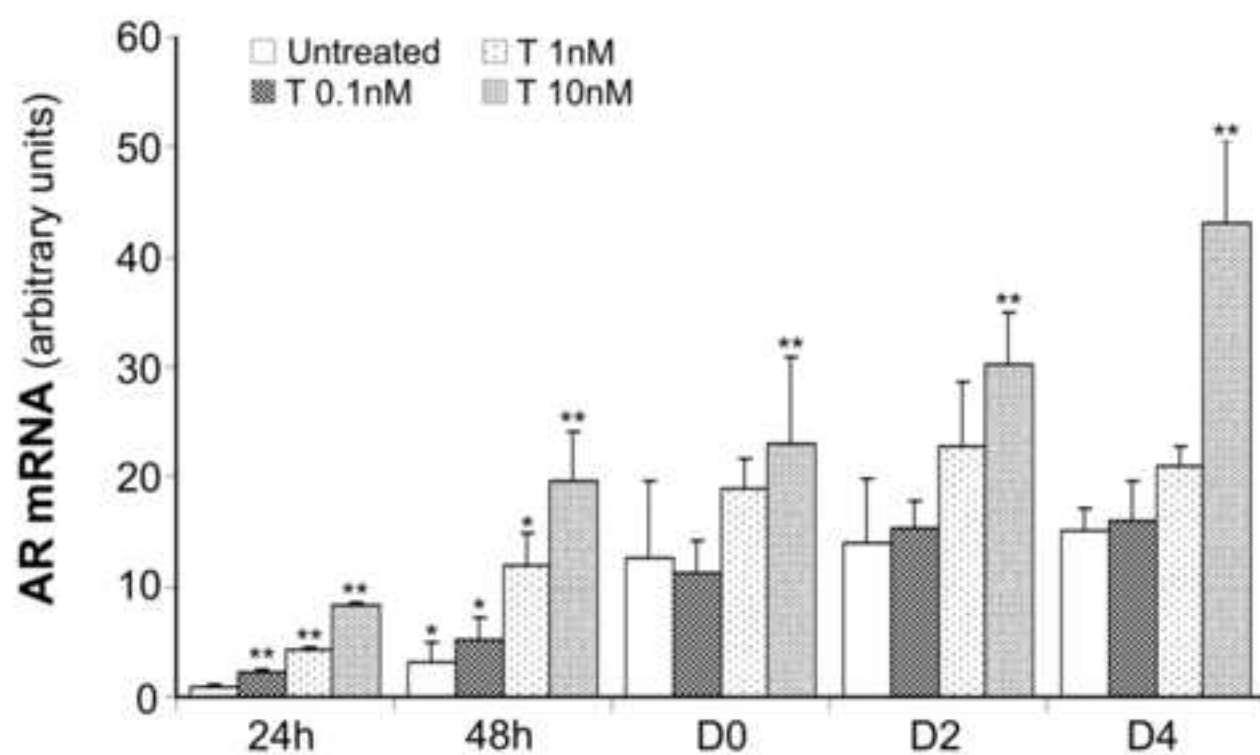
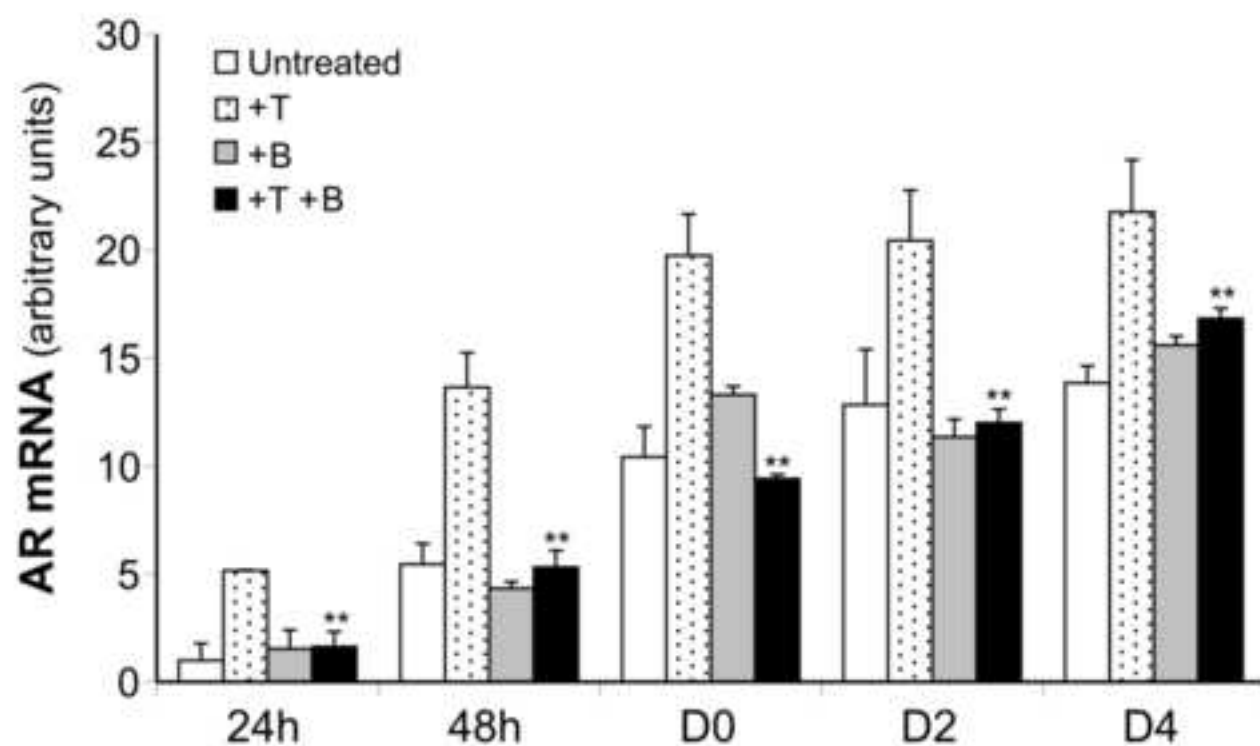
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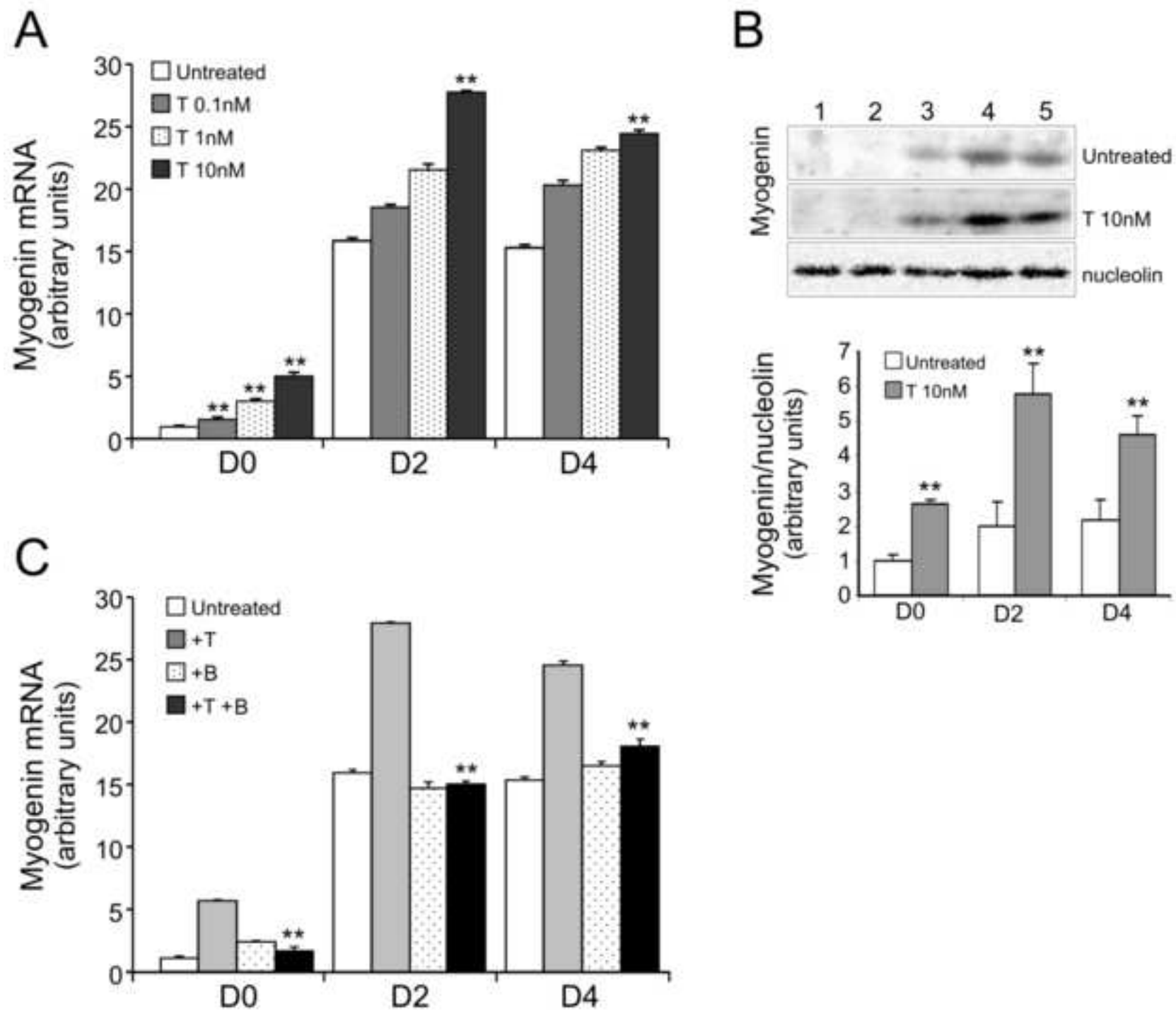


C





A**B**



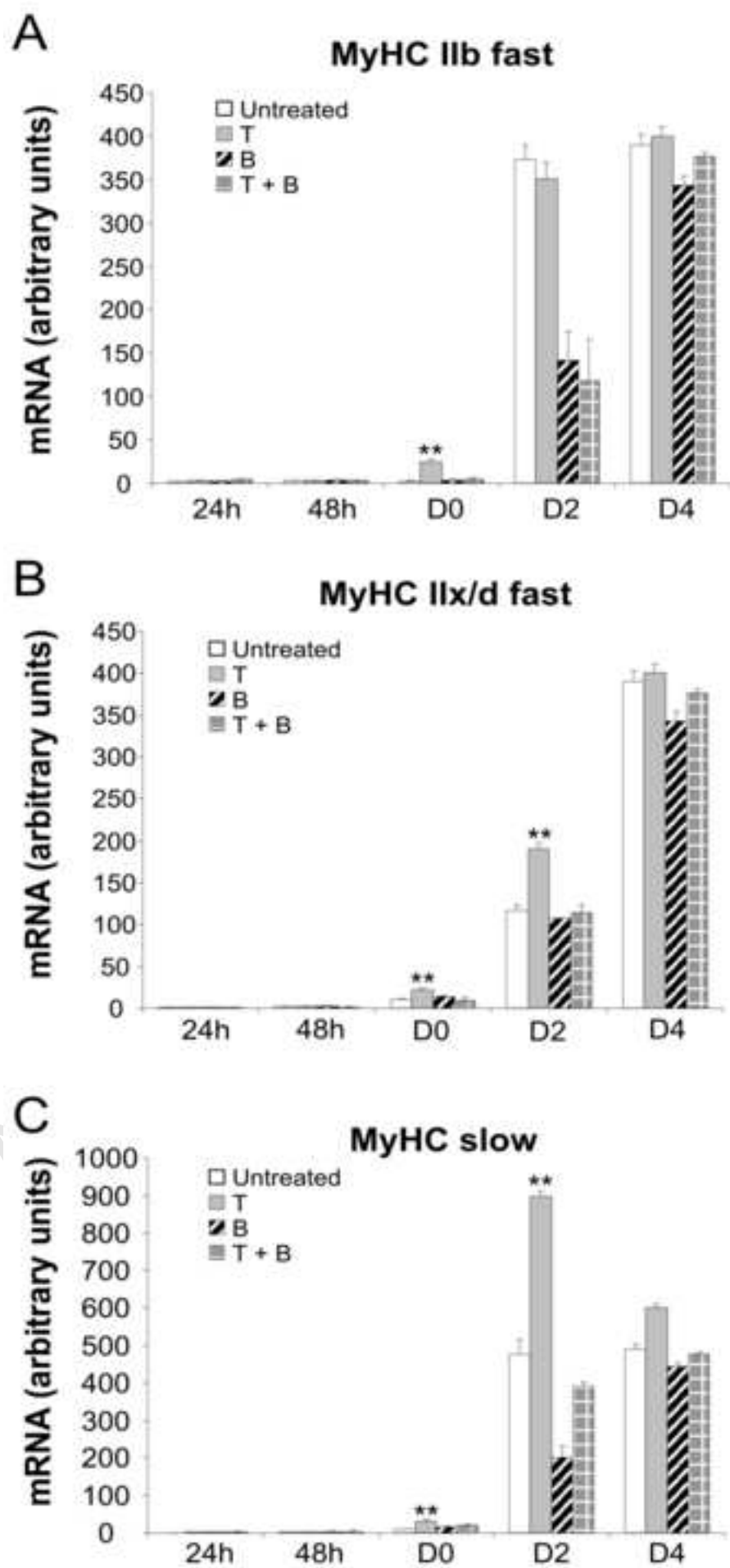


Figure 9

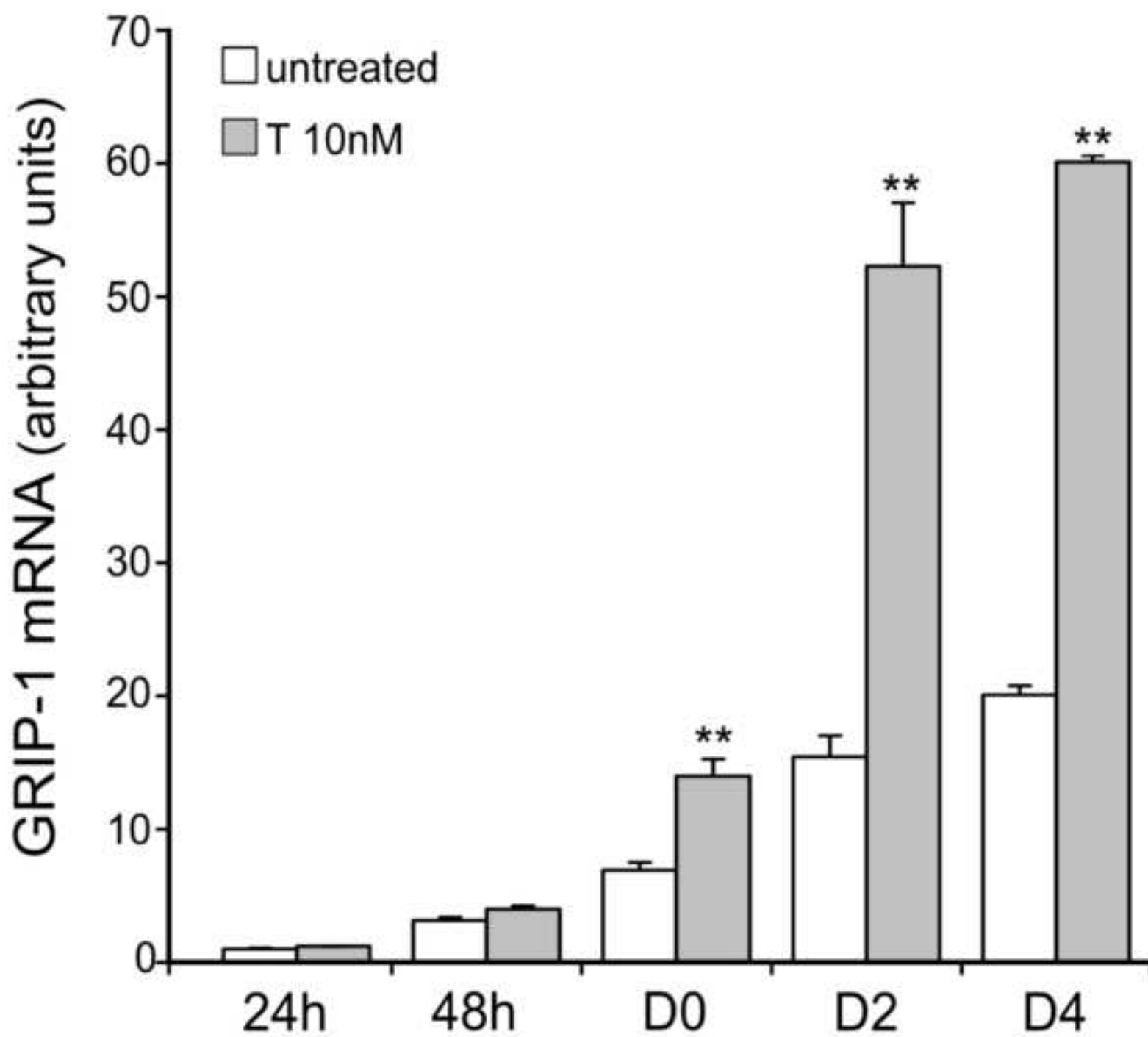


Table 1.
Sequences of primer pairs used for quantitative PCR measurements.

Name	Accession number	Sense primer	Antisense primer
Cyclophilin	NM0089071	CCCACCGTGTTCTTCGACAT	TTCTCTCCAGTGCTCAGAGC
Myogenin	NM0311891	AGCTGTATGAGACATCCCCC	TTCTTGAGCCTGCGCTTCTC
Cyclin D1	NM0076311	CGTGGCCTCTAAGATGAAGG	TGTTCTCATCCGCCTCTGGC
p21	U24173	CGGTGGAACCTTTGACTTCGT	CAGGGCAGAGGAAGTACTGG
AR	NM013476	TACCAGCTCACCAAGCTCCT	GATGGGCTTGACTTTCCCAG
GRIP-1	NM008678	ACAGAACCAGCCAAACCAAC	TGGTTGAGGATTTCCCTCTG
MyHC-IIb	BC052786	GAGCAGCTGGCGCTGAAGGG	GATTTCTCCTGTCACCTCTC
MyHC Ix/d	XM354615	TCAATGAGCTGACTGCGCAG	CAAGCTGCCTCTTCAGCTCC
MyHC-I	NM080728	AAGATCGTGTCCCGAGAGGG	TTGTACAGCACAGCCGGCTC

The name of the genes, their GENBANK accession number and 5' to 3' nucleotide sequences of the sense and antisense primers are presented. Abbreviation: MyHC, Myosin heavy chain