ABSTRACT

Title of Document: ANDROGEN RECEPTOR POLYGLUTAMINE REPEAT LENGTH AFFECTS RECEPTOR ACTIVITY AND C2C12 CELL MYOGENIC POTENTIAL

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Testosterone (T) has a strong anabolic effect on skeletal muscle and is believed to exert its local effects via the androgen receptor (AR). The AR harbors a polymorphic stretch of glutamine repeats demonstrated to inversely affect receptor transcriptional activity in prostate and kidney cells. However, longer AR glutamine repeat lengths are associated with greater lean body mass and higher serum T in humans. The effects of AR glutamine repeat length on skeletal muscle are unknown. *Purpose:* To determine the effects of AR glutamine repeat length on AR function in skeletal muscle cells. *Methods:* *AR* expression vectors carrying 14, 24, and 33 glutamine repeats, respectively, were

constructed and AR transcriptional activity was determined in transfected C2C12 myoblasts using an AR sensor plasmid. Each vector was subsequently stably transfected into C2C12 cells to create 3 independent cell lines: C2C12AR14, C2C12AR24, and C2C12AR33. Cellular proliferation and creatine kinase (CK) activity were determined. Gene expression was assessed via RT-PCR. Myosin expression, myotube formation, and myonuclear fusion index were examined immunohistochemically. *Results:* Transcriptional activity increased with increasing repeat length (3.91±0.26 vs. 25.21±1.72 vs. 36.08±3.22 relative light units in AR14, AR24, and AR33, respectively; $p<0.001$), in response to T. Ligand activation ratio indicated significant ligand-independent AR transcriptional activity. Significant AR protein expression was only detected in AR14 myoblasts. In contrast, AR mRNA expression was elevated in each stable line in the myoblast stage and throughout differentiation. The proliferation of AR33 cells was significantly decreased vs. AR14 (20512.3±1024.0 vs. 27604.17±1425.3, $p<0.001$) after 3 days. The CK activity of AR14 cells was decreased in comparison to AR24 and AR33 cells (54.9±2.9 vs. 68.3±2.2 and 70.8±8.1 units/ug protein, respectively; $p<0.05$) after 5 days of differentiation. The myonuclear fusion index was lower for both AR14 (15.21±3.24%, $p<0.001$) and AR33 (9.97±3.14%, $p<0.001$) in comparison to WT C2C12 cells (35.07±5.60%). Both AR14 and AR33 cells displayed atypical myotube morphology. RT-PCR revealed differences in the expression of genes involved in differentiation, cell fusion, and cell cycle progression. *Conclusion:* AR polyglutamine repeat length affects receptor activity and alters the growth and development of C2C12 cells. This polymorphism may explain some of the heritability of muscle mass in humans.

ANDROGEN RECEPTOR POLYGLUTAMINE REPEAT LENGTH AFFECTS RECEPTOR ACTIVITY AND C2C12 CELL MYOGENIC POTENTIAL

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2010

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Acknowledgments:

I would like to thank all of my committee members for agreeing to sit on this committee and for their overall support for this project. In particular, I would like to extend extra thanks to both Dr. Espen Spangenburg and Dr. Eva Chin for their extensive technical support and tolerance of my constant questioning.

To my advisor, Dr. Roth, I must extend my extreme gratitude for his support and guidance throughout my academic career, as well as his understanding of my Naval obligations.

To all of my fellow students, in particular Andy Ludlow, Erik Hanson, Nathan Jenkins and Lindsay Wohlers, you have my thanks for your advice, support, and brainstorming sessions.

Table of Contents

List of Tables

Table of Figures

List of Abbreviations

AF1/AF2- activation function 1/activation function 2
AIS- androgen insensitivity syndrome
AR- androgen receptor
ARE- androgen response element
ARKO- androgen receptor knockout
BMI- body mass index
CK- creatine kinase
DAPI- 6-diamidino-2-phenylindole
DBD- DNA binding domain
DEXA- dual energy X-ray absorptiometry
DHT- dihydrotestosterone
DM- differentiation medium
DMEM- Dulbecco's Modified Eagle Medium
DMSO- dimethylsulfoxide
EDL- extensor digitorum longus
ER- estrogen receptor
EtOH- ethanol
FBS- fetal bovine serum
FHL2- four and a half LIM domain protein 2
FI- fusion index
FITC- fluoroscein-isothiocyanate
FSH- follicle stimulating hormone
GFP- green fluorescent protein
GLN- glutamine
GM- growth medium
GnRH- gonadotropin releasing hormone
GR- glucocorticoid receptor
HDL- high density lipoprotein
HRE- nuclear hormone response element
HS- horse serum
IGF- insulin like growth factor
IGFBP- IGF binding protein
LA- levator ani
LBD- ligand binding domain
LH- luteinizing hormone
MHC- myosin heavy chain
MMTV- mouse mammary tumor virus promoter
MR- mineralcorticoid receptor
MRI- magnetic resonance imaging
NES- nuclear export signal
NFDM- non-fat dry milk
NLS- nuclear localization signal
NTD- amino terminal domain
PBS- phosphate buffered saline

PR- progesterone receptor
PSA- prostate specific antigen
RIA- radioimmunoassay
RT-PCR- reverse transcriptase polymerase chain reaction
SBMA- spinal bulbar muscular atrophy
SHBG- sex hormone binding globulin
T- Testosterone
TAU- transcription activation unit
TBST- TRIS-buffered saline w/ TWEEN
THG- tetrahydrogestrinone
TK- viral thymidine kinase promoter
WT- wild type

Introduction:

Androgens play an essential role in a number of physiological processes including muscle and bone development and the development and maintenance of secondary sexual characteristics. Testosterone (T) administration in otherwise healthy hypogonadal men increases fat-free muscle mass and decreases fat mass (1;2), increases fat-free mass in hypogonadal HIV-infected men (3), increases muscle cross-sectional area in healthy elderly men (4), and induces an improvement in self-perceived quality of life and mood (5). Supraphysiologic doses in healthy young men increase fat-free mass in a dose dependent manner (6), while muscle mass is increased even further when combined with resistance exercise (7). T administration also increases both bone density and bone mineral content in hypo-androgenic men (8) and women (9). As a result, testosterone therapy is becoming a more commonly accepted treatment for aging and disease-related muscle and bone wasting conditions. Moreover, there is evidence suggesting that serum androgen level is a largely heritable trait (10), and that there is significant variation in the inter-individual response to exogenous androgen administration (11). The mechanism(s) driving heritable differences in androgen levels and sensitivity are unknown, but could significantly affect the success of androgen replacement therapy.

Though the mechanisms behind T's anabolic effects in muscle are still relatively poorly understood, significant data exist supporting an anabolic action of T on skeletal muscle. T has been demonstrated to have a positive effect on muscle fractional protein synthesis (12;13), antagonizes the catabolic effects of glucocorticoids (14), and acts as an antagonist to the glucocorticoid receptor (15). Though the effect of T on muscle protein

breakdown is less clear, evidence suggests that T reduces protein breakdown in vivo but not in vitro (16-18). Another possible avenue by which T exerts its anabolic effects in muscle is the control of myonuclear addition via satellite cell activation. Adult skeletal muscle is post-mitotic and is largely dependent on satellite cell activity for growth and repair. T induces a dose-dependent increase in satellite cell number in the quadriceps muscle of elderly men (4) and increases in both satellite cell number and myonuclear count in the quadriceps of young men (19). T and its endogenous metabolite dihydrotestosterone (DHT) also increase in vitro C2C12 myoblast proliferation (20;21). Though the direct mechanism(s) responsible for these effects is unclear, it is believed that the majority of the anabolic effects of T are mediated via its interaction with the androgen-receptor (AR).

The AR is a ligand-activated nuclear hormone receptor that acts as a transcription factor to regulate expression of androgen responsive genes. Structurally, the AR consists of 3 functional domains; an amino-terminal transactivation domain (NTD), a central DNA binding domain, and a carboxy-terminal ligand binding domain (LBD). Located within the NTD and LBD are two short motifs known as activation function 1 and 2 (AF1 and AF2), respectively. Upon binding ligand the NTD and LBD fold over to allow contact between AF1 and AF2, a process that appears to be critical for co-factor recruitment, receptor stabilization and efficient gene transcription of certain target gene promoters (22). Variations within these motifs can result in altered receptor function (23;24). The NTD also harbors binding sites for a variety of additional cofactors (25-27) and mutational deletions demonstrate that the region is required for full receptor transcriptional activation (28), hence its designation as the transactivation domain.

Consequently mutations or polymorphisms within the region can be postulated to have an effect on AR activity.

The NTD harbors a polyglutamine repeat polymorphism that has been demonstrated to affect AR transcriptional activity, and has been associated with a number of androgen-related maladies including prostate cancer (29), prostate hypertrophy (30), and spinal bulbar muscular atrophy (31). AR transcriptional activity decreases with increasing polyglutamine repeat length in the prostate carcinoma LNCaP cell line (32), as well as the African green monkey kidney CV-1 cell line (33). Though well characterized in respect to prostate, the effect of AR polyglutamine length on skeletal muscle physiology is less clear; the only available data stemming from a handful of gene association studies. Walsh et al. (34) examined AR CAG repeat length in relation to skeletal muscle, demonstrating that longer repeat length is correlated with greater fat-free mass in men. Campbell et al. (35) reported that AR CAG repeat length was positively associated with fat-free mass in a subgroup of Kenyan men . The data of Lapauw et al. (36) suggest that AR CAG repeat length modulates the effect of T on body composition in elderly men. However, taking into account data from previous studies where AR activity is inversely correlated with repeat length, one could hypothesize that AR repeat length would be inversely related to fat-free mass in human subjects. Additionally, and in contrast to prior data where no association between serum testosterone and repeat length was found (37), Walsh et al. (34) reported that longer repeat length was associated with greater serum testosterone levels. Given these conflicting data, further clarification on the role of AR polyglutamine repeat polymorphism in skeletal muscle physiology is required.

Purpose of the Study

Testosterone has been demonstrated to be critical for the normal development and growth of skeletal muscle in human male subjects. Testosterone is believed to exert its anabolic effects on skeletal muscle via the AR, as deletion of the AR results in severe disruption of skeletal muscle development in male rodents. However, the mechanism by which testosterone signaling through the androgen receptor induces increases in muscle mass and strength are still largely unknown. Additionally, evidence suggests that the response to androgen signaling is a heritable trait (38). The AR polyglutamine repeat polymorphism has been demonstrated to influence AR transcriptional activity in non-muscle tissue (32;33;39;40). Though the effect of polyglutamine repeat length on AR transcriptional activity in skeletal muscle has not been addressed, a direct association of AR repeat length and lean body mass in humans has been demonstrated (34;35).

Therefore, the purpose of this study was to elucidate the mechanisms by which AR repeat length may affect skeletal muscle mass in humans by:

1) Determining the effect of polyglutamine repeat length on AR transcriptional activity in skeletal muscle myoblasts.
2) Evaluating the effect of AR harboring short, medium, and long polyglutamine repeat lengths on the growth and differentiation of skeletal muscle myoblasts.
3) Determining if AR repeat length affects the expression of select gene targets involved in normal skeletal muscle growth and development.

Specific Aims

Our central hypothesis is that AR harboring a shorter polyglutamine repeat length will be more transcriptionally active in skeletal muscle cells, and will induce effects in these cells that are consistent with skeletal muscle hypertrophy in vivo, in comparison to AR harboring a longer polyglutamine repeat length.

Specific Aim 1: To determine if polyglutamine repeat length alters AR transcriptional activity in skeletal muscle cells in vitro.

H_1: Androgen receptor harboring 14 glutamine repeats (AR14) will be more transcriptionally active than AR harboring 24 glutamine repeats (AR24), which will be more transcriptionally active than AR harboring 33 glutamine repeats (AR33) when expressed in C2C12 myoblasts.

H_2: AR protein and mRNA expression will not differ between C2C12 cells expressing AR14, AR24 and AR33.

Specific Aim 2: To determine if the transcriptional differences determined in Specific Aim 1 are accompanied by differences in nuclear translocation.

H_3: AR14 will translocate to the nucleus to a greater extent than AR24 and AR33 when treated with testosterone.

Specific Aim 3: To determine if AR polyglutamine repeat length affects the proliferation and differentiation of stably transfected C2C12 cells.

H_4: AR14 cells will have a greater rate of proliferation thanAR24 and AR33 cells when incubated in growth medium.

H_5: Once induced into differentiation, AR14 cells will have higher creatine kinase activity than AR24 and AR33 cells after 5 days of differentiation.

Specific Aim 4: To determine if the expression patterns of genes involved in myoblast determination, proliferation, and differentiation is altered by AR polyglutamine repeat length.

H_6: AR14 cells will display increased expression of myogenic genes in comparison to AR24 and AR33 cells.

H_7: AR14 will drive gene expression from selected myogenic gene promoters to a greater extent than AR24 and AR33.

Results

AR Transcriptional Activity in C2C12 cells

C2C12 murine myoblasts were transiently transfected with AR expression vectors harboring 14, 24, and 33 glutamine repeats, respectively. Additionally, a firefly luciferase reporter vector driven by the probasin gene promoter and a *Renilla* luciferase normalization vector driven by the viral thymidine kinase promoter were transfected. Cells were treated with 100nM testosterone (T) or ethanol control for 24 hours before being assayed for luciferase activity. In contrast to previous studies (32;33;39;40), we observed a positive relationship between AR repeat length and transcriptional activity in skeletal muscle tissue (*figure 1a*). With T treatment AR33 had 43% greater transcriptional activity than AR24 ($P<0.001$), while AR14 had transcriptional activity more than 5-fold lower than AR24 ($P<0.001$). Transcriptional activity of AR33 was more than 9-fold greater than AR14 ($P<0.001$). AR33 and AR24 were significantly more active than AR14 even in the absence of testosterone, but were not significantly different from each other ($P=0.201$). Mock transfections carried out with a promoter-less reporter vector displayed minimal luciferase activity (data not shown). A similar experiment was carried out in fully differentiated myotubes, where the data were similar apart from a lower ratio in AR24 cells (see Appendix, *figure 15*). Interestingly, the ligand activation percentage (i.e. firefly/*Renilla* luciferase activity ratio in testosterone treated cells vs. the firefly/*Renilla* luciferase activity ratio in ethanol vehicle treated cells) was considerably higher for AR33 in comparison to AR14 and AR24, which were relatively similar (110% vs. 53.9% and 57.3%, respectively). These data are supportive of a ligand-independent

effect of glutamine repeat length on AR transcriptional activity, but also demonstrate that AR33 responds to testosterone to a greater degree than either AR14 or AR24.

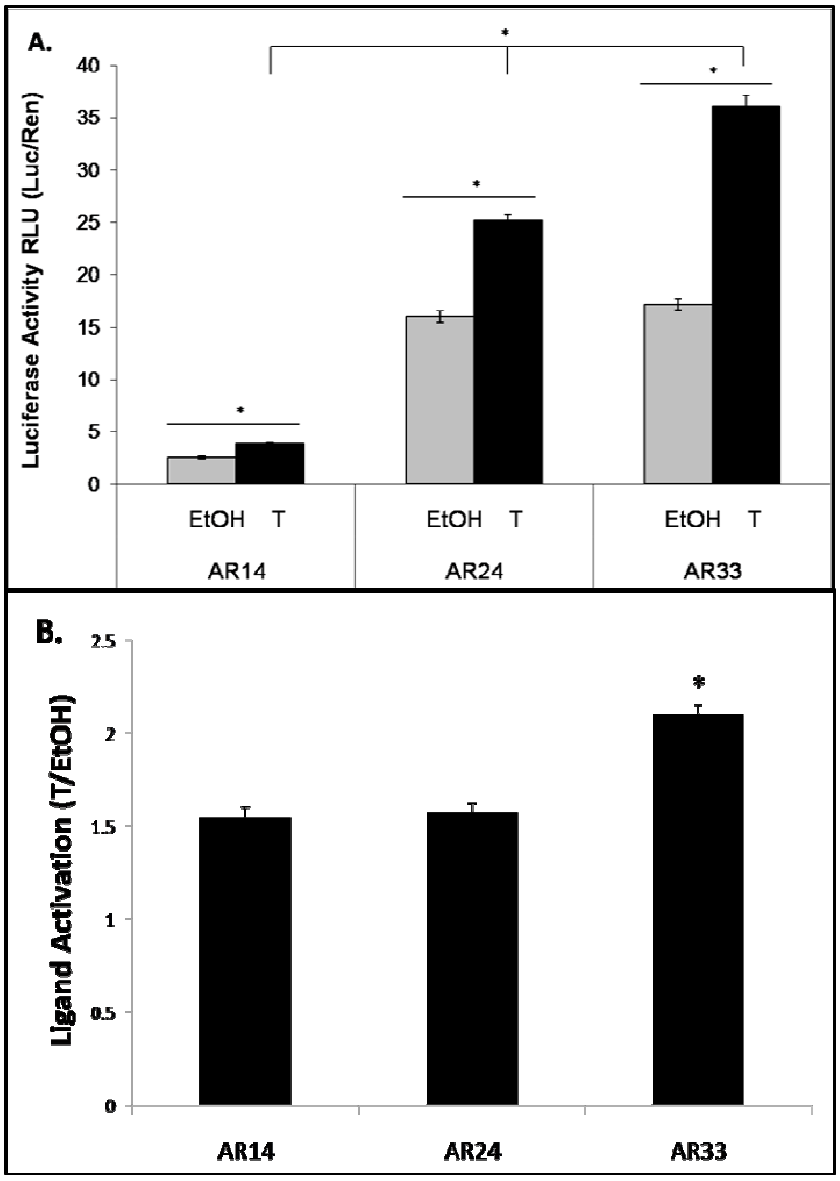


Figure 1: Effects of repeat length on AR transcriptional activity in C2C12 myoblasts. (A) C2C12 cells were transiently transfected with a human AR expression vector harboring 14, 24, and 33 CAG repeats, respectively, along with pPR-Luc reporter vector and pRL-TK normalization vector and were treated with 100nM T or ethanol vehicle for 24 hours. Data are expressed as the mean luciferase ratio, and bars represent the SE of 3 replicates over 3 separate experiments (*p<0.001). (B) Ligand activation ratio was determined by dividing the average firefly/*Renilla* luciferase ratio of T treated cells by the average firefly/*Renilla* luciferase ratio of ethanol treated cells. (* p<0.001)

AR Expression

We investigated AR protein and AR mRNA expression in wild type (WT) and stably transfected C2C12 myoblasts (see Appendix, *figure 14*, for verification of transgene expression*)* and fully differentiated myotubes (5 days cultured in differentiation medium (DM)) with and without T to determine if the transcriptional differences observed were due to variable AR expression, as current data are not in agreement on this issue. The data of Choong et al. (41) demonstrate a decrease in AR protein content with increasing repeat length, though in this case the repeat length was expanded into a range known to induce a pathological state of neurological degeneration (65 GLN residues) (42). In contrast, neither Beilin et al. (32) nor Tut et al. (40) observed any significant differences in AR protein content with variable repeat lengths spanning 15-31 GLN residues, well within the normal physiological range (43). Interestingly, both Beilin et al. and Tut et al. demonstrated a decrease in AR transcriptional activity with increasing length, while Choong et al. (41) did not observe such an effect.

AR protein content was assayed using anti-AR PG-21 (Millipore) and standard Western Blot techniques (*figure 2, A*) as well as in situ immunostaining. Nuclear and cytoplasmic protein fractions were isolated to determine AR nuclear localization. Whole LNCaP cell protein extract was used as a positive control. Appreciable AR protein was only detected in the nuclear fraction of T treated AR14 myoblasts, with slight indications of AR protein in the nuclear fraction of WT C2C12 myoblasts, and in the nuclear fraction of T-treated AR14 myotubes. Despite higher transcriptional activity, appreciable AR protein was not detected in AR24 nor AR33 cells under any conditions tested. In situ immunostaining did not reveal detectable AR protein in any of the cell lines (data not

shown). In contrast, AR mRNA expression in the stably transfected lines was readily detectable in myoblasts and throughout the differentiation process (*figure 2, B and C*), while AR mRNA expression was only apparent in WT cells 24 hours after switching to DM.

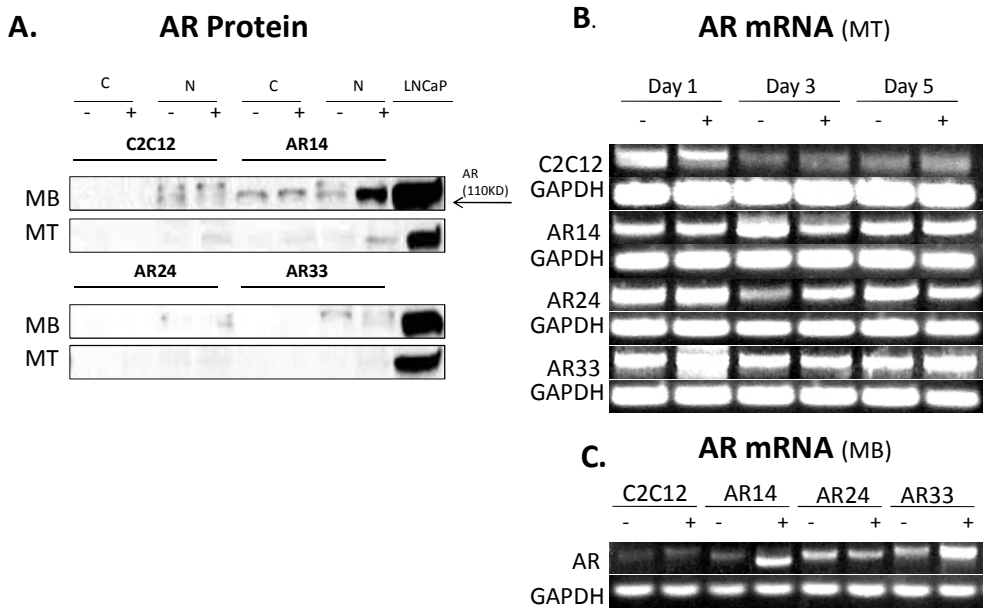


Figure 2 : (A) AR expression in WT and stably transfected C2C12 cells. AR protein content was determined simultaneously in nuclear (n) and cytosolic (c) protein fractions of fully differentiated myotubes and proliferating myoblasts, respectively, via Western blot using anti-AR PG-21. 30µg of total protein was loaded into each well. RT-PCR was performed on RNA extracted from myotubes after 24, 72, and 120 hours in DM (B) and from proliferating myoblasts (C). Cells were treated with ethanol (-) or 100nM T (+). All images are representative of at least 3 independent experiments

Rate of Proliferation

Qualitative differences in proliferation rate between the lines stably transfected with AR were observed during normal culturing. In order to better characterize these

differences a colorimetric cellular proliferation assay was performed (*figure 3*). Initial analysis revealed that there was no significant effect of testosterone on cell number among any of the lines; testosterone treatment was therefore dropped in subsequent analyses (see Appendix, *figure 17*, for data with T included). We observed a significant interaction of time and repeat length on cell number ($P<0.001$). 24 hours after plating there were greater numbers of both AR14 and AR33 cells in comparison to WT ($P<0.05$). No difference was observed between AR24 cells and any of the other lines on day 1. Likewise, no differences were apparent between any of the lines on day 2. By day 3 there were fewer AR33 cells in comparison to both AR14 and AR24 cells, 25.7% ($P=0.001$) and 20.3% ($P<0.05$) fewer cells, respectively.

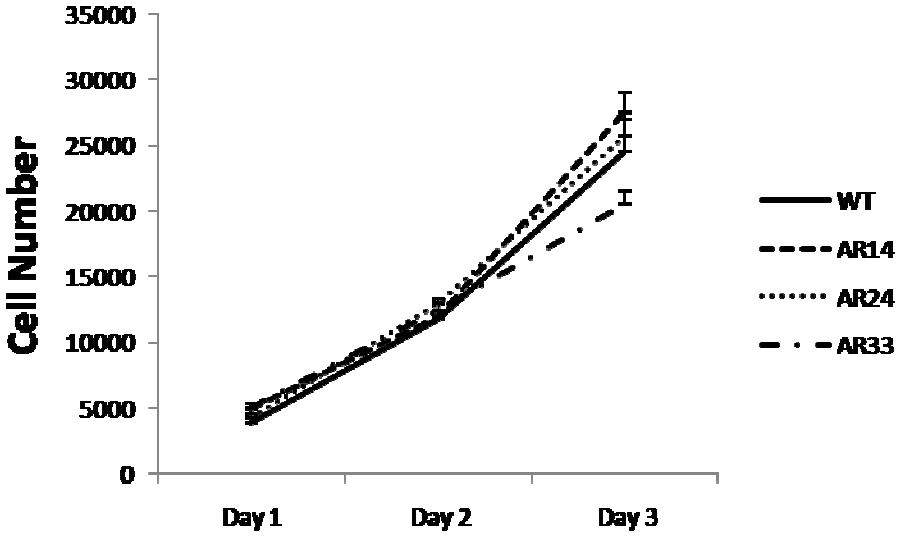


Figure 3: AR repeat length influences C2C12 cell proliferation. Cell growth of the stably transfected and WT C2C12 lines was measured using a colorimetric proliferation assay. Cell number was calculated by measuring formazan absorbance at 490nm and the data were plotted on a standard curve derived from known cell densities. Bars represent the SE of triplicates from 3 separate experiments.

Creatine Kinase Activity

To determine if the altered AR transcriptional activity has an effect on myoblast differentiation, the CK activity of each cell line was assessed over 5 days of incubation in DM (*figure 4*). Initial statistical analysis revealed a significant 3-way interaction of repeat length, length of incubation, and presence of testosterone ($p<0.001$). However, due to the obvious impact of length of incubation (Day) on CK activity this main effect was removed from subsequent analysis and multiple 2-way ANOVAs were performed with drug and repeat length as main effects. The overall effect of T was significant, increasing the CK activity in all lines by an average 4.46 units/μg protein across all conditions ($p<0.001$), which is in contrast to the previous assay where T had no effect on rate of proliferation. After 24-hours of incubation CK activity was greatest in the AR33 line; 30.9±1.2units/μg protein vs. 27.9±1.1, 26.1±1.4, and 22.8±1.7 in AR14, WT C2C12, and AR24 lines, respectively ($p<0.05$). After 72 hours of incubation an interesting reversal occurred, with both AR24 and WT lines (50.3±1.0 and 47.4±1.0 units/μg protein; respectively) having significantly greater CK activity than AR33 and AR14 (39.6±1.4 and 38.3±1.7; respectively), $p<0.001$. After 120 hours of incubation the CK activity of the AR14 line was decreased vs. all other lines; 54.9±1.2 vs. 70.8±3.3, 68.3±0.9, and 66.1±1.2 units/μg protein in AR33, AR24, and WT lines, respectively ($p<0.001$), whereas the AR33, AR24, and WT lines were not different from each other.

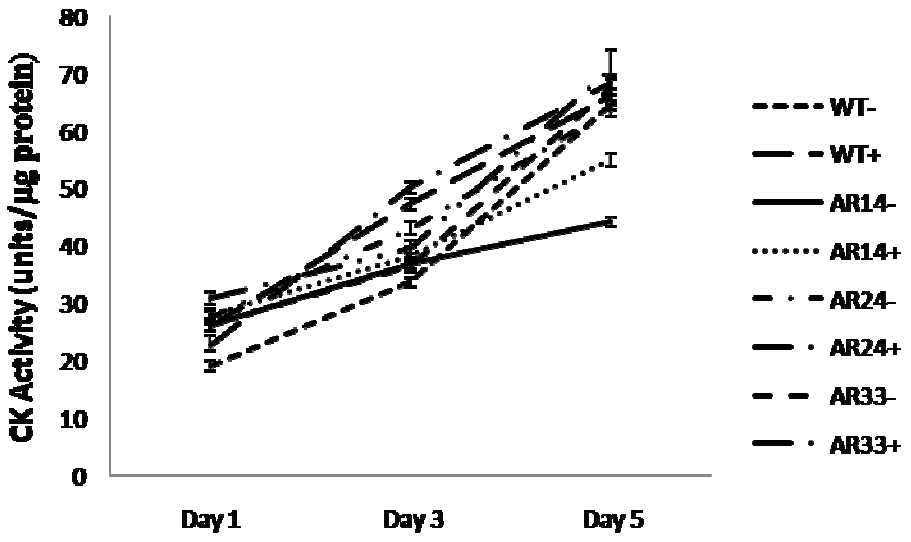


Figure 4: Influence of AR repeat length on CK activity. CK activity was assessed from whole cell lysates of each stably transfected line and WT C2C12 cells incubated in DM for up to 5 days in the presence of 100nM T (+) or ethanol vehicle (-). Bars represent the SE of duplicates from 3 independent experiments.

Myotube Development and Morphology

The differentiation and fusion processes were further examined via immunohistochemical staining. A representative image series from each field is shown for the AR14 line in *figure 5*. After 24 hours of incubation in DM very few myotubes were observed in any of the lines. However, the AR14 line displayed many myosin-positive, elongated, mono-nucleated cells (*figure 6*). After 72 hours, WT and AR24 lines displayed typical myotube morphology; large, thick, myosin-positive, multinucleated cells. In contrast, the AR14 line developed into very long, very thin cells with few, sparse nuclei, while the AR33 line developed into short, truncated myotubes with clustered nuclei. Interestingly, the morphology of the AR14 line mimics that seen in a line of NFATC2-/- murine myoblasts (44). A pattern similar to that at 72 hours was

noted after 120 hours; however the AR33 line displayed an increased number of small, myosin-positive cells with condensed nuclei and a round morphology in comparison to the 3 other lines.

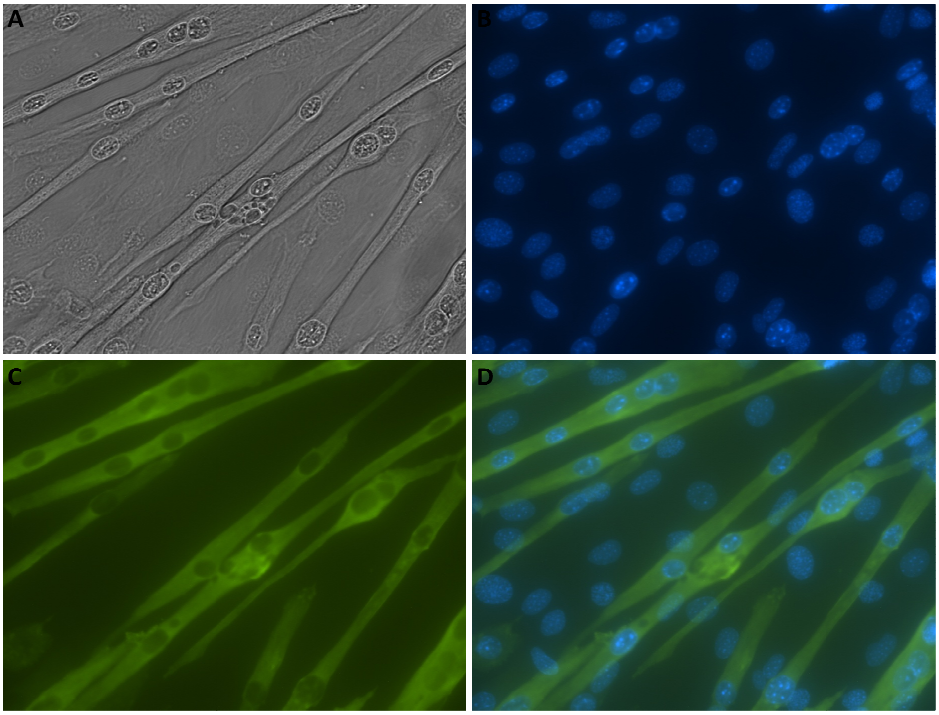


Figure 5: Representative image series from AR14 cells after 120 hours incubation in DM. A) bright-field B) DAPI nuclear stain C) sarcomeric myosin-FITC D) overlay of B+C

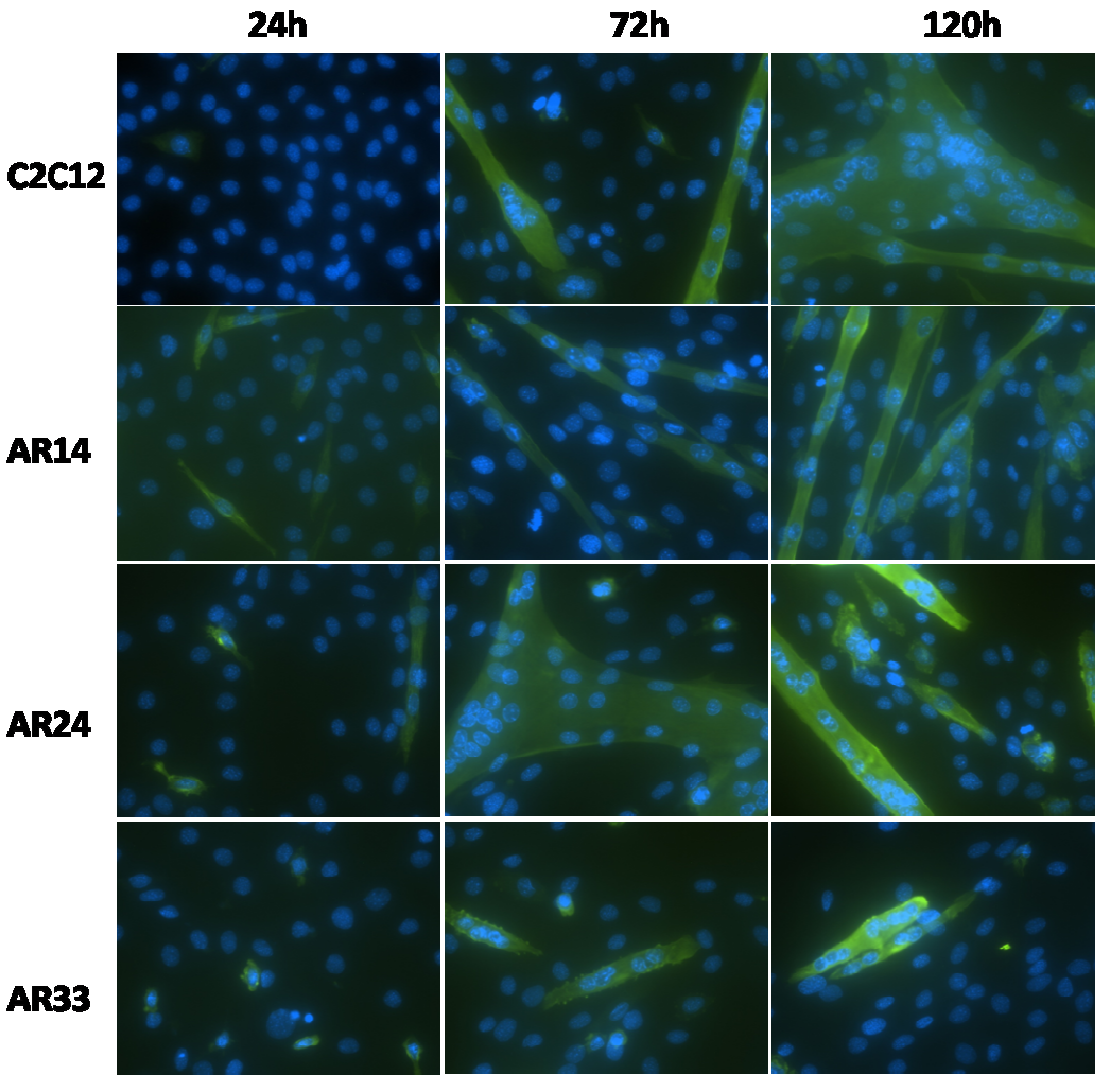


Figure 6: Immunohistochemical staining revealing differences in myotube development between the lines. Green = sarcomeric myosin protein. Blue = DAPI stained nuclei. Criteria for classification as a myotube included a myosin-positive stain and a minimum of 3 myonuclei. The above images are representative of a minimum of 5 fields per condition. All images were captured using the 20x objective

Significant differences in protein content between the lines were also observed (*figure 7*). After 1 day of differentiation, total protein content was elevated in all three stable lines in comparison to WT cells ($p<0.001$). By the 3rd day only the AR14 line had significantly greater protein content in comparison to WT. By the 5th day of differentiation significantly more protein was present in the AR14 line in comparison to the WT line ($p<0.001$), while the AR33 line contained significantly less total protein than the WT line ($p<0.001$). This data is in accordance with our proliferation data, where after 3 days of incubation a significantly lower cell number was observed for the AR33 line in comparison to WT cells. Interestingly, and in contrast to the data of Chen et al. (45), T treatment did not increase total protein content under any condition tested.

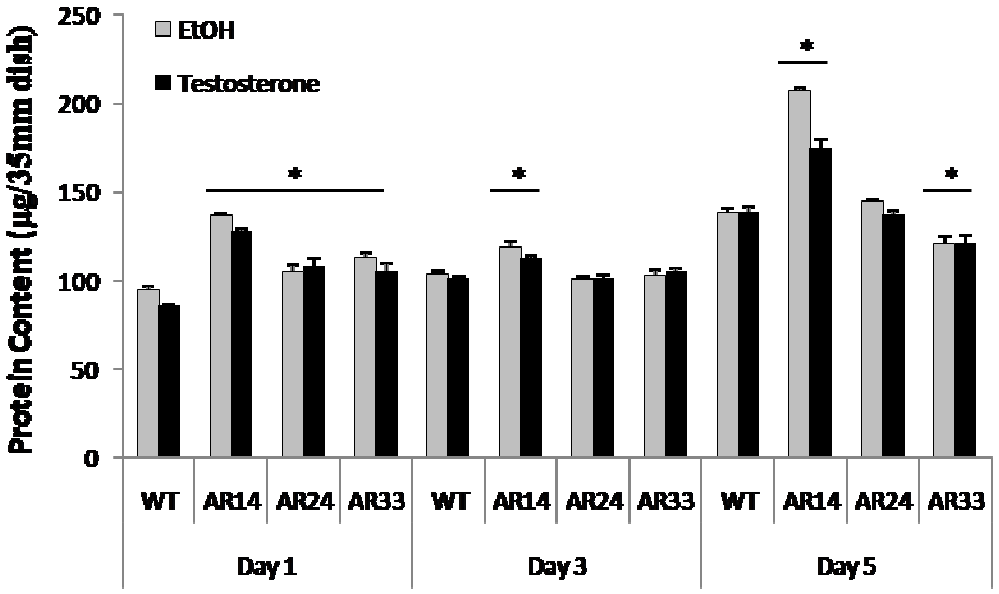


Figure 7: Total protein content in differentiating myotubes. (*) indicates significant difference from WT C2C12 at each respective time point ($p<0.001$)

Myonuclear Number and Myotube Fusion Index

Fusion index (FI) is a relative estimate of the efficiency of myoblast fusion by determining the percentage of intra-myotube nuclei within a given field. The images captured above suggest that fusion in both AR14 and AR33 lines is impaired, and FI analysis supports this finding (*figure 8a)*. By 72 hours the FI of the AR24 line was highest at 42.8±3.8%, followed by WT at 31±2.5%. FI of both the AR14 and AR33 lines was far lower; 11.8±2.3% and 13.1±3.8%, respectively ($p<0.05$). At 120 hours FI of the WT line was highest at 35.1±4.0%, followed by AR24 at 28.0±2.5, AR14 at 15.2±2.3, and AR33 at 9.8±2.2 ($p<0.05$).

Very few myonuclei were present after 24 hours in any field examined (*figure 8b)*. After 72 hours the average number of myonuclei per field was significantly reduced in both AR14 and AR33 cells: 6.5±1.3 and 6.2±1.6, respectively, vs. 23±3.1 and 28.3±2.7 in WT and AR24 cells, respectively ($p<0.001$). AR14 and AR33 lines were not different from each other. The same results were observed at 120 hours: 10.7±1.9 and 7.6±1.5 myonuclei/field for AR14 and AR33 cells respectively, vs. 35.3±4.2 and 20.3±1.9 in WT and AR24 cells, respectively ($p<0.001$).

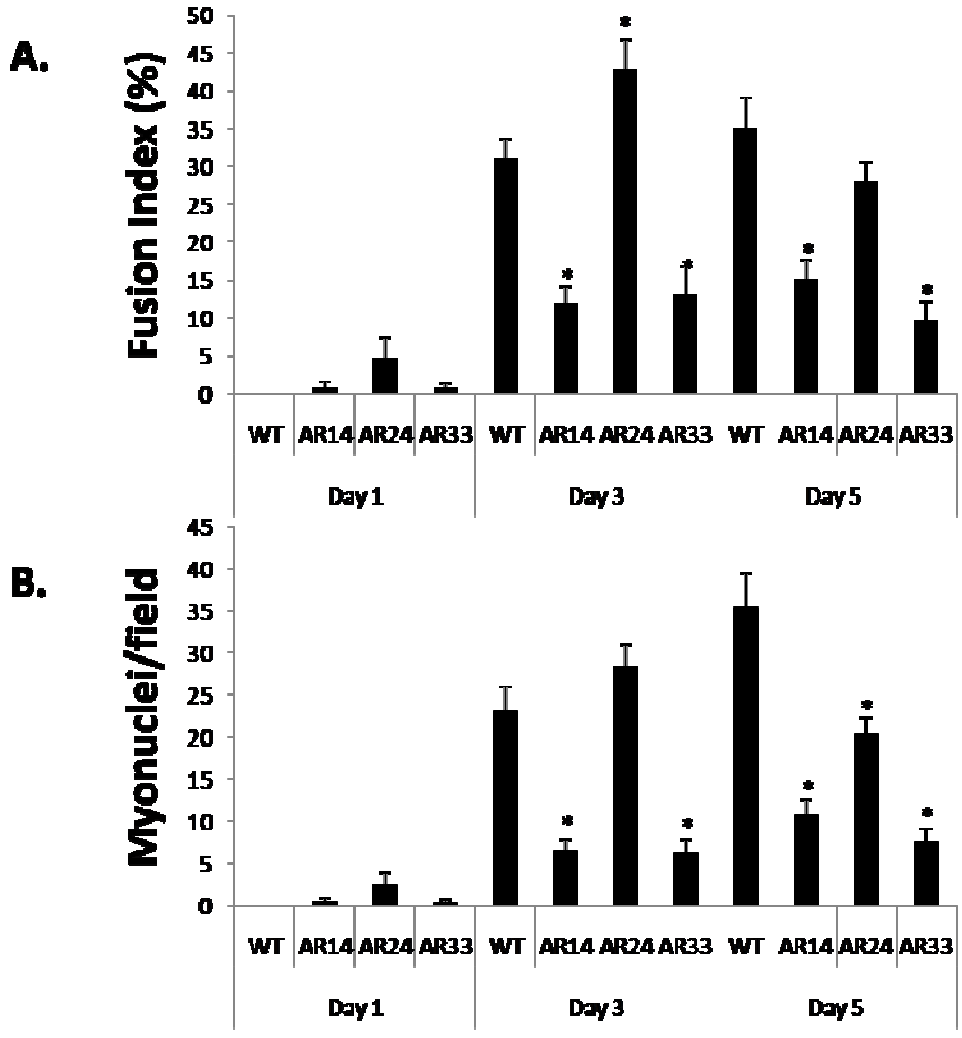


Figure 8: Myotube fusion index (a) and average myonuclei number per field (b) are reduced in AR14 and AR33 lines. Myonuclei were identified as nuclei within cells that 1) stained positive for myosin expression, and 2) contained at least 3 nuclei. Data are expressed as mean ± SE of 10 fields (*p<0.05). (*) indicates significant difference from WT C2C12 line at each respective time point.

These quantitative differences were accompanied by qualitative differences as well. While the WT and AR24 lines appeared similar, the AR14 and AR33 lines were very distinct despite having similar fusion indices and average myonuclei number per field. The low myonuclei numbers for the AR14 line were due to the highly elongated cells with sparse nuclei (see *figures 5* and *6*). In contrast, the low myonuclei numbers of

the AR33 line appear to be largely due to the small size of the myotubes formed (*figure 6*), as unlike the AR14 line, AR33 myotubes displayed the typical clustering of nuclei seen in WT myotubes.

Gene Expression

RT-PCR analysis was performed on RNA extracted from proliferating myoblasts and in differentiating myotubes 1, 3, and 5 days after switching to DM. The resulting cDNA was used to analyze the gene expression patterns of AR14, AR24, and AR33 lines as well as WT cells. Qualitative differences in the expression of several gene targets were found in differentiating myotubes (*figure 9 and table 1*) and in proliferating myoblasts (*figure 10*). Of note is the delayed onset of myostatin expression in all of the stably transfected lines, delayed myogenin expression in AR24 and AR33 lines, and altered NFATC2 expression in the stable lines during the myoblast stage.

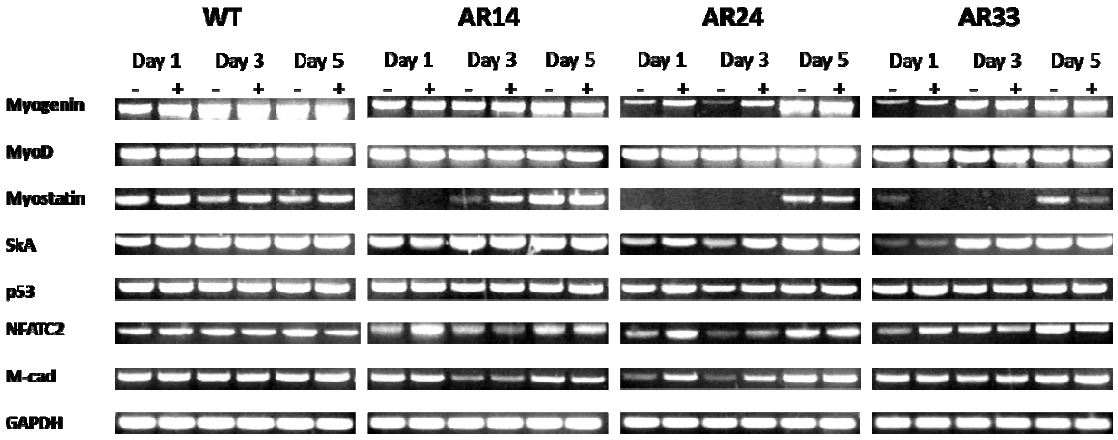


Figure 9: Myotube mRNA gene expression. RT-PCR was performed on RNA extracted from each line 24, 72, and 120 hours after switching to DM supplemented with either ethanol (-) or 100nM testosterone (+). GAPDH was included as a reference gene. All images are representative of 3 separate experiments.

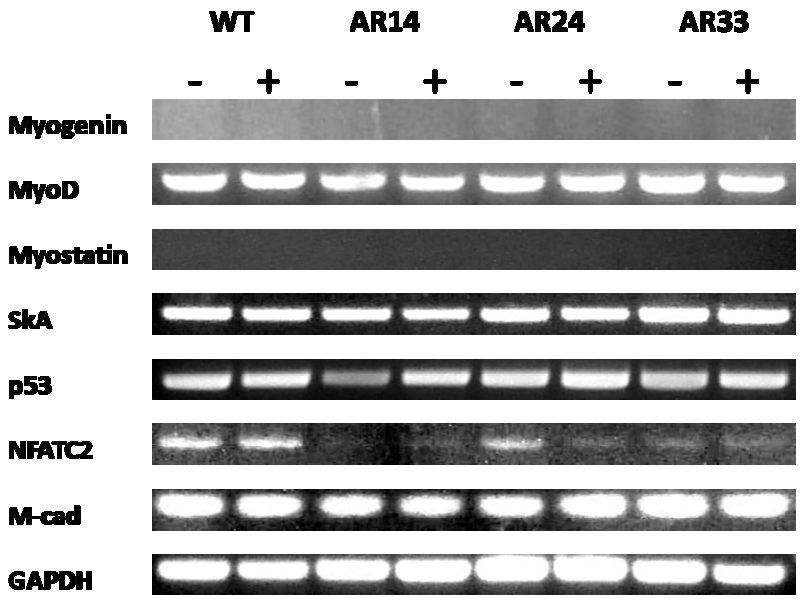


Figure 10: Myoblast mRNA gene expression. RT-PCR was performed on RNA extracted from cells harvested at ~75% confluency and after a 24 hour incubation with either ethanol (-) or 100nM testosterone (+). GAPDH is included as a reference gene. All images are representative of 3 separate experiments.

	Day 1						Day 3						Day 5					
	CK	MYOG	MSTN	ACTA1	AR	FI	CK	MYOG	MSTN	ACTA1	AR	FI	CK	MYOG	MSTN	ACTA1	AR	FI
AR14	↔	↔	↓	↔	↔	↔	↓	↔	↔	↔	↑	↓	↓	↔	↔	↔	↑	↓
AR24	↔	↓	↓	↔	↔	↔	↔	↓	↓	↔	↑	↑	↔	↔	↔	↔	↑	↔
AR33	↑	↓	↓	↓	↔	↔	↓	↔	↓	↔	↑	↓	↔	↔	↔	↔	↑	↓

Table 1: Summary of data collected from myotubes. (↑) indicates characteristic is increased in relation to WT C2C12 line; (↓) indicates characteristic is decreased in relation to WT line; (↔) indicates no change in relation to WT line. CK=creatine kinase activity, MYOG=myogenin, MSTN=myostatin, ACTA1=skeletal alpha actin, AR=androgen receptor, FI=fusion index

Discussion

A key finding of our work is that AR transcriptional activity increases with increasing polyglutamine repeat length in C2C12 skeletal muscle myoblasts. This contrasts with previous data demonstrating a decrease in AR transcriptional activity with increasing repeat length in prostate and kidney cells. Our results were not due to greater AR protein content in cells transfected with AR harboring 33 repeats, as appreciable AR protein was observed only in cells transfected with AR harboring 14 repeats. However, AR mRNA expression was elevated in all of the transfected lines in comparison to WT C2C12 cells. Another key finding of our work is that AR polyglutamine repeat length affects C2C12 myoblast proliferation and the development and morphology of myotubes. Striking morphological differences between the lines were apparent during differentiation, with the AR14 line developing into long, thin, sparsely nucleated myotubes and the AR33 line developing into truncated tubes with clustered nuclei. Both lines displayed decreased myonuclear number and fusion index in comparison to WT C2C12 and AR24 cells. Gene expression analysis indicates that these differences were accompanied by alterations in the expression profile of myogenin, myostatin, and NFATC2. Overall our data indicate that AR polyglutamine length is directly associated with AR transcriptional activity in skeletal muscle, and induces alterations in the growth and development of skeletal muscle myoblasts. These factors may explain some of the heritability of skeletal muscle mass in human subjects.

Glutamine Repeat Length Alters AR Transcriptional Activity in C2C12 Myoblasts

This is the first study to examine the relationship of AR glutamine repeat length and transcriptional activity in skeletal muscle cells. Previous studies have demonstrated that AR polyglutamine repeat length is inversely related to AR transcriptional activity in LNCaP cells, a highly differentiated prostate cancer line (32) and COS kidneys cells (32;40). Another study demonstrated a 3-fold increase in transcriptional activity of both human and rat AR in kidney cells upon removal of the entire repeat region, as well as decreased transcriptional activity of AR with 49 and 77 repeats in comparison to AR with both 25 and 35 repeats. (33). In contrast, repeat length did not have any effect on AR transcriptional activity in PC3 prostate cells, MCF-7 breast adenocarcinoma cells, CV-1 kidney cells or COS-1 kidney cells (32;41;46). Nenonen et al. (39) reported that when corrected for AR protein content, AR harboring 22 repeats had higher transcriptional activity than AR harboring 16 and 28 repeats, respectively, in COS-1 cells. We have demonstrated that increasing the number of glutamine repeats within the AR increases transcriptional activity in C2C12 myoblasts. Several factors may be contributing to these differing results.

First, the AR repeat length variations in previous studies were not identical. In the work of Beilin et al. (32), Nenonen et al. (39) and Tut et al. (40), repeat length varied from 15 to 31 residues, whereas our data was collected from AR vectors harboring 14, 24, and 33 repeats, respectively. These lengths are within the physiological range of 11-31 in healthy individuals (43), whereas Choong et al. (41) used AR vectors harboring 0,

14, 23, 43, and 65 repeats, and Chamberlain et al. (33) used AR vectors harboring 0, 25, 35, 49, and 77 repeats. Expansion of the repeat beyond 40 residues has been demonstrated to induce the formation of AR protein aggregates and is also the range for the onset of symptoms of Spinal Bulbar Muscular Atrophy (SBMA) (47). The formation of aggregates with expanded repeat lengths retards the translocation of receptor to the nucleus (48), effectively preventing or significantly reducing transcriptional activity, which could account for the decreased transcriptional activity reported by Chamberlain et al. with AR expression vectors harboring 49 and 77 repeats (33). It is also worth mentioning that the authors reported no differences in transcriptional activity between the 25 and 35 repeat vectors. Neuschmidt-Kaspar et al. (46) reported no differences in reporter gene activity between CV-1 cells transfected with WT AR and AR harboring 45 repeats. Choong et al. (41) did not report any differences in transcriptional activity between their AR constructs, but did report a significant decline in AR mRNA and AR protein content with increasing repeat length. In contrast, our data is not indicative of a decline in AR mRNA with increasing repeat. Additionally, both Beilin et al. and Tut et al. reported no changes in AR protein with increasing repeat length. The data of Choong et al. may be due to the fact that their "long" vectors contained far longer repeat lengths than our vectors or those of Beilin et al. and Tut et al, and the extended repeat length induced greater AR degradation and/or aggregate formation.

The second potential major source of variation between the studies is the choice of reporter and normalization vectors. Both Chamberlain et al. (33) and Nenonen et al. (39) reported inter-assay differences in transcriptional activity when the reporter and normalization vectors, respectively, were changed. The mouse mammary tumor virus

promoter (MMTV) (33;40;41), a HRE-triplet TATA minimal promoter (33), several ARE-TATA driven reporter constructs (46), PSA promoter ((39) and the probasin promoter (32), have all been used in similar studies. The MMTV promoter is androgen responsive, but is also responsive to other nuclear hormone receptors, including glucocorticoid, mineralcorticoid, and progesterone receptors (49), making findings utilizing this promoter difficult to interpret. Likewise, the minimal TATA promoter utilized by Chamberlain et al. uses 3-tandem general hormone response elements that are recognized by any of the class 1 nuclear hormone receptors. The strength of a minimal promoter such as this can be far lower in comparison; in fact Chamberlain et al. reported significantly different reporter gene activity from the HRE/TATA promoter in contrast to the MMTV promoter. Neuschmid-Kaspar et al. (46) used several promoter constructs driven by tandem AREs in conjunction with a TATA box sequence or the TK promoter. These constructs are relatively AR specific but also somewhat weaker than the MMTV promoter. On the other hand, the probasin promoter is both highly androgen responsive and displays preferential activation by AR (50). Though probasin is only expressed in prostate tissue, the promoter has been used effectively in studies of AR activity in a number of non-prostate tissues (22;32;51-54), indicating that the basal conditions required for activation of the probasin promoter are met in those tissues. While no studies were found using the probasin promoter in the study of AR function in skeletal muscle, the AR specific co-activator four and a half LIM domain protein 2 (FHL2) has been demonstrated to be a strong co-activator of the probasin promoter in yeast (55), and FHL2 is expressed in C2C12 myoblasts (56). Moreover, our results indicate that transcription from the probasin promoter can occur in skeletal muscle myoblasts. The

relative AR specificity of the probasin promoter also has the benefit of reducing the potential for co-activation of the reporter construct by other nuclear hormone receptors. Given the differences in strength and specificity of these promoter constructs, the inter-study transcriptional differences are not surprising.

Though data regarding AR repeat length and transcriptional activity are conflicting, ours is the first study to demonstrate a direct relationship between repeat length and transcriptional activity, and is also the first to examine AR repeat length in skeletal muscle tissue. The mechanism by which AR transcriptional activity is positively affected by repeat length in skeletal muscle while the opposite appears to be true in prostate and kidney cells is unclear. It is possible that alterations of the repeat length induce conformational changes in the AR NTD that enhance/suppress interactions with tissue-specific cofactors, leading to tissue-specific differences in transcriptional activity. For example, NTD-LBD interaction is required for the exposure of cofactor docking sites, and it is possible that the increased repeat length provides a stronger docking surface for cofactors specific to skeletal muscle. There is also the potential that nuclear localization is increased with expanded repeat lengths. NTD-LBD interaction is required for receptor activation in WT AR by allowing interaction of AF1 and AF2, stabilizing the ligand-binding pocket and exposing the nuclear localization signal (NLS) found in the hinge region (57). Expansion of the glutamine repeat may result in increased exposure of the NLS, facilitated by muscle specific cofactor interactions, and induce nuclear translocation and transcriptional activation in skeletal muscle cells but not in non-muscle tissues.

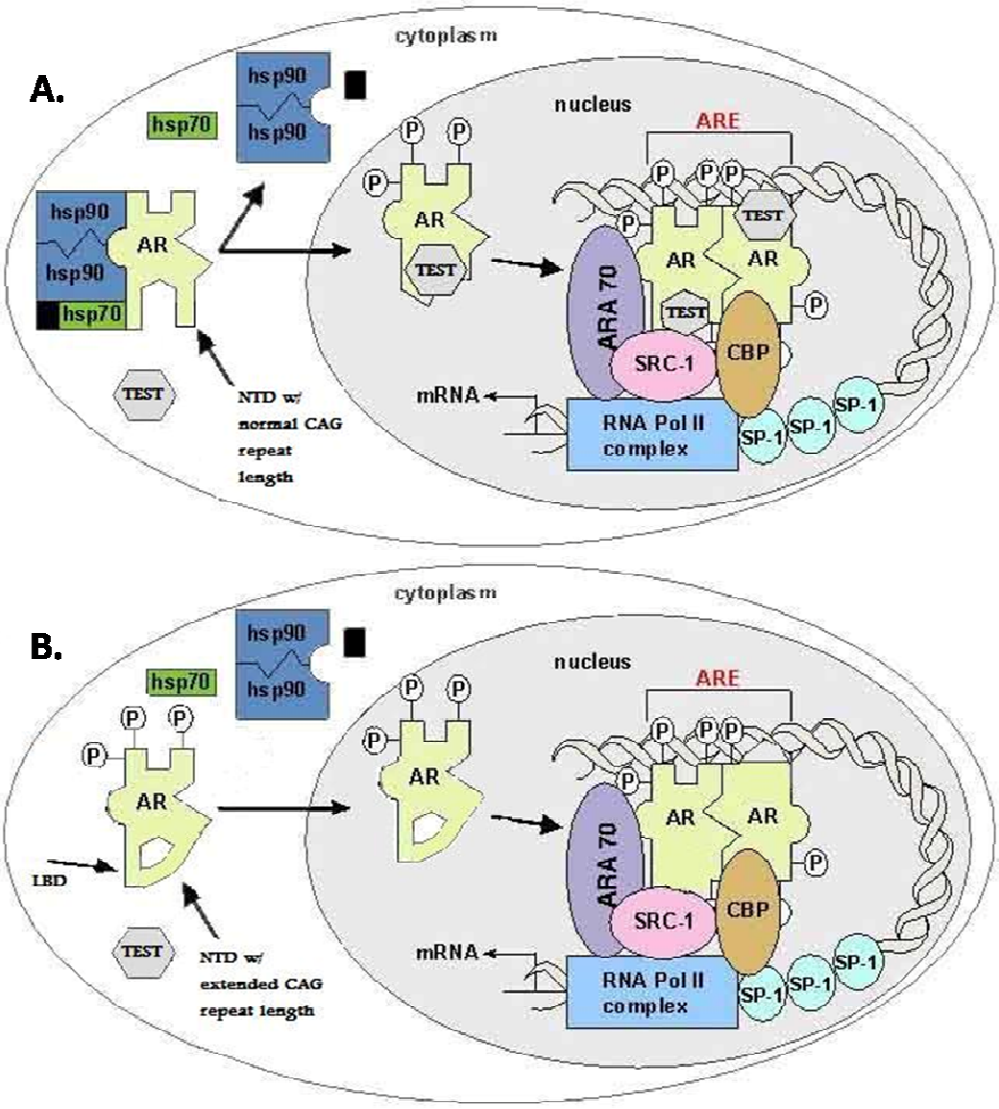


Figure 11: Possible mechanism of ligand-dependent vs. ligand independent AR transcriptional activation. (A) AR with normal polyglutamine repeat length binds testosterone and in a series of events dimerizes, dissociates from binding proteins, receives post-translational modifications including phosphorylation, acetylation, and sumoylation, translocates to the nucleus, and binds to co-activators before initiating transcription of gene targets. (B) Increased polyglutamine repeat length facilitates greater NTD-LBD interaction enabling subsequent activation steps and transcriptional activity even in the absence of ligand. Figure is modified from (58).

Although we were unable to determine AR cellular localization immunohistochemically, the fact that luciferase activity was elevated in both the AR33 and AR24 lines even in the absence of testosterone indicates ligand-independent AR

translocation. Our data also demonstrate that the differences in transcriptional activity between AR14 and AR24 are largely ligand-independent (though AR33 is activated by testosterone to a greater degree than AR14 or AR24; 110% vs. 53.9% and 57.3%, respectively), further supporting the possibility of increased ligand-independent nuclear localization with increased repeat length. Again, it is unlikely that the difference in transcriptional activity we observed was due to increased AR protein content in the AR33 and AR24 lines, as appreciable AR protein was only detectable in the AR14 line.

Additionally, the data of Siriett et al. (59) indicate that myostatin may negatively regulate AR transcriptional activity. Myostatin was demonstrated to suppress expression of the AR co-activator ARA70, which could effectively lower AR transcriptional activity. Though we did not assess ARA70 expression, our data revealed that the onset of myostatin expression in the stably transfected myotubes was delayed in a repeat length-dependent manner. The delayed myostatin expression in AR24 and AR33 myotubes may allow for greater ARA70 expression and hence contribute to the higher AR transcriptional activity of these lines. However, we were unable to detect any myostatin expression in myoblasts, so it is unlikely that the effects of myostatin were a contributing factor to the transcriptional differences between the lines while in a myoblast stage. Moreover, we did not examine the expression of other AR co-activators, so we cannot preclude the possibility that the absence/presence of such factors influenced our results.

Clearly more work is required to further determine the mechanism driving these differences. Though we have demonstrated a significant effect of AR repeat length on AR transcriptional activity, our data also indicate that AR repeat length affects the growth and differentiation of myoblasts in culture. Discovering the mechanism(s) of these *in*

vitro differences is important if we hope to elucidate the impact of AR repeat length on skeletal muscle mass and strength *in vivo*.

AR Protein Expression in Stably Transfected and Wild-Type C2C12 Cells

Despite the significant differences in AR activity, we were only able to detect appreciable AR protein in testosterone-treated AR14 myoblasts. All other cell lines and conditions tested displayed AR protein levels far below that of the AR-positive LNCaP cells, if in fact AR protein was detectable at all. Our results are not atypical, as the results of studies investigating AR protein content in skeletal muscle cell culture have been equivocal. Wannenes et al. (60) were able to detect AR protein in both proliferating C2C12 myoblasts and differentiating myotubes, and expression increased dose-dependently with testosterone administration. Lee et al. (61) detected AR protein in C2C12 myoblasts, and reported significant increases in AR protein when the cells were subjected to radial stretch. In contrast, both Chen et al. (45) and Lee (62) were unable to detect any AR protein in C2C12 myoblasts. Altuwaijri et al. (63) did not detect any AR protein in C2C12 myoblasts stably transfected with AR, though reported a slight increase AR protein in differentiating myotubes. However, all but one of these studies reported detectable AR mRNA in myoblasts with increased AR mRNA expression during differentiation. We observed very low AR protein content in all but the nuclear fraction of testosterone-treated AR14 cells, with AR protein nearly undetectable in fully differentiated myotubes. AR mRNA expression was low in WT C2C12 myoblasts, but increased after 24 hours of differentiation before dropping down again by 72 hours. In contrast, AR mRNA expression was observed in each of the stably transfected lines while

in rapid proliferation and throughout differentiation. Testosterone appeared to have little effect on AR mRNA expression in differentiating myotubes.

It is curious that though both the AR and AR33 lines displayed increased transcriptional activity in comparison to AR14, AR protein content was undetectable in either line. Several factors could potentially account for this discrepancy. First, the antibody used (rabbit polyclonal PG-21) is raised against residues 1-21 of the human AR. The glutamine repeat region begins at residue 58, so it is possible that the extended length of the repeat region affects antibody binding, and that AR protein was actually higher than what we were able to detect. It is also possible that the PG-21 antibody is just ineffective and that we did not get an accurate depiction of AR protein content. Secondly, though the 33 repeats present in our "long" AR construct are far fewer than the number known to induce aggregate formation and disease symptoms in vivo, it is not clear if this range is sufficient to induce aggregates or cytotoxic effects in skeletal muscle cells in vitro. If the AR33 protein is capable of inducing aggregate formation it is possible that antibody interactions would be disrupted, though AR protein aggregates would also likely reduce transcriptional activity, which our data does not support.

An unaddressed issue is the effect of glutamine repeat length on AR translation. Protein folding can be affected by the rate of translation, and it is possible that repeat length has an effect on translation efficiency that induces AR protein misfolding, leading to increased degradation via the proteasome. Our observation of increased AR mRNA in all of the stably transfected lines despite very low or undetectable AR protein content supports the possibility of reduced AR translation in C2C12 cells, regardless of repeat length.

Another possibility is the upregulation of an AR degradation mechanism, as several studies have demonstrated the presence of specific AR degradation complexes involving ubiquitination. Ubiquitin polymers are attached to misfolded and/or defective proteins via a three-part enzyme complex which targets them to the 26S proteasome for degradation (64). He et al. (65) demonstrated that the heat-shock protein 70 binding protein CHIP E3 ligase induces AR protein degradation in yeast by targeting ubiquitin polymers to the AR. Likewise, Lin et al. (66) report that AR protein is targeted for degradation via ubiquitination in a phosphorylation-dependant manner by an AKT-Mdm2 E3 ligase complex in prostate and kidney cells. Rodriguez-Gonzalez et al. (67) reported similar ubiquitin-mediated AR degradation in prostate cells. These studies demonstrate that a mechanism for degrading altered AR protein is functional in a number of cell types. Though we did not conduct any experiments intended to investigate this scenario, Lieberman et al. (68) reported that AR harboring 65 repeats was degraded via the ubiquitin-proteasome pathway to a far greater extent than WT AR in neuroblastoma cells. If the altered repeat length of our AR variants induced protein misfolding it is likely that ubiquitin-mediated AR degradation was also increased in our stable lines.

AR Glutamine Repeat Length and C2C12 Cell Proliferation

To our knowledge no studies have addressed the effect of AR repeat length on the growth and proliferation of cells in vitro, though numerous studies have examined the effects of AR and androgen administration on skeletal muscle cell proliferation and the results have once again been equivocal. We report that testosterone had no effect on cell

proliferation in any of the lines, indicating that the in vivo anabolic effect of androgens is not mediated by an increase in myoblast proliferation. This finding is consistent of the work of Chen et al. (45), where the treatment of C2C12 cells overexpressing the WT mouse AR with either DHT or testosterone had no effect on myoblast proliferation, and by Doumit et al. (69), where testosterone in doses up to 1uM had no effect on the proliferation of porcine myoblasts. In contrast, Benjamin et al. reported increased proliferation of C2C12 myoblasts overexpressing WT AR when treated with 10nM testosterone (21). In addition, Diel et al. (20) reported a slight increase in the percentage of C2C12 myoblasts in S-phase when treated with 10nM DHT , and Kamanga-Solio et al. reported increased H^3-thymidine incorporation in bovine myoblasts treated with up to 10nM of the synthetic androgen trenbolone (70). Finally, Lee (62) actually reported a decrease in proliferation of C2C12 cells stably expressing WT AR when treated with 10nM testosterone. These conflicting data can be attributed to a number of factors such as intra-assay variability, sensitivity of the measurements, quality of the culture serum, cell line, level of transgene expression, and the inherent variability of cell culture experiments. Our data demonstrating a lack of effect of testosterone on C2C12 cell proliferation is not particularly surprising considering that we were unable to detect significant AR protein content in all of our lines, and though limited, our gene expression analysis did not reveal testosterone-induced alterations that would indicate increased proliferation. Clearly work remains to be done in elucidating the effects of androgen signaling on skeletal muscle myoblast proliferation.

Results from our cell proliferation experiments demonstrate that there were significantly fewer cells of the AR33 line than all of the other lines after 3 days of growth

(*figure 3*). Morphologically, no differences between any of the lines were observed at any point during the proliferation phase (data not shown). RT-PCR did not reveal any significant differences in the expression of p53, a cell cycle regulator and initiator of apoptosis, the contractile protein skeletal alpha-actin, the adhesion molecule m-cadherin, nor the myogenic regulatory factor myoD (*figure 10*). Myostatin, a negative regulator of muscle growth, and the myogenic determination factor myogenin, were undetectable in any of the lines during proliferation. However expression of the calcium-sensitive transcription factor NFATC2, a protein demonstrated to be important for the growth of nascent myotubes by regulating myoblast-myotube fusion (71), was significantly decreased in both AR14 and AR33 lines, indicating potential differences in myoblast fusion and/or myotube formation. This conclusion was verified by immunohistochemical analysis.

AR Glutamine Repeat Length and C2C12 Cell Differentiation

As was the case with cell proliferation, differences in rate of differentiation among cell lines were apparent. In general, testosterone increased CK activity in all lines. This finding is in agreement with the work of Benjamin et al. (21) and Diel et al. (20), where testosterone and DHT, respectively, increased CK activity over ethanol vehicle during myoblast differentiation, though conflicts with Chen et al. (45) where testosterone had no effect on CK activity in AR stably transfected C2C12 cells, and Doumit et al. (69) where testosterone had a suppressive effect on porcine myoblast differentiation. In regards to our stably transfected lines, CK activity in the AR14 cells was significantly lower after 3 days and 5 days in comparison to C2C12 and AR24 cells. CK activity in the AR33 line was lower than both C2C12 and AR24 lines at 3 days, but was not significantly different at 5 days. These data indicate that shorter AR repeat lengths attenuate the differentiation process. The mechanism(s) behind these effects are partially elucidated by an examination of the expression patterns of genes involved in, or indicative of, the differentiation process, such as myogenin and myostatin.

The secondary myogenic regulatory factor myogenin is only expressed in differentiating myotubes. Not surprisingly myogenin expression increased in all lines with increasing time of incubation in differentiation media. However, distinct myogenin expression profiles were apparent between the stably transfected lines. Myogenin expression was detected in all lines after 24 hours in differentiation media, although expression was lower with increasing repeat length; C2C12>AR14>AR24>AR33. At 72 and 120 hours this difference had largely vanished; only AR24 cells at 72 hours expressed slightly ower levels of myogenin. However, the comparatively lower myogenin

expression in AR24 cells at 72 hours is not in accordance with comparatively higher CK activity at the same time point. Despite decreased CK activity in both AR14 and AR33 cells at 72 hours, myogenin expression at this time point was not different from C2C12 control cells. Testosterone had a slight stimulatory effect on myogenin expression in AR24 and AR33 lines at 24 hours and in AR24 cells at 120 hours, though appeared to otherwise have little effect on myogenin expression. Although no previously published data were found regarding myogenin expression and AR repeat length, Wannenes et al. (60) demonstrated an increase in myogenin expression in differentiating C2C12 cells with the addition of 10nM testosterone. This effect was abolished by the selective AR antagonist bicalutamide, indicating that the increase in myogenin was mediated via AR signaling. Accordingly, Lee (62) demonstrated a modest increase in myogenin expression in C2C12 cells overexpressing AR when treated with 10nM testosterone, though this effect was not observed in WT C2C12 cells. Though our data demonstrate only a very slight increase in myogenin with testosterone treatment at specific time points and in specific cell lines, the increased AR transcriptional activity of the AR24 and AR33 lines appeared to delay the onset of myogenin expression in differentiating cells.

Myostatin is a well know inhibitor of muscle cell proliferation and differentiation (72). Interestingly, after 1 day of differentiation myostatin was only detected in WT C2C12 cells; none of the 3 stably transfected lines had appreciable myostatin expression (*figure 9*). After 3 days of differentiation myostatin was detected in AR14 cells, but was still undetectable in AR24 and AR33 lines. Only after 5 days in DM was myostatin mRNA observed in all lines. These data suggest that transcriptionally active AR may delay the onset of myostatin expression in C2C12 cells. However, the effect of

testosterone was conflicting. There was a slight stimulation of myostatin at 3 and 5 days in C2C12 cells, and as well as in AR14 cells at 3 days. In contrast, testosterone suppressed myostatin in AR33 cells at 5 days. Once again, available literature on androgen regulation of myostatin expression in skeletal muscle is inconclusive. Diel et al. (20) reported a biphasic effect of DHT on myostatin expression in C2C12. Another paper by the same group reported increased myostatin in testosterone-treated rat gastrocnemius muscle (73). On the other hand, Mendler et al. (74) describe decreased myostatin protein expression in rat LA muscle after testosterone treatment. Interestingly, the data of Siriett et al. (59) suggests myostatin negatively regulates AR transcriptional activity by inhibiting expression of the AR co-activator ARA70. However, from our data it is not clear if transcriptionally active AR inhibits myostatin expression or if the reduced myostatin expression seen in AR24 and AR33 myotubes enables a more transcriptionally active AR. The data of Diel et al. (20) demonstrate that myostatin expression in differentiating C2C12 cells follows a complex biphasic expression pattern. We only tested samples from 1, 3 and 5 days post-switching to differentiation media and it is possible we may have detected more subtleties in the relationship between AR and myostatin with a more thorough data set. We did not evaluate the expression of ARA70, though a thorough comparison of ARA70 expression with that of AR and myostatin during differentiation may elucidate the relationship with more certainty.

AR Glutamine Repeat Length and C2C12 Myotube Development and Morphology

The representative images in *figure 6* highlight the striking morphological differences observed between each line during the differentiation process. Though each line appeared very similar during the proliferation phase, both AR14 and AR33 cells adopted a highly irregular morphology soon after differentiation was initiated. Both WT and AR24 lines formed very large, thick myotubes with large clusters of nuclei. The thin, elongated, sparsely nucleated appearance of the AR14 line is very similar to that of NFATC2-/- cells described by Horsley et al. (44). Though NFATC2 expression in the AR14 line was only slightly lower than WT cells during differentiation it was nearly undetectable during proliferation. In addition, the AR14 line displayed a large number of myosin positive cells with fewer than 3 myonuclei (data not shown). Both CK activity and myotube fusion index in AR14 cells were also significantly lower than WT and AR24 cells at 72 and 120 hours. Myogenin expression was not significantly different from WT cells, though the onset of myostatin expression was delayed. Collectively these data indicate that AR14 myoblasts proliferate more rapidly and begin the differentiation process sooner than either the AR24 or AR33 line. Our conclusion is that the initial myoblast-myoblast fusion process occurs normally, but subsequent myonuclear addition is retarded. As described by Pavlath and Horsley (71), NFATC2 appears to have a vital role in myotube-myoblast fusion as cells lacking functional NFATC2 display impaired myonuclear addition. Moreover, our AR14 myoblasts displayed markedly lower NFATC2 expression in comparison to both WT and AR24 lines, though the difference was very slight in myotubes. The mechanism by which NFATC2 alters myoblast fusion

has been hypothesized to occur downstream via the cytokine IL-4 (75), with NFATC2 itself stimulated upstream by the prostaglandin PGF2α (76). Though an examination with the program TFSEARCH did not reveal any ARE's in the NFATC2 or IL-4 promoters, we did not examine the expression or production of either IL4 or PGF2α and we cannot preclude a significant effect of transcriptionally active AR on their expression. Analysis of NFATC2 protein content and its phosphorylation status in each of the lines may provide additional information regarding the effect of AR transcriptional activity on NFATC2 expression and activation.

In contrast to the AR14 line, the AR33 line formed very short, cylinder-like myotubes with clustered nuclei. Incubation in DM also resulted in a large number of myosin-positive, mono-nucleated cells, and the number of dead and detached cells was far greater by day 5 than that of any other line (data not shown). Like the AR14 line, both the average number of myonuclei per field and myotube fusion index were significantly lower in the AR33 line than either WT or AR24 lines. NFATC2 expression was also drastically reduced in AR33 myoblasts in comparison to WT myoblasts, though like the AR14 line this difference was largely abolished in myotubes. It is unclear if reduced NFATC2 expression in the AR33 line is responsible for the decreased FI and average myonuclei number per field in differentiating myotubes, as the morphology of the AR33 line was not similar to the myotubes described by Horsley et al. (44), nor to that of the AR14 line. On day 1 of differentiation expression of myogenin and myostatin was decreased in comparison to WT cells. These data, in addition to significantly lower CK activity after 3 days in DM, indicate a decreased rate of differentiation in the AR33 line, though differentiation of the AR33 line seems to have "caught up" to that of WT

cells after 5 days in DM. CK activity of the AR33 line was not significantly lower than the WT line by day 5, though total protein content was significantly lower in comparison to all other lines. Though we did not conduct an extensive examination of cytotoxic or apoptotic mechanisms, the presence of comparatively larger numbers of "floaters" in the AR33 line as well as what can only be described as an unhealthy appearance during differentiation may be indicative of a toxic condition. In addition, our proliferation data indicate that fewer cells of the AR33 line were present after 3 days incubation in GM, in comparison to WT cells. Total protein content of the AR33 line was significantly lower after 5 days of differentiation in comparison to WT cells and immunohistochemical analysis indicates that fewer myosin-expressing AR33 cells are present after 5 days in DM, yet CK activity normalized to protein was not different from WT and markers of differentiation were not different after 5 days in DM. These data suggest that despite the unusual appearance of the AR33 myoblasts, they are differentiating properly (if more slowly), yet fewer of them are present after 5 days. It is possible that 33 glutamine repeats, though lower than the number known to induce cytotoxic effects in vivo, is sufficient to induce an apoptotic response in C2C12 cells when exposed to the low serum environment of differentiation media.

Conclusions

If we consider AR signaling to induce an anabolic effect in tissues where it is expressed, our data are supportive of the work of Walsh et al. (34), where a positive association between AR repeat length and lean body mass were found in 2 independent human cohorts. In this case, the lower transcriptional activity demonstrated by our AR14 variant in comparison to both the AR24 and AR33 lines could result in lower skeletal

muscle mass in vivo. In addition, it has been reported that serum testosterone levels are significantly higher in men with longer AR glutamine repeat lengths (34;77). Whether the increased lean body mass in men with longer repeat lengths reported by Walsh et al. and Campbell et al. (35) are due to higher transcriptional activity, as our *in vitro* data would suggest, or simply due to higher circulating androgen levels has not yet been determined. However, our data also demonstrate that skeletal muscle myoblast gene expression is altered by AR repeat length is cells stably transfected with AR. We observed that the onset of myostatin expression is delayed by AR with longer glutamine repeat length, indicating that myoblasts in human skeletal muscle harboring longer AR repeat lengths may take longer to exit the cell cycle, leading to greater numbers of skeletal muscle fibers in these individuals. Additionally, the decrease in NFATC2 expression observed in our AR14 line provides a possible mechanism driving the formation of numerous thin, elongated myotubes with few myonuclei. Decreased expression of NFATC2 in developing skeletal muscle harboring shorter AR repeat lengths may result in larger numbers of activated satellite cells that are defective in contributing additional myonuclei, leading to smaller myofibers with a more limited potential for hypertrophy.

Overall, our data suggest several possible mechanisms that could lead to greater skeletal muscle mass in humans with longer AR glutamine repeats. However, despite significant differences in AR transcriptional activity, we were unable to demonstrate appreciable AR protein in WT or stably transfected C2C12 myoblasts. This inability is not uncommon in studies of AR action in skeletal muscle, and it is hard to argue that the anabolic effects of T in skeletal muscle are directly mediated via AR signaling when AR

protein cannot be detected. Though data exists supporting a direct effect of T on skeletal muscle cells *in vitro*, evidence that these effects are mediated via AR signaling in skeletal muscle is weak or largely non-existent. Additionally, though the anabolic effects of T on skeletal muscle mass and strength in animal and human subjects are very well documented, large increases in mass and strength are generally dependent upon the combination of exogenous T and muscular overload. Anecdotal reports from athletes suggest that exogenous T administration results in very rapid increases in force production, far more quickly that what could be expected from significant tissue remodeling. As the AR is highly expressed in motor neurons and in the central nervous system (78;79) the possibility exists that the main action of T may in fact occur via AR signaling in neural tissue. An increase in AR-mediated neurotransmitter release, for example, could result in an increase in force production potential, thereby overloading the muscle and stimulating increased protein synthesis and remodeling via traditional mechanisms.

Future Experiments

Given the results of our current data, several follow-up experiments would address some of our unanswered questions.

1) Assess the relationship between AR glutamine repeat length and myofiber number/cross sectional area (CSA) in human skeletal muscle. Our data suggest that NFATC2 expression is differentially regulated by AR repeat length, and that subsequent alterations to myofiber number and myofiber size may be observable in human skeletal muscle tissue. Quadriceps muscle biopsies would be obtained from male subjects (AR is X-linked and male subjects remove the difficulty of accounting for X-chromosome silencing) with AR glutamine repeat lengths near the min/max of normal physiological range. Samples would be fixed and stained with an anti-myosin heavy chain antibody and visualized using immunofluorescence. Myofiber number per field would be determined by direct counting and fiber-CSA determined using the appropriate software.
2) Conduct a comprehensive gene expression analysis using an Affymetrix and/or microarray approach on the biopsies obtained above. While we observed significant differences in several genes, our scope was very limited and it is likely differences in the expression of other genes involved in AR signaling and regulation were missed.
3) Perform the same AR transcriptional activity experiments in additional non-muscle cell lines. Though available data indicates a tissue-specific effect of glutamine repeat length on AR transcriptional activity, no single study has

demonstrated an opposite effect of long and short glutamine repeat lengths on AR transcriptional activity in different tissues. We propose repeating our luciferase experiments in prostate (LNCaP, PC3), kidney (COS-1), cardiac muscle (HL-1), and breast (MCF-7) cell lines in addition to our C2C12 lines, and comparing the results among lines. This experiment would provide powerful evidence for a tissue-specific regulation of AR transcriptional activity, if in fact such a mechanism exists.

4) Assess the ability of the different AR constructs to drive luciferase expression from the NFATC2 and myostatin promoters. These experiments would further strengthen our data suggesting that NFATC2 and myostatin expression are differentially regulated by AR with variable glutamine repeat length.

Limitations

1) Our study relied upon the use of cultured myoblasts. While C2C12 cells are a very commonly used cell type in the study of muscle physiology, they are certainly not a perfect model. While they do not accurately represent the characteristics of quiescent human satellite cells, they are a reasonable model of activated satellite cells in vivo. Access to human skeletal muscle tissue would have provided interesting options but was not feasible during the conduct of the present dissertation.
2) Our study was limited to the use of AR constructs harboring repeat lengths within the typical physiological range. While this range is certainly the most physiologically relevant, several studies have included repeat lengths far outside the normal range, and inclusion of these ranges in our study would have allowed us to compare our results to the previously published literature.
3) We were unable to confirm our luciferase data from C2C12 cells in the LNCaP prostate cell line, for which the effect of AR repeat length on transcriptional activity has previously been reported, due to technical problems. Demonstration of a tissue-specific effect of AR repeat length on transcriptional activity would have been very powerful.
4) We were unable to test our hypothesis that AR nuclear translocation efficiency would be altered by glutamine repeat length. We hypothesized that a greater affinity for nuclear import/reduced affinity for nuclear export could account for the transcriptional activity differences reported in previous studies, but we were unable to detect AR protein immunohistochemically.

5) Though we observed interesting differences between the lines in the expression of several genes, our analysis was limited in scope. Microarray analysis may have uncovered additional differences that we missed with our experiments.

Review of Relevant Literature

Introduction

The documented history of the use of testosterone as an ergogenic aid goes back to the late 1800's, where extracts from dog testicles were used as a "rejuvenating" elixir (80). It has also been suggested that Nazi Germany began administering the newly identified testosterone to soldiers in the 1930's as a performance enhancer (81). The muscle mass and strength enhancing effects of testosterone have been known to athletes for many years, though not until recent decades has the same consensus reached scientific circles. What is also apparent is the fact that the individual response to androgen administration, even in tightly regulated clinical studies, is highly variable, suggesting that there is a genetic component involved (38). This review will discuss in depth recent clinical studies demonstrating the anabolic effects of androgens in vivo. In addition, though the anabolic potential of androgens is now well know, the mechanism by which these physiological effects are manifested is still a matter of great interest and uncertainty. Included in this review is a look at the effects of androgens at the cellular level, including effects on protein balance and cellular growth and development. It is believed that the majority of these physiological effects of androgens are mediated via the androgen receptor. We will discuss data supporting this view, including an analysis of the structure and function of the androgen receptor itself, and possible interactions with other hormone systems. Finally, this review will discuss the impact of genetic variation within the androgen receptor, and whether these variations could account for some of the heritability of skeletal muscle mass.

Clinical Effects of Androgen Administration

In recent years testosterone (T) administration has become a more accepted treatment for a variety of conditions affecting both young and aged men alike. Though historically prescribed primarily for the treatment of anemia, hypoandrogenemia, and severe wasting conditions such as burn trauma and AIDS, T has gained favor as a treatment for muscle wasting and "anti-aging" (82;83). Due to the fact that T and its synthetic derivates have androgenic effects in addition to anabolic effects, that men have endogenous androgen levels in excess of 10-fold greater than those of women (84;85), and that women do not experience the steady decline in serum T seen in men (86), treatment of these conditions with T has been largely limited to men. Following is a review of the current literature regarding androgen administration and its effects on skeletal muscle mass. Until relatively recently the anabolic action of T on skeletal muscle mass was controversial. Though athletes and bodybuilders in particular have been using T and its synthetic derivates since the 1960's in order to increase their muscle mass and strength (81), many studies up until the mid 1990's demonstrated little effect of T on fat-free mass. The disconnect between athletes, whom were keenly aware of the anabolic properties of androgens, and the scientific community can be attributed to study design. Variables such as nutritional status, daily activity, resistance training, androgen doses, baseline hormonal status, etc., that are not properly controlled can greatly influence results from studies designed to tease out potential anabolic effects of androgens.

Effect of Testosterone Replacement in Males with Hypogonadism

Normal healthy males secrete between 4mg and 10mg of T/day, equating to 300-1200ng/dL, though production begins to decline in the 5th decade of life, so that by the age of 65 the average man secretes ~4mg/day (87). This decline is such that 20% of men aged 60 are considered hypogonadal (<10nM serum T), 30% by age 70, and 50% by age 80 (88). Interestingly this decrease in serum T closely mirrors the onset and progression of sarcopenia, or the loss of muscle mass and strength that occurs with aging (89). Hypogonadism is also associated with increased fat mass, decreased fat-free mass and decreased bone mineral density in comparison to aged-matched eugonadal men (8). Artificial suppression of endogenous T production in healthy young men via a gonadotropin releasing hormone agonist results in a condition similar to normal hypogonadism, including increases in fat mass and decreases in lean mass (90). Katznelson et al. (8) treated hypogonadal men with weekly 100mg intramuscular injections of the long-acting T enanthate for 18 months. Using quantitative computed tomography, dual-energy X-ray absorptiometry and bioelectrical impedance, these men demonstrated a 14% decrease in total body fat, 13% decrease in subcutaneous fat, 7% increase in fat-free muscle mass, and 14% increase in trabecular bone mineral density. Another study administered 200mg T enanthate injections bi-weekly for 3 months to hypogonadal men, mean age 76, and though there was no measurable decrease in body fat percentage, significant increases in total T and free T, as well as grip strength, were noted (91). Bhasin et al. (92) also administered 100mg weekly T enanthate injections to hypogonadal men aged 19-47 years. These subjects gained an average 5kg of lean body mass measured by underwater weighing over a 10 week period and increased arm and leg

circumference measured by MRI. Another method of T treatment involves the use of topical gel and transdermal patches. Wang et al. (93) treated hypogonadal men aged 19-68 with unesterified T via topical gel, designed to deliver 50mg or 100mg/day, respectively, as well as with transdermal T patches delivering 5mg T/day. All subjects gained lean body mass, though the 100mg/day gel group had the greatest gains, 2.7kg vs. 1.3kg and 1.2 in the 50mg/day gel and 5mg/day patch groups, respectively. All subjects experienced increases in hematocrit levels, hemoglobin levels, and upper and lower body muscle strength, though only the 100mg/day gel group decreased body fat. These gains in muscle strength were achieved without any resistance training.

Thus, and despite the absence of resistance training and/or dietary intervention, a 100mg/week injection of a long-acting T preparation appears sufficient to stimulate changes in body composition in hypogonadal men. A 100mg T enanthate injection in hypogonadal men increases serum T to ~1400ng/dL within 2 days, and serum levels decline to ~800ng/dL 7 days after administration (94). This varies from slightly above, to right in the middle of the normal range. Though effective, T concentrations with patch treatment tend to be more variable than with injections, ranging from >250ng/dL to 1000ng/dL with a patch designed to deliver 6mg T/day (95).

Testosterone Administration in Men with HIV

Severe weight loss and muscle wasting is a common occurrence in subjects afflicted with AIDS. Approximately half of men with advanced stage AIDS are also hypogonadal (96) and those afflicted by AIDS lose a proportionally greater amount of lean body mass (97), therefore T replacement has become common among AIDS

patients. In one randomized, double-blind study men (mean age 42 years) afflicted with HIV-induced muscle wasting whom were also androgen-deficient were treated with 300mg T enanthate injections every 3 weeks for 6 months (98). Compared to age-matched placebo control subjects, men treated with T gained lean body mass (2.0kg vs. -0.6kg) and reported feeling better with a higher quality of life. In another double-blind randomized study, 49 HIV-infected men with significant weight loss were assigned to 4 treatment groups; placebo, T alone, resistance training alone (RE), or T plus exercise (TRE) (3). T treatment groups received 100mg weekly T injections for 16 weeks, while the RE and TRE groups underwent a resistance training program for 16 weeks. The placebo subjects did not change in any measures, but T, RE, and TRE groups demonstrated improvements in muscle strength in all 5 exercises tested (17-28%, 29-36%, and 10-32%, respectively), though the TRE group was not significantly different than either single intervention. T, RE, and TRE groups all increased lean body mass (2.9kg, 2.0kg, 1.6kg, respectively), though the TRE group did not differ significantly than either single intervention. All three treatment groups experienced increases in thigh muscle volume (40cm^3, 62cm^3, and 44cm^3, respectively) though again the TRE group did not show gains greater than either treatment alone.

Effects of Supraphysiological Testosterone Doses on Healthy Subjects

The goal of T replacement therapy for hypogonadal men is simply to return serum T levels to the 300-1200ng/dL range. Though returning hypogonadal men to the normal range of serum T levels does induce changes in body composition, it does not result in lean mass gains exceeding the level seen in normal, healthy, eugonadal subjects. Athletes and bodybuilders using exogenous T or other synthetic androgens generally administer

doses in great excess of those required to return hypogonadal men to the normal range. In one survey 50% of androgen users reported administering 500mg of T per week or more (99), and in another survey of competitive athletes 25% reported administering 1000mg of T per week or more (100). Though these subjects are likely to be an extreme example, it is also likely that "recreational" users will administer more than simply replacement doses of T. It is therefore important to understand the effects of supraphysiological doses of T on otherwise healthy subjects. Additionally, many studies examining the effects of T on lean body mass fail to mimic the dietary regimens or resistance training protocols followed by athletes. Likewise, in order to accurately assess the effects of T on body mass in human subjects, studies must provide the appropriate dietary and training conditions.

One of the first and to date most comprehensive studies to examine the effects of supraphysiological amounts of T on healthy human subjects was done by Bhasin et al. (7) in 1996. Forty men aged 19-40 were assigned to one of 4 treatment groups and evaluated over 10 weeks: placebo, testosterone (T), exercise (E), and testosterone plus exercise (TE). T treatment consisted of 600mg intramuscular injection per week, and exercise consisted of a free-weight program of semi-progressive overload. Dietary intake was monitored for total calories, protein, and vitamin/mineral content. Fat-free mass (underwater weighing), muscle size (MRI), and muscle strength (1-rep max) were assessed, as well as hormone levels and full blood chemistry at various time points. The T, E, and TE groups increased fat-free mass by 3.2kg, 1.9kg, and 6.1kg, respectively, while body fat did not increase significantly in any group. Muscle size of the triceps and quadriceps increased significantly in both T and TE groups, and bench press and squat

strength increased in T, E, and TE groups (*figure 11)*. LH, FSH, and SHBG were all suppressed in T and TE groups. Total cholesterol and LDL did not change in any of the groups, though interesting HDL did decrease in the E group. PSA levels did not change in any group, and digital prostate examination did not reveal any abnormalities.

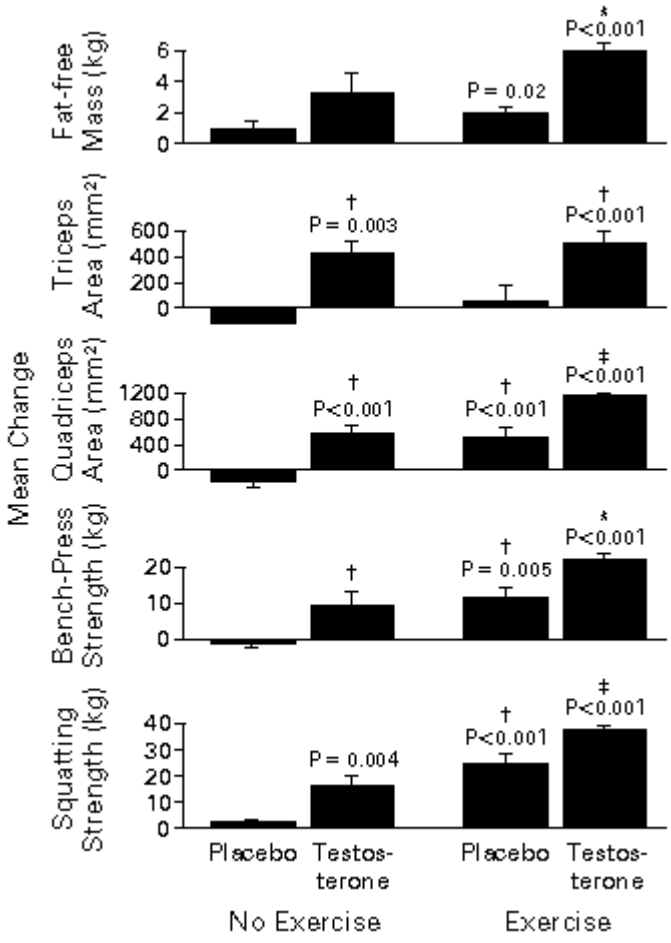


Figure 12: Changes in Fat-free mass, muscle area, squat strength, and bench press strength. Adapted from (7)

Despite the lack of a true progressive overload exercise program and a diet providing relatively low levels of protein and calories, T induced significant changes in

muscle area, lean mass and muscle strength, and these effects were greatly enhanced by exercise training.

In another study of 39 healthy men aged 18-35 (101), subjects were administered from 25mg to 600mg of T in weekly intramuscular injections for 20 weeks, in conjunction with a GnRH agonist to suppress endogenous T production. No other interventions were performed. Muscle volume (MRI) changes ranged from -4ml in the group receiving 25mg injections, to +48ml in the group receiving 600mg injections. Significant changes in muscle fiber type size (distinguished immunohistochemically via MHCII antibody) occurred in the subjects receiving 300mg and 600mg injections. Type I fiber size increased ~1000um^2 and ~1650um^2 in the 300mg and 600mg groups, respectively. Type II fibers increased ~1470um^2 in the 600mg group. Fiber type proportion did not change significantly for either group. Type I fiber myonuclear number increased in 300mg and 600mg groups (+0.7 and +0.8myonuclei/fiber, respectively), and type II fiber myonuclear number increased only in the 600mg group (+1.3 myonuclei/fiber). Measurements of body weight or lean mass were not made, yet despite a lack of resistance training clear improvements in muscle volume and individual muscle fiber size were demonstrated with a minimum 300mg weekly intramuscular injection.

In another set of experiments conducted by the same research group, young (6) and aged (11) men, aged 19-35 and 60-75 years respectively, with normal T levels were treated with graded, supraphysiological doses of T ranging from 25mg to 600mg for 20 weeks in order to determine any differences in T responsiveness between the young and the aged. Subjects were prohibited from engaging in any intensive resistance or endurance training and were instructed to follow a diet standardized for total calorie and

protein content. Body composition and muscle volume were determined as in (7) , muscle strength via 1-rep maximum leg press, and T, free T, LH, FSH, and hemoglobin via radioimmunoassay (RIA). T, free T and hemoglobin increased to a greater extent in the elderly in comparison to the young at doses of 125, 300, and 600mg, likely due to reduced T clearance in the elderly. Fat-free mass increased similarly in both young and elderly, was correlated with T dose and was not significantly different between groups. Fat mass decreased in the young and elderly, and the decrements were inversely correlated with T dose. Leg press strength increased in direct correlation to T dose, but did not differ between young and elderly. Both young and elderly men experienced increases in PSA level and decreases in HDL, though there was no significant effect of age for either. Older men did experience greater incidence of high hematocrit values (greater than 54%).

The above experiments demonstrate that testosterone is capable of inducing increases in lean mass and muscle strength as well as simultaneous decreases in fat mass in both young and aged subjects. These effects are observable without resistance training, but are enhanced by the combination of T and resistance exercise. Detrimental side effects include decreases in HDL levels and increases in PSA values. Elderly subjects run the risk, at doses in excess of 300mg/week, of hematocrit values exceeding the recommended range (>54%). Though none of these studies address the mechanism by which T induces increases in lean body mass and strength, the anabolic actions of T on skeletal muscle have become widely accepted.

Heritability of Serum Androgen Levels and the Response to Exogenous Administration

From a clinical perspective serum androgen levels are a concern not only for the aging male, but also for young men and women. As discussed above, the decline in T that occurs with aging is associated with decreased bone mineral density, increased fat mass, and decreased muscle mass. Low serum T is also associated with an increased risk of beta amyloid formation and Alzheimer's Disease-like neuropathology (102). In contrast, elevated androgen levels and androgen replacement are associated with an increased libido in both men and women (103-105). It is also of clinical relevance that serum androgen/T level appears to be a largely heritable trait. In analysis of 532 subjects from the National Heart, Lung, and Blood Institute Twin Study, genetic factors accounted for ~57% of the variation in T levels (10) in male twins. Another study by Meikle and Bishop (106) found that of 75 monozygotic and 88 dizygotic male twins, genetic factors accounted for 63% of the variation in serum T levels. Similar results were reported by Hong et al. (107) and by Bogaert et al. (108), whom found genetic factors accounted for 69% and 65% of variability in serum T levels .

Though the heritability of serum hormone levels is well characterized there is also evidence, albeit not as concrete, suggesting that the magnitude of response to exogenous androgen treatment may be a heritable trait. In a survey of competitive athletes and bodybuilders, Evans (99) reported the exogenous androgen regimes utilized during different phases of competition. Despite similar experiences and competitive aspirations,

subjects reported highly variable doses and drug combinations, suggesting a variable individual response to exogenous androgens. In a study of the responsiveness of older vs. young men to T administration, Bhasin et al. (11) report high standard deviations in respect to changes in muscle mass, though the authors do not address this variability in discussion. Another study by the same group investigating the T dose-response relationships in young men demonstrated high standard deviations in a number of variables, including fat-free mass, leg strength, and muscle volume (6). Choi et al. administered T to HIV-infected women and demonstrated a high standard deviation with regard to changes in lean mass (0.7kg±0.4kg) or fat mass (0.3kg±0.7kg) in the T treated groups (109). In 262 HIV-infected men treated with the synthetic androgen oxandrolone, changes in body weight were highly variable (1.1 +/- 2.7, 1.8 +/- 3.9, 2.8 +/- 3.3) in men receiving 20, 40, and 80mg daily, respectively (110). A longitudinal study by Krithivas et al. (111) found that the decline in serum testosterone that occurs with aging is correlated with CAG repeat length within the androgen receptor, a finding supported by Walsh et al (34) where cross-sectional analysis revealed a positive association of serum testosterone and increasing CAG repeat length.

It is difficult to discern the inter-individual differences in the response to androgen administration from the literature as most studies do not provide data from each individual subject, leaving standard deviation/standard error as the only available measure. Only one study was found that acknowledged inter-individual differences in androgen responsiveness and attempted to directly identify predictors of response to androgen administration. Woodhouse et al. (38) review and highlight these inter-study differences, and conducted their own study with 54 healthy men receiving graded doses

of T over 20 weeks. A wide range of independent variables such as subject age, height, weight, BMI, lean mass, leg strength, leg fatigue resistance, fiber type, fiber area, serum and free T, SHBG, LH, FSH, IGF-1, IGFBP-3, lipid profile, PSA, and T dose were examined. Univariate and multivariate analysis revealed that testosterone dose was most highly correlated with changes in fat-free mass, and that a 3-way interaction of dose, subject age, and PSA level could explain 67% of the variability in response to T. Androgen receptor polymorphisms were also examined, including the polyglutamine repeat in exon 1, but found only weak correlations between AR polyglutamine repeat length and changes in lean body mass. This is in contrast to the data of Walsh et al. (34), where statistically significant, though perhaps physiologically minor, associations were found between polyglutamine repeat length and lean body mass as well as in serum T levels. Woodhouse et al. (38) do not explain how subjects were grouped in regards to polyglutamine repeat length nor do they provide mean repeat numbers, but they do acknowledge that genetic variation could account for a significant percentage of the difference in androgen responsiveness. Nonetheless, the above studies provide support to reports from athletes that significant differences exist in the inter-individual response to exogenous androgen treatment.

Mechanisms of the Anabolic Actions of Testosterone on Skeletal Muscle

Though the clinical effects of T have been well established, the cellular and molecular mechanism(s) by which T exerts its anabolic effect on skeletal muscle tissue are less clear. Skeletal muscle adapts to overload and other damaging stimuli primarily by increasing the size of existing muscle fibers, or in more rare cases (such as severe injury) by the formation of additional muscle fibers. Evidence suggests that T affects

both of these processes. However, many early studies of T action in animal models used the very highly androgen responsive levator ani muscle. Though a skeletal muscle, this tissue is abnormal in that it expresses much higher levels of the androgen receptor (AR) than other skeletal muscle (112) , and is more sensitive to the actions of testosterone and synthetic androgens than other skeletal muscle (113;114). It is also constructive to highlight differences in gene expression between the LA and other skeletal muscle. For instance, the synthetic androgen fluoxymesterone greatly increased skeletal alpha actin mRNA expression in rat LA muscle but had no effect on skeletal alpha actin mRNA expression in gastrocnemius muscle (115). It is therefore important to consider the data from studies using the levator ani muscle carefully, as these results may not be entirely applicable to other skeletal muscles.

Influences of Testosterone on Skeletal Muscle Protein Balance

Evidence of T's positive effects on protein synthesis has been apparent for some time. In animal studies, Grigsby et al. (116) demonstrated increased uptake of H^3-leucine into myofibrillar proteins of the semitendinosus muscle of rabbits treated with T implants for 15 days, when compared to untreated control animals. Rogozkin (117) determined that male rats treated with the synthetic androgen methandrostanolone had increased levels of myofibrillar and sarcoplasmic protein, via C^{14}-leucine incorporation. In humans, Griggs et al. (118) administered 3mg/kg of T to healthy men for 12 weeks. Protein synthesis measured by C^{13}-leucine incorporation in quadriceps muscle increased a mean 27%, where total body protein synthesis did not change significantly. In elderly hypogondal men T replacement increased fractional protein synthesis a mean 57%, measured by C^{13}-leucine infusion (2). Similar increases were seen in elderly men with

low-normal serum T levels (119). On the short term, Ferrando et al. (13) using a rather complex method of quadriceps muscle biopsy and arteriovenous sampling measuring labeled, mixed amino acid uptake, demonstrated that a single 200mg T injection to healthy men increased fractional protein synthetic rate from 1.6%/day to 3.35%/day after 5 days, while fractional protein breakdown was unchanged. Using similar methodology, Sheffield-Moore et al. (120) administered 15mg/day of the synthetic androgen oxandrolone to healthy men for 5 days and found that fractional protein synthesis increased from 1.38%/day to 1.96%/day, with no change in the rate of protein breakdown. In both cases (13;120) it was calculated that androgen treatment significantly increased the rate of intracellular amino acid reutilization. On the other hand, suppression of endogenous T production has the opposite effect. Though skeletal muscle protein synthesis was not measured, healthy young men treated with a GnRH agonist demonstrated near complete suppression of serum T (mean 535ng/dL to 31ng/dL after 10 weeks), and a concomitant 13% decrease in whole body protein synthesis measured via C^{13}-leucine disposal (90). Though the effects of T on muscle protein degradation are less clear, Ferrando et al. (121) administered T at 200mg/wk to severe burn victims, a condition characterized by extreme tissue wasting. Though protein synthesis was unchanged (likely due to an already very high rate of protein synthesis in compensation for the severe tissue damage), protein breakdown was decreased nearly 200%. At the cellular level, Zhao et al. (122) determined that in C2C12 myoblasts expression of the proteolytic muscle atrophy factor MAFbx is heavily suppressed by T. Although in two distinct studies T and synthetic androgens do not appear sufficient to suppress the proteolytic and catabolic conditions induced by dexamethasone treatment in

C2C12 myoblasts (123) nor L6 myoblasts (124), these results may be attributed to the doses of dexamethasone and testosterone (10:1 ratio) used, and the fact that protein was isolated without the expressed use of protease inhibitors.

Effects of Testosterone on Myogenic Cell Activity

In recent years T has been demonstrated to have an effect on adult skeletal muscle satellite cells *in vivo* as well as cultured myoblasts *in vitro*. The satellite cell, first described by Mauro (125), is a mononucleated cell that lies under or within the basal lamina of a mature myofiber. These cells are normally held in a quiescent state with minimal levels of protein synthesis or expression of myogenic genes. They become activated in the presence of stimuli resulting from muscle damage or mechanical stress (126), move outside the basal lamina and begin to co-express the myogenic determination factor MyoD and paired-box transcription factor Pax7 (127). The activated satellite cells, now myoblasts, can then be followed into two separate factions. Most commence multiple rounds of cell division, lose Pax7 expression, initiate expression of contractile and other muscle proteins, differentiate, and either fuse to existing myofibers or fuse together with other myoblasts to form new myofibers. A small percentage of the activated satellite cell population will continue to proliferate and express Pax7, but will down regulate MyoD expression and return to a quiescent state, repopulating the satellite cell pool (128).

Recent evidence has demonstrated that T has an effect on satellite cell activation and cycling. In a series of experiments, Joubert and Tobin (129-131) demonstrated that T induces an increase in satellite cell number and myonuclei number, detectible by

morphological observation, within 3 days of treatment. All of these experiments were conducted on the LA muscle however, so the results must be interpreted with caution. Nnodim (132) determined that satellite cell number in the denervated LA muscle of castrated rats was increased 2-fold in animals treated with T, suggesting that T has a powerful effect on satellite cell proliferation in response to denervation. Doumit et al. (69) determined that the proliferation of porcine myoblasts was not affected by T, but that differentiation was slowed ~20% by T after 4 days of treatment. Kadi et al. (133) acquired biopsies from the trapezius and quadriceps muscles of 17 elite-level powerlifters, 9 or whom had a history of anabolic steroid use, and 6 untrained subjects. Those with a history of steroid use had a significantly higher myonuclear number in both muscles in comparison to non-user powerlifters and the untrained subjects. In addition, Powers and Florini (134) reported a ~25% increase in H^3-thymidine labeling in the nuclei of rat L6 myoblasts treated with T, indicating a stimulation of DNA replication. Interestingly, this effect was not apparent in cells treated with dihydrotestosterone, indicating a that testosterone alone may be responsible for the actions of androgens on skeletal muscle. In a clinical study of healthy young men, T induced a dose-dependent increase in satellite cell number and myonuclear number in quadriceps muscle after 20 weeks of treatment with 300mg and 600mg/week of T enanthate (19). In another similar study by the same group (4), 300mg/week of T for 20 weeks increased satellite cell number in quadriceps muscle of elderly men. Interestingly, T at 300mg/week (the only dose where gene expression was tested) increased the number of satellite cells expressing Notch, and the number of satellite cells expressing myogenin. This indicates that T may increase both proliferation and differentiation of satellite cells in vivo. This is in

agreement with the findings of Diel et al. (20), whom demonstrated via cell flow cytometry and creatine kinase activity that androgens (T, DHT, or tetrahydragestrinone (THG)) significantly increase the rate of proliferation and differentiation of mouse C2C12 myoblasts. There were also accompanying changes in gene expression; androgens increased the Pax7 and myostatin expression in a time-dependant manner during differentiation. Lee (62) examined the effects of T on C2C12 cells transfected with an AR expression plasmid. In normal C2C12 cells, treatment with 10nM testosterone had no effect on proliferation, determined via formazan production. However proliferation in AR-transfected cells was decreased ~50% by T in comparison to control cells when grown in low serum media (3% FBS). T did not affect proliferation of AR-transfected cells when grown in high serum media (15% FBS). Myogenin expression was also slightly increased in AR-transfected cells when treated with T, and morphological examination revealed increased fusion in T-treated cells grown in differentiation conditions. In contrast, Chen et al. (45) examined the effect of T on normal C2C12 cells and those transfected with an AR expression vector. They reported no change in proliferation or differentiation for either cell line when treated with 100nM DHT, but did report a ~20% increase in protein content of differentiated myotubes.

These conflicting results can be attributed to a number of factors. First, studies looking at the effects of androgens using the LA muscle must be interpreted carefully, as the LA muscle is abnormally androgen responsive and may not adequately model the response of other skeletal muscles to androgens. Secondly, in human studies one must be aware of the subject's history both in terms of training and potential androgen use, both of which may skew the muscular responses to androgen treatment. Third, cultured

myoblasts studied in vitro are not a true representation of satellite cells in vivo. Though they are the activated descendants of satellite cells, differences in the culture environment and in gene expression make inferring results from studies of myoblasts to satellite cells in vivo difficult at best. Inter-batch differences in the growth serum (which contains androgens) could significantly affect the results of such studies. Lastly, the use of different androgens and doses may contribute to the observed differences. Skeletal muscle does not express detectable levels of 5-alpha reductase (135), the enzyme that converts T into DHT. In vivo exposure of skeletal muscle to DHT is likely very low; therefore stimulation of muscle cells with DHT in culture likely does not represent a physiological situation.

There is also evidence suggesting that androgens may influence the fate of another type of myogenic precursor cell, the pluripotent mesenchymal stem cell. C3H 10T1/2 cells are mouse pluripotent fibroblasts that have the capacity to differentiate into muscle, fat, bone, and cartilage tissue when exposed to the proper stimuli (136;137). Singh et al. (138) treated C3H10T1/2 cells with increasing doses of T and DHT (3-300nM and 1-30nM, respectively) in order to assess the ability of androgens to drive the fibroblasts into the myogenic lineage and inhibit adipogenic differentiation. Under basal conditions 10T1/2 fibroblasts expressed undetectable levels of AR, but expression was increased dose dependently in response to androgen treatment. Both T and DHT increased the expression of myosin heavy chain slow and MyoD in a dose dependent manner (measured via Western blot and RT-PCR), and the number of MHC+ cells after 12 days of incubation (immunohistochemistry). Both compounds also reduced the number of adipocytes formed (determined via oil-red-O) and the expression of

transcription factors required for adipogenic differentiation, PPARγ2 and C/EBPα (via Western Blot and RT-PCR). All of the effects of T and DHT were blocked by the AR antagonist bicalutamide, suggesting the pro-myogenic and anti-adipogenic effects are mediated via the AR. Though T did appear to induce a greater proportion of 10T1/2 cells into the myogenic lineage, differentiation of CH310T1/2 fibroblasts into the myogenic lineage has so far only been achieved in vitro when the cells are pre-treated with the demethylating agent 5-azacytidine or when transfected with MyoD. It is undetermined if T is capable of inducing pluripontent fibroblasts to enter the myogenic lineage in vivo, though the above study does provide an intriguing potential mechanism for the effects of T on body composition.

Structure and Function of the Androgen Receptor

T and its reduced metabolite DHT are the main endogenous ligands for the androgen receptor (AR). The AR is a nuclear hormone receptor that functions as a transcription factor to initiate transcription of certain target genes. The majority of the effects of T are thought to be mediated via its interaction with the AR, though there is evidence that non-AR mediated pathways may contribute to the physiological actions of T. The AR-mediated effects of T are initiated upon binding to cytoplasmic AR, which in turn initiates a cascade of events resulting in transcription of AR responsive gene targets. Unbound AR resides primarily within the cytoplasm where it is associated with several molecular chaperones including heat shock proteins 40, 70 and 90 (139;140), that help keep the AR in an inactive state. Upon binding of androgen, the chaperone proteins disassociate and the receptor changes conformation to allow contact between 2 receptor domains and to expose surfaces containing signals for nuclear localization (141). The

AR receives further post-translational modifications (142) and co-factor recruitment including the p160 family, p300-CBP family, AR associated co-activator 70 (ARA70) , steroid receptor co-activator 1 (SRC), and transcription initiation factor 2 (TIF2) (27;143-145) to name a few. The AR is then recruited to specific DNA sequences known as androgen response elements (AREs) where, when complexed with cellular transcriptional machinery, it initiates transcription of androgen responsive gene targets. The AR is a protein of 920 residues with 3 main functional domains; an amino-terminal domain (NTD, residues 1-555) that contains sequences vital for transcriptional activation and cofactor binding, a DNA-binding domain (DBD, residues 556-624) that regulates the binding of the AR to specific sequences in the promoters of target genes, a small hinge region (residues 625-670), and a ligand-binding domain (LBD, residues 671-919) that is responsible for interaction with free androgens (140).

AR Amino-Terminal Domain and Transcriptional Activation

Though nuclear hormone receptors display a high level of conservation in their DBDs, this is certainly not the case for the NTDs, which displays only ~15% sequence homology (146) between the AR, glucocorticoid receptor (GR), mineralcorticoid (MR) and progesterone (PR) receptors. The NTD is also referred to as the transactivation domain due to the presence of 2 regions (the first being highly conserved) required for full receptor activation. These sites serve as binding sites for additional cofactors that facilitate interaction of sequences in the LBD with the NTD in an androgen dependant fashion.

The NTD activation functions were identified when Jenster et al. (147) created a series of deletion mutants from wild-type human AR and a constitutively active human AR. The researchers showed that a large portion of the NTD is required (residues 1-485) for transcriptional activity in wild-type AR, though the constitutively active form only required a 168-bp segment designated transcription activation unit 1 (TAU1). Chamberlain et al. (28) inserted a series of point mutations/deletions into the rat AR and determined that deletion of a 14-bp segment (part of the larger TAU1 segment) designated AF1a, resulted in loss of ~60% of AR transcriptional activity. Simultaneous deletion of a 65-bp segment, designated AF1b, resulted in loss of >90% of AR transcriptional activity. Callewaert et al. (148) later identified several overlapping functional motifs (residues 177-203) within TAU1 that serve as recognition sequences of a number of co-activators. A highly conserved 13-bp sequence (residues 234-247) downstream of the TAU1 core region was shown by He et al. (149) to form the binding site for the E3 ligase CHIP, which has the effect of increasing AR protein degradation. Alen et al. (27) determined that the AF1a region interacts with the co-activator p160, which in turn interacts with sequences in AF2 within the LBD. Bevan et al. (144) confirmed these data by demonstrating that the co-activator SRC1 interacts with AF1a motifs and recruits the NTD to AF2 in the LBD in an androgen dependant manner. Meanwhile deletion of a glutamine rich region of SRC1 that interacts with AF1a abolished AR transcriptional activity, while deletion of the *LXXLL* motif within SRC1 had no effect on AR activity. He et al (150) later demonstrated that AF2 forms overlapping binding sites for the *FXXLF* and *LXXLL* motifs common in p160 co-activators. Though interaction of AF1a and AF2 induced at least in part via p160 co-

activator binding appears to be required for AR transcriptional activity, additional factors appear to be important, as deletion of the *FXXLF* motifs within the p160 co-activators ARA70 and ARA55 prevents AF2 binding but does not significantly reduce AR transcriptional activity (151). This may be due to effects of beta-catenin, which has multiple binding sites for the p160 co-activators TIF2 and GRIP1 as well as binding sites for AF2. Data from Song and Gelmann (152) indicates that beta-catenin forms a 3-way complex with AR AF2 and TIF2 in CV-1 cells, such that over-expression of beta-catenin was capable of partially rescuing transcriptional activity of a mutant AR lacking almost the entire NTD. Incidentally, beta-catenin expression is increased in cases of prostate cancer (153).

Because the *FXXLF* and *LXXLL* binding motifs in AF2 are so weak, yet p160-coactivator binding is essential for full AR activity, another transcription activation unit, TAU5 (residues 361-490) residing within the NTD is thought to be the main p160 interacting domain (144). TAU5 also harbors a *WXXLF* motif that itself was demonstrated by Dehm et al. (154) to alter AR transcriptional activity. In LNCaP cells grown in the presence of androgens the motif has the function of repressing AR activity, though the motif enhances ligand-independent AR activity in ADI cells, suggesting a possible tissue-specific regulatory role of this motif. As with TAU1, deletional mutants show that TAU5 is required for full AR transcriptional activity in COS-7 monkey kidney cells (155). Finally, a recently discovered endogenous variant lacking the entire NTD was demonstrated to have greatly reduced transcriptional activity, and when expressed simultaneously with wildtype AR was demonstrated to reduce AR transcriptional activity by forming a heterodimer with the WT receptor (156). This indicates that while the

various motifs and sub-domains of the NTD are important, full AR activity is generally only possible in vivo when the entire NTD is present.

Given the poor level of sequence conservation, the presence of numerous cofactor binding sites, and core motifs demonstrated to be required for NTD-LBD interaction, the AR NTD is considered to be the primary regulatory site of AR function. Mutations or alterations within this region, such as the NTD polyglutamine repeat which we will discuss shortly, are likely to have a considerable impact on AR function.

AR DNA Binding Domain and Androgen Response Elements

The AR DBD in approximately 80 residues in length and comprises all of exons 2 and 3. There is a high degree of evolutionary conservation in the DBD of AR, GR, PR, and MR, such that each consists of 2 zinc-finger domains where each zinc atom stabilizes 4 cysteine residues (157). In dimerized receptors these motifs recognize and bind to inverted repeats of the sequence *TGTTCT*, known as a steroid hormone response element (HRE), with each repeat separated by 3 nucleotides (49). The exception to the *TGTTCT* motif is the estrogen receptor, which instead recognizes a *TGACCT* motif, and has so far shown minimal interaction with the other HREs (158). These inverted repeats are very sensitive, such that a single point mutation within the sequence severely reduces binding affinity (159), and several natural mutations to the sequence result in complete clinical androgen insensitivity syndrome in humans (160). Though the *TGTTCT* sequence is recognized by AR, GR, PR, MR, and is found within the promoters of genes know to be responsive to each respective hormone, there are slight variations of the consensus sequence that are AR specific. Rennie et al. (50) identified 2 regions within the rat

probasin gene which were selective for AR and which induced reporter gene activation in PC3 cells to a far greater degree in the presence of AR rather than GR or PR. Cleutjens et al. (161) discovered an AR specific response element in an enhancer region upstream of the PSA promoter, and deletion of this element completely abolished PSA promoter activity in LNCaP cells. Zhou et al. (162) conducted random binding assays using an AR DNA-binding domain fusion protein and identified an overlapping direct repeat of the *TGTTCT* core motif with high AR specificity in CV-1 cells. Most selective AREs have been identified as imperfect direct repeats of the *TGTTCT* core motif, rather than the inverted repeat sequence common to non-selective HREs (163) (*figure 12*).

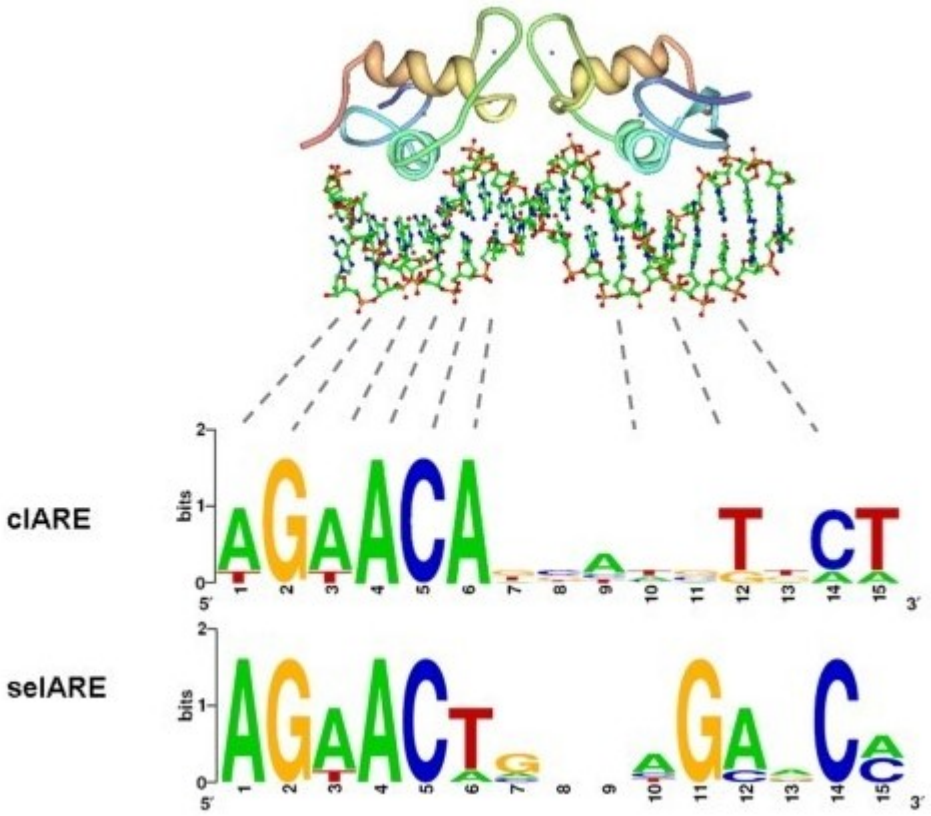


Figure 13: Image adapted from (163) demonstrating a HRE sequence recognized by all class I nuclear hormone receptors (clARE) and an androgen receptor-selective ARE (selARE).

The selective AREs are also very sensitive to sequence variation. Verrijdt et al (164) introduced point mutations within the core ARE of the mouse sex-limited protein enhancer, and demonstrated that an A to T switch completely abolished AR selectivity of the enhancer. Though the HREs and specific AREs are helpful tools to aid in the identification of potential androgen responsive genes, there are cases where genes can be responsive to androgen treatment despite a lack of general or specific AREs. Using a luciferase expression vector driven by the skeletal alpha-actin (ACTA1) gene promoter, Vlahopoulos et al. (165) demonstrated that androgen treatment increased gene expression ~90 fold in C2C12 cells transfected with human AR. They also demonstrated that AR was associating with the ACTA1 promoter via an interface with serum response factor (SRF), a ubiquitous transcription factor that recognizes DNA binding elements known as serum response elements and acts like a docking protein for a variety of proteins and transcription factors (166). This data precludes one from thinking only of AREs when considering the possibility of androgen regulation of target genes, and provides an additional layer of complexity in determining AR function.

AR Ligand Binding Domain

The main function of the AR LBD is, of course, the binding of ligand. Among nuclear hormone receptors the LBD is partially conserved, with some regions being nearly identical while others are highly variable (167). Though structures vary, the process of ligand activation is similar among receptors. In the case of AR, upon binding

to androgen the ligand binding pocket closes to expose the previously mentioned activation function 2 (AF2) (168). AF2 forms a docking interface for a number of co-activators including the p160 family and CBP/p300 family, and the ability of AF2 to interact with AF1 within the NTD, either directly or via interfacing with p160 co-activators, is essential for stabilizing the ligand binding pocket (169). Surprisingly given current crystallography techniques there appears to be little difference in the conformational shifts induced by ligands despite their variable structures (170). However, Askew et al. (171) demonstrated that DHT stabilizes the ligand binding pocket to a greater extent than T, which could account for the increased potency of DHT in prostate tissue. Despite the designation of AF2 and the LBD hydrophobic cleft as important domains for ligand interaction, there is evidence of other LBD surface motifs affecting AR activation. Estebanez-Perpin et al. (172) in an attempt to identify novel AR antagonists, identified a surface motif dubbed BF3 that when bound by the novel inhibitors abolished the recruitment of co-activators to AF2. Mutations to BF3 had the ability to block AF2 activity and AR activation. This is the first evidence suggesting a strong regulatory region within the LBD and independent of AF2, that can affect AR activity. Surprisingly, differences in the physiological effects of various AR agonists/antagonists have not been directly studied to any degree, but they likely result from differential pharmacokinetics, co-factor recruitment, metabolism, and potentially non-AR mediated effects.

The LBD may also harbor a novel nuclear exportation signal domain. Saporita et al. (173) identified a region (residues 742-817, approximately) of the LBD that is responsible for AR nuclear export in PC3 cells. The nuclear export signal (NES) is

repressed in the presence of ligand, but overwhelms the nuclear import signal located in the DBD in the absence of ligand. This is a significant finding as cellular localization plays an important role in the transcriptional activity of most nuclear hormone receptors. Kesler et al. (174) demonstrated that AR localization is highly transient in PC3 and COS-7 cells even in the presence of androgen, but that forced localization of AR to the cytoplasm or nucleus by the fusion of localization motifs was sufficient to initiate or repress AR transcriptional activity independently of the presence of androgens . The work of Farla et al. (175) also indicates that the NES identified by Saporita et al. may be involved in the "turning off" of the AR, as mutant AR lacking the entire LBD demonstrated increased nuclear localization and higher transcriptional activity when expressed in Hep3B hepatoma cells.

Another notable characteristic of the AR LBD is the high number of point mutations identified that severely disrupt or completely abolish AR transcriptional activity. The majority of clinically identified point mutations in the region result in complete or partially androgen insensitivity syndrome, a condition where despite normal levels of androgen receptor and elevated levels of serum androgens, subjects have severely reduced or abolished AR transcriptional activity. Another interesting and particularly well studied mutation is the T→A shift at residue 877. This site is interesting as it alters the normal pharmacodynamics of many AR agonists/antagonists. Ligand binding and disassociation, as well as transactivation studies, conducted by Ozers et al. (176) determined that the potency of most AR agonists was increased by the T→A shift, however the ability of several well-known antagonists such as hydroxyflutamide to block co-factor recruitment and AR transcriptional activity was disrupted. In fact the T877A

mutation resulted in hydroxyflutamide as well as cyproterone acetate *increasing* AR transcriptional activity. Southwell et al. (153) found that the T877A mutation induced greater sensitivity of AF2 for the *LXXLL* and *FXXLF* motifs found in the p160 co-activators. Consequently this mutation is very common in cases of androgen-independent prostate cancer. Many individuals with prostate cancer are placed on androgen-deprivation therapy, including treatment with AR antagonists such as flutamide. The T877A mutation not only removes the oppressive effect of flutamide on AR activity, it actually induces flutamide and other AR antagonists to stimulate AR activity, which can have disastrous consequences for prostate cancer.

AR Hinge Region

The final major domain of the AR is the so-called hinge region, as small flexible linker sequence that connects the DBD to the LBD. Its primary feature is the nuclear import signal (NIS) motif (residues 617-633) identified by Zhou et al. (57). Though the hinge region is poorly conserved among nuclear hormone receptors, it contains the NIS for GR, PR, MR, and AR. Deletion of this region abolishes nuclear import while deletion of either the NTD or LBD, respectively, induces the receptor to be preferentially located in the nucleus independent of the presence of androgens. Interestingly, though it would appear that elimination of the NIS would negatively affect AR transcriptional activity; this does not appear to be the case. Haelens et al. (177) tested the functional significance of 2 point mutations (R629Q, and K630T) located within the AR NIS and reported to be found in cases of human prostate cancer (178). The researchers deleted residues 628-646 of the hinge region and found that transcriptional activity was increased in the mutant AR in comparison to WT AR. Other than the presence of the NIS, comparatively little is

known of the function of the hinge region. Additionally, little is known of the transport proteins and/or co-activators that are involved in mediating the nuclear localization signaled by the NIS. More work is required to elucidate the mechanisms of AR nuclear import and export.

AR Signaling and the Anabolic Effects of Testosterone on Skeletal Muscle

It is widely accepted that T administration results in increases in skeletal muscle mass and strength, especially in conjunction with resistance training and/or mechanical stress. What is not known is how these effects are mediated.

AR Expression in Skeletal Muscle

Expression levels of the AR in select tissues could be perceived as an indicator of its relative importance to the normal growth and maintenance of those tissues. Surprisingly, available data on AR expression in skeletal muscle is somewhat equivocal. Several studies have examined AR expression in muscle cell culture. Doumit et al. (69) identified the presence of AR protein via Western Blot using anti-AR PG-21 in cultured porcine myoblasts. In proliferating C2C12 myoblasts, Wannenes et al. (60) were able to detect low levels of AR mRNA, with levels increasing significantly over 5 days of differentiation in low serum media. AR protein, measured by Western Blot using anti-AR N-20 was also detectable in both myoblasts and myotubes, increased dose-dependently by T. In contrast, Lee (62) was unable to detect any AR protein in C2C12 myoblasts, and contrary to data in vivo, treatment with T did not increase AR expression and no AR transcriptional activity was detected. Chen et al. (45) reported similar results from another study of C2C12 cells. RT-PCR detected low levels of AR mRNA in C2C12 myoblasts (<0.1% of that found in gastrocnemius lysate), with slightly increased AR mRNA in differentiated myotubes. AR protein expression, determined via Western Blot using anti-AR PG-21, was undetectable in C2C12 cells. Ruizveld de Winter et al. (179) conducted an immunohistochemical analysis of AR expression in a variety of human

tissues using a mouse monoclonal antibody targeted against residues 301-320 of the human AR. AR protein was not detected in the skeletal muscle tissue, though the authors do not specify which skeletal muscle was examined. In contrast, Ferrando et al. (180) were able to detect AR protein in quadriceps muscle lysate from healthy men using an unspecified anti-human AR antibody. Maclean et al. (181) found that AR mRNA expression was variable within the skeletal muscles of male mice, with gastrocnemius levels being significantly higher than TA or EDL, and much higher than the slow-twitch soleus. However, all hind limb muscles expressed far less AR mRNA than the LA muscle, further evidence that studies of AR function using the LA muscle must be interpreted with caution. Lee et al. (61) examined AR protein expression in rat plantaris muscle subjected to functional overload and/or the synthetic androgen nandrolone. Baseline AR protein, determined via Western Blot using the N20 anti-AR antibody, was low but detectable. However, expression increased significantly in response to both overload and androgen treatment. This increased mRNA expression in response to overload was also demonstrated by Willoughby et al. (182), where AR mRNA and protein increased in the quadriceps after 3 bouts of resistance training. Finally, Sinha-Hikim et al. (183) et al examined AR expression in the quadriceps muscle of healthy young men at baseline and after treatment with 600mg of T for 20 weeks. Muscle sections were stained with anti-CD34 antibody, anti-syndecan-4 (stains myogenic cells) and anti-AR N-20. The authors claim that AR staining was localized to the satellite cells and largely to the nuclei of mature muscle fibers, but that T increased nuclear AR staining ~25%. "Enriched" cultures of human satellite cells (obtained commercially) were also examined for AR protein expression, and all cells that stained positive for C-

met (satellite cell marker) also stained strongly for AR. This work by the laboratory of Shalender Bhasin is the first to show apparently strong AR protein expression in satellite cells. It is somewhat curious that quiescent cells would demonstrate such high levels of AR expression, and certainly warrants further study into AR expression in skeletal muscle.

Again data supporting AR expression in skeletal muscle is not completely in agreement. This may be due to differences in study design. First, the use of satellite cells is problematic due to their highly transient nature (i.e. transition into myoblasts) in vitro. The use of myoblasts in culture, while considered an appropriate method of studying skeletal muscle in vitro, does not approximate the conditions of mature skeletal muscle in vivo, and may not accurately reflect conditions of the androgen system in vivo. Second, the presence of AR mRNA does not necessarily mean AR protein is being expressed. In fact many studies do demonstrate the presence of AR mRNA in skeletal muscle under basal conditions, but AR protein appears to be expressed in comparatively smaller amounts. This could be partially due to available AR antibodies, as immunohistochemical and immunoblotting techniques are limited by the quality of the antibodies in use.

Another point to consider regarding AR expression in muscle is that fact that androgen treatment results in significantly increased AR protein content. This effect is almost universally demonstrated in studies of androgen action where AR protein is in fact detectable (20;60;61;69;120;133;183;184), and is likely due to the strong AREs within the *AR* gene promoter (159). It also provides a mechanism for the effects of supraphysiological effects of T. Work by the Bhasin group, as well as anecdotal

evidence from athletes, has demonstrated that exogenous doses of T far exceeding saturation of the AR continue to increase gains in muscle mass and strength. This suggests that T can potentiate its own effects on muscle by positively regulating AR protein expression in a feed-forward mechanism.

AR Knockout, Genetic Mutation, and Pharmacological Repression

Another factor to consider is the data available from cases of pharmacological AR repression or genetic defects in AR expression. Maclean et al. (181) studied the effects of AR deficiency on a line of transgenic AR-knockout (ARKO) mice. Male ARKO mice had ~12% reduced bodyweight in comparison to WT males. The mass of all hind limb muscles was significantly reduced in ARKO males, ~20% in all muscles. ARKO males also had significantly lower tetanic force in the fast-twitch EDL muscle, though no change in specific force, indicating loss of force was due to a loss in mass. No differences in tetanic force were observed in the soleus muscle. Hormone analysis revealed ARKO males had lowered T levels, but no changes in IGF-1. Incidentally, similar effects to those seen in the ARKO males were observed in orchidectomized male mice in a previous study by the same group (185), and in that study T replacement abolished the decreases in muscle size and force. Additionally, ARKO females displayed no apparent physiological differences from WT females, indicating that while androgen signaling is important in males, it is either redundant or not required for normal muscle development in females.

There are a rather large number of known mutations within the *AR* gene that severely affect the normal function of the AR and result a number of symptoms. Most of these mutations result in an inhibited response to androgens and are broadly defined as androgen insensitivity syndrome (AIS). Most men with AIS (which is largely limited to males due the fact that the *AR* gene is located on the X-chromosome) have non-functional AR protein or lack it altogether, though generally have greater than normal serum T levels (186). The most obviously and common effects of AIS are hypovirilization, or underdeveloped urogenital organs and secondary sexual characteristics, infertility, and decreased muscle and bone mass.

Lastly but importantly, many studies examining T and skeletal muscle report that co-treatment with an AR antagonist such as flutamide or bicalutamide largely repress any effects induced by T or other synthetic androgens (20;32;138), as well as muscle mass and strength gains induced by resistance training (187). Such compounds are AR-specific and block AR transcriptional activity. The ability of selective AR antagonists to suppress the physiological effects of T indicates that the AR plays an important role in mediating the function/s of T.

Androgen Interaction with other Nuclear Hormone Systems

While evidence suggests that many of the effects androgens have on skeletal muscle is mediated via the AR, there are distinct mechanisms by which androgens can affect skeletal muscle that do not involve traditional AR signaling.

As discussed previously, nuclear hormone receptors share a certain degree of homology in their recognition of hormone response elements. Early studies of AR

binding before the discovery of specific AREs often focused on GREs due to the sequence similarity. This sequence similarity between AR and GR response elements suggests a degree of interaction between AR and GR signaling. Though other nuclear hormone receptors have not been shown to bind to specific AREs, a mechanism hypothesized to be due to the imperfect direct repeat nature of the core TCTTGT sequence present in selective AREs (188), AR can interact with other HREs. Takai et al. (189) demonstrated this interaction in ROS17/2.8 osteosarcoma cells. AR overexpression increased transcription of bone sialoprotein, a marker of osteoblast differentiation, via binding to cAMP and glucocorticoid response elements in the BSP promoter. Holterhus et al. (190) demonstrated that a variety of endogenous and synthetic androgens induced strong activation of a GRE-driven reporter gene hamster ovary cells when co-transfected with AR. Conversely, Zhao et al. (191), co-transfecting AR and GR into CV-1 cells, demonstrated that AR activated by the synthetic androgen oxandrolone strongly suppressed GR-mediated activation of a GRE-driven reporter construct. Repression was not apparent where AR was lacking, indicating the effects were not due to competitive inhibition of the GR by oxandrolone but due to another AR-mediated mechanism. These data indicate that an interaction exists between the androgen and glucocorticoid signaling systems.

Although sequence similarity between androgen and estrogen response elements is relatively small, evidence suggests that some of the effects of testosterone in males are potentially due to a direct effect of testosterone on the estrogen receptor and/or conversion of testosterone to estradiol and signaling through the ER.

Androgens have a reputation for increasing aggressiveness in males (192). However, evidence suggests that while AR is highly expressed in most brain tissue (193), signaling through the ER is responsible for this so-called aggressive behavior. Schlinger and Callard (194) demonstrated that T-induced aggressive behavior in male quails is suppressed by an aromatase inhibitor, while an AR antagonist had no effect on aggression. In contrast, aggressiveness was reduced by an ER antagonist, and DHT, which is not a substrate for aromatase, did not induce aggressive behavior. Additionally, Wersinger et al. (195) observed that T-induced aggressive behavior in WT mice was abolished in mice lacking functional ER. Finally, the data of Vagell et al. (196) indicate that both testosterone stimulated AR and functional aromatase are required for normal sexual drive in male mice. These data highlight the functional interplay of androgen and estrogen signaling in the male brain. At a molecular level, Crostan et al. (197) co-transfected AR and ER into HepG2 hepatocytes and assessed activation of the LDL receptor promoter. While ER overexpression stimulated transcription from an LDL receptor promoter reporter, AR overexpression alone had no effect on reporter activity, but suppressed ER-stimulated reporter gene activity when co-transfected. This apparent AR-mediated repression of reporter activity was confirmed by the fact that repression was independent of ligand concentration, and that the addition of the AR-antagonist casodex removed repression of reporter gene activity. This indicates that androgen and estrogen signaling may act in an antagonistic manner on certain gene targets. In an in vivo model using ERβ-null mice, Glenmark et al. (198) demonstrated that male mice lacking ERβ had lower levels of fatigue and a faster return of force production after exercise than did WT males, indicating that estrogen receptor signaling does play an

important role in the skeletal muscle physiology of male animals. On the other hand, and unlike testosterone suppression, direct suppression of estrogen levels in men seems to have little effect on body composition or muscle strength. Mauras et al. (199) administered the powerful aromatase inhibitor anastrozole to men for 10 weeks and measured lean body mass, hormone profiles, bone density, muscle strength, and protein synthesis. Aromatase converts testosterone to estradiol and accounts for a large proportion of total estrogen production in men, and both aromatase mRNA and enzymatic activity are present in skeletal muscle (200;201). There was no significant change in lean body mass, body fat, bone calcium levels, or muscle strength in any of the subjects. Though estrogen levels dropped by ~50%, T increased by a mean of 58% while SHBG levels did not change. LH and FSH levels both significantly increased, likely due to the decline in estrogen and the fact that serum estrogen in a negative regulator of LH release from the pituitary. Together the above data indicate that estrogen itself is either not critical for skeletal muscle function in males, or that there is sufficient excess that a 50% decrease in serum levels does not adversely affect normal function in males. However, signaling through the ER does appear to play some part in male skeletal muscle function, and the ability of AR to affect ER function further confirms a significant interaction between nuclear hormone systems.

Genetic Variation within the Androgen Receptor

As stated previously, there are several known mutations that have been demonstrated to alter AR functionality. The majority of these are point mutations that result in a dysfunctional receptor or a completely non-functional, truncated receptor. In most cases there is no treatment, and subjects are afflicted with androgen insensitivity syndrome. The most widely studied AR polymorphism that does not entirely ablate AR function is a polyglutamine repeat sequence that resides within the NTD. The repeat begins around residue 58 in humans with a mean repeat number of 22. The repeat is also present in rodents, though it begins around residue 174 with a mean repeat number of 17. The repeat in rodents is also not perfect, containing 2-3 histidine residues, and its impact on receptor function has not been extensively studied. The repeat is of significant interest in humans however, as it has been associated with certain forms of prostate cancer (29;202;203), and it appears to be the primary determinant of Spinal Bulbar Muscular Atrophy (SBMA), a neuromuscular degenerative disorder that typically manifests with repeat lengths in excess of 40 (204;205).

The development and progression of prostate cancer is highly dependent on the action of androgens, such that the primary pharmacological treatment involves AR blockade or suppression of endogenous testosterone production. Consequently, a mutation common to prostate cancer is the AR T877A point mutation, which abrogates AR-antagonist suppression, and enables AR transcriptional activity even in the presence of antagonists (176). Studies associating polyglutamine repeat length with prostate cancer have found an inverse relationship, with shorter repeat lengths being more common in those displaying early onset and more aggressive forms. Beilin et al. (32) examined the

impact of repeat length on AR transcriptional activity in several transiently transfected cell types using a luciferase reporter assay. Both LNCaP prostate carcinoma cells and COS-7 kidney cells demonstrated higher transcriptional activity with decreasing repeat length, while PC3 prostate cells and MCF7 breast cancer cells did not display any differences in AR transcriptional activity with variable repeat length. This is the first study to demonstrate tissue-specific differences in the effect of repeat length on AR activity. Tut et al. (40) and Chamberlain et al. (33) reported similar findings, with AR transcriptional activity being inversely related to glutamine repeat length in COS and CV-1 cells, respectively. Interestingly, another AR mutation found in human prostate tumors included 2-leucine residues within the polyglutamine tract (206). This mutation disrupts NTD-LBD interaction, but significantly increases transcriptional activity. In contrast, Neuschmidt-Kaspar et al. (46) reported no difference in transcriptional activity between WT AR and AR harboring 45 glutamine residues when expressed in CV-1 cells. Another study by Choong et al. (41) did not find any differences in transcriptional activity with variable repeat length, but reported that AR mRNA and protein expression is inversely related to repeat length. Lieberman et al. (68) also reported decreased AR protein expression with highly expanded repeat length (65gln) in MN-1 neuroblastoma cells. In addition, aberrant gene expression profiles were observed. AR65 was hyper phosphorylated and acetylated on several sites, and Affymetrix analysis revealed different expression profiles of 11 genes in comparison to AR24. These data indicate that AR polyglutamine repeat length can have a significant effect on receptor activity in certain tissues.

SBMA results from a toxic gain-of-function effect via misfolded, expanded polyglutamine repeat AR. The condition is largely limited to males as females are typically asymptomatic due to low endogenous androgen levels and the low likelihood of possessing two high repeat number copies of the *AR* gene. Stenoien et al. (207) reported that electron microscopy revealed AR aggregates in HELA cells transfected with a 48 repeat-containing AR. The aggregates were dependent on the presence of androgen and included a number of heat shock proteins and demonstrated defects in proteolytic processing. This defect in proteolysis could contribute to increased apoptotic signaling as demonstrated by Ellerby et al. (208). Here it was demonstrated that increased repeat length enhanced AR cleavage by caspase 3, a potent protease involved in apoptosis, and enhanced apoptosis. SBMA pathology does not appear due solely to toxic gain of function however, but also due to loss of normal AR function. Thomas et al. (209) generated transgenic mice expressing 100 and 20 repeats respectively, in AR-null and WT strains. Neurodegeneration and impaired muscle function was greater in AR-null mice transfected with AR100 in comparison to WT mice transfected with AR100, indicating that a lack of normal AR signaling in addition to expanded repeat toxicity contributes to the SMBA progression and pathology. Finally, though loss of endogenous AR function with highly expanded repeat length may to contribute to SBMA, neurotoxity appears to be the prime mechanism. Overexpression of the ubiquitin-ligase CHIP selectively degraded expanded repeat AR and greatly decreased aggregate formation and toxicity in neural cells (210). Similar methods involving up regulation of AR-selective ubiquitin ligases are currently being developed as promising treatments for SBMA.

Though well studied in respect to prostate cancer and SBMA, the impact of AR polyglutamine repeat length on AR function in skeletal muscle has not been clearly addressed. Walsh et al. (34) found an association between lean body mass and increased repeat length in men from two independent cohorts. Men aged 19-93 from two independent cohorts cohorts carrying >22 polyglutamine repeats demonstrated 1.2kg and 3.4kg greater lean mass measured by DEXA, respectively, than men carrying <22 repeats. No differences were observed in women. Men with >22 repeats also has significantly higher serum T levels than men with <22 repeats. These results appear to be counter to the results of previous studies demonstrating greater transcriptional activity with shorter repeat length, if we assume that a transcriptionally active AR contributes an anabolic effect in muscle tissue.

The mechanism by which AR repeat length affects transcriptional activity has not been thoroughly addressed, particularly in skeletal muscle. As discussed previously, AR activation requires a series of events involving ligand binding, NTD-LBD interaction, exposing transcription factor docking sites, etc. Davies et al. (211) performed an extensive series of experiments to determine the effect of repeat length on AR protein structure. They determined that increased repeat length (AR45) enhanced NTD alpha-helix formation, while complete removal of the repeat reduced it. In addition the ligand binding pocket of AR45 demonstrated a more hydrophobic nature, and the protein in general was more susceptible to unfolding. Because the primary purpose of ligand binding is believed to be the stabilization of NTD-LBD interaction and to provide a docking site for cofactors required for nuclear import, any alteration to the ligand binding pocket or changes to NTD structure could seriously alter AR function. The alpha-helix

structures demonstrated to be affected by repeat length also harbor AF1, a primary site of protein-protein interaction and cofactor recruitment. The previously discussed data of Lieberman et al. (68) suggests the possibility that these folding changes may in fact alter ligand-induced gene expression. Here, ligand-activated AR65 induced changes in 11 genes, where ligand-activated AR24 induced changes in 54 genes. This could indicate that increased repeat length eliminated ARs ability to induce transcription of 43 genes, or that the expanded repeat length resulted in partial constitutive activity, such that some transcriptional activity remained even in the absence of ligand. The data of Davies et al. would suggest that this is a possibility. Finally, Becker et al. (48) demonstrated that AR with an expanded repeat length formed cytoplasmic aggregates to a far greater extent that WT AR when exposed to androgens. Since nuclear translocation is required for AR transcriptional activity, the inability of AR with extended repeat length to penetrate the nucleus, due to aggregate formation, could largely explain the decreased transcriptional activity of AR with extended repeat length (32;40).

Summary

There is overwhelming evidence the androgens induce an increase in skeletal muscle mass and strength in humans, especially when combined with resistance training. This effect appears to have a genetic component, as the magnitude of each individual response to exogenous androgens can vary considerably. Androgens have a positive effect on protein accretion in skeletal muscle, and have been demonstrated to alter the proliferation and activity of satellite cells in vivo, though the mechanisms are not completely understood. It is believed that most of the clinical effects of androgens are mediated via the AR, as AR blockade or knockout ameliorate many, if not all, of the

physiological effects of androgens. Data regarding AR expression in skeletal muscle is not entirely in agreement, though significant data exist demonstrating an increase in AR expression in skeletal muscle in response to training and/or exogenous androgen administration. The AR protein is relatively complex, with 4 primary domains, each known to have a unique and important function. Variation within these domains can have a significant impact on AR function. The polyglutamine repeat polymorphism within the NTD is of interest as it has been demonstrated to alter AR transcriptional activity in certain cell types. The relevance of this polymorphism to skeletal muscle function is unknown, but presents a potential mechanism to explain differences in individual response to androgens.

Experimental Procedures

Cell Culture

The murine myoblast line C2C12, first subcloned by Blau et al. (212) from the C2 line developed by Yaffe (213) was selected as the experimental cell line. These cells are very proliferative, with a doubling time of roughly 16 hours, and differentiate rapidly when exposed to low serum culture conditions. Cultured myoblasts are the activated decendants of satellite cells, and though they certainly do not mirror the physiology and gene expression of whole muscle in vivo, when differentiated they generally reestablish the phenotype of the muscle from which they were derived. C2C12 myoblasts have an advantage over the MM14 cell line developed by Linkhart et al. (214) in that they do not require fibroblast growth factor (FBF) for propagation. They were also chosen over the rat L6 line derived by Yaffe (213) as the L6 line proliferates and differentiates more slowly and does not express local IGFs, making them dependent on the presence of IGF in serum (215). The L6 line was also avoided due to it being demonstrated to have 5-alpha reductase activity (216), which has not been shown in any other muscle cell type or even in skeletal muscle in vivo. The disadvantage of the C2C12 line is a decline in the rate of differentiation after multiple passages. C2C12 cells experience inhibition of growth upon contact with nearby cells and must be passaged at 50-70% confluency to ensure a lack of contact inhibition and early differentiation. In addition, a subpopulation of rapidly differentiating cells is gradually lost with repetitive passaging, and for this reason only populations with passage number of 5-20 were used for all experiments.

Cells were kept in exponential growth by maintaining them in growth medium (GM) consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. Cells were seeded at ~10^5 cells per 100mm dish and kept undisturbed at 37^{o}C and 5% CO_2 for ~48 hours before subsequent passaging. Differentiation was initiated once the cells had reached ~90-100% confluency by switching to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (HS) and antibiotics as above. Where applicable, cells were treated with 100nM testosterone in ethanol vehicle or an equal volume of ethanol control. Ethanol concentration did not exceed 0.1% of total volume. Stably transfected cell lines were maintained as above with the addition of 500μg/ml of G418 sulfate (GIBCO). Excess cells were frozen down in 95% growth medium supplemented with 5% DMSO at -80^{o}C overnight before transfer to liquid N_2 for long term storage.

AR Vector Creation

The human AR expression vector pCMV5-hAR was a generous gift from Dr. Elizabeth Wilson (University of North Carolina). This vector was sequenced (Applied Biosystems 3730 DNA Sequencer) upon receipt and found to harbor 24 CAG repeats. The human AR gene is under control of the constitutively active cytomegalovirus promoter and supports robust expression in eukaryotic cells even in minimal growth factor environments (217). DNA previously isolated from human subjects involved in the Baltimore Longitudinal Study of Aging was isolated and sequenced for AR CAG repeat

length, and DNA harboring 14 and 33 CAG repeats, respectively, was selected for amplification via PCR (conditions described below). The genomic DNA was amplified using primer set (F primer 5'-tgcacctacttcagtggacac-3'; R primer 5'-gtatcttcagtgctcttgcctgcg-3') and reaction conditions to include 95^{o}C for 2 minutes, followed by 40 cycles of 95^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 75 seconds, and a final 5 minute incubation at 72^{o}C. The ~1220-1280bp fragment was resolved and purified from 1% agarose gels using the PureLink quick gel extraction kit (Invitrogen) according to manufacturer instructions, and then subjected to phenol-chloroform extraction. This step was necessary to remove residually active polymerase from the reaction mixture prior to restriction enzyme digestion. Purified PCR products and pCMV5hAR backbone were subsequently serially restriction enzyme digested with BglII and BsmI (New England Biolabs) at 37^{o}C overnight and at 65^{o}C for 2 hours, respectively, using NEB buffer 2. The digested genomic inserts and vector backbone were again resolved and purified from agarose gels. The purified products were treated with shrimp alkaline phosphatase (SAP, Fermentas) at 37^{o}C for 1 hour to remove the 5' phosphate groups from DNA ends and prevent undesired re-ligation. SAP was inactivated by incubation at 65^{o}C for 15 minutes. Insert and vector fragments were combined in a 10:1 ratio (calculated via moles of DNA ends) and ligated together using T4 DNA ligase (Invitrogen) and incubated overnight at room temperature. Resulting products were transformed into DH5α competent E.coli bacteria (Invitrogen) according to manufacturer instructions. Bacterial suspensions were spread onto 100mm agar plates supplemented with 100μg/mL ampicillin and grown overnight at 37^{o}C. The pCMV5 vector contains the ampicillin-resistance gene; cells successfully incorporating a

functional vector will hydrolyze ampicillin on the agar plates and form viable colonies, while cells lacking an intact vector will not grow. After overnight incubation all resulting colonies were removed by picking with a pipet tip and transferring to 15mL tubes filled with 5mL LB broth, and expanded by overnight incubation at 37^{o}C with vigorous shaking. Resulting bacterial suspensions were collected, and plasmid DNA was isolated using Qiagen Plasmid Mini Kit (Qiagen) according to manufacturer instructions. The isolated plasmid DNA was amplified via PCR and a primer set designed around the AR CAG repeat (see *table* 2) and sequenced to verify the presence of the appropriate number of repeats. The successfully cloned vectors as well as the original are designated pCMV5-hAR14, pCMV5-hAR33, and pCMV5-hAR24, respectively.

Stable Line Creation

The above cloned AR expression vectors were used to create stably transfected C2C12 lines expressing the human AR. The benefit of using stably transfected lines is a much higher expression level of the transfected gene in comparison to transiently transfected lines; where only a small, variable percentage of cells in the culture vessel can be expected to take up the vector of interest in a transient transfection, stably transfected cell lines should be close to 100% gene expression of the target gene. A stable line is created by co-transfecting the vector of interest along with an antibiotic resistance gene and culturing the line in the presence of said antibiotic. The assumption is that all transfected vectors are taken up equally, so any cell in the culture vessel that has survived

antibiotic selection is assumed to have taken up the vector of interest as well as the vector carrying the antibiotic resistance gene.

C2C12 cells were seeded into 6-well tissue culture plates at ~10^4 cells per well and incubated in GM overnight. Cell confluency was kept low to avoid cell-cell contact over the several days following transfection. The three AR expression vectors were transfected into low passage number cells using Lipofectamine and Plus Reagent (Invitrogen) according to manufactures instructions. Cells were co-transfected with a vector carrying the neomycin resistance gene, pCI-neo (Promega), which also conveys resistance to the aminoglycoside antibiotic G418 sulfate. G418 blocks protein synthesis in both prokaryotic and eukaryotic cells by interfering with peptide elongation (218). Each well received 1.5μg of each vector suspended in 1mL of Lipofectamine/PLUS reagent mixture in serum/antibiotic-free DMEM. Following a three-hour incubation with the transfection mixture, each well received 1ml of GM for an overnight incubation. The transfection medium was removed the following morning and was replaced with fresh GM supplemented with 1000μg/mL of G418. This concentration was previously determined to result in the death of >95% of non-resistant C2C12 cells within 7 days (data not shown). The cells were observed closely for several days for the appearance of small, proliferating clusters of cells. C2C12 cells do not form the tight colonies common to other cell types, but instead form a monolayer across the culture vessel surface. This characteristic makes it problematic to select a clonal colony derived from a single cell, as the cells do not grow well if spaced too far apart. Accordingly, small clusters of healthy cells were selected at first appearance as candidates for subcloning. Once these clusters reached a number of cells deemed sufficient for removal, sterile cloning rings were used

to remove the clusters for subculturing. A thin layer of sterile silicone grease was applied to each cloning ring, and the rings were carefully placed around each cluster until a seal was formed. Media within the ring was aspirated, the cluster rinsed with PBS, and covered with 100μL of 0.05% trypsin solution. The cells were detached and mixed with gentle pipetting and transferred to fresh 24 well plates. The subcultured cells were incubated in GM with G418 until the cells approached 70% confluency or until cell-cell contact was imminent, at which point they were passaged to ~15-20% confluency and the incubation was continued. This process was repeated for approximately 14 days, or until the no additional dead/detached cells were observed in the medium, an indication that all non-stably transfected cells had been selected out by G418. At this point aliquots of each line were removed for RNA extraction and cDNA generation (described below). The presence of the appropriate cloned AR transcript was verified via RT-PCR (primers and conditions listed in *table* 2) and sequencing (see appendix, *figure 14*). Once verified each line was expanded until a sufficient number of cells were obtained for freezing and long term storage in liquid N_2.

Nucleic Acid Isolation, Quantification, and RT-PCR

RNA was extracted from cells using the guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (219). Myoblasts and or myotubes were aspirated and rinsed with PBS to remove traces of medium. TRIzol (Invitrogen) reagent was added to the culture plates (2ml for 100mm dishes and 1ml for 35mm dishes) for 3 minutes before homogenizing the cell mixture with vigorous pipetting for several minutes. The mixture was transferred to centrifuge tubes and set to gentle rotation for 15 minutes at room temperature. 0.2ml of chloroform was added per

1ml of TRIzol, and the tubes were again set to gentle rotation for 10 minutes at room temperature. The mixture was then centrifuged at 13,000g for 12 minutes at 4^{o}C. The top 80% of the aqueous layer (containing the RNA) was carefully transferred to a fresh centrifuge tube along with 5ul of glycogen solution to assist in subsequent precipitation. 2x volume of isopropanol was added and the tubes were set to gentle rotation for 10 minutes at room temperature. Tubes were centrifuged at 13,000g for 10 minutes at 4^{o}C, and the supernatant was removed. 0.5ml of 70% ethanol was added for 5 minutes, and the tubes centrifuged at 7000g for 5 minutes. The supernatant was carefully removed and the remaining RNA pellet was air-dried until slightly transparent. The pellet was resuspended in 50µl of TE buffer and stored at -80^{o}C until further use. Aliquots from each sample were removed and diluted 1:25 in TE buffer and quantified via spectrophotometry by measuring absorbance at 260nm. To isolate DNA, cells were rinsed with PBS and treated with 0.05% trypsin for 3-5 minutes. The cells were mixed by repetitive pipetting, the trypsin neutralized by an 2x volume of GM, and transferred to a 15ml tube before spinning a 150g for 5 minutes. The supernatant was poured off, and the cell pellet resuspended in 0.5ml cell lysis solution. The cells were set to gentle rotation for 20 minutes at room temperature. 0.25ml of protein precipitation solution was added and the samples were vortexed vigorously for 30 seconds before centrifuging at 13000g for 10 minutes at 4^{o}C. The supernatant was poured into 2x volumes of 100% isopropanol and set to rotation for 10 minutes at room temperature. The remainder of the procedure was the same as that for RNA isolation described above.

The isolated RNA was used to generate cDNA for gene expression analysis. Before reverse transcription each sample was treated with DNAase1 (Fermentas) for 30

minutes at 37^{o}C to degrade any trace remaining DNA. cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to instructions and 2μg of RNA per 40μl reaction. Cycling consisted of 10 minutes at 25^{o}C, 120 minutes at 37^{o}C, and a final 85^{o}C for 5 seconds. The cDNA was collected and stored at -20^{o}C for future analysis.

AR Transcriptional Activity

A common method to assess that activity of a transcription factor, such as nuclear hormone receptors, is to measure the appearance of an easily quantifiable reporter gene whose expression is known to be driven by a promoter that is sensitive to the transcription factor of interest. Reporter genes such as luciferase, chloramphenicol acetyltransferase, GFP, and β-galactosidase are introduced into the cell via transfection, and when stimulated via the addition of an enzymatic substrate produce a signal that can be quantified. Luciferase, an enzyme that catalyzes the conversion of the pigment luciferin into oxyluciferin + light (220), is a very common reporter gene in molecular biology. One benefit of the luciferase system is that neither the firefly, nor *Renilla* luciferase proteins require any post-translational modifications, meaning that both proteins are fully active immediately following translation and in most cell types (221). The Dual-Luciferase Reporter Assay System (Promega) uses a double luciferase substrate system that allows for the quantification of reporter gene activity while "normalizing" the reporter gene of interest to another reporter gene that should remain unaffected by the treatments of the experiment. In this case, the LARII solution includes beetle luciferin, a substrate for the firefly (*Photinus pyralis)* luciferase enzyme. Luciferin is converted into oxyluciferin and emits light that can be measured in a luminometer and reported as

relative light units (RLUs). The Stop&Glo reagent is then added which quenches the firefly luciferase signal, and provides the substrate for sea pansy (*Renilla reniformis)* luciferase. Renilla luciferase converts coelenterazine into coelenteramide + light.

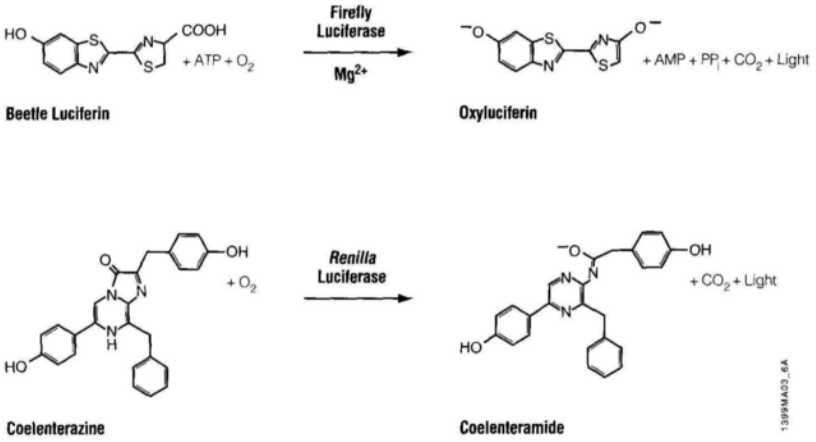


Figure 14: Luciferase reactions. Taken from Dual-Luciferase Reporter Assay System Technical Manual (Promega).

Both luciferase enzymes must be introduced into the experimental cell type. For the assay to work effectively, the firefly luciferase vector must be driven by a promoter that is sensitive to the treatment in question, while the renilla luciferase vector is driven by a promoter that is insensitive to the treatment in question. This system allows one to account for differences in transfection efficiency, cell number, quantity of lysate, etc. The luminescence obtained from the firefly luciferase is normalized to the luminescence obtained from the renilla luciferase, and that resulting ratio is considered to be normalized RLU's.

In this experiment, a firefly luciferase vector driven by the highly androgen responsive rat *Probasin* gene promoter was utilized to measure AR transcriptional

activity (preliminary experiments using a minimal promoter containing a dual-ARE sequence were unsuccessful, see appendix). Probasin, also known as prostatic basic protein, is a prostate-specific secretory protein with heparin-binding capacity (222;223) that has been heavily studied as a model for androgen specific regulation of gene expression. The basic probasin promoter covers positions (-426/+28) upstream of the transcription start site and contains 2 regions termed androgen receptor binding site 1 and 2 (ARBS1/ARBS2) (50). Both sites are protected by AR during DNAase footprinting assays and are required and sufficient for AR induced gene expression in PC3 cells (224), though another upstream enhancer region (-705/-426) containing 2 additional sites, ARBS3 and ARBS4, has been shown to further enhance AR-mediated gene expression in LNCaP and MCF-7 cells (225). Though very androgen responsive in a number of prostate cell lines (32;226), the probasin promoter has also been shown to be AR responsive in liver (54), kidney (22;32;51), fibroblast (52), and breast (32;53;225) cell lines. Though not addressed in muscle, the fact that the probasin promoter is activated by AR in a number of tissues with certainly variable transcription factor profiles suggests that the same may be true for skeletal muscle. Many of the known AR cofactors and associated proteins shown to be involved in AR signaling in prostate, such as the p160 family, CBP/p300 family, SRC-1, TIF2, heat shock proteins, etc., are ubiquitously expressed and would be expected to fulfill a similar role in skeletal muscle. Another major benefit of the probasin promoter is AR specificity; no other nuclear hormone receptor has shown the ability to initiate significant transcription, unlike the mouse mammary tumor virus (MMTV) promoter, which though AR responsive is also responsive to progesterone, glucocorticoid and mineralcorticoid receptor-mediated

transcription (49). Consequently, the p159pPr-luc vector (Addgene plasmid 8392) developed by the laboratory of Jeffery Green (227) was used as the luciferase reporter vector, driven by the basic rat *probasin* gene promoter. A *Renilla* luciferase normalization vector driven by the herpes simplex virus thymidine kinase promoter, pRL-TK (Promega), was used because of its stable, low to moderate levels of luciferase expression and because it displayed little to no response to AR or androgen treatment in preliminary experiments. Initially a simian virus-40 driven vector was chosen, pRL-SV40 (Promega), but this construct proved to be overly active in C2C12 cells and provided a level of *Renilla* luciferase expression many orders of magnitude greater than the firefly luciferase vector, in addition to showing a response to T administration.

Transient transfections were carried out in C2C12 cells seeded at 4000 cells/well in 24-well culture plates and cultured until ~75% confluent. The transfection reagents of choice were Lipofectamine enhanced with Plus Reagent (Invitrogen), and the transfection procedure was carried out according to manufacturer instructions. 400ng of the each respective AR vector in addition to 400ng of pPr-luc were added to each well, as well as 50ng of pRL-TK normalization vector. A control transfection was also performed consisting of a promoter-less firefly luciferase construct in a pGL3-basic vector (the parent backbone of the pPR-luc reporter plasmid), 400ng of pCMV5-hAR24 vector, and 50ng of pRL-TK. Each transfection mixture was diluted in serum-free/antibiotic free medium and added to each respective well for 3 hours at 37^{o}C, after which an equal volume of GM was added and the cells were incubated overnight. The following morning the transfection mixture was removed and replaced with fresh GM supplemented with 100nM T or ethanol vehicle and incubated for 24 hours. A 100nM T

concentration was chosen as preliminary dose-response luciferase experiments demonstrated this dose to drive luciferase activity to peak levels (see appendix, *figure 17*). Total ethanol volume did not exceed 0.1%. The following morning all media was removed and the cells were washed 2x with PBS. Passive lysis buffer was added for 20 minutes with gentle rocking at room temperature, and the plates were then spun at 3000g for 10 minutes. 20µL of the supernatant was removed and added to 100µL of LARII reagent, mixed by repetitive pipetting until homogenous, and luminescence was measured in a Modus Fluorometer for 5 seconds with a 2 second delay. The reaction (firefly luciferase) was then quenched with the addition of 100µL of Stop&Glo reagent (mixed until homogenous) and luminescence (*Renilla* luciferase) was again read for 5 seconds with a 2 second delay. All data is expressed as the ratio of firefly/*Renilla* luminescence. Each experimental condition was performed in triplicate, and in three separate experiments. The exact procedure was carried out in the cell lines stably expressing each AR construct, with the exception that only pPR-luc and pRL-TK vectors were transiently transfected.

Western Blot

Western blot approach was used to determine AR protein expression and assess potential differences in AR nuclear translocation efficiency. The 3 lines stably

transfected with hAR and standard C2C12 controls were grown to ~75% confluency in 100mm dishes in GM. Cytoplasmic and nuclear protein fractions were isolated by scraping the cells into cell lysis buffer (20mM HEPES pH7.5, 10mM NaCl, 1.5mM $MgCl_2$, 0.1% Triton-X100, 10% glycerol, 1mM DTT, protease inhibitor cocktail) using a sterile plastic cell scraper, incubated at 4^{o}C for 30 minutes with gentle rotation, and spun at 5000g for 10 minutes. The supernatant (cytosolic fraction) was removed from the pelleted nuclei, which was then lysed in cell lysis buffer + 500mM NaCl at 4^{o}C for 1 hour with gentle rotation. The lysate was then spun at 15000g for 10 minutes, and the supernatant containing the nuclear fraction was removed. Protein content in the cytosolic and nuclear fractions from each sample was quantified via the bicinchoninic acid protein (BCA) assay (Pierce) according to manufacturer instructions. Samples were prepared by diluting 30µL of protein from each sample in sample buffer (250mM Tris-HCL, 2% SDS, 2.5% 2-ME) and water to 25µL and boiling for 5 minutes. A positive control sample consisted of 30µg of LNCaP whole cell lysate. LNCaP cells, as mentioned previously, are a highly differentiated prostate carcinoma cell line with high endogenous AR expression. Samples were cooled and loaded onto 7.5% polyacrylamide gels and resolved for approximately 1 hr at 150V. The gels were carefully removed from the gel assembly and transferred to polyvinylidene fluoride (PVDF) membranes in ice-cold transfer buffer (25mM Tris Base, 192mM glycine) for ~1 hour at 60V. The membranes were removed from the transfer apparatus and rinsed several times with Tris-buffered saline with 0.05% Tween (TBS-T). The membranes were blocked with 3% non-fat dry milk (NFDM) dissolved in TBST for 1 hour at room temperature, to prevent non-specific antibody interactions. The anti-AR antibody PG-21 (Millipore) was selected as the primary

antibody. PG-21 recognizes residues 1-21 of the human AR, and is commonly used in studies of the AR in various cell lines and tissues (32;45). PG-21 was diluted 1:300 in 3% NFDM-TBST and the membranes were incubated overnight at 4^{o}C. The membranes were then washed 3X for 5 minutes with TBST and a horse-radish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody diluted 1:2000 in 3% NFDM-TBST was applied to the membranes for 1 hour at room temperature. The HRP conjugated secondary antibody is used to detect the presence of AR-bound primary antibody by producing an easily quantifiable luminescent signal. HRP catalyzes the conversion of luminol to 3-aminophthalate in the presence of intermediate substrates, producing light (228). The SuperSignal West Pico Chemiluminescent Substrate (Pierce) kit provides the necessary substrates to greatly enhance the HRP reaction and enable detection of picograms of antigen. The secondary antibody solution was washed from the membranes 3X for 5 minutes with TBST, and the Supersignal West Pico solution was applied to the membranes for ~ 3 minutes at room temperature in darkness. The membranes were visualized using a GeneGnome Bioimager (Syngene).

Cell Proliferation

As discussed previously, evidence suggests that androgens may alter the proliferation and differentiation of both satellite cells and myogenic precursor cells in vivo. We therefore hypothesized that the greater lean body mass observed in humans with increased AR repeat length (34) could be partially due to altered rates of myogenic cell proliferation and differentiation. Consequently, the proliferative rate of each of the C2C12 lines stably transfected with AR was assessed to determine if AR repeat length, and subsequent differences in AR transcriptional activity, might affect the growth and

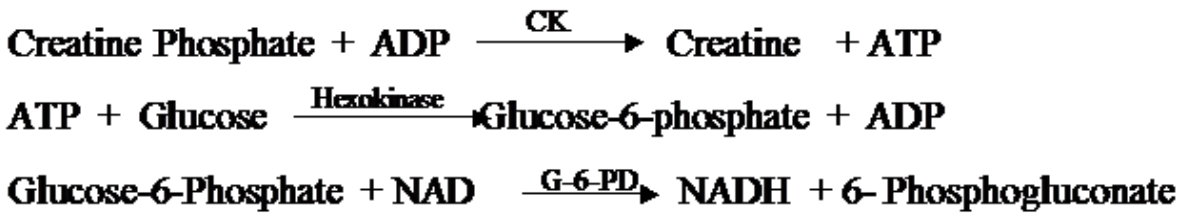
proliferation of myoblasts in culture. The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit was used to determine cell numbers. The basis of the kit is a colorimetric substrate that can be measured by assessing absorbance of light at 490nm. The solutions in the kit provide a tetrazolium salt, MTS, and an electron-coupling reagent (phenazine methosulfate, PMS). Dehydrogenase and reductase enzymes from living cells catalyze the conversion of MTS to formazan, a chromogenic dye that is soluble in cell culture media. The rate of reduction of tetrazolium salt to formazan is directly proportional to the number of living cells in a sample (229). The advantage to this particular kit is the inclusion of MTS rather than other tetrazolium salts such as XTT or MTT, which unlike MTS are either not stable in solution or require additional processing steps due to product insolubility (230;231).

This experiment was designed to measure the proliferation of myoblasts over a 3-day period. Each cell line was passaged normally and the concentration of suspended cells in the passage medium was carefully determined using a hemocytometer (Fisher). Cell number was obtained by loading 10μL of cell suspension into the device, counting 6 entire squares, averaging the number obtained and multiplying the result by 10,000 to arrive at # of cells/mL. This was the most critical step in the experiment, as results were dependant on the assumption that all plates started with an identical number of cells per well. The experiment was initially performed by seeding 250 cells/well into 96-well plates. However, the low volume, low cell number, and small well size resulted in the initial runs having drastic intra-assay variability between replicates of the same cell line. Consequently, the experiment was switched to a 24-well format seeding 1000 cells/well in triplicate with either 100nM T or ethanol vehicle, on 3 separate plates (one for each

day). At 24 hour intervals, 80µL of MTS solution was added to 400µL of fresh GM and the cells were incubated at 37°C for 3 hours. The plates were read at 490nm using a Wallac 1420 Multiplate Reader (Perkin Elmer) and the absorbance values were recorded. Cell number per well was calculated by plugging each absorbance value into an equation derived from a standard curve of a known number of cells from control wells. The control curve was obtained by counting out and plating C2C12 cells ranging 1000-30,000 cells/well (on the same plate to be assayed), between 6 and 8 hours before the assay was performed. This 6-8 hour delay was chosen to allow the cells time to adhere to the plate surface, but not to allow enough time for cell division to occur, which is approximately 16 hours (212). Each experimental condition was observed in triplicate over 3 separate experiments.

Cell Differentiation

Rate of differentiation of the stably transfected lines was investigated by assessing the activity of the enzyme creatine kinase (CK), one of the commonly used methods to identify myoblast differentiation along with visual verification of myotube formation, and the appearance of contractile proteins such as sarcomeric myosin and sarcomeric actin (232). CK catalyzes the conversion of the high energy substrate creatine phosphate + ADP into creatine and ATP, and the reverse reaction, as shown:



The subsequent reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase produce NADPH, the production of which is directly proportional to the activity of CK and can be read via spectrophotometry at 340nm (233). CK is highly expressed in tissues which rapidly consume ATP, including skeletal muscle (234), however CK is detectible only upon the initiation of differentiation, not in proliferating myoblasts (235). The EnzyChrom Creatine Kinase Assay Kit (BioAssay Systems) was used to measure CK activity in each of the cell lines. An assay buffer, substrate solution and enzyme mixture were combined immediately before testing and added to cell lysate as a single working solution.

The 3 stably transfected cell lines as well as C2C12 control cells were seeded into 35mm plates at ~ 25,000 cells per well. Cells were incubated in GM until ~90% confluent, at which point the medium was changed to DM and either 100nM T or ethanol vehicle was added and the cells were incubated for up to 5 days with fresh media applied every 24 hours. At the indicated time points, the cells were washed 2X in PBS, gently removed via a sterile plastic cell scraper and spun for 5 minutes at 150g and 4^{o}C. The supernatant was removed and the cell pellet was resuspended in 50µL lysis buffer (20mM HEPES pH7.5, 10mM NaCl, 1.5mM $MgCl_2$, 0.1% Triton-X100, 10% glycerol, 1mM DTT, protease inhibitor cocktail) for 30 minutes at 4^{o}C with gentle rotation. The lysate was spun at 5000g for 10 minutes at 4^{o}C and the supernatant was transferred to fresh microcentrifuge tubes. 10µL of supernatant from each sample was diluted 1:5 in PBS (this step was necessary in order to not exceed the threshold for kit accuracy) transferred to 96-well plates and 100µL of assay working solution was added. Each sample plate included a set of calibrator samples consisting of 110µL of water, and 10µL calibrator

solution + 100μL water, respectively. The sample plates were transferred to an incubator at 37°C for 10 minutes before being read at 340nM using a Wallac 1420 Multiplate Reader (Perkin Elmer). The values were recorded and the plates were immediately returned to the incubator. After an additional 30 minute incubation, the plates were read again and the values recorded. CK activity was calculated via the following formula:

$$\mathbf{CK\ (U/L) = OD_{40min}\text{-}OD_{10min}/OD_{calibrator40min}\text{-}OD_{H2O40min}\ X\ 100}$$

(equivalent activity of the calibrator) X 5 (dilution factor)

This equation provides CK activity in units/liter. These values were normalized to protein content of each sample (which was determined using the BCA assay as described previously), and the final results are expressed as CK units/μg of protein. Each experimental condition was performed in duplicate over 3 independent experiments.

AR in situ localization

The relative proximity of the polyglutamine repeat to activation function-1 of the AR-NTD, the importance of AF-1 in AR stabilization and NTD-LBD interaction (144), and the presence of a mutation of the polyglutamine tract that inversely alters NTD-LBD interaction and AR transcriptional activity (206) indicate that polyglutamine repeat length variations may alter AR nuclear translocation efficiency. Additionally, our preliminary transcriptional activity data demonstrating higher activity with longer repeat length even in the absence of ligand could potentially be explained by an increased rate of AR nuclear translocation with increased repeat length. We used in situ immunohistochemical staining of AR in each stably transfected cell line to test this hypothesis.

Cells were seeded onto 35mm plates in GM with 100nM T or ethanol vehicle and were grown until ~80% confluent. The medium was removed, the cells were washed 2X in PBS, and were fixed with ice-cold methanol for 10 minutes at -20^{o}C. After another rinse in PBS, 8% NFDM in PBS was added for 1 hour at room temperature to block non-specific binding. Cells were again rinsed in PBS, and the anti-AR primary antibody (PG-21, Millipore) diluted 1:50 in 1%BSA in PBS was applied to the cells and incubated overnight at 4^{o}C. The following morning the primary antibody solution was removed and the cells were again rinsed in PBS. An Alexa Fluor 430 fluorescent conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) diluted 1:500 in PBS was applied for 2 hours at room temperature in darkness. Cells were then washed 2x in PBS and visualized using a Nikon TI-U inverted fluorescent microscope (Nikon) using the appropriate filters. Unfortunately this procedure ultimately proved unsuccessful in detecting any appreciable AR protein, so a series of alterations to the procedure were made including switching blocking solutions, incubation times, fixing agent, antibody dilutions, etc. None of these conditions proved successful and AR protein was not detectible in any of the samples tested.

Myotube Development and Morphology

The formation of mature muscle fibers is a complex series of events involving the expression of myogenic genes, differentiation of myoblasts into myotubes, and the fusion of 2 myotubes to each other or to existing myofibers. However, evidence suggests that myoblast-myoblast fusion and myoblast-myofiber fusion are in fact different processes, and that different signaling molecules are involved (reviewed by Pavlath and Horsley (71;236)). Myoblast-myofiber fusion is the more common process in human skeletal muscle, as this type of fusion occurs throughout life as part of normal maintenance, where the myoblast-myoblast fusion typically only occurs in embryonic development or in response to severe muscle injury that requires the creation of new muscle fibers (237). As a post-mitotic tissue, mature skeletal muscle appears to be reliant upon the fusion of activated satellite cells (myoblasts) for growth and repair (238). Myoblast fusion contributes additional nuclei to growing or repairing myofibers, and the growth of myofibers is tightly associated with changes in myonuclear number (239).

During the culturing of the C2C12 stable lines, significant differences were observed in the rate of differentiation and in the morphology of the resulting myotubes. To determine if variable AR transcriptional activity was responsible for these observed differences, an attempt was made to characterize and quantify the differences using immunohistochemical staining techniques. Of particular interest were the identification of differences in the appearance of contractile proteins, myotube fusion, and myonuclear number. Sarcomeric myosin was used as the contractile protein of choice, as it is a marker of myotube formation and has been shown to be expressed in differentiated C2C12 cells (240). Cells expressing sarcomeric myosin were identified immunohistochemically using the MF20 sarcomeric myosin antibody (Iowa Hybridoma).

Myonuclear number was determined by staining nuclei with 4', 6-diamidino-2-phenylindole (DAPI), a commonly used fluorescent stain that binds double-stranded DNA. In conjunction with MF20 staining, myonuclei present in myotubes were counted separately from nuclei outside of myotubes. Myotube fusion index is often used as a relative estimate of fusion efficiency, and was determined by comparing myotube nuclei to total nuclei within a given field. The inclusion criteria for designation as a myotube were a positive staining for sarcomeric myosin, and a minimum of 3 nuclei per fiber.

Cells from each stable line as well as C2C12 controls were seeded onto 35mm plates in GM and grown until ~90-95% confluent, at which point the culture media was switched to DM supplemented with 100nM T or ethanol vehicle. Cells were incubated for 24, 72, or 120 hours in DM, respectively, before being fixed in ice-cold methanol for 10 minutes at -20^{o}C. Cells were rinsed 3X for 5 minutes in 3% NFDM in TBST to block non-specific interactions. The MF20 anti-sarcomeric myosin antibody was applied diluted 1:50 in PBS for 1 hour at room temperature. Cells were washed 3X in PBS, and a fluoroscein-isothiocyanate (FITC) conjugated anti-mouse IgG secondary antibody was applied diluted 1:500 in PBS for 1 hour at room temperature. Cells were washed 3X in PBS and DAPI was applied at 500ng/mL for 5 minutes at room temperature in darkness. The cells were again washed in PBS, and visualized using a TI-U inverted fluorescent microscope (Nikon) using the appropriate filters. Each plate was analyzed from a minimum of 5 fields.

Gene Expression

Table 2. Primer sets and PCR conditions for each gene target

Gene	F primer (5'-3')	R primer (5'-3')	Anneal Temp. (C)	Cycle #	Expected Size (bp)
AR	accgaggagctttccagaat	cagctgagtcatcctcgtccg	55	30	420 (21CAGs)
Myogenin	tccctgtccaccttcagggcttcg	taaggagtcagctaaattccctcg	59	30	804
Myostatin	taaccttcccaggaccagga	cactctccagagcagtaatt	55	30	225
MyoD	gtggcagaaagttaagacga	agtcgaaacacgggtcatca	50	25	170
ACTA1	gcgcaagtactcagtgtgga	cacgattgtcgattgtggtc	55	22	182
P53	gggacagccaactctgttatgtgc	ctgtcttccagatactcgggatac	62	25	300
M-cadherin	agccctgagttcttcagcat	ccttcaaggatggtgaacct	55	22	320
NFATC2	cgacgccttctactctggac	cttggttggctctttgaagc	50	30	427
GAPDH	gtgtccgtcgtggatctg	cctgcttcaccaccttcttg	55	25	90

The AR primer set was designed to amply the region of exon 1 containing the CAG repeat of the human AR, though PCR performed on murine DNA also resulted in a band slightly smaller than that observed in human DNA, indicating a high degree of sequence homology between the two species. Myogenin was selected as a molecular marker of differentiation, as myogenin is typically not observed in rapidly proliferating myoblasts but its expression is both necessary and sufficient to induce myotube formation (241). In contrast, MyoD is expressed during myoblast proliferation and has been demonstrated to be vital for myogenic determination (242), and primary myoblasts derived from MyoD-/- mouse satellite cells are differentiation deficient (243). Skeletal alpha-actin was chosen because of its status as a contractile protein whose expression is known to be sensitive to androgen administration and which has an AR-responsive region in its promoter (115). Myostatin is a negative regulator of skeletal muscle development, and its expression has been shown to be modulated by androgen treatment (20;73). M-

cadherin is a muscle specific, calcium dependent cell-cell adhesion molecule that plays a role in both the proliferation and differentiation of embryonic and adult skeletal muscle(244), and was selected due to apparent differences in fusion between the stably transfected C2C12 lines. NFATC2 was investigated due to the similarities in morphology between the AR14 stable line and a line of NFATC2-/- primary murine hindlimb myoblasts (44), both of which display a long, thin myotube with sparse nuclei, indicating a possible effect of AR on NFATC2 or its downstream signaling. Due to apparent differences in cell survival between lines upon initiation of differentiation, an investigation of p53 expression was performed. p53 is a cell cycle mediating protein that is a strong initiator of apoptosis if DNA damage becomes severe (245). GAPDH was selected as a normalization gene due to its constitutive, stable expression as a "housekeeping" gene.

Statistical Analysis

Pair-wise comparisons of means were performed by two-tailed Student's t-test. Two-way ANOVA was used for multiple comparisons in dose-response experiments (i.e. luciferase activity) with drug and cell line as main effects. Three-way ANOVA with repeated measures was used for time course experiments with drug, cell line, and day as main effects, and Tukey's post-hoc for comparison of means. All experiments were performed in triplicate and were repeated on 3 separate occasions, unless otherwise noted. All comparisons were carried out using the SPSS software package (SPSS Inc.).

Appendix

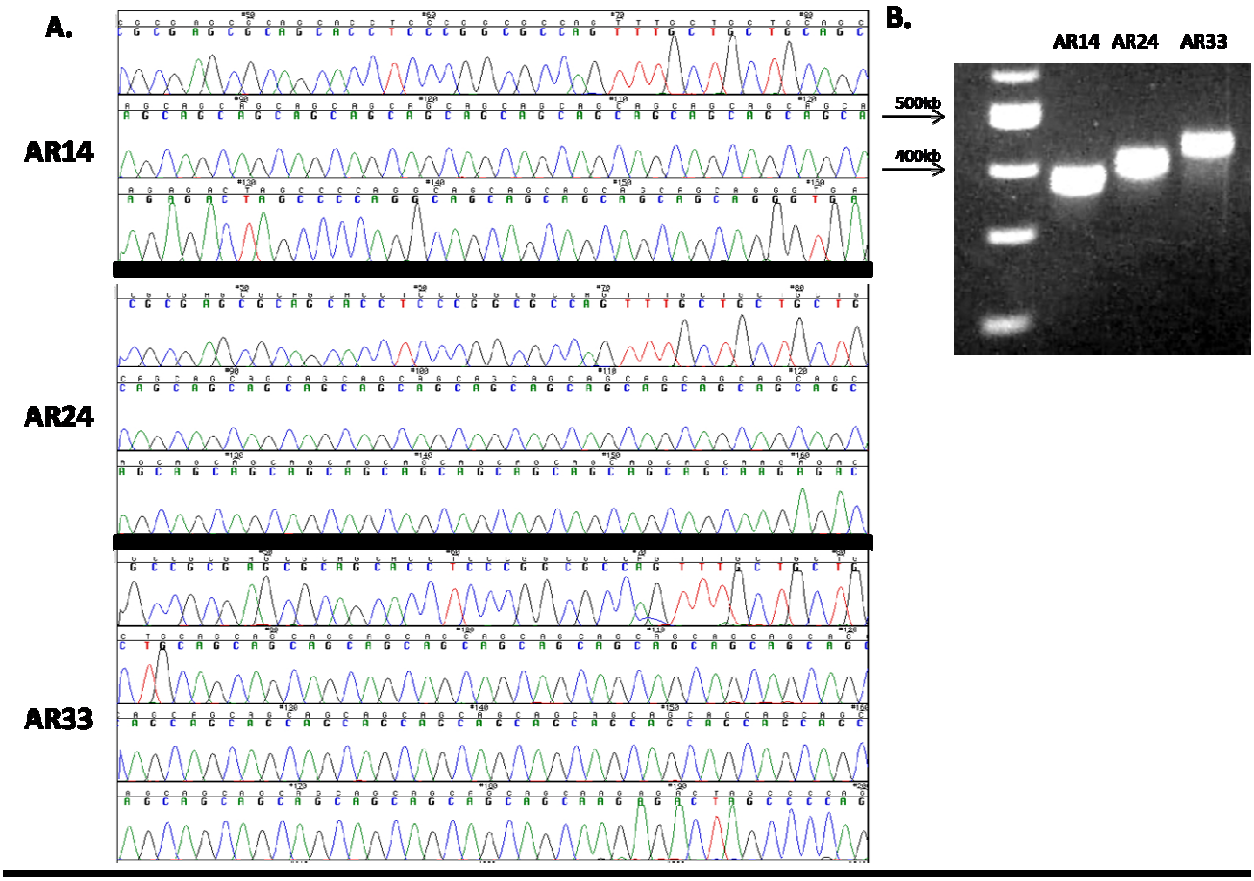


Figure 15: A) Chromatograms of sequencing analysis of AR constructs showing 14, 24, and 33 CAG repeats, respectively. B) RT-PCR performed on RNA from stably transfected lines demonstrating AR size differences.

Figure 14, panel A displays the sequencing chromatograms for each of the AR vectors. After cloning and expansion (see experimental procedures, AR vector creation), the glutamine repeat region was amplified via PCR, resolved via agarose gel electrophoresis (*figure 14b*) and purified via PureLink Gel Extraction Kit (Invitrogen). Samples were resuspended in dH_2O and sequenced with a 3730 Applied Biosystems

DNA Sequencer with the forward AR primer listed in *table 2*. In *panel B*, RNA was extracted from stably transfected C2C12 cells and the AR repeat region was amplified via RT-PCR and resolved on a 1.5% agarose gel.

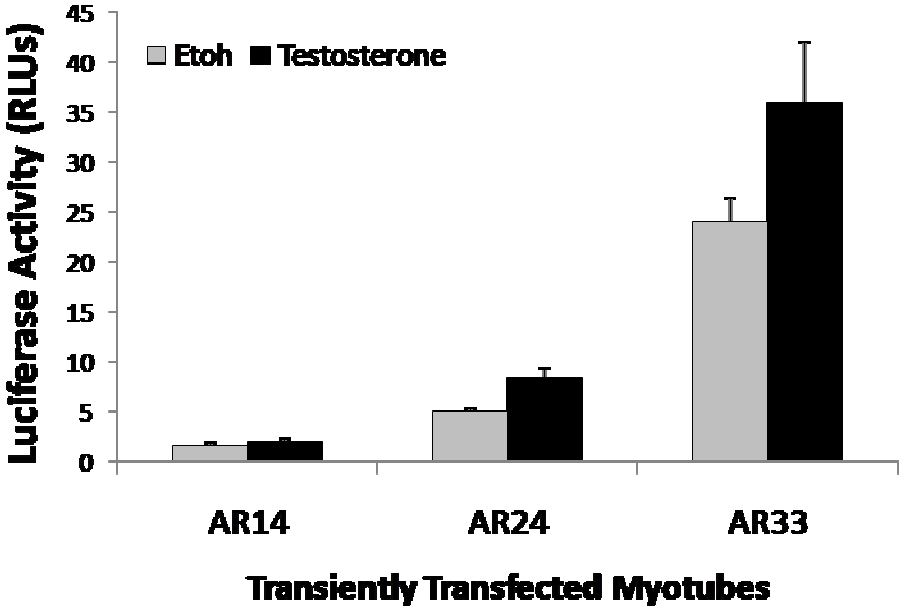


Figure 16: Relative luciferase activity of transiently transfected myotubes. Cells were grown to ~75% confluency before being transfected with the respective AR vector, pPR-Luc reporter vector, and pRL-TK normalization vectors as described. Cells were then incubated for 72 hours in DM. DM was supplemented with ethanol or 100nM testosterone 24 hours before being assayed for luciferase activity. (*p<0.05)

Because myoblasts and differentiating myotubes have divergent gene expression profiles, the effect of glutamine repeat length on AR transcriptional activity was also examined in myotubes (*Figure 15).* The experiment was carried out as described (see experimental procedures**)** with the exception that myoblasts were grown to ~75% confluency before transfection, and were subsequently incubated in DM for 72 hours to allow for differentiation before the luciferase assay was carried out.

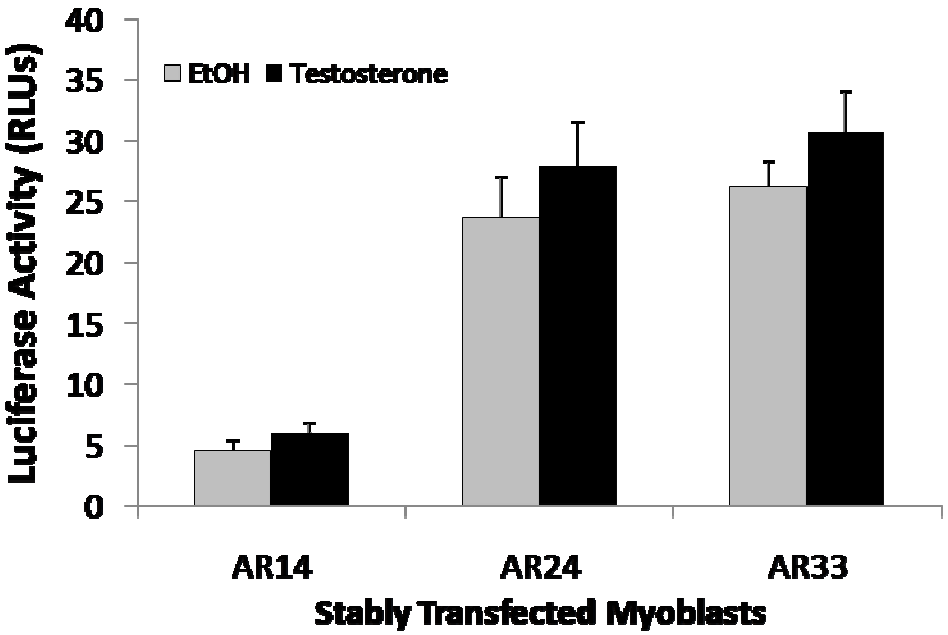


Figure 17: Relative luciferase activity of stably transfected myoblasts.

Figure 16 displays the results from the luciferase assay carried out in stably transfected myoblasts. The assay was conducted as described (see experimental procedures) with the exception that only pPR-luc and pRL-TK vectors were transiently transfected. Unlike the results from transiently transfected myoblasts and myotubes, no significant differences in transcriptional activity were observed between AR24 and AR33, though AR14 transcriptional activity was still significantly lower ($p<0.001$).

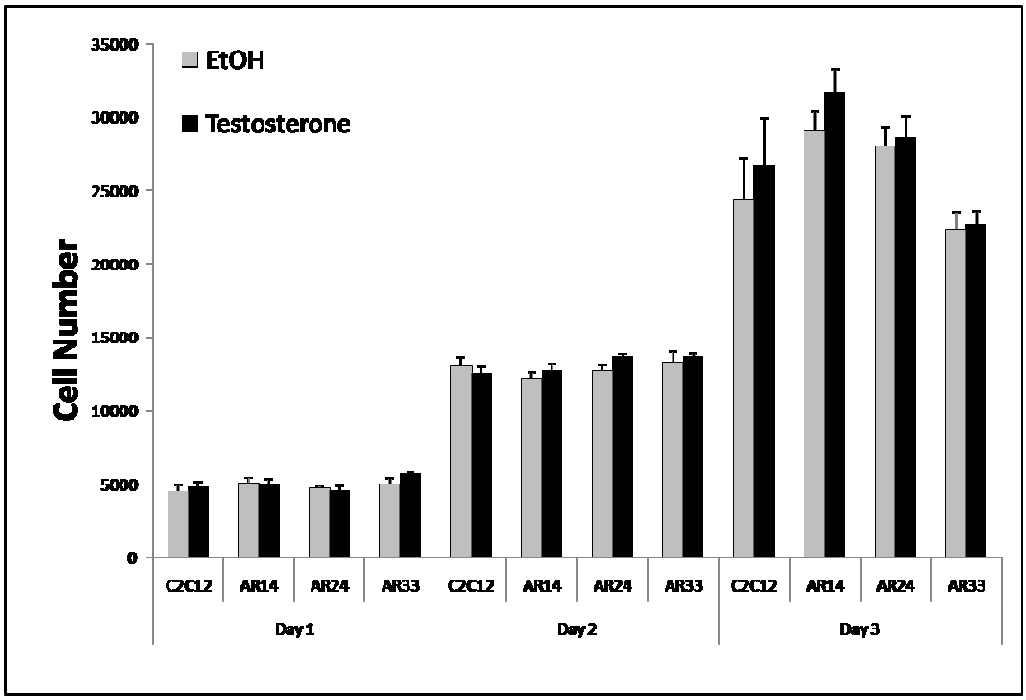


Figure 18: Influence of AR repeat length on C2C12 cell proliferation with testosterone included in the analysis.

Figure 17 represents the data from the cell proliferation experiments with testosterone included in the analysis. Statistical analysis indicated that testosterone did not have a significant effect on the proliferation of any of the lines, nor at any time point, and was subsequently removed from the final analysis for simplification.

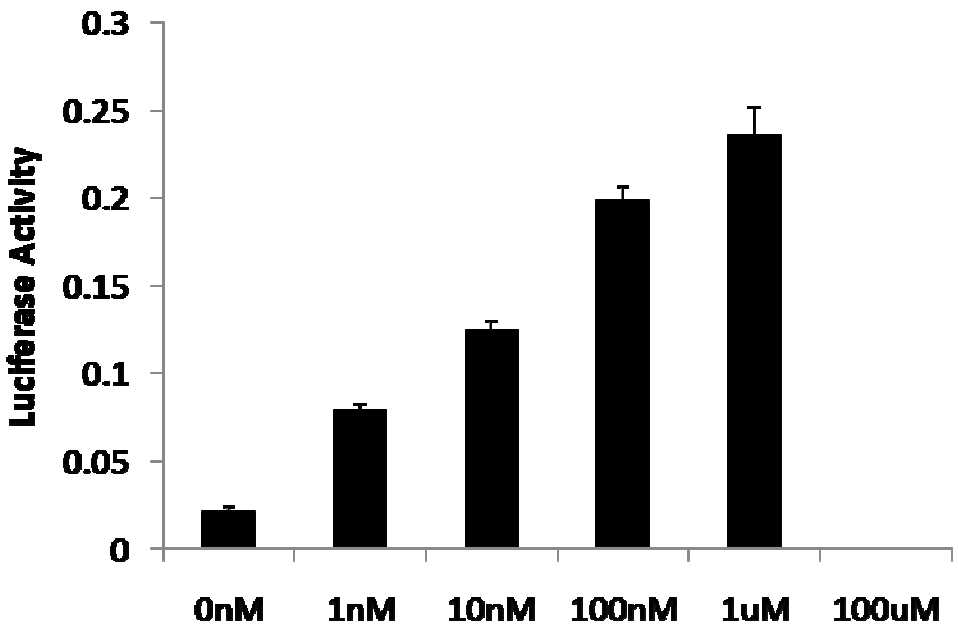


Figure 19: Testosterone dose-response curve. C2C12 cells were grown to ~50-60% confluency and were transiently transfected with AR24, pPR-Luc and pRL-SV40 vectors and incubated in the presence of the indicated amount of testosterone or ethanol vehicle for 24 hours before being assayed for luciferase activity. The 100uM dose was 100% fatal.

We applied testosterone at 100nM in all of our experiments based on the results from a preliminary dose-response curve. Unlike the subsequent luciferase experiments, the normalization plasmid used here, pRL-SV40, was extraordinarily active in C2C12 cells, and resulted in firefly:*Renilla* luciferase ratios that were much smaller. However, as shown in *figure 17*, luciferase activity of AR24 transfected cells continued to rise with testosterone doses increasing up to 1uM. The 100uM dose resulted in 100% cell fatality; hence the assay was not performed at this dose. Though serum testosterone ranges from 10-30nM in health human males, we selected the 100nM as the experimental dose as luciferase activity at this dose was significantly higher than the 10nM dose, but luciferase values were only slightly greater at the 1uM dose.

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