Award Number: DAMD17-02-1-0099

TITLE: Humanizing the Mouse Androgen Receptor to Study Polymorphisms and Mutations in Prostate Cancer

PRINCIPAL INVESTIGATOR: Diane M. Robins, Ph.D.

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, MI 48109-1274

REPORT DATE: January 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Humanizing the Mouse Androgen Receptor to Study Polymorphisms and Mutations in Prostate Cancer

Androgen receptor (AR) plays a critical role in prostate cancer. Length of AR’s N-terminal glutamine (Q) tract has been associated with disease risk, and AR somatic mutation may influence disease progression. To study the basis for these effects in an animal model, we converted the mouse AR gene to the human sequence, using homologous recombination in embryonic stem cells. Mice bearing “humanized” AR alleles with 12, 21, or 48 Qs are normal in growth and fertility; subtle differences in some traits and gene expression were within normal biological variability. Differences are more apparent when crossed with TRAMP, providing a prostate oncogene. In intact mice, age at initiation is similar for wild type and 21Q hAR mice, but tumors in 12Q mice arise earlier and those in 48Q mice are significantly delayed. This corroborates epidemiological data showing inverse correlation of Q tract length with risk of disease. Remarkably, disease progression in castrated mice, mimicking androgen-independence, shows the opposite effect – 12Q AR is strongly protective against onset of disease. The basis of this has been assessed histologically by tissue microarray, at the molecular level by sequencing AR cDNAs for mutations, and we are pursuing Q-tract effects on ligand-independent AR activation.
INTRODUCTION

Development and progression of prostate cancer (PCa) depend on genetic and environmental factors that are still poorly defined. One thing all tumors share, however, is an initial dependence on androgen for growth [1]. Polymorphisms in androgen receptor (AR) may impact risk of disease, and somatic mutations may affect progression and response to therapy [2-4]. In most cases, hormonal therapy is initially successful, but tumors ultimately become androgen-independent and resist further treatment. Despite androgen independence, AR levels in the tumor remain high and the AR signaling pathway is intact, revealing a continued role of AR in the disease process. AR molecular genetics underlie two crucial problems in PCa: 1) Do polymorphisms in AR lead to differential risk of disease? 2) Do somatic mutations in AR during tumor growth circumvent hormone ablation, and are they selected by treatment? This project addresses these questions for the human receptor in a transgenic mouse prostate cancer model, which allows initiation, progression and treatment of disease to be integrated experimentally. The mechanisms by which genetic variation alters AR action may reveal new molecular markers in treatment, and, ultimately, novel targets for therapy.

BODY

Our hypothesis is that genetic variation in human AR affects the initiation and progression of prostate cancer. Germline variation may affect initiation of disease, while somatic mutations may drive androgen-independent growth. To confirm this hypothesis, our first major objective was to replace the mouse AR with human alleles to study their effects in general mouse physiology and in a transgenic tumor model (Transgenic Adenocarcinoma of the Mouse Prostate, or TRAMP [5]). Since Q (glutamine) tract length is thought to enter into PCa risk, we are comparing hAr alleles that vary in Q tract length. We also are comparing mutations arising in androgen-dependent vs. –independent disease, to identify sites correlating with AR function. Our aims underlying the Statement of Work were as follows:

Aim I. To study the role in PCa of polymorphisms in human Ar, mAr will be “humanized” by homologous recombination in embryonic stem cells, to create three h/mAr alleles differing in glutamine tract length (12Q, 21Q, 48Q). Differences in androgen action (fertility, behavior, molecular markers) and spontaneous prostate cancer will be studied in mice with h/mAr alleles.

Aim II. To determine the role of human AR variants on PCa initiation, h/mAr alleles will be placed on the TRAMP background for transgene-induced oncogenesis. Effect of the Q tract will be assessed on prostate pathophysiology and gene expression by cDNA microarray.

Aim III. To determine the role of AR variation on PCa progression, spontaneous mutations will be identified in AR cDNAs from castrated (androgen-independent) vs. intact h/mAr-TRAMP mice. The effect of mutations will be determined by introduction into ARs for transfection analysis, with and without coactivators. The effect of mutations on the oncogenic potential of prostate cells will be tested by tumor formation and metastasis in SCID mice.

Each Aim corresponds to a Task within the Statement of Work. Aim I is complete as planned and the significant results are reported in the appended manuscript, “Replacing the mouse androgen receptor with human alleles demonstrates glutamine tract length dependent effects on physiology and tumorigenesis in mice”. Experiments for Aim II are mostly complete,
although the data analysis is still ongoing. For Aim III, the AR sequencing is complete; most functional analysis remains to be performed. Significant findings are summarized below, with figures and ms. included in the appendix.

Polymorphism in AR’s N-terminal Q tract has been implicated in affecting aspects of male health from fertility to cancer. Extreme expansion underlies Kennedy disease, a late-onset neurodegenerative disorder. Length of the Q tract corresponds inversely with capacity of the AR to activate transcription in vitro. However, whether variation in Q tract length within a nonpathological range influences physiology or the etiology of disease is controversial and has not been resolved by epidemiological studies. To assess directly the functional significance of Q tract variation, we converted the mouse AR to the human sequence by germline gene targeting, introducing alleles with 12, 21 or 48 glutamines. These three “humanized” (h/mAR) mouse lines were grossly normal in growth, behavior, fertility and reproductive tract morphology (see appended ms.). Phenotypic analysis revealed subtle trends, particularly for the 48Q mice in which slightly increased body fat and lower seminal vesicle weight suggested borderline androgen insensitivity. Upon molecular analysis, tissue specific differences in AR levels and target gene expression were detected between mAR and h/mAR lines, and between Q tract alleles, but largely within the range of normal biological variation. For example, in prostate, AR mRNA showed a trend to decreased levels with increasing Q tract length, but the differences did not reach statistical significance. Similarly, expression of the AR target gene Nkx3.1, which is critical in prostate growth and differentiation, was higher for short compared to long Q tract alleles (see Fig. 6 in ms.), likely reflecting the greater transcriptional strength of the short Q tract. However, when crossed with transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, striking genotype-dependent differences in prostate cancer initiation and progression were revealed (described in greater detail below). This link between Q tract length and prostate cancer establishment, likely due to the differential transcriptional strength of these AR alleles and thus the differential activation of key target genes, corroborates human epidemiological studies linking shorter Q tracts to increased risk of disease and longer tracts to resistance.

The characterization of these mice also revealed some findings noteworthy in a broader context. First, although differences in physiological and molecular traits between the Q tract variant mice fall within the range of normal phenotypic variation, these differences are amplified when the homeostatic balance is upset, in this case by the stress of cancer, and distinct patterns of disease progression are produced for each allele. Second, these differences are evident in the homogeneous mouse genetic background, but are not readily demonstrable in a human population, in part due to the genetic heterogeneity and confounding gene-gene and gene-environment interactions. Third, one might say that the more rapid disease initiation with the transcriptionally stronger short Q tract AR might be predicted in this model simply due to its earlier or more robust activation of the androgen-driven oncogenic transgene in the TRAMP model. In a sense this is exactly the point – while human PCa does not initiate with T-antigen, early disease is androgen-dependent and downstream events are similar for both. Further, additional stochastic androgen-dependent events are required for tumorigenesis and differential activation of multiple genes are likely involved. Thus, analogous activities in these mice should prove extremely useful for revealing not only AR-dependent (and possibly androgen-independent) mechanisms in prostate cancer initiation or progression, but Q tract influence on other physiological traits as well. Further, these mice provide an animal model to test novel drugs targeted specifically to the human, rather than mouse, AR.

Results of Aim I validate the effort of creating these mice, and their value for future studies. Still in progress are aging studies, and additional gene profiling analysis. Previously we presented microarray data on an early generation of these lines, for testis, liver and kidney
RNA. Now that the lines have been backcrossed several additional generations to the C57BL/6 strain, we are doing a more detailed analysis of prostate gene expression profiles.

For Aim II, the Q tract variant mice were crossed onto the TRAMP background, in order to initiate cancer via prostate epithelial-driven T antigen expression. At the age of twelve weeks one cohort was castrated and another left intact, for each genotype including mAR littermates. Twelve weeks was chosen as the time for castration since at that age prostatic intraepithelial neoplasia (PIN) has developed, but mice do not yet have overt disease. Tumor initiation and progression were followed by external abdominal palpation, and in some cases by magnetic resonance imaging (MRI). The MRI analysis allows tumor detection about two weeks earlier than manual palpation, but otherwise agrees with qualitative assessments, and was prohibitively time-consuming and expensive. Mice were sacrificed when they became moribund, and tumors collected for histological and molecular analysis. Tissue microarrays have been constructed and their analysis is ongoing, for AR presence and subcellular localization, as well as presence of particular coactivators and signaling molecules. Intriguing results with respect to tumor progression are described below.

As noted by others working with the TRAMP model, castration (or treatment with flutamide) may delay, but does not prevent, prostate cancer, for about 70% of the treated (with wild type mAR) mice [6]. However, for about 30% of the mice, more aggressive disease occurs following androgen ablation. This finding bears remarkable similarity to results of the SWOG prevention trial in which men were treated with finasteride [7]. Tumor progression is heterogeneous in TRAMP mice as it is in men, despite the genetic homogeneity of the mouse model, likely because following the first transgene-induced oncogenic hit, additional stochastic genetic hits must occur. When intact TRAMP mice bearing the 21Q-h/mAR allele were compared to littermates with wild type mAR, no significant differences in disease initiation (as indicated by age of first palpable tumor) or progression (as indicated by survival time) were evident (see Fig. 1, Appendix). At a 29 week time point, when slightly more than half the mice with mAR or 21Q-h/mAR alleles had palpable tumors, fewer than 30% of the 48Q-h/mAR mice had tumors but nearly all the 12Q-h/mAR mice did. This supports the notion from human studies that the short Q tract AR confers greater risk of disease; in mice, the corollary that the long Q tract is protective is even more pronounced. Remarkably, the most significant difference between alleles is in progression, or length of disease, as indicated by time of palpation until death. As might be expected for the 48Q-h/mAR mice, which show very delayed initiation, disease course is very slow and mice survive well past one year of age. More intriguingly, although the 12Q-h/mAR mice show early disease initiation, tumors progress on average even more slowly that in the 48Q mice. Thus each allele dictates a different disease course. While this may differ in some specifics from human pathology, these mice represent an allelic series with regard to AR transcriptional strength, and provide a unique opportunity to explore further in vivo the role of AR in tumor initiation versus progression.

Further support of the value of these mice in offering novel insights comes from the observation that each allele confers a distinct pattern of progression in the castrated TRAMP mice, which mimic androgen-independent disease progression, as well as in intact mice (Fig. 2). As noted for TRAMP mice previously, following castration a subset develop early aggressive disease while many are delayed in onset relative to intact. The proportion of tumors initiating early versus late varied with AR allele, despite the absence of hormone. Somewhat surprisingly, 12Q-h/mAR mice developed disease significantly later, and had a disease course significantly longer, than 21Q- and 48Q-h/mAR mice. The striking difference in early disease and progression for AR alleles in the absence of androgen suggests Q-tract length is affecting androgen-independent, as well as androgen-dependent, tumor growth. This has not been
demonstrated previously, and we will be pursuing the underlying mechanism by which Q tract affects ligand-independent AR activity, both by analysis of mouse tumor samples and by experiments in tissue culture.

Perhaps even more striking than the differences in disease initiation conferred by each allele were the pronounced differences in mean length of disease, as determined from age at first palpation of a tumor until death (Fig. 3). In intact mice, length of disease in mAR and 21Q-h/mAR mice is comparable, averaging about 5-6 weeks from tumor detection until death. However, for 12Q and 48Q intact mice, disease length spans a broader range and is much longer in both groups, with a mean disease length of greater than 14 weeks. Given the delayed initiation of 48Q mice, slow progression may not be surprising. However, finding similar slow progression in the more rapidly initiating 12Q mice suggests different pathways of progression may be in operation. In all castrate groups, length of disease is significantly shorter than in intact mice (approximately 3-4 weeks), with the 12Q mice again somewhat surprisingly showing a longer mean length of disease (7.5 weeks) than the other genotypes. The lack of linearity in the relationship between length of disease and Q tract length suggests that there may be something “optimal” about the wild type mAR and hAR alleles, and disease course diverges from this at the gross level for both the short and long extremes of Q tract length. Whether disease progression is similar in 12Q and 48Q mice at the cellular and molecular level is an important question. Clues to underlying mechanisms may be found by examination of the tissue microarray, which is in progress since only recently have the 12Q tumors become available.

By way of summarizing PCa progression in the Q tract variant mice, Fig. 4 compares tumor status for all groups at a time point of 29 weeks. In each histogram, the red portion represents the percent of mice in that group that have died of PCa, the yellow portion represents those with a palpable tumor, and the blue portion are those that are tumor free at 29 weeks of age. In intact mice, about half the mAR and 21Q-h/mAR mice have overt disease (death or palpable tumor) by 29 weeks, whereas 85% of the 12Q mice have cancer and less than 30% of the 48Q mice. This again corroborates human data that Q tract length influences disease initiation [8]. In castrated mice the genotype-dependence presents the inverse image – mAR and 21Q-hAR mice are again similar to each other, with approximately one-third of the mice having overt disease, whereas over half the 48Q but less than one-quarter of the 12Q show PCa. Genetic variation of the AR thus plays a role in initiation and progression of PCa, both in the presence and in the absence of androgen.

Thus far we have grouped all mice within a genotype together, but there may be significant differences in mechanisms of progression within a genotype that may correlate with early versus late disease onset. To begin to characterize these differences, we are analyzing the tissue microarray by immunohistochemistry, first for intensity and subcellular localization of AR (Fig. 5). Fig. 5 simply portrays the heterogeneity of staining we obtain within a group, confirming that we will need to correlate this data with time of disease initiation as well as staining for additional markers such as T-antigen, coactivators, neuroendocrine markers, and a panel of signaling molecules. The microarrays, generated by the University of Michigan Tissue Microarray Core, should prove generally valuable for mouse tumor studies and we have already shared slides with several collaborators.

Finally, we have analyzed AR mutations arising in these tumors, by sequencing the equivalent of 10 full-length cDNAs per tumor. We initially had some technical difficulties due to the highly GC-rich nature of the N-terminus, but have optimized reactions to overcome this. We also have performed all sequencing from two independent Reverse Transcriptase reactions, and only consider mutations as “real” if they occur multiple times within a tumor, or in multiple
mice. Although this duplicated the amount (and cost) of the work, it verifies that base differences are mutations in the tissue, not RT or PCR errors in processing. Because of the large amount of effort, and because we were not finding strong evidence of colocalization of mutation site dependent on hormonal status, we completed sequencing for 21Q-h/mAR samples, but have only done some 48Q tumors and none of the 12Q at this point. Should interesting results occur as we proceed with analysis, we can sequence more samples in future.

We are finding 1-3 mutant ARs per tumor, in accord with previous observations [9], and thus far they are a mix of previously noted as well as novel mutations. A pictorial summary of mutations found thus far is shown in Fig. 6, Appendix. We have not found the strong correlation between mutation site and hormonal status reported previously [9]. Experimental differences could enter into this (we have examined a larger number of mice, and doubled the sequencing per tumor), or it could be differences due to the mouse compared to human gene. Supporting the notion that these mutations are not just random is that many have been noted previously in PCa, and are gain rather than loss of function. We have found significant variation around the Q tract itself, which tends to vary plus or minus a few Qs in many tumors (Fig. 6, lower panel). It is disappointing that we have not found more mutations in the LBD in intact mice and more in the N-terminus in castrated animals. It is possible that such differences would be more pronounced at earlier tumor stages, rather than for the end-of-life necrotic samples we obtained. Regardless, our findings agree with increasing data from human studies that while AR mutations certainly occur with progression of PCa, they are but one of many mechanisms involved in resistance to treatment and frequent or common mutations are not evident.

In sum and in accord with our original plan, this project has focused on establishing a human AR allelic series in mice, primarily to analyze effect of Q tract variation in initiation and progression of prostate cancer. These strains function as predicted, in being physiologically normal but having subtle phenotypes when hormonal effects are examined in greater detail. These phenotypes become pronounced in the context of a physiological stress, such as cancer. Thus we are encouraged that these mice will be valuable for assessing the role of androgen receptor in prostate cancer initiation and progression, may provide better subjects for preclinical testing, and may lead to new treatments for disease.

KEY RESEARCH ACCOMPLISHMENTS

- 12Q, 21Q, and 48Q h/mAR mouse strains were established and have been crossed onto the C57BL/6 background for at least 7 generations, with no abnormalities noted. F5 individuals at 6 months, 18 months and 2 years have been compared for molecular and endocrine markers, and phenotypic characterization of these strains has been submitted for publication.
- 12Q, 21Q and 48Q h/mAR mice were crossed with TRAMP to generate prostate tumors, and all tumors were collected when mice became moribund, except for a few remaining 12Q mice that exhibit very slow progression. In intact mice, the Q tract length inversely correlates with time of disease initiation. Remarkably, in castrated mice, the opposite is found – in particular the short Q tract has a distinctly protective effect in tumor initiation and/or progression relative to median or long Q tracts.
- A tissue microarray has been created from normal prostate and tumor samples of these mice and is being analyzed for presence and subcellular localization of AR, coactivators, and other biomarkers to characterize differences underlying distinct tumor behaviors.
- AR cDNAs from tumors from intact vs castrated 21Q mice have been sequenced. Mutations that could aid in androgen resistant progression are found with frequency, but do not strongly colocalize dependent on hormonal status as previously reported.
REPORTABLE OUTCOMES

We generated three lines of “knock-in” mice for this study, carrying “humanized” AR alleles varying in glutamine tract length and designated as follows:

- 12Q-h/mAR (short Q tract humanized AR allele)
- 21Q-h/mAR (median “ “ “
- 48Q-h/mAR (long “ “ “

The following manuscript is in review at Molecular Endocrinology:

“Replacing the mouse androgen receptor with human alleles demonstrates glutamine tract length dependent effects on physiology and tumorigenesis in mice”; M. Albertelli, A. Scheller, M. Brogley, and D.M. Robins.

We have presented our results at local poster sessions and informal seminars, and also as part of departmental colloquia. Since the last report, these include:

- Dept of Cell Biology and Molecular Genetics, University of Maryland, Baltimore, 5/13/05
- Cancer Center and Vattikuti Urology Institute, Henry Ford Hospital, Detroit, 11/03/05

Talks or posters were (or will be) presented at the following meetings; the unpublished abstracts for the Interprostate SPORE meeting from last year as well as the one next week are included in the Appendix, along with last year’s and the upcoming Endocrine Society Abstract:

- Interprostate SPORE Conference, Houston, 1/31/05
- " " " , 2/4/06
- Endocrine Society 87th Annual Meeting, San Diego, 6/3/05
- " " " Boston, 6/17/06

The 21Q h/mAR mice are proving a valuable resource and are being used for a project within the University of Michigan SPORE in Prostate Cancer, to determine effects of antiandrogens (flutamide, bicalutamide) on incidence of AR mutations in the humanized mice compared to in patient samples. Information on that grant is as follows:

NIH/NCI P50 CA69568, S.P.O.R.E. in Prostate Cancer
P.I. Kenneth J. Pienta
7/1/03 – 6/30/08
P.I. of Project #5 – D. Robins; ~150,000/yr direct costs
“Multiple Mechanisms of Antiandrogen Resistance in Prostate Cancer Progression”

The targeting vector used to create the 21Q h/mAR mice has been modified in collaboration with two junior investigators to create mouse models for other studies. With Andrew Lieberman, a mouse model of Spinal and Bulbar Muscular Atrophy (SBMA, Kennedy disease) has been created bearing a CAG tract expanded to 113 Q. A paper characterizing these mice and elucidating their testis phenotype has been published:

Yu Z, Dadgar N, Albertelli M, Scheller A, Albin RL, Robins DM, Lieberman AP:
CONCLUSIONS

Mouse strains carrying human rather than mouse AR sequences have been constructed. This allows direct testing of the role of AR glutamine tract variation in initiation of prostate cancer, which may help clarify contradictory results from epidemiological studies. Further, tumors initiated by transgenes in these mice will allow tracking the role of AR, and AR mutants, in resistance to antiandrogen therapy and androgen independent growth. The site of mutations in human AR sequence may lead to downstream interacting proteins that will be novel and perhaps more effective targets in new treatment strategies.

REFERENCES

APPENDICES

1) List of all publications, meeting abstracts, and personnel receiving pay from this research effort.

2) Abstracts for meetings, as listed in appendix 1, # 2.1 – 2.8.

3) Figure 1 – tumor initiation and survival curve, intact AR variant TRAMP mice

4) Figure 2 – “ “ “ “ “ “ , castrated “ “ “ “

5) Figure 3 – Length of disease in h/mAR-TRAMP mice

6) Figure 4 – Proportion with disease or disease-free at 29 weeks

7) Figure 5 – Example of tissue microarray, stained with AR antibody

8) Figure 6 – Mutation summary for 21Q-h/mAR; somatic mutations in PCa

9) Manuscript submitted, “Replacing the mouse androgen receptor with human alleles demonstrates glutamine tract length dependent effects on physiology and tumorigenesis in mice”.
Publications from this Work (several additional in preparation):
Albertelli M, Scheller A, Brogley M, and Robins DM. “Replacing the mouse androgen receptor with human alleles demonstrates glutamine tract length dependent effects on physiology and tumorigenesis in mice”, submitted to Molecular Endocrinology, 1/11/06. (Appendix #3)

Meeting Abstracts from this Work (appended, with authors on each abstract):
1. “Genetic Variation of Androgen Receptor in Prostate and Breast Cancer”
   Sex and Gene Expression, San Jose, 4/6/02

2. “Androgen Receptor Genetic Variation and Prostate Cancer in Mice”
   2nd International Conference on Prostate Cancer Research, Iowa City, 10/12/02

3. “Humanizing the Mouse Androgen Receptor”
   4th Annual Symposium of Michigan Prostate Research Colloquium, Grand Rapids, 4/10/04, and Great Lakes Nuclear Receptor Conference, Toledo, 11/15/04

4. “Human Androgen Receptor Genetic Variation and Prostate Cancer in Mice”
   10th Prouts Neck Meeting on Prostate Cancer, Portland, 11/4/04

5. “Genetic Variation of the Human Androgen Receptor and Prostate Cancer in Mice”
   Interprostate SPORE Conference, Houston, 1/31/05

6. “Replacing the Mouse Androgen Receptor with Human Alleles Varying in Glutamine (CAG) Tract Length”, Endocrine Society 87th Annual Meeting, San Diego, 6/3/05

7. “Human Androgen Receptors Varying in Q-Tract Length Differentially Influence Initiation and Progression of Prostate Cancer in Mice”, Interprostate SPORE Conference, Houston, 2/4/06

8. “Variation in Q-tract Length of the Androgen Receptor Influences Initiation and Progression of Prostate Cancer in “Humanized” AR Mice”, Endocrine Society 87th Annual Meeting, San Diego, 6/17/06

Personnel receiving pay from this grant:
Diane M. Robins, Ph.D., P.I.
Rosie Alfinito, M.S.
Arno Scheller, Ph.D.
Sara Peters, B.A.
Michele Brogley, M.S.
Leslie Larkins, M.S.
Shaema Khan, M.S.
Jennifer Gerber
**Genetic Variation of Androgen Receptor in Prostate and Breast Cancer**

Diane M. Robins  
Department of Human Genetics  
University of Michigan Medical School  
Ann Arbor, MI 48109-0618

Despite diverse genetic and environmental factors contributing to prostate and breast cancer, both tumors initially are steroid hormone dependent. This in part reflects crucial developmental roles of androgen in the prostate and estrogen in the breast, working via their intracellular receptors (AR, ER) to regulate gene expression. The steroid receptors are critical in initiation and progression of disease, and therefore are key targets in therapy. For AR in prostate cancer (unlike ER in breast cancer), genetic polymorphisms have been associated with disease risk, and somatic mutations are observed in later stages. The significance of these variations has been difficult to study, however, due to the lack of patient material from early stage disease and the paucity of animal models to address underlying mechanisms.

To test the oncogenic role of AR polymorphisms, and the function of somatic mutations in disease progression, we have “humanized” the mouse by converting its AR gene to the human sequence, using homologous recombination in embryonic stem cells. The mouse and human ARs are identical in DNA and ligand binding domains, but diverge by 15% in the N-terminal transactivation domain, most notably in the extent and position of a glutamine tract. The length of this tract is inversely correlated with tumor risk in man and receptor strength *in vitro*. The effect of AR alleles differing in glutamine tract length is being tested in the context of a transgenic mouse tumor model, TRAMP, developed by Norm Greenberg at Baylor. This model also allows us to examine mutations that arise in AR during progression. AR mutations in TRAMP tumors occur in different receptor domains dependent on hormonal status. Such mutations may be associated with acquisition of androgen-independent growth which, in man, leads to resistance to therapy, a major clinical problem. The sites of mutation in human AR sequence, particularly within the transactivation domain, may identify proteins that, by their interaction with receptor, are potential therapeutic targets. These mice also allow preclinical testing of innovative anti-androgen regimens, suggested by differences *in vitro* in the mechanism of action of the common antagonists, flutamide and bicalutamide. Further, these mice may be informative for breast cancer studies, since long glutamine tract ARs appear to increase the risk of breast cancer in men.

To assess broad interactions of androgen and estrogen, some of which underlie side effects of hormonal therapies, we are also studying mice deficient in one or both receptors. Perhaps surprisingly, mice deficient in both AR and ER are generally healthy, but effects in testes, bone, and liver underscore the importance of estrogen in males. These mice highlight cell and tissue specificity of enhancing and opposing actions of sex steroids, and may reveal species-specific differences in AR function in diverse pathways, helping to define human physiology.
Appendix 2.2 – abstract for 2nd International Conference on Prostate Cancer, Iowa City, 10/02

Androgen Receptor Genetic Variation and Prostate Cancer in Mice.
Arno Scheller, Ivelisse Gonzalez, Michele Brogley, Mara Steinkamp and Diane M. Robins.
Dept of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109-0618.

Despite diverse genetic and environmental factors contributing to prostate cancer, tumors initially depend on androgen for growth, reflecting crucial roles of the intracellular hormone receptor (AR) in regulating gene expression. Because of its importance in disease initiation and progression, AR is a key target in therapy. Genetic polymorphisms in AR have been associated with disease risk, and somatic mutations are observed in later stages. The significance of these variations has been difficult to study, however, due to the lack of patient material from early stage disease and the paucity of animal models to address underlying mechanisms.

To test the oncogenic role of AR polymorphisms, and the function of somatic mutations in disease progression, we have converted the mouse AR gene to the human sequence, using homologous recombination in embryonic stem cells. The mouse and human ARs are identical in DNA and ligand binding domains, but diverge by 15% in the N-terminal transactivation domain, including in the extent and position of a glutamine tract. The length of this tract is inversely correlated with tumor risk in man and receptor strength in vitro. The effect of AR alleles differing in glutamine tract length is being tested in the context of the transgenic mouse tumor model, TRAMP, developed by Norman Greenberg at Baylor. This model also allows us to examine mutations that arise in AR during progression. AR mutations in TRAMP tumors occur in different receptor domains dependent on hormonal status. Such mutations may be involved in acquisition of androgen-independence, which, in man, leads to resistance to therapy, a major clinical problem. We found in vitro that the two most commonly used antiandrogens, flutamide and bicalutamide, differ subtly in their antagonism of AR and in their partial agonist activity. This suggests that mechanisms of resistance may vary, and that mutant ARs resistant to one drug may not have a similar advantage against the other. Experiments are underway to understand in molecular detail the mechanisms of androgen resistance, in hopes that this will translate to improved clinical treatment.
“HUMANIZING” THE MOUSE ANDROGEN RECEPTOR
Megan Albertelli, Arno Scheller, Michele Brogley, Mara Steinkamp, Diane Robins
Department of Human Genetics, University of Michigan

Androgen exposure is a major risk factor in prostate cancer development and polymorphisms in the androgen receptor (AR) may influence prostate cancer risk. Polymorphism in the glutamine (Q) tract length of AR has been associated with prostate cancer risk in some epidemiological studies. Somatic mutations in AR may also influence prostate cancer progression, allowing AR signaling to occur in the absence of androgen and resulting in androgen independent tumor growth. A mouse model would be useful to further study the effect of glutamine tract length and AR mutations on prostate cancer risk and progression. However, the mouse AR is significantly different from the human AR in the N-terminal region. The mouse N-terminal region has a displaced glutamine tract and about 15% sequence difference when compared to human AR, making it difficult to study the role of the AR N-terminal region in prostate cancer.

Therefore, the first exon of the mouse AR was replaced with the first exon of the human AR by homologous recombination in embryonic stem cells. The resulting “humanized” mice (h/mAR) have a human AR N-terminal region while the remainder of the AR consists of mouse sequence. Three lines of h/mAR mice were created, each containing 12, 21, or 41 glutamines in the polyglutamine tract. The h/mAR mice are viable and fertile, with no differences in behavior or gross reproductive tract morphology when compared to wildtype littermates, and no significant differences were seen by microarray analysis of testis and kidney RNA.

To identify AR mutations that arise during androgen dependent and independent disease and to compare the influence of the polyglutamine tract, h/mAR 21Q, 41Q, and wildtype mice were crossed with TRAMP mice in order to initiate prostate tumor growth in the progeny. Half of the male progeny were then castrated at 12 weeks of age to drive androgen independent tumor growth. In intact animals, h/mAR 41Q TRAMP mice had fewer tumors than wildtype or h/mAR 21Q TRAMP mice, suggesting a protective effect of the longer polyglutamine tract during tumor initiation. In castrated animals, h/mAR 21Q and 41Q TRAMP mice both had more aggressive tumors that arose earlier than tumors in wildtype TRAMP mice, suggesting that the humanized AR results in more severe androgen independent disease. RNA was isolated from harvested tumors and sequence analysis is in progress. Breeding of h/mAR 12Q mice is also currently in progress.
Appendix 2.4 – abstract for Prouts Neck Meeting, 11/04

Abstract for the 10th Prouts Neck Meeting on Prostate Cancer
Nov 4-7, 2004

Human Androgen Receptor Genetic Variation and Prostate Cancer in Mice.
Megan Albertelli, Arno Scheller, Michele Brogley, Mara Steinkamp and Diane M. Robins.
Dept of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109-0618.

Despite diverse genetic and environmental influences in prostate cancer, tumors initially depend on androgen for growth, reflecting crucial roles of the hormone receptor (AR) in gene regulation. Because of its importance in tumor progression, AR is a key target in therapy. Genetic polymorphisms in AR have been associated with risk, and somatic mutations occur in late disease. The significance of these variations has been difficult to study, partly due to lack of early stage patient samples and lack of animal models that address underlying mechanisms.

To test the oncogenic role of AR polymorphisms, and the function of somatic mutations in disease progression, we have converted the mouse AR gene to the human sequence, by gene targeting in embryonic stem cells. The mouse and human ARs are nearly identical in DNA and ligand binding domains, but diverge by 15% in the N-terminal transactivation domain, including in the extent and position of a polymorphic glutamine (Q) tract. The length of this tract is inversely correlated with tumor risk in man and receptor transactivation ability in vitro. The effect of AR alleles differing in glutamine tract length (12Q, 21Q and 41Q) is being tested in the context of the transgenic mouse tumor model, TRAMP, developed by Norman Greenberg. This also allows us to examine mutations that arise in AR during progression, in order to confirm and extend Greenberg’s observation that AR mutations in TRAMP tumors occur in distinct receptor domains dependent on hormonal status. An intriguing result thus far is that in intact mice the mAR and 21Q hAR alleles allow similar PCa progression, whereas the 41Q hAR is more resistant to tumor development, in accord with human epidemiology. Thus these mice may reveal the basis of Q tract differences in PCa initiation. In contrast, in castrated mice, both human alleles produce more aggressive disease than mAR, suggesting N-terminal sequences other than the Q tract may influence androgen-independent progression. These mouse models may accentuate androgen-dependent and –independent disease and may distinguish roles of AR that vary with disease stage and liganded state of receptor.
Appendix 2.5 – abstract for Interprostate S.P.O.R.E. meeting, 1/05

Genetic Variation of the Human Androgen Receptor and Prostate Cancer in Mice.
Megan Albertelli, Arno Scheller, Michele Brogley, Orla O’Mahoney and Diane M. Robins.
Dept of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109-0618.

Despite diverse genetic and environmental influences, prostate tumors initially depend on androgens for growth, reflecting crucial roles of the hormone receptor (AR) in gene regulation. Because of its importance in tumor progression, AR is a key target in therapy. Genetic polymorphisms in AR have been associated with risk, and somatic mutations occur in late stage disease. The significance of these variations has been difficult to assess, in part due to lack of animal models that address underlying mechanisms.

To test the oncogenic role of AR polymorphisms, and the function of somatic mutations in disease progression, we have converted the mouse AR gene to the human sequence, by gene targeting in embryonic stem cells. The mouse and human ARs are nearly identical in DNA and ligand binding domains, but diverge by 15% in the N-terminal transactivation domain, notably in the extent and position of a polymorphic glutamine (Q) tract. The length of this tract is inversely correlated with tumor risk in man and receptor transactivation strength in vitro. The effect of AR alleles differing in glutamine tract length (12Q, 21Q and 41Q) is being tested in the context of the transgenic mouse tumor model, TRAMP, developed by Norman Greenberg. This also allows us to examine mutations that arise in AR during progression, in order to confirm and extend Greenberg’s observation that AR mutations in TRAMP tumors occur in distinct receptor domains dependent on hormonal status. We have noted that in intact mice the mAR and 21Q hAR alleles allow similar PCa progression, whereas the 41Q hAR is more resistant to tumor development, in accord with human epidemiology. In contrast, in castrated mice, both human alleles produce more aggressive disease than mAR, suggesting N-terminal sequences other than the Q tract may influence androgen-independent progression. Level of AR expression in these tumors is being compared in relation to level of SV40 T-antigen expression, and their growth rates are being tracked by MRI. In addition, we are examining effects of antagonist treatment on the humanized AR mice, to determine how mutations in AR vary with treatment. These mouse models may accentuate androgen-dependent and –independent disease and may distinguish roles of AR that vary with disease stage and liganded state of receptor.
Appendix 2.6 – abstract for Endocrine Society, 6/05

Replacing the Mouse Androgen Receptor with Human Alleles Varying in Glutamine (CAG) Tract Length

Megan Albertelli, Arno Scheller, Mara Steinkamp, Michele Brogley, Diane Robins

Within the androgen receptor (AR), polymorphic variations in the length of an N-terminal glutamine (Q) tract (CAG repeat) are associated with disease states including prostate cancer, infertility and, for extreme lengths, spinobulbar muscular atrophy. To determine how AR’s Q tract length may influence prostate cancer initiation or progression and whether acquired mutations lead to androgen independence, we have converted the mouse AR sequence to that of man.

While mammalian ARs are nearly identical in the DNA and ligand binding domains, they differ by about 15% in the N-terminal amino acid sequence. The mouse AR Q tract is shifted by about 100 amino acids carboxy-ward of the human position and is disrupted by several histidines, while a glycine tract before the DBD is greatly abbreviated. To “humanize” the mouse AR gene, we created a targeting vector in which the human AR N-terminus (amino acids 35-466) replaced the equivalent mouse region of exon 1, and was flanked by mouse 5’ untranslated and first intron sequences derived from genomic 129/SV DNA. Homologous recombination in embryonic stem cells resulted in an AR transcription unit in which human coding sequences are controlled by mouse regulatory elements.

Three mouse lines were created with short, average, or long Q tracts (12, 21, and 41 Q). At a gross level, humanized AR mice display normal fertility, body weight, reproductive anatomy, and behavior, indicating that human AR can functionally replace the mouse gene. Testosterone levels and prostate histology are not significantly different between glutamine tract variants at six months of age. However, studies of AR levels and expression of AR target genes show subtle differences between the glutamine tract variants. Further, studies evaluating the effect of Q tract length on prostate tumor onset and progression in the transgenic TRAMP model amplify the differences between these variants and in comparison to wild type mouse AR. Therefore, variation in glutamine tract length within the “normal” range may not greatly impact steady-state physiology, but may play a role in androgen-dependent diseases such as prostate cancer. These humanized AR mice are useful for modeling the role of the AR N-terminus in disease and for developing new AR-targeted therapeutic agents.
Human Androgen Receptors Varying in Q-Track Length Differentially Influence Initiation and Progression of Prostate Cancer in Mice. M. Albertelli, A. Scheller, M. Brogley, O. O’Mahoney, M. Steinkamp, J. Tosoian, and D. M. Robins. Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109-0618

The androgen receptor (AR) plays a critical role in prostate development and disease, and differences in AR function are likely to impact these processes. Variable length of the N-terminal glutamine (Q) tract (CAG repeat) is linked to infertility and, for extreme lengths, spinobulbar muscular atrophy, but association with prostate cancer risk has been controversial. To assess the functional significance of the Q-tract polymorphism in an animal model, we converted the mouse AR gene to the human sequence by germline gene targeting.

The mouse and human ARs are nearly identical in their DNA and ligand binding domains, but diverge by about 15% in the N-terminus. The mouse AR Q tract is displaced relative to human and disrupted by several histidines, and a glycine tract near the DBD is greatly abbreviated. We created genomic targeting vectors in which amino acids 34 to 467 of mAR exon 1 were replaced with the equivalent hAR region, including tracts of 12, 21 or 41 Qs. Homologous recombination in ES cells placed these human AR coding sequences under the control of mouse regulatory elements. The three “humanized” mouse lines are normal in fertility, growth, and behavior. While subtle differences in particular traits and target gene expression were detected between mAR and hAR lines, and between Q-tract alleles, they all fell within a normal range of variation.

To test the effect of Q-tract length on prostate cancer, mice were crossed with the oncogene-induced TRAMP model, created by Norm Greenberg. In intact male progeny, the age of tumor initiation and length of disease was similar for mAR and 21Q hAR alleles. Tumors in 41Q mice, however, were significantly delayed whereas those in 12Q mice arose earlier. In 41Q mice, the time from initiation to death was also significantly prolonged. When mice were castrated at 12 weeks, prior to detection of overt cancer, a subset exhibited early aggressive disease while many were delayed relative to intact. The proportion initiating early vs. late varied with the AR allele, despite the absence of hormone. Further, castrated 12Q mice developed disease significantly later than 21Q and 41Q groups. The striking difference in disease course for AR alleles in the absence of androgen suggests Q-tract length also affects androgen-independent growth, or Q-tract-dependent events that determine cancer progression occur prior to 12 weeks of age. Mechanisms underlying these differences are being explored.

This study corroborates, in an animal model, epidemiological data correlating Q-tract length with prostate cancer initiation and progression. In man, detection is complicated by numerous additional quantitative traits. The homogeneous mouse background reveals the effects of variant genes under conditions of stress (e.g., cancer) that are not evident in the steady state. This AR allelic series should prove useful in discerning mechanisms of androgen resistance.
Variation in Q-tract Length of the Androgen Receptor Influences Initiation and Progression of Prostate Cancer in “Humanized” AR Mice.

M. A. Albertelli, A. Scheller, M. Brogley, O. A. O’ Mahony, M. P. Steinkamp, J. Tosoian, and D. M. Robins.  Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI  48109-0618

The androgen receptor (AR) plays a critical role in prostate development and disease. Length of a polymorphic N-terminal glutamine (Q) tract correlates inversely with transcriptional strength and has been associated with prostate cancer risk, although this is controversial. To assess the functional significance of Q-tract polymorphism in an animal model, we converted the mouse AR gene to the human sequence by germline gene targeting.

Mouse and human ARs are nearly identical in the DNA and ligand binding domains, but differ by 15% in the N-terminus. In addition, mAR’s Q tract is displaced relative to hAR and disrupted by histidines, while a glycine tract is abbreviated. mAR exon 1 was replaced by homologous recombination with the equivalent hAR region, with 12, 21 or 48 Qs. The three resulting “humanized” mouse lines showed normal growth, fertility and behavior. Subtle differences in physiological traits and target gene expression between genotypes fell within normal biological variation.

To test the effect of Q-tract length on prostate cancer, mice were crossed with the oncogene-induced TRAMP model. In intact male progeny, the age of tumor initiation and length of disease was similar for mAR and 21Q hAR alleles. Remarkably, tumors in 48Q mice were significantly delayed whereas those in 12Q mice arose earlier. In 48Q mice, the time from initiation to death was also significantly prolonged. When mice were castrated at 12 weeks, prior to detection of overt cancer, a subset exhibited early aggressive disease while many were delayed relative to intact. The proportion of tumors initiating early vs. late varied with AR allele, despite the absence of hormone. Further, castrated 12Q mice developed disease significantly later than 21Q and 48Q groups. The striking difference in disease course for AR alleles in the absence of androgen suggests Q-tract length affects androgen-independent growth. Mechanisms underlying these differences, such as Q tract effects on ligand-independent AR activity, are being explored.

This study corroborates in an animal model epidemiological data correlating Q-tract length with prostate cancer initiation and progression. In man, detection is complicated by numerous additional quantitative traits. The homogeneous mouse background reveals the effects of variant genes under conditions of stress (e.g., cancer) that are not evident in homeostasis. This AR allelic series should prove useful in discerning mechanisms of androgen resistance.
Fig. 1 – Initiation and Progression of Prostate Cancer in Intact Q Tract Variant h/mAR Mice. Male progeny from TRAMP sires crossed to dams heterozygous for wild type mouse AR (mAR, blue) and one of three humanized AR alleles [21Q (h21, green), 48Q (h48, yellow) or 12Q (h12, red)] were palpated weekly for tumor growth. The top panel indicates percent of mice at indicated times with no palpable tumor (tumor-free). Lower panel indicates survival (i.e., age at death from prostate disease) for these same mice.
**Fig. 2 – Initiation and Progression of Prostate Cancer in Castrated Q Tract Variant h/mAR Mice.** A cohort of mice as in Fig. 1 were castrated at 12 weeks of age and tumor progression followed by palpation for age of palpable tumor (top panel) compared to survival (lower panel). TRAMP mice with wild type mAR are shown in blue, 21Q-h/mAR mice in green, 48Q-h/mAR in yellow, and 12Q-h/mAR in red.

**Tumor Incidence: Castrated Mice**

**Survival: Castrated Mice**
**Fig. 3 – Mean Length of Disease Differs in Q Tract Mice.** For the mice in Fig. 1 and 2, length of disease was calculated as time from first palpable tumor to time of death. In intact mice, both long and short Q tracts lead to significantly longer disease course than wild type mouse or human AR. Disease in castrated mice generally progresses much more quickly, although tending to somewhat slower in the short Q tract mice.

**Fig. 4. Histogram comparing tumor phase at 29 weeks of age.** As a way of comparing progression across groups, intact (upper panel) and castrated (lower panel) mice are compared with regard to whether they have no detectable tumor (blue), have a palpable tumor (yellow) or have already died of prostate cancer (red). The effect of Q tract length is evident, oppositely for intact and castrated mice.
**Fig. 5 – Immunohistochemical Staining with Anti-AR antibody of TRAMP Tumors.** A tissue microarray was prepared by the University of Michigan Microarray Core from fixed and embedded prostate tumors of TRAMP mice. Microarray blocks were sectioned and slides stained with various antibodies following standard procedures. Below as an example is the AR staining for several tumors from intact [I] or castrated [C] mAR, 21Q-h/mAR and 48Q-h/mAR mice. AR staining is very distinct in well-differentiated tumors but is less so and heterogeneous in poorly differentiated tumors. We are currently correlating tumor grade and staining patterns with time of initiation and aggressivity of disease progression per mouse within each genotype.
**Fig. 6 – Location of Somatic Mutations in AR Arising During Progression in 21Q-h/mAR Mice.** The upper panel shows a diagram of AR domains, with PCR primers indicated as paired colored arrows. 10 AR cDNAs were sequenced, from two independent reverse transcriptase reactions, from each of 10 tumors from intact and castrated 21Q-h/mAR mice. Roughly shown are positions of mutations that were found multiple times within a tumor (balls) or in multiple tumors (stars). Below is a portion of N-terminal AR sequence showing specific mutations, and extensive variation around the Q-tract itself. Q70R is a particularly interesting mutation that has been noted and functionally characterized as gain-of-function in a PCa patient [10].

E.g. - Q70R – repeated in intact mice, and found in a patient before therapy. Q tract disrupted by leu has higher transactivation despite lower N/C interaction and lower protein levels [10].
REPLACING THE MOUSE ANDROGEN RECEPTOR WITH HUMAN ALLELES DEMONSTRATES GLUTAMINE TRACT LENGTH DEPENDENT EFFECTS ON PHYSIOLOGY AND TUMORIGENESIS IN MICE

Megan A. Albertelli, Arno Scheller, Michele Brogley, and Diane M. Robins

Department of Human Genetics, University of Michigan Medical School. Ann Arbor, Michigan 48109-0618

Corresponding author:
Diane M. Robins
Department of Human Genetics, 4909 Buhl Bldg
University of Michigan Medical School, Ann Arbor, MI 48109-0618
Phone: 734-764-4563; Fax: 734-763-3784; Email: drobins@umich.edu

Running Title: Q Tract Variants in Humanized AR Mice

Keywords: androgen receptor, polyglutamine tract, testis, prostate, humanized mice

(Required statement from NIH will be added to the final manuscript.)
ABSTRACT

Polymorphism in length of the N-terminal glutamine (Q) tract in the human androgen receptor (AR) has been implicated in affecting aspects of male health from fertility to cancer. Extreme expansion of the tract underlies Kennedy disease, while the AR Q tract length in vitro correlates inversely with transactivation capacity. However, whether normal variation influences physiology or the etiology of disease has been controversial. To assess directly the functional significance of Q tract variation, we converted the mouse AR to the human sequence by germline gene targeting, introducing alleles with 12, 21 or 48 glutamines. These three “humanized” (h/mAR) mouse lines were grossly normal in growth, behavior, fertility and reproductive tract morphology. Phenotypic analysis revealed subtle trends, particularly for the 48Q mice in which increased body fat and lower seminal vesicle weight suggested borderline androgen insensitivity. Upon molecular analysis, tissue specific differences in AR levels and target gene expression were detected between mAR and h/mAR lines, and between Q tract alleles, but largely within the range of biological variation. However, when crossed with transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, striking genotype-dependent differences in prostate cancer initiation and progression were revealed. This link between Q tract length and prostate cancer establishment, likely due to differential activation of key AR target genes, such as Nkx3.1, corroborates human epidemiological studies. This h/mAR allelic series in a homogeneous mouse genetic background allows testing of numerous physiological traits for Q tract influences, and provides an animal model to test novel drugs targeted specifically to the human AR.
Abbreviations used:

AR, androgen receptor
hAR, human androgen receptor
mAR, mouse androgen receptor
h/mAR, humanized androgen receptor
Q tract, polyglutamine tract
G tract, polyglycine tract
INTRODUCTION

The androgen receptor (AR) is a recently evolved, highly conserved member of the superfamily of nuclear receptor transcription factors (1). In mammals, AR is responsible for male primary and secondary sexual differentiation, and also influences numerous physiological processes not directly linked to reproduction. Moreover, development, homeostasis and tumorigenesis of the prostate depend on androgen acting via its receptor. Like the other nuclear receptors, AR function relies on three major structural regions: the most conserved central DNA binding domain (DBD), the moderately conserved C-terminal ligand binding domain (LBD), and the N-terminal transactivation domain (NTD) that bears little similarity among receptors. Because the AR gene (Ar) is located on the X chromosome, males are hemizygous and mutations are phenotypically evident. This allows extensive receptor structure/function correlations to be drawn from cases of partial to complete androgen insensitivity (2). Polymorphisms of Ar that influence its function within a nonpathological range may underlie phenotypic variation in male traits and could modify susceptibility to androgen-dependent diseases.

The most studied polymorphisms in the NTD of the AR are variations in length of two polyamino acid tracts comprised of glutamine (Q) or glycine (G) residues (3, 4). Glutamine rich regions are functionally important domains in a number of transcription factors, including Sp1, TATA binding protein (TBP) and glucocorticoid receptor (GR) (5). The human AR polyglutamine tract, encoded by a CAG trinucleotide repeat, has 9 - 37 residues in the normal population, with the majority between 15 and 30, while the G tract, encoded by GGN repeats, varies between 8 and 18 residues (6). The Q tract has received significant attention, in part because expansion of the CAG repeat beyond 40 residues is associated with the late-onset neurodegenerative disease, spinal and bulbar muscular atrophy (SBMA, Kennedy disease) (7).
The mechanisms by which Q tract length affects AR function have been well explored experimentally. Expanded Q tract lengths result in decreased mRNA and protein levels, both in vivo and in vitro (8). Transfection studies that take into account differences in AR levels have shown an inverse relationship between Q tract length and AR transactivation (9-14), with one study linking maximum AR activity to a tract length of 15 or 17 Qs (15). The greater transcriptional efficacy for shorter Q tract lengths may sum over several activities, including increased interaction between the AR N terminal and ligand binding domains, and increased association with p160 coactivators or SWI/SNF chromatin remodeling components (9, 16, 17). Finding Q tracts in numerous transcription factors also suggests they may be sites for cofactor interactions. A candidate coactivator for AR, the Ras-related nuclear protein (RAN)/ARA24, interacts directly with the Q tract, with both interaction and consequent AR activation diminished with increasing Q tract length (18).

While extensive expansion of the Q tract in man results in SBMA, it is argued whether variation within the normal range has a detectable physiological outcome or influences androgen-dependent disease. Longer Q tract length has been associated with defective spermatogenesis and male infertility in some populations (19, 20). Associations have been found between Q tract length and body fat mass (21), testosterone levels (22) and HDL cholesterol levels (23). Greater effort has focused on discerning an effect in prostate cancer, especially since shorter tracts occur in the higher risk African-American population (24). While several studies link shorter Q tract length with increased risk, earlier age of onset, or greater aggressivity of prostate cancer (24-28), other studies have found no association (29-37). These conflicting results may be due to patient selection bias, small sample size, genetic heterogeneity of the human population, or confounding gene-gene or gene-environment interactions.
To address whether the Q tract plays a role in male phenotypic variation or disease origins, we created a mouse model containing human AR alleles varying in Q tract length. To enhance relevance to human studies, we swapped the entire human and murine AR NTDs, which diverge about 15% in N-terminal amino acid sequence. Further, mAR has a Q tract disrupted by several histidines and displaced 120 amino acids towards the C terminus, and a greatly abbreviated G tract (38). In contrast, the DBD and LBD are identical between man and mouse, excluding a few amino acid differences in the hinge region. Reduced transcriptional activity of rodent relative to human AR in vitro has been mapped to the NTD (39), suggesting sequence differences therein are functionally significant. The mouse NTD was replaced by homologous recombination in embryonic stem cells to generate three lines of “humanized” AR mice containing short (12Q), median (21Q) and long (48Q) glutamine tract lengths. At a gross level, these mice were indistinguishable from wild type littermates. Upon molecular analysis, some subtle tissue specific distinctions occurred between alleles, but within the range of normal biological variation. However, when the homeostatic balance was perturbed by introduction of an oncogenic transgene, dramatic allele-specific effects became evident in initiation and progression of prostate cancer. This AR allelic series in genetically homogenous mice provides a model to assess the controversial role of the Q tract in the etiology of disease.

RESULTS

Generation of Humanized AR Mice

Humanized AR (h/mAR) mice were created by recombining a DNA fragment encompassing most of human Ar exon 1 (from amino acid 31 to 484) with the mouse Ar gene
(Fig. 1). Targeting vectors to accomplish this included a selectable marker for neomycin resistance \((\text{neo}')\) flanked by loxP sites (40). A fragment of mouse chromosomal \(Ar\), containing 4.3 kb 5’ flanking sequence, the entire exon 1 coding region and 120 bp of intron 1, was cloned on one side, and 2 kb of the contiguous intron 1 sequence on the other side of the oppositely oriented \(\text{neo}\) expression cassette. Human AR cDNA sequences (kindly provided by M. McPhaul and M. Marcelli, (41)) were exchanged via conserved restriction sites, resulting in h/mAR exon 1 embedded within mouse chromosomal (regulatory) sequence and differing from hAR in only 4 codons (Fig. 1A). Three targeting vectors were created containing 12, 21, or 48 Q residues in the N-terminal tract, as the median human Q tract has 21 residues while tract lengths of 12 and 48 Qs represent extremes. Although 50 Q can result in SBMA in man, introduction of transgenic ARs with as many as 65 Qs have not led to detectable phenotypes in mice, where gene defects often must be more severe to model a human syndrome (42).

Since the mouse AR fragment was obtained from a J1 embryonic stem (ES) cell DNA library, CJ7 ES cells that derive from the same 129/Sv mouse substrain were selected to optimize recombination (43). Correctly targeted clones were identified by Southern blot (Fig. 1B), examined for euploidy, and transiently transfected with cre recombinase to remove the \(\text{neo}\) cassette. Two independent ES cell clones per Q tract allele were injected into C57BL/6J blastocysts to create chimeric animals. Male chimeras were bred to C57BL/6J females and daughters carrying the targeted allele were bred to C57BL/6J males. Progeny from this cross were genotyped (Fig. 1C, D) and h/mAR males were bred to heterozygous females to generate the h/mAR males, mAR males, and homozygous h/mAR female mice used in this study.

**h/mAR Mice are Grossly Normal and Fertile**
Neither sex of h/mAR mice showed any remarkable differences from mAR littermates in appearance or behavior, despite a reported two-fold greater transcriptional activity of the human receptor (39, and our unpublished data). h/mAR males were fertile, as were homozygous females, and had grossly normal reproductive tract anatomy, indicating full functionality of the h/mAR gene in the mouse. Since Q tract length may affect fertility in men (14, 44, 45), h/mAR alleles were compared by breeding homozygous h/mAR females to males with the same AR genotype. Each h/mAR allelic variant produced similar numbers of pups per litter (Table 1) at similar frequency (not shown), with average numbers similar to those reported for the C57BL/6J and 129 parental strains (46).

Body mass and composition in men have been reported to be affected by Q tract length (21, 47), therefore body weights of male h/mAR mice and wild type littermates were measured at 3 weeks, 9 weeks, 6 months and 18 months of age. No statistically significant differences were found at any time point, although 12Q-h/mAR and 48Q-h/mAR mice consistently weighed more than 21Q-h/mAR mice (Table 1). Interestingly, body composition analysis performed by dual-energy X-ray absorptiometry (DEXA) of male 24 month old 21Q-h/mAR and 48Q-h/mAR mice showed a trend toward higher percentage body fat in the latter (P = 0.08) while total body weight remained similar (Table 1). This could indicate that the purportedly weaker 48Q AR leads to a reduction in androgen activity, and/or possibly reduced estrogen opposition, increasingly with age.

To determine whether hormone levels were affected, either directly or by feedback mechanisms, testosterone was measured in serum of male h/mAR mice and mAR littermates at 6 months of age. Testosterone levels varied widely between individuals and for the same individuals tested at different times, as reported previously for mice (48), masking an ability to
detect statistically significant differences (Table 2). Therefore, as a more direct physiological marker of testosterone activity (49, 50), seminal vesicle weight was measured at 6, 18 and 24 months of age (Table 2). Increasingly with age, 12Q-h/mAR mice had higher mean seminal vesicle weights and 48Q-h/mAR mice had lower mean seminal vesicle weights, although this again was of borderline statistical significance. Nevertheless, similar to body composition, this could indicate slightly reduced AR activity in the long Q tract mice and increased activity in the short Q tract, in accord with in vitro analysis.

**Testis Physiology of h/mAR Mice is Normal but Variations Occur at the Molecular Level**

h/mAR activity in the testis was examined as morphology and function of this organ, in both spermatogenesis and androgen synthesis, is highly sensitive to androgen regulation. Testes from 6 months old h/mAR mice and mAR littermates (n=6 per genotype) were fixed, sectioned, stained with hemotoxylin and eosin (H & E) and examined by light microscopy. Morphology was normal for all h/mAR alleles, with seminiferous tubules similar in size and density to those in mAR mice, normal interstitial cells and presence of all stages of spermatogenesis (Fig. 2A). Upon immunohistological examination for AR, strong nuclear staining was present in interstitial and Sertoli cells in mAR and h/mAR mice (Fig. 2B).

Because AR expression is affected by Q tract length in vitro (8), AR mRNA levels were measured by real-time PCR. In 6 months old mice, testis AR mRNA was 1.3 - 1.6 fold higher in all h/mAR compared to mAR mice, with the most statistically significant difference occurring between mAR and 21Q-h/mAR mice (P < 0.05) (Fig. 3A). Testis AR protein levels, determined by western blotting of crude lysates, were also somewhat higher in h/mAR than mAR mice (Fig. 3B). As the expanded Q tract in SBMA leads to AR aggregation, the pellet fraction of the total
protein isolate was examined to assess levels of protein complexes or denatured protein. In fact, 48Q-h/mAR was more abundant in the pellet than supernatant fraction, suggesting possibly problematic protein folding. However, cytoplasmic particles or intranuclear aggregates were not detected in testis by immunohistochemistry (Fig. 2B), nor in muscle, although an equivalent 113Q-h/mAR knock-in allele produced substantial aggregation ((51), Lieberman et al., in preparation). Thus the 48Q-h/mAR may have a capacity for increased aggregation, which may be exacerbated during cell lysis and sample preparation.

To pursue further a basis for the subclinical androgen insensitivity in the 48Q-h/mAR mice, we compared Leydig cell function to that in testicular feminized (tfm) animals that lack functional AR. Leydig cells in tfm mice fail to differentiate full mature function, and so are deficient in adult androgen synthesis (52). Testis RNA was analyzed by semiquantitative RT-PCR for expression of the testosterone synthetic enzymes Hsd3b1, a marker of fetal Leydig cell function, and Hsd17b3, characteristic of mature Leydig cells (53) (Fig. 4). While Hsd3b1 mRNA did not vary significantly with AR allele, Hsd17b3 levels did. mAR, 12Q-h/mAR, and 21Q-h/mAR mice expressed similar levels of the adult Hsd17b3, which were significantly higher than tfm levels (P < 0.05), as expected for normal adult Leydig cell populations. The 48Q-h/mAR mice had levels of Hsd17b3 intermediate to the tfm and other mice, although the difference to any other genotype did not reach statistical significance, similar to trends in other traits noted above.

**h/mAR Prostates Appear Normal but Expression of AR and Target Genes is Slightly Altered**
Prostates of h/mAR mice were examined as AR plays a critical role in development of this organ and the Q tract has been implicated in its tumorigenesis. At the light microscopy level, prostates from 6 months old h/mAR mice and mAR littermates (n=6 per genotype) appeared normal in all lobes (Fig. 5A). Low levels of hyperplasia and mouse prostatic intraepithelial neoplasia (PIN) increase with age (54), but in a preliminary scan of prostates from 12, 18 and 24 month old males (n=3 per genotype) the levels of hyperplasia and PIN were equivalent in mAR and h/mAR mice (not shown). When stained for AR by immunohistochemistry, mAR and h/mAR prostates both showed strong nuclear staining in epithelia and stroma, as expected (Fig. 5B).

To probe molecular regulation, expression in the prostate of mRNAs for AR, Foxa1 and Nkx3.1 were measured by real-time RT-PCR in 6 months old h/mAR mice and mAR littermates. Foxa1 is indirectly regulated by AR but interacts with AR in transcriptional regulation of numerous other prostate genes (55, 56), whereas Nkx3.1 is a prostate-specific homeobox factor under direct transcriptional AR control (57). In contrast to testis, h/mAR mice had somewhat lower levels than mAR mice of AR mRNA in prostate, that appeared to correlate inversely with Q tract length, but again this was below the level of statistical significance (Fig. 6). Foxa1 expression was uniform across genotypes, suggesting little sensitivity to AR levels or activity. Despite h/mAR levels being somewhat less than mAR, Nkx3.1 expression was similar in mAR, 12Q-h/mAR, and 21Q-h/mAR mice. 48Q-h/mAR mice, however, had significantly lower Nkx3.1 expression compared to mAR mice (P < 0.05), suggesting that the longer Q tract led to decreased AR activation, a finding in accord with in vitro data. There was a broad range of Nkx3.1 expression in the 12Q-h/mAR mice, with most at or significantly above mean levels,
while a subset were consistently lower. The basis of this variability is not clear but was also seen in additional mice (not shown).

**Q Tract Length Affects Prostate Tumor Initiation in a Transgenic Model**

Prostate cancer does not occur spontaneously at an appreciable level in mice, even with h/mAR alleles. Therefore, in order to investigate the effect of Q tract length in oncogenesis, h/mAR females were crossed with a transgenic model of prostate adenocarcinoma, TRAMP (58). TRAMP mice carry the SV40 T antigen (Tag) driven by a prostate epithelium-specific probasin promoter. Males develop PIN by 12 weeks of age and die of prostate cancer within 4 months to one year. h/mAR-TRAMP mice were followed for tumor development by abdominal palpation performed weekly by two independent observers. Mice were euthanized when moribund and prostate tumors and metastases preserved for analysis. We compared disease status at 29 weeks of age, since at this time about half of the mice had evident disease; complete survival curve data and additional analyses will be presented when all mice have succumbed. At 29 weeks, 65% of wild type mAR mice and 52% of 21Q-h/mAR littermates had a palpable tumor or had died due to prostate cancer. In contrast, overt disease was present in 100% of 12Q-h/mAR mice and only 28% of 48Q-h/mAR mice (Fig. 7). Because the time from palpation of tumor until death was generally only a few weeks, differences in numbers of mice that died per group may be less informative than comparing the sum of mice with palpable cancer and cancer death to those without palpable tumor. Thus while cancer in the mice with an average human Q tract length AR progressed similarly as in wild type mice, the short Q tract AR resulted in significantly earlier tumor development while the long Q tract appeared protective. This striking dependence of disease progression on Q tract length corroborates several epidemiological studies, and
discerns a difference not detected in others, probably accentuated by the relatively homogeneous mouse genetic background. Further, modest AR-dependent gene expression differences in the prostate, such as detected for Nkx3.1 and suggested for AR itself, likely contribute to these Q tract effects in oncogenesis.

DISCUSSION

Whether AR Q tract length variation in a nonpathogenic range influences androgen-dependent traits ranging from fertility to prostate cancer has been difficult to resolve due to conflicting epidemiological data and lack of experimental models. In order to test Q tract length effects directly, we “humanized” the murine AR by replacing exon 1 with the equivalent human region, including 12, 21 or 48 CAG repeats. The resulting AR variant strains support proper anatomy, reproduction and behavior, indicating these h/mARs are fully functional in mice, regardless of differences in transcriptional capacity in vitro (39, 41). However, detailed analyses reveal allele-specific variance, albeit within a normal range, in some physiological indicators. Moreover, when homeostasis is perturbed by activation of an oncogenic pathway, remarkable Q tract length-dependent disease progression is evident.

In creating these mice, we chose Q tract lengths at the extremes of those found in the normal human population to optimize the ability to obtain informative phenotypes. Further, in models of pathology mice often require more severe alleles than those that cause the disease in man. A relevant example is Kennedy disease, which in man is caused by expansion of the AR Q tract beyond 40 residues, but in mice an AR cDNA transgene containing 65 CAG repeats produces no abnormal phenotype (42). Other polyglutamine expansion disorders such as
Huntington disease are also not obtained in mice by modest Q tract expansions expressed at endogenous levels (59, 60). Absence of severe phenotypes, as well as stability of CAG repeat number over time unlike in man, may be augmented by the short lifespan of the mouse. The 48Q-h/mAR mice show no muscle weakness or wasting with age, and have morphologically normal testes with no Sertoli cell pathology or disruption of spermatogenesis. In contrast, these traits are exhibited by 113Q-h/mAR mice created in parallel, which prove to be an effective model of Kennedy disease ((51) and A. Lieberman, unpub.).

While the 48Q-h/mAR mice show no evidence of Kennedy disease, they are at the low end of the normal range (compared to 21Q-h/mAR mice and mAR littermates) for some androgen-dependent traits, such as seminal vesicle weight. By comparison, the 12Q-h/mAR mice are at the high end of the normal range in seminal vesicle weight. Body fat percentage is slightly increased in 48Q-h/mAR mice, similar to men with longer Q tracts (21), but this does not develop into late-onset obesity as observed in AR null male mice (61). These traits likely stem, directly or indirectly, from the reduced transcriptional activity of the longer Q tract AR.

Nevertheless, the 48Q-h/mAR still has sufficient potency to produce healthy fertile males essentially indistinguishable from the other Q tract variant mice or their wild type littermates. The reduced activity may be compensated for by numerous factors responsive to hormone levels and to feedback mechanisms within the organism.

At the molecular level, AR expression and downstream regulation differs somewhat between these mouse lines, dependent on organ site. In testis, there is slightly more AR mRNA with h/mAR alleles than for wild type mouse littermates, corresponding to somewhat higher AR protein levels. In prostate, the inverse difference appears, with mAR mRNA levels somewhat higher than for the humanized alleles. Since the genes differ only within exon 1, this may reflect
regulatory elements that differ between man and mouse within the coding region, operating at transcriptional or post-transcriptional levels in cell-specific manners. Further, mouse cofactors that interact with the NTD may have differential interactions with human AR, affecting autogenous as well as downstream differences. In the prostate, where the AR mRNA levels are equivalent amongst the humanized alleles, expression of the AR target gene Nkx3.1 is sensitive to Q tract length. Q tract length effects are likely to vary between tissues as they do between cell lines, suggesting involvement of differentially expressed accessory factors (10). Again, however, these differences are not sufficient to manifest at the light microscope level and the prostates appear normal.

The clinically important question of whether Q tract length influences prostate cancer risk has been difficult to confirm in man due to factors including genetic heterogeneity, limited statistical power, and bias to earlier detection of pathology by increasing use of PSA testing (34, 62). To test experimentally Q tract association, a prostate-targeted transgenic oncogene was introduced by crossing the humanized AR mice to TRAMP (58). In contrast to physiological traits of h/mAR mice that have intriguing variations but rarely reach statistical significance, the Q tract length dramatically affects prostate tumor initiation or early stage growth. At a time point of 29 weeks, when about half of the mAR and 21Q-h/mAR mice have palpable tumors or have died, all of the short Q tract mice have tumors but only a quarter of the long Q tract mice do. This striking inverse correlation between Q tract length and detection of disease becomes more pronounced over time, as 48Q-h/mAR mice can live longer than 1 year prior to tumor development (Albertelli et al., in prep). This result in a genetically simplified model corroborates the notion that Q tract length impacts initiation and/or progression of prostate cancer.
Existing evidence is consistent with the idea that differences in receptor transcriptional activity mediate the effect of AR Q tract length variation. These differences could be modest but additive over time, since the increased risk to prostate cancer caused by androgen exposure is cumulative over the lifespan. In the TRAMP model, an obvious candidate for a critical sensor of differential AR strength is the androgen-dependent probasin promoter driving the T-antigen oncogene. The 12Q-h/mAR might produce critical levels of the oncogene transcript earlier, and conversely the 48Q-h/mAR might lag in this process, relative to the other alleles. While this differential gene activation can be shown in vitro, it is difficult to demonstrate in vivo at the relevant early time points and in a subset of cells, when the oncogenic event must actually occur. At later times, T-antigen levels in tumors are similar amongst strains of h/mAR mice (not shown), in agreement with other studies in mouse cancer models that find little correlation between level of expression of transgenic oncogene and tumor progression (63).

While prostate cancer in man does not initiate with T-antigen, early disease is androgen-dependent and downstream events are similar for both. Thus differential activation of T-antigen by Q tract length variants of AR may not be simply a trivial explanation of the effects we see but instead analogous to differential oncogenic activation in man, where the androgen-dependent causative events are still unknown. A recent candidate for such an event comes from finding common translocations in prostate cancer that place members of the ETS transcription factor family under the control of androgen-dependent promoters (64). Further, additional stochastic androgen-dependent events are required for tumorigenesis and differential activation of multiple genes are likely involved. We have shown Q tract length inversely correlates with differential activation of Nkx3.1, a factor critical for both prostate differentiation and oncogenesis. Finding reduced Nkx3.1 expression in the 48Q-h/mAR mice that are more resistant to tumorigenesis may
be somewhat paradoxical since loss of Nkx3.1 function is frequently associated with prostate cancer (65). However, both tumorigenesis and activation of Nkx3.1 downstream target genes are exquisitively sensitive to gene dosage in mice, and it is not clear when these differences in Nkx3.1 levels exert an effect or in which of many interacting pathways. It may be that in our model, Nkx3.1 levels accurately indicate AR transcriptional strength, but are less critical for TRAMP tumorigenesis where potent T-antigen dominance may supplant usual cooperative mechanisms. Identification of additional androgen-dependent targets sensitive to Q tract length by expression array analysis may shed light on this question.

For the most part, the h/mAR Q tract variant mice show only subtle differences in physiological or molecular characteristics, within the range of normal phenotypic variation. Yet when the homeostatic balance is upset by the stress of cancer, this variation is amplified and distinct patterns of disease progression are produced. It may be that mechanisms that limit the effects of genetic variation (e.g., in AR activity) and buffer the organism from environmental change (e.g., in androgen levels) are abrogated by the global disregulation that accompanies oncogenesis. One such compensatory mechanism is provided by chaperone molecules. Intriguingly, HSP90 deficiency in Drosophila is known to reveal otherwise cryptic genetic variation (66). Since HSP90 interacts with AR, as well as most components of signal transduction cascades involved in development and cancer, it may play a role in amplifying subclinical phenotypes made manifest by stress. Another example of a phenotype unmasked by stress is in the steroid receptor coactivator-1 (SRC-1) deficient mouse, which has normal function of the hypothalamic-pituitary-steroidogenic axis until placed under chronic food deprivation (67). These allelic variations that lead to dramatic stress-response differences may
be more readily highlighted against the relative homogeneity of the mouse genetic background, again validating the utility of this genetic model.

These mice demonstrate that a functional difference in AR activity within the normal range of phenotypic variation can affect prostate cancer biology. Whether one or a few CAGs more or less would have a detectable effect in this model is impractical to test. In man there is evidence that receptors with Q tracts outside a critical range of 16-29 residues may mediate disease phenotype more significantly than length differences across the entire range (16). More important than the absolute length is determining the downstream effects of Q tract variation, which is possible in the experimental system of the h/mAR mice. On a broader level than just Q tract association, these mice represent an allelic series of ARs titrated for transactivational strength, and thus may provide clues to the endocrine basis of infertility and cardiovascular disease, as well as prostate cancer. Finally, these humanized AR mice provide a preclinical model for testing essential new antiandrogen therapies designed to overcome the critical problem of androgen-independent but AR-dependent prostate tumor growth.
ACKNOWLEDGEMENTS

We thank Dr. Norman Greenberg (Fred Hutchinson Cancer Center, Seattle, WA) for advice and encouragement throughout this study, Michael McPhaul (UT Southwestern University, Dallas, TX) and Marco Marcelli (Baylor College of Medicine, Houston, TX) for hAR cDNAs used in targeting vectors, Kent Christensen (University of Michigan, Ann Arbor, MI) for help assessing testis morphology, Andy Lieberman for numerous helpful discussions and assays comparing 48Q h/mAR mice to his 113Q h/mAR mice, Martin Sanda (Harvard University, Boston, MA) and Jennifer Loveridge for the gift of TRAMP mice and training in their abdominal palpation, and Shaema Khan, Jeff Tosoian, Salina Olmsted, and Jennifer Gerber for technical assistance. Ron Koenig and Gary Hammer provided helpful comments on the manuscript.

This work was supported by grants to D.M.R. from the DOD (DAMD17-02-1-0099), the NIH (NIDDK56356), and the NCI (S.P.O.R.E in Prostate Cancer, P50 CA69568). M.A. was supported by a NCRR/NIH training program (T32-RR07008). DNA sequencing by the University of Michigan Sequencing Core and blastocyst injections by the University of Michigan Mouse Models Core were supported by the University of Michigan Cancer Center Support Grant (5 P30 CA46592) and the Michigan Diabetes Research and Training Center (NIH5P60 DK20572).
MATERIALS AND METHODS

Construction of Targeting Vector and Generation of h/mAR Mice

Targeting vectors were constructed using the backbone vector pGEM5zf+LP1 (40), a mouse Ar genomic clone isolated from a J1 mouse ES cell library in phage EMBL3 (the library was kindly obtained from Tom Glaser, University of Michigan), and hAR cDNAs obtained from MJ McPhaul and M Marcelli (41). In the vector, a BamHI site within the loxP-PGKneo<sup>r</sup>-loxP cassette was eliminated, a HindIII site in the polylinker was converted to an NheI site to one side of the neo cassette, and BamHI and HindIII sites were introduced into the polylinker on the other side. This allowed insertion 3’ to the neo<sup>r</sup> cassette of a 5.8 kb SaeI-SpeI fragment, extending from upstream of mAR to 120 bp into intron 1; the contiguous 2 kb BamHI-HindIII fragment of intron 1 was placed 5’ to the cassette (see Fig. 1A). hAR sequences were introduced in place of mouse exon 1 coding information by exchanging homologous SmaI-Bsu36I fragments (nt 93-nt 1453 of 21Q-hAR, nt 93-nt 1433 of mAR). This fragment includes 88 amino acids that differ between man and mouse, not counting the polymorphic Q and G tracts, leaving one different mouse residue near the N terminus and three near the end of exon 1 in the now humanized AR exon 1. In the vector there are 4 kb of homology upstream and 2 kb downstream for recombination, with the neo<sup>r</sup> cassette oppositely oriented to Ar. Three vectors were created, with ARs containing 12, 21 or 48 glutamines in the Q tract.

CJ7 ES cells, derived from the same mouse substrain as provided the genomic library, were electroporated with vectors linearized at the MluI polylinker site. Over 1000 G418-selected ES cell colonies were screened for recombination by Southern blot hybridization relying on diagnostic SphI fragments created by an additional SphI site in the vector, visualized by 5’ and 3’ probes to genomic sequences outside the extent of the vector (see Fig. 1B). For the 21Q-h/mAR
allele, for example, 22 colonies were correctly recombined, and of these, two colonies had more than 80% euploid cells as determined by analysis of chromosome spreads. These two clones underwent transient transfection with a cre recombinase expression plasmid (pMC-Cre, (68)). The majority of colonies showed correct excision of the neo' cassette, first by PCR analysis and confirmed by Southern blotting for correct AR gene structure as above. Two independent targeted clones per h/mAR allele were injected into C57BL/6J blastocysts by the University of Michigan Transgenic Animal Models Core.

Mouse Breeding and Care

Male chimeric mice that had significant agouti contribution were crossed with C57BL/6J females. Resulting agouti female progeny, indicating transmission of the targeted paternal X chromosome, were bred to C57BL/6J males. Offspring from this cross (genotyped as in Fig. 1C, D) were then interbred in order to create homozygous female and hemizygous male mice used in this study. Homozygous TRAMP mice on the C57BL/6J background (gift of Martin Sanda) were crossed to heterozygous h/mAR female mice (generation F4) in order to obtain male h/mAR-TRAMP and mAR-TRAMP mice used in this study. All mouse procedures were approved by the University of Michigan Committee on Use and Care of Animals, in accord with the NIH Guidelines for the Care and Use of Experimental Animals.

Genotyping

DNA was isolated from tail biopsies by standard procedure and analyzed by PCR using primers that amplify both mouse and h/mAR regions encompassing the G tract (forward primer: 5’-CCACGTTGTCCCTGCTGGGCCAC-3’, reverse primer: 5’-
GACACTGCTTTACACAACTCCTTGGC-3’) in a 10 µl reaction containing 1.25 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM each primer, and 1 U Taq polymerase (Invitrogen). Cycling conditions consisted of 40 cycles of 94°C for 25 seconds, 67°C for 30 seconds, and 72°C for 30 seconds. PCR products were visualized on 2.5% agarose gels stained with ethidium bromide. Product lengths were 289 bp for mAR and 247 bp for h/mAR (Fig. 1C). Additional genotyping of Q tract length was performed by PCR using primers that amplified the h/mAR but not mAR Q tract region (forward primer 5’-ACCCAGAGGCGCCGAGCGC-3’, reverse primer 5’-GACTCCAGGGCGACTGCG-3’) in a 20 µl reaction similar to above but containing 1.5 mM MgCl₂. PCR products were visualized on 4% agarose gels and product lengths were 221 bp for 12Q-h/mAR, 248 bp for 21Q-h/mAR, and 329 bp for 48Q-h/mAR. TRAMP genotyping was performed as described (69).

Serum Hormone Levels

Serum testosterone levels were determined by radioimmunoassay (Diagnostic Systems Laboratories, Inc.), following manufacturer’s instructions.

Real Time PCR Analysis

Total RNA was isolated from tissues using RNeasy columns (Qiagen) and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). Taqman assays were performed using 5 ng cDNA and FAM-TAMRA or VIC-TAMRA labelled primers and probes designed by Applied Biosystems. Duplicate samples were assayed at least twice independently, values normalized to 18S rRNA, and relative expression was calculated by the standard curve method.
**Leydig Cell Marker Semi-quantitative RT-PCR**

Total testis RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and cDNA was amplified using primers and conditions as reported previously (53). Amplification products were visualized on agarose gels stained with ethidium bromide, and quantified by densitometry.

**Western Blotting**

Tissues were homogenized in RIPA buffer containing complete protease inhibitors (Roche) and centrifuged at 15,000 x g for 15 minutes in order to obtain supernatant and pellet fractions. Pellet fractions were homogenized in RIPA buffer for further analysis. Protein concentrations were determined by the Bradford method (Bio-Rad Dc Protein Assay). 30 µg testis protein was boiled in RIPA buffer for 10 minutes and examined by 7% SDS-PAGE. Protein was transferred to PVDF membrane using a tank transfer system. Nonspecific sites were blocked with 5% nonfat dry milk in PBS/05% Tween 20 for 1 hour at room temperature. Membranes were probed with AR (N-20, Santa Cruz Biotechnology) or β tubulin (H-235, Santa Cruz Biotechnology) primary antibodies and horseradish peroxidase-conjugated secondary antibody (anti-rabbit, Amersham Biosciences) at 1:2000 dilution in 5% nonfat dry milk/PBS. Bands were visualized using chemiluminescence.

**Histology and Immunohistochemistry**

Tissues were fixed in 10% buffered formalin for 24 hours, paraffin embedded, sectioned at 5 µM and stained with hematoxylin and eosin using standard methods.
Immunohistochemistry was performed as reported previously (70), using antibodies against AR (N20, Santa Cruz Biotechnology) at 1:500 dilution.

**Tumor palpation**

Starting at 12 weeks of age, mice were abdominally palpated once a week by two independent observers. The age of tumor initiation was recorded as the age at which both observers initially noted palpable tumor presence, which was approximately at 1 cm in diameter with a firmer density than normal prostate.

**Statistical analysis**

Data are presented as mean ± standard deviation. Statistical analysis was performed by ANOVA and Tukey’s studentized range (HSD) test for multiple comparisons. P<0.05 was considered significant.
REFERENCES


38. Gaspar ML, Meo T, Tosi M 1990 Structure and size distribution of the androgen receptor mRNA in wild-type and Tfm/Y mutant mice. Mol Endocrinol 4:1600-10


46. The Jackson Laboratory 1991 Handbook on Genetically Standardized JAX Mice, 4th ed, Bar Harbor, ME


49. Chubb C 1987 Animal models of physiologic markers of male reproduction: genetically defined infertile mice. Environ Health Perspect 74:15-29


55. Gao N, Zhang J, Rao MA, Case TC, Mirosevich J, Wang Y, Jin R, Gupta A, Rennie PS, Matusik RJ 2003 The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and
androgen receptor in transcriptional regulation of prostatic genes. Mol Endocrinol 17:1484-507


57. Yoon HG, Wong J 2005 The corepressors SMRT and N-CoR are involved in agonist- and antagonist-regulated transcription by androgen receptor. Mol Endocrinol


62. Giovannucci E 2002 Is the androgen receptor CAG repeat length significant for prostate cancer? Cancer Epidemiol Biomarkers Prev 11:985-6


68. Gu H, Zou YR, Rajewsky K 1993 Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell 73:1155-64


FIGURE LEGENDS

Fig. 1. **Generation of humanized AR mice.** (A) The AR targeting vector contains a 1.6 kb SmaI-Bsu36I fragment of hAR exon 1, with polyglutamine (Q) and polyglycine (G) tracts, flanked by mAR 5’ upstream and intron 1 sequences, and a Pkg-neo expression cassette flanked by loxP sites. Homologous recombination in ES cells results in an AR gene nearly identical to hAR in coding regions but under control of mAR regulatory sequences. (B) SphI sites from the targeting vector produced 13 and 4 kb fragments with the 5’ and 3’ AR probes, respectively, in correctly targeted (1, 2) relative to wild type (WT) ES cell clone DNA in blot hybridization. (C) Genotyping was performed with PCR primers that amplified a 289 bp fragment from mAR and a 247 bp fragment from h/mAR mice, with both bands readily detected in heterozygotes (M=marker, Het=heterozygote). (D) Additional PCR primers distinguished Q tract lengths in h/mAR mice.

Fig. 2. **Testis morphology, spermatogenesis and AR cellular localization appear normal in h/mAR mice.** A) H&E staining of testes from 6 month old mice (genotype shown above) shows normal seminiferous tubule size and density (upper panels, 400x original magnification), and normal interstitial cells (filled arrowhead), and all stages of normal spermatogenesis from spermatogonia (open arrowhead) to mature spermatids (arrow) (lower panel, 1000x original magnification). B) Testes from 6 month old mice were immunohistochemically stained for AR. Sertoli and interstitial cells show nuclear staining in all genotypes (400x original magnification).
Fig. 3. **Testis AR levels are slightly higher in humanized AR mice.** (A) Real time RT-PCR was used to quantitate AR mRNA levels in testes of 5-6 mice per genotype and results were calibrated to mAR. Groups designated “a” or “b” above are significantly different (P<0.05), whereas “ab” is not significantly different from “a” or “b”. (B) Total protein samples of testes from 3 mice per genotype were separated into supernatant and pellet fractions and analyzed by western blot. AR levels are higher in h/mAR mice, most notably in the 48Q-h/mAR pellet fraction, indicating a greater potential for aggregation in ARs with longer Q tracts.

Fig. 4. **Leydig cell maturation is normal in h/mAR mice.** Semiquantitative RT-PCR for Leydig cell markers was performed with testis RNA from 3 mice per genotype. Testis RNA from *tfm*, a mouse model of androgen insensitivity with impaired Leydig cell maturation, was used as a control. (A) *Hsd3b1*, a marker of fetal Leydig cells, is not significantly different in all groups. (B) *Hsd17b3*, a marker of mature Leydig cells, is normal in 12Q-h/mAR and 21Q-h/mAR mice as compared to mAR littermates while 48Q-h/mAR show levels between *tfm* and mAR mice. Groups with different letter superscripts are significantly different (P<0.05).

Fig. 5. **Prostate morphology and AR subcellular localization is normal in h/mAR mice.** A) H&E staining of prostates from 6 month old mice shows normal multilobular structure in mAR, 12Q-h/mAR, 21Q-h/mAR, and 48Q-h/mAR mice at 100x original magnification. VP = ventral prostate, LP = lateral prostate, DP = dorsal prostate. B) 6 months old mice show nuclear immunohistological stain for AR in epithelium and stroma of prostates from mAR, 21Q-h/mAR and 48Q-h/mAR mice at 400x original magnification.
Fig. 6. **Prostate AR gene expression levels are lower in h/mAR mice but AR target gene expression is similar to mAR mice.** Real time RT-PCR was used to quantitate AR, Foxa1, and Nkx3.1 mRNA levels in prostates of 3 mice per genotype. Results were calibrated relative to mAR levels. Differences in AR and Foxa1 mRNA levels between genotypes did not reach statistical significance, whereas Nkx3.1, a direct AR target, showed a significant decrease in 48Q-h/mAR mice.

Fig. 7. **H/mAR Q tract length affects initiation of prostate tumors.** Intact or castrated h/mAR-TRAMP mice were abdominally palpated weekly to track tumor initiation and necropsied at death to confirm the presence of a tumor. The status of each genotype at 29 weeks of age is shown, with those mice already dead represented by the black portion of the bar, those with tumor but alive represented in gray, and those with no palpable tumor in white. Longer Q tract length is protective in prostate tumor initiation, while shorter Q tract length promotes earlier disease.
Table I. Normal fertility, body weight and body fat of h/m AR mice.

<table>
<thead>
<tr>
<th>Litter Size</th>
<th>Body wt, 6 mos</th>
<th>Body wt, 18 mos</th>
<th>Body fat, 24 mos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>n pups/litter</td>
<td>n g</td>
<td>n g</td>
</tr>
<tr>
<td>mAR</td>
<td>n.d.</td>
<td>10 29.1±4.4</td>
<td>6 31.9±3.0</td>
</tr>
<tr>
<td>12Q-hAR</td>
<td>10 7.1±2.2</td>
<td>11 31.7±2.4</td>
<td>5 33.1±1.7</td>
</tr>
<tr>
<td>21Q-hAR</td>
<td>15 6.0±2.7</td>
<td>6 29.5±2.5</td>
<td>6 30.2±1.1</td>
</tr>
<tr>
<td>48Q-hAR</td>
<td>10 7.1±3.3</td>
<td>10 31.9±3.2</td>
<td>6 31.2±4.7</td>
</tr>
</tbody>
</table>

n.d. = not done
Table II. Testosterone levels and seminal vesicle weights with age in h/mAR mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Testosterone</th>
<th>SV wt, 6 mos</th>
<th>SV wt, 18 mos</th>
<th>SV wt, 24 mos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>ng/ml</td>
<td>range</td>
<td>n</td>
</tr>
<tr>
<td>mAR</td>
<td>17</td>
<td>1.59±2.71</td>
<td>0.14-9.00</td>
<td>5</td>
</tr>
<tr>
<td>12Q-hAR</td>
<td>9</td>
<td>1.91±3.34</td>
<td>0.18-10.45</td>
<td>5</td>
</tr>
<tr>
<td>21Q-hAR</td>
<td>10</td>
<td>1.41±1.72</td>
<td>0.24-4.88</td>
<td>n.d.</td>
</tr>
<tr>
<td>48Q-hAR</td>
<td>9</td>
<td>2.45±2.97</td>
<td>0.34-9.27</td>
<td>5</td>
</tr>
</tbody>
</table>

n.d. = not done
Figure 1
Figure 3
Figure 4
Figure 6