DNA-binding-dependent androgen receptor signaling contributes to gender differences and has physiological actions in males and females

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Abstract

We used our genomic androgen receptor (AR) knockout (ARKO) mouse model, in which the AR is unable to bind DNA to: 1) document gender differences between males and females; 2) identify the genomic (DNA-binding-dependent) AR-mediated actions in males; 3) determine the contribution of genomic AR-mediated actions to these gender differences; and 4) identify physiological genomic AR-mediated actions in females. At 9 weeks of age, control males had higher body, heart and kidney mass, lower spleen mass, and longer and larger bones compared to control females. Compared to control males, ARKO males had lower body and kidney mass, higher splenic mass, and reductions in cortical and trabecular bone. Deletion of the AR in ARKO males abolished the gender differences in heart and cortical bone.

Compared with control females, ARKO females had normal body weight, but 14% lower heart mass and heart weight/body weight ratio. Relative kidney mass was also reduced, and relative spleen mass was increased. ARKO females had a significant reduction in cortical bone growth and changes in trabecular architecture, although with no net change in trabecular bone volume. In conclusion, we have shown that androgens acting via the genomic AR signaling pathway mediate, at least in part, the gender differences in body mass, heart, kidney, spleen, and bone, and play a physiological role in the regulation of cardiac, kidney and splenic size, cortical bone growth, and trabecular bone architecture in females.

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Introduction

Males and females show sexual dimorphism in a number of tissues. Males tend to be taller, with higher muscle and bone mass and lower fat mass than females, with differences also observed in the immune system, nervous system, and in erythropoiesis (Mooradian *et al.* 1987). These sex differences are likely to be due to differences in the concentrations of estrogen and testosterone as well as the expression of their receptors. Androgens, predominantly testosterone and dihydrotestosterone (DHT), have traditionally been thought of as the male sex hormones, which are essential for masculinization of the developing male fetus and development of secondary sexual characteristics at puberty. The non-reproductive actions of androgens in males have been investigated more recently, particularly the anabolic actions

of androgens in the musculoskeletal system. Both skeletal muscle mass and bone mineral density are androgen responsive, as demonstrated in studies in human subjects (Behre *et al.* 1997, Chen *et al.* 2005) and mouse models (Wakley *et al.* 1991, Axell *et al.* 2006).

The classic paradigm of androgen action is that androgens act via the androgen receptor (AR), a ligand-dependent nuclear transcription factor (MacLean *et al.* 1997). This view is supported by the fact that loss of function mutations in the AR gene causes androgen insensitivity syndrome (AIS), in which affected males are androgen resistant and have a female external phenotype (MacLean *et al.* 2004). However, more recently, it has become apparent that the actions of androgens are more complex than this simple model suggests. It is now clear that in males, some androgen actions also arise through aromatization of androgens to estrogens and activation of

the estrogen receptor (ER). This is evidenced by the fact that men with ER or aromatase gene mutations lack the pubertal growth spurt, fail to fuse their epiphyses, and have osteopenia (Smith *et al.* 1994, Grumbach & Auchus 1999). Similarly, male mice with targeted deletion of the aromatase gene show loss of male-type sexual behaviors, increased adiposity, reduced bone length, and mineralization (Jones *et al.* 2000, Oz *et al.* 2001, Robertson *et al.* 2001).

In addition to the transcriptional actions of the AR, non-DNA-binding-dependent pathways have been identified *in vitro* (Kousteni *et al.* 2001, Estrada *et al.* 2003, Gill *et al.* 2004, Kang *et al.* 2004, Norris *et al.* 2009). The physiological relevance of this AR signaling is unclear, as previous studies in AR-null knockout (ARKO) mouse models have not distinguished between DNA-binding-dependent and -independent actions of the AR (Yeh *et al.* 2002, Kawano *et al.* 2003, De Gendt *et al.* 2004).

In addition to the ER-dependent actions of androgens in males, the potential physiological actions of androgens in females are also being identified. The pharmacological effects of exogenous androgens in females range from hirsutism and masculinization to muscle and bone anabolism (Strauss *et al.* 1985, Buchanan *et al.* 1988), and pathological effects of excess androgen occur in polycystic ovarian syndrome (Nisenblat & Norman 2009). Human bone cells from male and female donors have similar concentrations of ARs, suggesting that androgens may play an important role in the maintenance of bone mass in both sexes (Colvard *et al.* 1989). Further supportive evidence for a role of the AR in females is provided by analyses of the reproductive system of ARKO female mice demonstrating a physiological role for androgens in ovarian folliculogenesis, uterine development, and mammary gland development (Yeh *et al.* 2003, Hu *et al.* 2004, Shiina *et al.* 2006, Walters *et al.* 2007, 2009). Other physiological actions of androgens in females have yet to be identified.

Since males and females express the AR at similar levels in many tissues (Rinn *et al.* 2004), but the production rate of testosterone is 20–30-fold higher in men (Handelsman 2010), we hypothesize that phenotypic differences between males and females are attributed at least in part to androgen actions via the genomic AR pathway. Furthermore, we also propose that androgens acting via the genomic AR signaling pathway play an important physiological role in non-reproductive tissues of both males and females. In this study, we have utilized our global ARKO mice, which have an in-frame deletion of the second zinc finger of the DNA-binding domain that abolishes genomic AR signaling (Notini *et al.* 2005). To test the hypotheses, we have examined the non-reproductive tissues in control and genomic ARKO males and females to: 1) determine the differences between males and females; 2) identify the genomic (DNA-binding-dependent) AR-mediated actions in males; 3) determine the contribution of genomic AR-mediated actions to these gender differences; and 4) identify physiological genomic AR-mediated actions in females.

Materials and Methods

Mice

ARKO males and females were studied on a C57BL/6 background, with all lines of mice used in this study backcrossed to a C57BL/6 background for >8 generations prior to experimental analysis. CMV-cre transgenic (CMV-cre.Tg+) mice were obtained with permission from Dr Ursula Lichtenberg, Institute for Genetics, University of Cologne. The *AR* gene is on the X chromosome, and genomic ARKO have exon 3 of the *AR* gene deleted (AR^{Δ} ; Notini *et al.* 2005). ARKO males ($AR^{\Delta/Y}$) were offspring of heterozygous $AR^{\Delta/+}$ females crossed with wild-type (WT) $AR^{+/Y}$ males, and ARKO females were generated by crossing $AR^{\Delta/+}$ and CMV-cre.Tg+ heterozygous females with AR floxed ($AR^{fl/Y}$) hemizygous males as described, which deletes >99.9% of the AR in genomic DNA from ARKO females (MacLean *et al.* 2008). Control littermates were used for both males (WT) and females ($AR^{fl/+}$ and CMV-cre.Tg+). Mice were housed in a conventional facility with a 12 h light:12 h darkness cycle, and standard chow and water were provided *ad libitum*. Studies were conducted in accord with accepted standards of humane animal care, and performed with the approval of the Austin Health Animal Ethics Committee.

Tissue collection and serum analysis

Adult mice were studied at 9 weeks of age. For dynamic bone histomorphometry experiments, mice received two i.p. injections of 20 mg/kg per BW calcein (Sigma) at 10 and 3 days prior to killing. On the day of tissue harvest, mice were anesthetized by isoflurane and blood collected via cardiac puncture, and mice were killed by cervical dislocation. Blood was clotted on ice, and centrifuged $2 \times 1400 g$ to collect serum, which was stored at $-20^{\circ}C$ prior to analysis. Tissues were dissected post-mortem, and wet weight of tissues was determined to an accuracy of 0.1 mg from the mean mass bilaterally. Body mass and organ mass were measured in genomic ARKO and WT male littermates ($n \geq 24$ /group), and in genomic ARKO females and $AR^{fl/+}$ and CMV-cre.Tg+ female littermates ($n \geq 12$ /group). Data from $AR^{fl/+}$ and CMV-cre.Tg+ female controls were pooled, as there was no statistical difference between all parameters in these groups (data not shown). Since body weight was decreased in ARKO and as the weights of most individual organs scale as a constant fraction of body mass (Lindstedt & Schaeffer 2002), organ mass was analyzed as the absolute mass, and also relative mass, calculated by correcting the mass of individual organs by body weight. The C-terminal telopeptide $\alpha 1$ chain of type I collagen (X-laps) was determined in serum by ELISA (RatLaps ELISA; Nordic Bioscience Diagnostics, Herlev, Denmark).

Bone histomorphometry

Femur length was measured using a digital calliper (Etalon, Renens, Switzerland). Distal femora were prepared for quantitative histomorphometry, using established resin-embedding techniques as described previously (Notini *et al.* 2007). Trabecular bone volume, thickness, and number were calculated in the metaphyseal region below the growth plate excluding the primary spongiosa, using a Leica Quantimet Image Analysis System and QWin software (Cambridge, UK). The region of the metaphysis analyzed was a function of femur length as described previously (Davey *et al.* 2006, Notini *et al.* 2007). Dynamic markers of bone turnover (mineralizing surface, mineral apposition rate, bone formation rate, osteoclast surface, and osteoid surface) were estimated in the same region of the secondary spongiosa of the distal femoral metaphysis used for the static histomorphometry parameters as described (Davey *et al.* 2006, Notini *et al.* 2007).

Quantitative micro-computed tomography

The midshaft of the femur was evaluated using a microtomographic imaging system (Viva CT40; Scanco Medical AG, Bassersdorf, Switzerland) as described previously (Chiang *et al.* 2009). Fifty transverse computed tomography slices of the mid-femoral diaphysis were acquired using 10.5 µm isotropic voxel size. Cortical bone properties were evaluated in a region commencing 56% of the femur length distal to the femoral head and extending 0.5 mm distally. Images were identified automatically, filtered, and reconstructed with a specimen-specific threshold of 240. Morphometric parameters were computed using a direct 2D and 3D approach that does not rely on any assumptions about the underlying structure (Hildebrand *et al.* 1999). Cortical thickness, periosteal circumference, and medullary circumference were determined.

Statistical analysis

When comparing the mean of two groups, two-tailed unpaired Student's *t*-test was used for equal or unequal variance where appropriate. A value of $P < 0.05$ was considered significant. All analyses were performed using SPSS 16 for Mac (Chicago, IL, USA).

Results

Control males versus control females

To identify sexually dimorphic characteristics, we compared the mass of a number of potentially androgen-sensitive tissues in control male and female mice at 9 weeks of age. Males weighed 23% ($P < 0.001$) more than females (Fig. 1A), and absolute heart mass and kidney mass were higher in males than females (19% higher, $P < 0.001$ and 25% higher,

Figure 1 Body and organ mass in control males and females at 9 weeks of age. (A) Body mass, (B) absolute heart mass, (C) absolute kidney mass, and (D) absolute spleen mass. $n \geq 15$ /group, ** $P < 0.001$ versus control male, Student's *t*-test.

$P < 0.001$ respectively; Fig. 1B and C). Heart mass and kidney mass were proportional to body mass, as there was no difference in heart weight/body weight ratio or kidney weight/body weight ratio between males and females (data not shown). Absolute spleen mass was not significantly different between males and females (Fig. 1D); however, relative spleen mass was 36% lower in males than females (mean $\pm$ S.E.M.; control male: 2.99 ± 0.09 mg/g, $n = 22$ versus control female: 4.08 ± 0.12 mg/g, $n = 25$, $P < 0.001$). Control males had increased femur length ($P < 0.001$), periosteal circumference ($P < 0.005$), cortical thickness ($P < 0.05$), medullary circumference ($P < 0.05$), trabecular bone volume ($P = 0.05$), and trabecular number ($P < 0.001$) compared with control females (Fig. 2).

Genomic ARKO males versus control males

To establish the actions of androgens mediated via the genomic AR signaling pathway in males, we compared ARKO males with their WT male littermate controls. Body mass was decreased by 16% ($P < 0.001$) in ARKO males compared to control males (Fig. 3A). Heart, kidney, and testis mass were reduced by 17% ($P < 0.001$), 28% ($P < 0.001$), and 94% ($P < 0.001$) respectively in ARKO males versus control males (Fig. 3B, C and E), whereas spleen mass was increased by 35% ($P < 0.001$; Fig. 3D). In ARKO males, the decrease in heart mass was proportional to the decreased body mass (heart weight/body weight, mean $\pm$ S.E.M.; control male: 5.59 ± 0.14 mg/g, $n = 30$ versus ARKO male: 5.56 ± 0.12 mg/g, $n = 29$), but relative kidney, spleen, and testis

Figure 2 Bone parameters in control males and females at 9 weeks of age. (A) Femur length, (B) cortical thickness, (C) periosteal circumference, (D) medullary circumference, (E) trabecular bone volume (BV/TV), (F) trabecular number (TbN), (G) trabecular thickness (TbTh), (H) mineralizing surface (MS), (I) mineral apposition rate (MAR), and (J) bone formation rate (BFR). $n \geq 6/\text{group}$, ** $P < 0.001$, * $P < 0.05$ versus control male, Student's t -test.

mass were all significantly different to control males (mean $\pm$ S.E.M.; kidney, control male: 6.64 ± 0.08 mg/g, $n = 36$ versus ARKO male: 5.74 ± 0.09 mg/g, $n = 36$, $P < 0.001$; spleen, control male: 2.99 ± 0.09 mg/g, $n = 22$ versus ARKO

male: 4.81 ± 0.15 mg/g, $n = 26$, $P < 0.001$; testis, control male: 3.25 ± 0.08 mg/g, $n = 18$ versus ARKO male: 0.22 ± 0.01 mg/g, $n = 21$, $P < 0.001$).

ARKO males had decreases in both cortical and trabecular bone compared to control males, while femoral length was unchanged (Fig. 4A). Cortical thickness ($P < 0.005$), periosteal circumference ($P < 0.005$), and medullary circumference ($P < 0.05$) were decreased in ARKO males by 14, 11, and 9% compared to control males respectively (Fig. 4B–D). Trabecular bone volume was also decreased by 33% in ARKO males ($P < 0.001$) compared to control males (Fig. 4E), due to a reduction in trabecular number ($P < 0.01$; Fig. 4F), not thickness (Fig. 4G). The reduced trabecular bone in ARKO males was associated with an increase in unmineralized matrix as measured by osteoid surface (mean $\pm$ S.E.M.; control male: $10.9 \pm 2.7\%$, $n = 5$ versus ARKO male: $23.9 \pm 5.4\%$, $n = 6$; $P < 0.05$), and a reduction in mineralizing surface ($P < 0.01$; Fig. 4H). Mineral apposition rate (MAR) was unaffected (Fig. 4I), and while the mean values for bone formation rate were lower in ARKO

Figure 3 Body, organ, and reproductive tissue mass in ARKO males and control males at 9 weeks of age. (A) Body mass, (B) absolute heart mass, (C) absolute kidney mass, (D) absolute spleen mass, and (E) absolute testis mass. $n \geq 15/\text{group}$, ** $P < 0.001$ versus control male, Student's t -test.

Figure 4 Bone parameters in ARKO males and control males at 9 weeks of age. (A) Femur length, (B) cortical thickness, (C) periosteal circumference, (D) medullary circumference, (E) trabecular bone volume (BV/TV), (F) trabecular number (TbN), (G) trabecular thickness (TbTh), (H) mineralizing surface (MS), (I) mineral apposition rate (MAR), and (J) bone formation rate (BFR). $n \geq 6$ /group, $**P < 0.001$, $*P < 0.05$ versus control male, Student's t -test.

males compared to controls, this did not reach statistical significance ($P = 0.058$; Fig. 4J). Bone resorption as measured by osteoclast surface (mean $\pm$ S.E.M.; control male: $7.3 \pm 2.1\%$, $n = 6$ versus ARKO male: $11.9 \pm 3.8\%$, $n = 5$) and serum X-laps (mean $\pm$ S.E.M.; control male: 32.7 ± 3.5 ng/ml, $n = 14$ versus ARKO male: 41.2 ± 5.1 ng/ml, $n = 14$) were unchanged in ARKO males compared with controls.

Genomic ARKO males versus control females

To determine the contribution of the androgen/genomic AR pathway to the differences between males and females, we compared ARKO males with control females. Despite the decrease in body mass of ARKO males compared to control males, they were still 8% heavier than control females (Table 1). Both absolute heart mass and heart weight/body weight ratio did not differ between ARKO males and control females (data not shown). In contrast, while absolute kidney mass did not differ between ARKO males and control females, kidney weight/body weight ratio was 14% lower in ARKO males (Table 1), and both absolute and relative spleen mass were higher in ARKO males (Table 1). Femur length was increased in ARKO males (mean $\pm$ S.E.M.; control females: 14.8 ± 0.1 , $n = 15$ versus ARKO males: 15.5 ± 0.2 mm, $n = 9$ versus $P < 0.001$), while trabecular bone volume was decreased (mean $\pm$ S.E.M.; control females: 30.3 ± 2.0 , $n = 8$ versus ARKO males: 20.3 ± 1.2 , $n = 10$, $P < 0.05$), due to decreased trabecular thickness in ARKO males (mean $\pm$ S.E.M.; control females: 21 ± 2 μ m, $n = 9$ versus ARKO males: 23 ± 1 μ m, $n = 8$, $P < 0.05$). Cortical bone and the dynamic bone parameters did not differ between ARKO males and control females (data not shown).

Genomic ARKO females versus control females

To identify physiological actions of androgens in females, we compared ARKO females with control females. There was no difference in the body mass of ARKO females and control females (Fig. 5A). Despite the fact that total body mass did not differ, absolute heart mass was decreased by 14% in ARKO females (mean $\pm$ S.E.M.; control female: 122.0 ± 4.9 mg,

Table 1 Comparison of 9-week-old genomic ARKO male mice with control female mice. Data represent mean $\pm$ S.E.M.

	Control female	ARKO male
Body mass (g)	20.8 $\pm$ 0.3 (37) ^a	22.6 $\pm$ 0.3** (53)
Kidney weight/body weight ratio (mg/g)	6.56 $\pm$ 0.12 (26)	5.74 $\pm$ 0.09** (36)
Spleen weight (g)	83.4 $\pm$ 3.1 (25)	109.6 $\pm$ 3.9** (26)
Spleen weight/body weight ratio (mg/g)	4.08 $\pm$ 0.12 (25)	4.81 $\pm$ 0.15** (26)

ARKO, androgen receptor knockout.
^aNumber in parenthesis denotes sample size.
^{**} $P < 0.001$ versus control females, Student's t -test.

Figure 5 Body and organ mass in ARKO females and control females at 9 weeks of age. (A) Body mass, (B) relative heart mass, (C) relative kidney mass, and (D) relative spleen mass. $n \geq 15/\text{group}$, * $P < 0.05$ versus control female, Student's *t*-test.

$n = 25$ versus ARKO female: 104.9 ± 2.2 mg, $n = 12$, $P = 0.001$), and heart weight/body weight ratio was also reduced by the same amount ($P < 0.01$; Fig. 5B). Relative kidney mass was decreased by 6% ($P < 0.05$) in ARKO females (Fig. 5C), with a trend for reduced absolute kidney mass (mean $\pm$ S.E.M.; control female: 134.5 ± 3.8 mg, $n = 26$ versus ARKO female: 125.8 ± 3.6 mg, $n = 12$, $P = 0.082$). Similarly, relative spleen mass was increased by 10.5% ($P < 0.05$) in ARKO females (Fig. 5D), with a trend for increased absolute spleen mass (mean $\pm$ S.E.M.; control female: 83.4 ± 3.1 mg, $n = 25$ versus ARKO female: 92.4 ± 3.9 mg, $n = 12$, $P = 0.095$). There was no difference between ARKO females and control females in absolute or relative ovary or uterus mass (data not shown).

Periosteal circumference and medullary circumference were decreased by 4 and 5% respectively in ARKO females compared to controls ($P < 0.05$), while cortical thickness was unaffected (Fig. 6B–D). Although trabecular bone volume was unaffected in ARKO females compared to controls (Fig. 6E), trabecular number was decreased by 26% ($P < 0.01$; Fig. 6F), and trabecular thickness was increased by 15% ($P < 0.05$) (Fig. 6G). No differences were observed in measurements of bone formation (mineralizing surface, mineral apposition rate, and bone formation rate; Fig. 6H–J).

Discussion

To determine the contribution of androgens acting through the genomic (DNA-binding-dependent) AR signaling pathway in males and females to gender differences, we

generated and characterized male and female mice with ablation of the genomic actions of the AR. Other global ARKO mouse models are completely AR null, because they have frameshift deletions that result in no detectable AR

Figure 6 Bone parameters in ARKO females and control females at 9 weeks of age. (A) Femur length (B) cortical thickness, (C) periosteal circumference, (D) medullary circumference, (E) trabecular bone volume (BV/TV), (F) trabecular number (TbN), (G) trabecular thickness (TbTh), (H) mineralizing surface (MS), (I) mineral apposition rate (MAR), and (J) bone formation rate (BFR). $n \geq 6/\text{group}$, * $P < 0.05$ versus control female, Student's *t*-test.

protein, and therefore have deletion of both genomic (DNA-binding-dependent) and DNA-binding-independent AR signaling pathways (Yeh *et al.* 2002, Kawano *et al.* 2003, De Gendt *et al.* 2004). In contrast, our ARKO mice express mutant AR protein, lacking the second zinc finger of the DNA-binding domain (Notini *et al.* 2005). We previously showed that our genomic ARKO mice have a modest reduction in serum testosterone levels (Notini *et al.* 2005); therefore, their phenotype arises through a combination of loss of genomic AR signaling and testosterone deficiency. However, treatment of genomic ARKO males with supraphysiological levels of testosterone for 6 weeks has no effect on any phenotypic parameter examined (Lim *et al.* 2008), and H E MacLean & R A Davey, unpublished data), supporting the hypothesis that the ARKO male phenotype is caused by the loss of DNA-binding-dependent AR actions. Therefore, our study is strongly suggestive that androgens act through the genomic AR signaling pathway to play a physiological role in a number of tissues in both sexes.

Body mass of ARKO males was between that of normal males and females. We previously showed that ARKO males have normal levels of serum insulin-like growth factor 1 (MacLean *et al.* 2008), and the fact that femoral length did not differ between ARKO and control males suggests that skeletal growth rate is normal. We have also previously demonstrated that both absolute and relative skeletal muscle mass are decreased in ARKO males versus control males (MacLean *et al.* 2008). Since body mass is contributed by lean body mass (muscle and visceral organs), fat mass, and skeletal size and density, the reduction in ARKO male body mass is therefore most likely to be caused by the reduction in mass of skeletal muscle and other organs. Body weight and femoral length were still higher in ARKO males than control females, in contrast to previous studies showing no difference between young AR-null ARKO males and control females (Sato *et al.* 2003, Lin *et al.* 2005). A major difference between AR-null ARKO models and our genomic ARKO mice is that the genomic ARKOs retain potential DNA-binding-independent AR actions. These include activation of second-messenger signaling pathways by the AR (Heinlein & Chang 2002), and indirect gene repression in the absence of direct binding to DNA, via tethering of transcription factors (Schneikert *et al.* 1996). While our study was not designed to identify AR actions independent of DNA binding, the differences in ARKO male body mass between the different models suggest the possibility that this could contribute to promoting body growth in males. However, to definitively identify the role of non-DNA-binding-dependent AR actions, direct comparisons between the different ARKO models would be required to be performed in the same environment, and with the mice on the same genetic background. The fact that genomic ARKO males were larger than females could also be analogous to the height of patients with complete AIS, which has been reported as increased compared to average female height (Marcus *et al.* 2000). Alternatively, the increased longitudinal growth in ARKO

males compared with control females may be attributed to the lower levels of circulating testosterone in the ARKO males that is available for conversion to estradiol (Yamazaki & Yamaguchi 1989, Notini *et al.* 2005). Further supportive evidence for this hypothesis is the lower trabecular bone volume and thickness in the ARKO males compared with control females, which are changes consistent with estrogen deficiency in the rodent (Wronski *et al.* 1986, Yamazaki & Yamaguchi 1989). However, in combination with these previous studies, our data suggest that factors other than the genomic AR pathway contribute to the size and height differential between males and females.

Cardiac mass is sexually dimorphic, with our data showing that males had larger hearts than females, in proportion to their differences in body weight. Androgens promote cardiac myocyte hypertrophy *in vitro* (Marsh *et al.* 1998), and male mice that are AR null have small hearts, with a 14% decrease in heart weight/body weight ratio, and are susceptible to angiotensin II-induced cardiac remodeling (Ikeda *et al.* 2005). In contrast, our ARKO males had a 17% decrease in absolute cardiac mass compared with control males, but heart weight/body weight ratio was unchanged. The milder cardiac phenotype in our ARKO males compared with AR-null ARKO males may reflect the effects of different genetic backgrounds (homogenous C57BL/6 in our study versus mixed C57BL/6 and CBA in the previous study). Alternatively, these differences could also indicate the possibility that a DNA-binding-independent AR signaling pathway acts in concert with the classical genomic pathway in the heart to promote cardiac growth and development. This latter hypothesis is supported by the fact that rapid androgen signaling pathways have been identified in cardiomyocytes *in vitro* (Vicencio *et al.* 2006), and a recent study has showed that DHT treatment of isolated rat left atria causes a non-transcriptional cardiotonic response associated with increased ornithine decarboxylase (ODC) activity (Bordallo *et al.* 2009). The fact that there was no difference in absolute or relative heart mass between ARKO males and control females indicates that the sex differences in cardiac mass are determined solely by androgens acting through the genomic AR pathway in males.

In mice, the kidney is one of the most androgen-sensitive non-reproductive tissues, with kidney mass closely related to androgen levels in males (Berger & Watson 1989). Kidney mass was 25% lower in female mice than males, proportional to the lower body mass in females. This may reflect the fact that lower body mass in females is caused by the decrease in both visceral organ and skeletal muscle mass compared to males. ARKO males also had a reduction in both absolute and relative kidney mass compared to control males, and ARKO male kidney mass was reduced to that of normal females, supporting the hypothesis that the gender differences in kidney mass are due to androgen actions in males. Androgens are thought to regulate kidney hypertrophy in part through AR-dependent regulation of polyamine levels, demonstrated by the fact that androgens regulate the genes

encoding polyamine biosynthetic enzymes including ODC in the kidney (Levillain *et al.* 2005). Androgen response elements have also been identified in the *Odc* gene promoter (Crozat *et al.* 1992). Together, these data suggest that androgens promote kidney hypertrophy in males through the genomic AR pathway.

Females have a higher incidence of autoimmune diseases than males, and sex hormones regulate the immune system (McCombe *et al.* 2009). Castration of male mice increases splenic and thymic weights, due to an increase in newly formed B cells and mature B lymphocytes (Ellis *et al.* 2001). Male *Tfm* mice, with the naturally occurring AR mutation, also have increased B cell precursors in the bone marrow and mature B cells in the spleen (Smithson *et al.* 1998), and our data demonstrating increased splenic weight in genomic ARKO males compared to control males suggest that the suppression of B lymphopoiesis by androgens occurs via the genomic AR pathway. ARKO males also had higher absolute and relative splenic weights than control females. Female hypogonadal (*hpg*) mice, which lack GnRH and therefore have a deficiency in both estrogen and androgen production, have elevated B lymphopoiesis and increased splenic B cell number, which can be normalized by estrogen supplementation (Smithson *et al.* 1994). Therefore, the differences in splenic weight between males and females are likely to be due to androgen actions in males and also estrogen-mediated suppression of lymphopoiesis in females.

Males have larger bones than females, in part due to the anabolic actions of androgens on outward bone growth via stimulation of periosteal apposition (Seeman 2001). In females, estrogens inhibit this process while stimulating endocortical deposition of bone (Seeman 2001). Consistent with this, cortical thickness, periosteal circumference, and medullary circumference were decreased in ARKO males compared to control males. Furthermore, the loss of genomic AR action in ARKO males resulted in a 33% reduction in trabecular bone volume compared to controls, and was due to fewer trabeculae, while trabecular thickness was unaffected. A similar reduction in trabecular bone volume has been observed in AR-null males, due to increased bone resorption (Yeh *et al.* 2002, Kawano *et al.* 2003, Venken *et al.* 2006). Despite the decreased trabecular bone volume in our ARKO males, we did not detect any increase in bone resorption markers. It is possible that bone resorption is also increased in our ARKO males prior to 8 weeks of age, as suggested by our osteoblast-specific ARKOs (Notini *et al.* 2007, Chiang *et al.* 2009). The decreased trabecular bone in the ARKO males was also attributed to an increase in unmineralized bone matrix and a decrease in the number of mineralizing surfaces reflected by osteoid surface and mineralizing surface respectively, providing evidence for a role of androgens via the AR in the coordination of bone matrix synthesis with its subsequent mineralization. The trabecular bone phenotype of the ARKO males is consistent with our previous findings that deletion of genomic AR signaling specifically in mature and mineralizing osteoblasts results in trabecular bone loss with

increased bone resorption (Notini *et al.* 2007, Chiang *et al.* 2009), and that deletion in mineralizing osteoblasts results in disruption of the bone matrix synthesis and mineralization processes (Chiang *et al.* 2009). Taken together, these data indicate the importance of genomic AR signaling in males to maintain cortical and trabecular bones.

The physiological actions of androgens via the genomic AR signaling pathway in females have not previously been well understood, due to the lack of good knockout mouse models, and the inability to distinguish androgen and estrogen effects in gonadectomy studies. Although females have low levels of circulating androgens, the local androgen concentrations in tissues can be higher, and most tissues express the AR at levels similar to that found in males (Rinn *et al.* 2004), providing potential for significant androgen actions in females. The use of the cre/lox approach has enabled the generation of global ARKO females to identify the physiological actions of androgens. Previous studies in ARKO females have focused only on reproductive actions, including identifying a role for androgens in folliculogenesis, mammary gland development, and uterine morphology and development (Yeh *et al.* 2003, Hu *et al.* 2004, Shiina *et al.* 2006, Walters *et al.* 2007, 2009). Despite the defect in late follicular development, our genomic ARKO female mice have normal levels of serum LH, FSH, estradiol, and testosterone (Walters *et al.* 2007). Our current study demonstrates that in addition to these reproductive actions, androgens also act through the genomic AR pathway in female kidney, heart, bone marrow, and bone.

In females, there was no difference in body mass between ARKOs and controls, despite the change in mass of some visceral organs, indicating that the effects of androgens on body mass are male specific. This is consistent with the findings in AR-null female models (Yeh *et al.* 2003, Shiina *et al.* 2006). ARKO females had a 14% decrease in absolute cardiac mass and heart weight/body weight ratio compared to control females. Therefore, androgens also play a physiological role in promoting cardiac growth in females. Relative kidney mass was reduced by 6% in ARKO females compared to control females. While this reduction is only half of that observed in ARKO males compared to control males, it nevertheless demonstrates that androgens also promote kidney hypertrophy in normal females. ARKO females also had increased relative splenic mass compared to control females. Therefore, this suggests that androgens acting via the genomic AR pathway play a physiological role to down-regulate B lymphopoiesis in females.

Femoral length was unaffected in our ARKO males and females, consistent with observations in AR-null mouse models (Kawano *et al.* 2003, Venken *et al.* 2006). Of interest was the small, but significant decrease in bone size of ARKO females, with decreases in periosteal circumference and medullary circumference. This is the first report of a possible role of the AR in cortical bone growth in female mice, as previous characterization of the bone phenotype of AR-null ARKO female mice has been limited to soft X-ray and bone

mineral density analyses (Kawano *et al.* 2003). Androgen actions result in the outward growth of bone via stimulation of periosteal apposition in males, while estrogens inhibit this process and stimulate endocortical deposition in females (Seeman 2001, Jarvinen *et al.* 2003). Although the actions of estrogen are predominant in females, these data also provide evidence for a contribution of androgen action via the genomic AR pathway in cortical bone growth and expansion in females.

While trabecular bone volume of ARKO females did not differ from control females, we observed a reduction in trabecular number and increase in trabecular thickness, suggestive of increased bone turnover. Therefore, while estrogen is the main sex steroid regulating bone accrual and maintenance in females, we have provided evidence that androgens also play a physiological role in bone in females via the genomic AR pathway. Supportive evidence for this is provided by observations of increased bone mineral density in hirsute women with increased circulating levels of testosterone (Buchanan *et al.* 1988), in woman following treatment with the synthetic non-aromatizable androgen nandrolone decanoate (Need *et al.* 1987, Johansen *et al.* 1989), and in female ovariectomized rats treated with the non-aromatizable androgen, DHT (Tobias *et al.* 1994).

In conclusion, we have identified that the genomic AR signaling pathway mediates, at least in part, the gender differences in body mass, heart, kidney, spleen, and bone. In addition, this study provides the first evidence that androgens acting via the genomic AR pathways in females play a physiological role in the regulation of splenic mass, cardiac growth, kidney hypertrophy, cortical bone growth, and trabecular bone architecture. We have shown that DNA-binding-dependent AR actions play a role in cortical and trabecular bone accrual and maintenance in females, and we have also identified that heart, kidney, and spleen are key areas of AR action outside the musculoskeletal system. In-depth analysis of these other organs will provide further information regarding the mechanisms via which genomic AR signaling regulates their function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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