Corrections

COMMENTARY. For the article “Extracting functional information from microarrays: A challenge for functional genomics,” by Michael Q. Zhang, which appeared in number 20, October 1, 2002, of Proc. Natl. Acad. Sci. USA (99, 12509–12511; First Published September 23, 2002; 10.1073/pnas.212532499), Fig. 1 appeared incorrectly. The locants for B and C were reversed. In addition, the locant for the description of D was omitted from the legend. The corrected figure and its legend appear below.

Fig. 1. Relations among different concepts in the SP-analysis method. (A) Expression profile matrix (table). t = (t_1, t_2, ... ) is the experimental condition index; in this example it indicates a set of time points. (B) Expression profiles (patterns). g_1 and g_x are not strongly correlated directly, but both are strongly correlated with the correlated set (g_y, g_z). g_i, g_j are the transitive genes interpolating the two terminal genes along SP_1 (see C and D); similarly, g_j is the transitive gene interpolating g_1 and g_5 along SP_2. (C) GO biological process tree. The Ps are process annotations for genes at a particular node. A gene may belong to more than one node (“multiple-function,” such as g_2). (D) Expression profile space. g_i is on the short path SP_1 terminated by the known genes g_1, g_2, and hence is assigned a function of P_{1,1,1,1} (level L0) according to the GO tree in C. g_j is on SP_2 terminated by g_1, g_4, and hence is assigned a function of P_{1,1,1} (level L1). g_i is shared by both SPs and may be involved in both processes, which means the processes represented by SP_1 and SP_2 actually crosstalk to each other. The linked gene network can be formed by the subgraph SP_1+SP_2.

www.pnas.org/cgi/doi/10.1073/pnas.242607999
CHEMISTRY. For the article “Creating nanocavities of tunable sizes: Hollow helices,” by Bing Gong, Huaiqiang Zeng, Jin Zhu, Lihua Yu, Yaohua Han, Shizhi Cheng, Mako Furukawa, Rubén D. Parra, Andrey Y. Kovalevsky, Jeffrey L. Mills, Ewa Skrzypczak-Jankun, Suzana Martinovic, Richard D. Smith, Chong Zheng, Thomas Szyperski, and Xiao Cheng Zeng, which appeared in number 18, September 3, 2002, of Proc. Natl. Acad. Sci. USA (99, 11583–11588; First Published August 12, 2002; 10.1073/pnas.162277099), the accession numbers for the crystal structure data were omitted from the paper. The omitted footnote appears below.

Data deposition: The atomic coordinates have been deposited in the Cambridge Structural Database, Cambridge Crystallographic Data Centre, Cambridge CB2 1EZ, United Kingdom, www.ccdc.cam.ac.uk [reference nos. 188141 (2b), 188142 (4c), 188143 (5b), 188144 (8), and 188145 (9)].

In addition, the author name Lihua Yua should have appeared as Lihua Yuan. The online version has been corrected. The corrected author line appears below.

Bing Gong, Huaiqiang Zeng, Jin Zhu, Lihua Yuan, Yaohua Han, Shizhi Cheng, Mako Furukawa, Rubén D. Parra, Andrey Y. Kovalevsky, Jeffrey L. Mills, Ewa Skrzypczak-Jankun, Suzana Martinovic, Richard D. Smith, Chong Zheng, Thomas Szyperski, and Xiao Cheng Zeng

www.pnas.org/cgi/doi/10.1073/pnas.222533199

BIOCHEMISTRY. For the article “Generation and characterization of androgen receptor knockout (ARKO) mice: An in vivo model for the study of androgen functions in selective tissues,” by Shuyuan Yeh, Meng-Yin Tsai, Qingquan Xu, Xiao-Min Mu, Henry Lardy, Ko-En Huang, Hank Lin, Shauh-Der Yeh, Saleh Altuwaijri, Xinchang Zhou, Lianping Xing, Brendan F. Boyce, Min-Chie Hung, Su Zhang, Lin Gan, and Chawnshang Chang, which appeared in number 21, October 15, 2002, of Proc. Natl. Acad. Sci. USA (99, 13498–13503; First Published October 7, 2002; 10.1073/pnas.212474399), the author name Min-Chie Hung appeared incorrectly. The correct name is Mien-Chie Hung. The online version has been corrected. The corrected author line appears below.

Shuyuan Yeh, Meng-Yin Tsai, Qingquan Xu, Xiao-Min Mu, Henry Lardy, Ko-En Huang, Hank Lin, Shauh-Der Yeh, Saleh Altuwaijri, Xinchang Zhou, Lianping Xing, Brendan F. Boyce, Mien-Chie Hung, Su Zhang, Lin Gan, and Chawnshang Chang

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IMMUNOLOGY. For the article “Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation,” by Maximilian Diehn, Ash A. Alizadeh, Oliver J. Rando, Chih Long Liu, Kryn Stankunas, David Botstein, Gerald R. Crabtree, and Patrick O. Brown, which appeared in number 18, September 3, 2002, of Proc. Natl. Acad. Sci. USA (99, 11796–11801; First Published August 23, 2002; 10.1073/pnas.092284399), the figures in the printed article are of low resolution. The online article contains high-resolution figures (www.pnas.org/cgi/reprint/99/18/11796.pdf).

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Generation and characterization of androgen receptor knockout (ARKO) mice: An \textit{in vivo} model for the study of androgen functions in selective tissues


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Contributed by Henry Lardy, July 29, 2002

By using a cre-lox conditional knockout strategy, we report here the generation of androgen receptor knockout (ARKO) mice. Phenotype analysis shows that ARKO male mice have a female-like appearance and body weight. Their testes are 80% smaller and serum testosterone concentrations are lower than in wild-type (wt) mice. Spermatogenesis is arrested at pachytene spermatoocytes. The number and size of adipocytes are also different between the wt and ARKO mice. Cancellous bone volumes of ARKO male mice are reduced compared with wt littermates. In addition, we found the average number of pups per litter in homologous and heterozygous ARKO female mice is lower than in wt female mice, suggesting potential defects in female fertility and/or ovulation. The cre-lox ARKO mouse provides a much-needed \textit{in vivo} animal model to study androgen functions in the selective androgen target tissues in female or male mice.

Androgen receptor (AR), a member of the nuclear receptor superfamily, was first cloned in 1988 (1–3). It contains an N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal ligand-binding domain (4). AR may form a dimer and interact with many coregulators to modulate androgen target genes (5). In addition to its natural ligands, testosterone (T) and dihydrotestosterone, 17β-estradiol can also induce AR transactivation in the presence of some selective coregulators in some selective tissues (6, 7). The increasing evidence shows that androgen and AR may also play important roles in female physiological processes, including folliculogenesis (8), bone metabolism (9), autoimmune diseases (10), maintenance of brain functions (11), and several female cancers, including breast, ovary, and endometrium (12–14).

In the cartilage and bone, AR is expressed in chondrocytes, osteoblasts, osteocytes (15), and osteoclasts (16). Clinical studies suggested that combined therapy of estrogens plus androgens may enhance bone mineral density and bone mass to a more significant degree than estrogen therapy alone in postmenopausal women (17, 18). However, the mechanism of androgen action on bone metabolism remains controversial. Some studies suggest that the effect is mainly through the effects of aromatase, which is critical for male fertility. To generate tissue-specific ARKO mice or female ARKO mice, a cre-lox strategy for conditional KO is necessary. The cre-lox system utilizes the expression of P1 phage cre recombinase (Cre) to catalyze the excision of DNA located between flanking lox sites (25). This strategy differs from the standard targeted disruption procedure in that embryonic stem (ES) cells are generated in which the targeted segment is not disrupted but flanked by lox sites (flanked). The target gene thus functions normally and mice can be bred to homozygosity for the targeted locus. Here we describe the generation and characterization of ARKO mice. The potential \textit{in vivo} application of this model for the study of androgen functions in selective tissues is also discussed.

\textbf{Experimental Procedures}

The steps leading to the birth of the female mice carrying the Cre and the homozygous floxed AR genes in both X chromosomes are illustrated in Figs. 1–3. We first constructed the targeting vector followed by generation of the founder mice carrying the floxed AR fragment. The founder mice were then mated with the female Cre mice to generate the F3 offspring that carries ARKO with Cre expression.

\textbf{Construction of Targeting Vectors.} Two genomic clones containing exon 2 of mouse AR were isolated from an ES129/SVJ bacte riophage λ genomic library (Stratagene) by using the mouse AR exon 2 sequence as the probe. The flanking region was sequenced and cloned into the PKI vector (26). Fig. 1 details the procedure for the construction of targeting vectors.

\textbf{Generation of the Chimera Founder Mice.} The ES cell line 129/SEVE was grown according to the conditions described (27). For electroporation, 40 \( \mu \)g of the targeting vector was linearized by NotI and suspended together with \( 1 \times 10^7 \) ES cells in 1 ml of Dulbecco's modified Eagle's medium. They were electropolarized at 300 F, 0.4 ms in the Gene Pulser II system (Bio-Rad). The neo colonies were selected in the presence of 300 \( \mu \)g/ml G418. Homologous recombinations were identified by genomic Southern blot hybridization. The clones with homologous recombination were amplified and reelectropolarized to introduce pCMV-Cre vector into the cells. The transient expression of the Cre in the cells resulted in three types of recombination, which was checked by Southern blot hybridization (27). The ES cells with

Abbreviations: AR, androgen receptor; KO, knockout; ES cell, embryonic stem cell; wt, wild type; T, testosterone; Tfm, testicular feminization; Cre, cre recombinase.

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type 1 recombination were then injected into the inner cell mass of blastocysts, which were then implanted into the uterus of foster mothers for further development.

Mating of the Chimera Founder Mice with the Homozygous Cre Mice.

The mating strategy is illustrated (see Fig. 3). The strain of the mosaic founder was C57BL/6J−129/SEV (B6/129). The mating between the founder and the female B6 mice create agouti female offspring carrying the heterozygous floxed AR (F1). The F1 offspring mate with the B6 male mice to create male mice carrying the floxed AR in the X chromosome (F2), or mate with the homozygous ACTB/Cre male that are carrying the Cre under the control of β-actin promoter to generate female mice carrying both the heterozygous floxed AR and Cre (F2). Mating these two genotypes of the F2 mice finally generate female mice carrying the homozygous ARKO and Cre. β-Actin is a housekeeping gene and is universally expressed in every tissue. Therefore, the β-actin promoter-driven Cre will express and delete the floxed AR fragment in all of the cells.

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Primer Design and Genotyping of ARKO Mice. Based on the sequence information we obtained for the AR genomic DNA, two pairs of primers have been designed to distinguish the wild-type AR (wtAR), ARKO, and floxed AR X chromosome on mice. For examining the ARKO on the X chromosome, the 5′ primer named “select” is located in the intron 1 and its sequence is 5′-GTGCTAACTTACTCTGC-3′, and the 3′ end primer is 2–9, which is located in intron 2 and its sequence is 5′-CCTACATGTACTGAGAG-3′. If the mice carry ARKO, the PCR product size from this pair of primers would be 238 bp. If the mouse contains wtAR, this pair of primers will amplify a PCR product with 580 bp. For examining the floxed AR X chromosome, primer “select” and primer “2–3” are used. 2–3 primer is the 3′ end primer which is located in the exon 2 with the sequence 5′-TTCAGCGGCTTTTGAAG-3′. This pair of primers will amplify a product with 444 bp. For examining wt AR, the PCR product will be 410 bp. The expression of Cre and internal control IL2 were confirmed by PCR during genotyping. The primer design and PCR conditions of Cre and IL2 follow The Jackson Laboratory’s suggestions.

Histologic Analysis of Bones. Preparation and analysis of bones were performed, as described (28). In brief, bones were fixed in 10% buffered formalin, decaclified in 10% EDTA, and embedded in paraffin. Sections were stained with hematoxylin and eosin for assessment of bone volume and for tartrate-resistant acid phosphatase activity for quantitation of osteoclasts. Bone volume (amount of bone matrix expressed as a percentage of the cancellous space) and osteoclast numbers (expressed per square millimeter of the cancellous bone volume) in the cancellous space of the long bones were measured. Bone samples were examined from a minimum of four wt and KO mice. Double labeling of bone-forming surfaces was performed by s.c. injection of calcine (20 mg/kg) given 7 days and 1 day before killing. Bone formation rate was measured, as described (29).

Generation of Osteoclasts. Splenocytes from ARKO mice and their wt littermates were used to generate osteoclasts in the absence of osteoblast/stromal cells. These cells were incubated with ammonium chloride solution for 10 min on ice to lyse red blood cells, and then cultured (1.75 × 10^6) cells per well in 96-well plates) in α-MEM (GIBCO/BRL) supplemented with 10% FCS (HyClone) in the presence of RANKL (100 ng/ml) and M-CSF (10 ng/ml). Cultures were maintained for 5–6 days at 37°C in an atmosphere of 5% CO₂/air and the media were changed every 2 days by replacing half of the spent media with fresh media supplemented with RANKL/M-CSF. Cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts, as described (30).

Results and Discussion

Construction of Targeting Vectors and Electroporation of the ARKO Plasmid in ES Cells. To disrupt the AR gene, exon 2 is targeted for loxP/Cre-mediated excision. Exon 2 encodes the second zinc finger of the DNA-binding domain, and deletion of the DNA-binding domain has been reported to result in the complete androgen-insensitive syndrome (31). As shown in Fig. 1, the PKI vector was modified from the pBluescript vector and a thymidine kinase-selective marker (MCT-TK) was inserted at the 5′ end of the multiple cloning site. The other neomycin-resistant marker (PKG-Neo) flanked by two loxP sequences was inserted at the 3′ end of the PKI cloning site. A lox sequence plus XhoI site at the 5′ end, two multiple cloning sites (MCS), two lox sites (−), a positive Neo selective marker (PKG-Neo), and a negative thymidine kinase selective marker (MCT-TK) for the cloning, the XhoI site at the 5′ end MCS was first destroyed. A 3-kb intron 2 fragment was introduced into the 3′ EcoR1 cloning site (R1), followed by a fragment containing intron 1, exon 2, and a small fragment of intron 2 sequences into 5′ XhoI site (X). A lox sequence plus an artificial KpnI site were finally inserted into the XhoI site shortly 5′ to the beginning of exon 2. The constructed plasmid was linearized by NotI before being electroporated into ES cells.
The transient transfection of pCMV-Cre would result in type 1 AR-chimera male mice, respectively. ES clones, A9 and K7, we were able to obtain 6 and 12 floxed AR-chimera male mice. With blastocyst injections of two wt ES clones, B4 to B8 are the wt ES clones that displayed signal only at the 9-kb position. (C) Southern blot screening of the floxed AR clone transfected with pCMV plasmid. The pCMV-Cre restricted the sequence between two lox sites and generated four types. Lane 1 is without recombination (7 kb), lane 2 is type I recombination (5 kb), lane 3 is type II (11 kb), and lane 4 is the type III recombination (9 kb).

**Screening of ARKO ES Cells and Genotype Screening of Floxed AR Chimera Male Mice.** For the screening of ES cell clones with ARKO, we designed two pairs of primers to distinguish between the wt and floxed AR locus (Fig. 2B). Southern blotting was applied to verify the floxed AR construct in ES cells (Fig. 2B). The transient transfection of pCMV-Cre would result in type 1, type 2, and type 3 recombinations. As determined by Southern blot analysis illustrated in Fig. 2C, we successfully screened type 1 recombinant that represents the floxed AR without Neo. This type 1 recombinant, containing loxP sites flanking the AR exon 2, was then used for the blastocyst injection to generate floxed AR-chimera male mice. With blastocyst injections of two ES clones, A9 and K7, we were able to obtain 6 and 12 floxed AR-chimera male mice, respectively.

**Generation of ARKO Mice.** To generate mice with the disruption of AR, the floxed AR male mice were crossed to female mice carrying Cre under the control of the β-actin (ACTB). ACTB-Cre transgene will be activated in all tissues to generate mice lacking functional AR. As shown in Fig. 3, after two matings, we obtained F2 mice with male floxed AR mice (floxed AR/Y male) and heterozygous female ARKO on one X chromosome (ar/AR-ACTB/Cre female). We then bred them together to obtain F3 ARKO female (ar/AR-ACTB/Cre+/−) and male (ar/AR-ACTB/Cre−/−) mice.

**Genotype Screening of ARKO Mice.** Alternatively, we were able to obtain ARKO male mice by mating floxed AR female and ACTB/cre male mice. This strategy will generate pups of four possible genotypes (ar/Y-ACTB/Cre-male, ar/AR-ACTB/Cre-female, AR/Y-ACTB/Cre-male, AR/AR ACTB/Cre-female) with ratio of 1:1:1:1. Three primers, select, exon 2–3, and exon 2–9 (for the relative position of each primer in the AR gene see Fig. 4), were synthesized to amplify mice genomic DNA to distinguish the floxed AR, ARKO, and wt mice. As shown in Fig. 4, we were able to identify ARKO male mice by using select and exon 2–9 primers to PCR amplify the 238-bp DNA. In contrast, wt mice can produce 580-bp PCR-amplified DNA fragments by using select and 2–9 primers (Fig. 4B).

**Phenotype Characterization of ARKO Male Mice.** Six 5- and 8-week-old ARKO mice were killed for the comparison of their phenotype with wt male and female mice. We first noticed that ARKO male mice have female-like appearance and body weight. In the 5-week-old ARKO male mice, the genito-anal distance is 0.55 cm, similar to female mice, yet is shorter than the wt male mice of 1.05 cm. In the 8-week-old ARKO male mice, the genito-anal distance is 0.59 cm, which is shorter than that of wt male sibling of 1.12 cm and similar to their female siblings (Fig. 5A). The ARKO male mice have testes, yet the size is much smaller, only 20% as compared with the same age of wt male mice (Fig. 5B–D).

**Male Reproductive Organs.** The external genitalia in male ARKO mice showed ambiguous or feminized appearance. The penis seems microphallus and the urethra shows hypospadia. The scrotum is poorly developed and looks like the labia major in the female. All of vas deferens, epididymis, seminal vesicle, and prostate are agenesis (Fig. 5B and C). Testes are small in size and cryptorchid in the low abdominal area close to the internal inguinal ring, and are similar in Tfm mice or humans with complete androgen-insensitive syndrome (Fig. 5B–D). No vaginal opening is found, and also no fallopian tubes and uterus can be observed.

**Analysis of Testes.** To dissect further the testes morphology in the ARKO mice, immunostaining was performed. As shown in Fig,
6A and B, we could observe obvious AR positive signals in testes from 8-week-old wt mice (Fig. 6A). In contrast, no AR signal could be detected from 8-week-old ARKO mice (Fig. 6B).

We then compared the histology of testes from ARKO mice and wt mice. As shown in Fig. 6C and D, in the testes from wt mice, spermatogenesis sequentially developed round spermatids and elongated spermatids. In addition, mature spermatocytes can be clearly observed (Fig. 6C). However, testes from ARKO mice are less cellular and thin in the seminiferous tubules. Some tubules contain Sertoli cells only and the others contain a few germ cells (Fig. 6D). In the tubules with germ cells, the spermatogenesis is hypoplastic. Occasionally, some spermatocytes at the pachytene stage are found (Fig. 6E). No round spermatids, elongated spermatids, or mature spermatocytes occur in the whole testis, suggesting spermatogenesis may be arrested at pachytene spermatocyte. The Sertoli cells show fibrillary degeneration in the cytoplasm of Sertoli cells. (G) Some cells in the tubule contain condensed and pyknotic nuclei. They may represent apoptotic bodies. (H) Leydig cells located in interstitial space are hypertrophic.

**Fig. 6.** Immunostaining of AR and histologic analysis of testes from wt and ARKO male mice. Immunostaining of AR in testes from wt (A) and ARKO mice (B). For AR staining, anti-AR antibody and Vectorstain ABC-AP kit were used according to the manufacturer’s procedures. Testes from wt show positive signal (A, arrows), and testes from ARKO mice show no positive staining. (C–D) The histology of the testis from wt (C) and ARKO (D) shown by hematoxylin and eosin stain. Spermatogenesis in ARKO mice arrested in pachytene spermatocyte (P) stage. (E–F) Rs, round spermatids; Es, elongated spermatids; P, pachytene spermatocytes. In further analysis of testes in complete ARKO mice we obtained the following information (E–H): (E) The tubule contains hypoplastic spermatogonia and pachytene spermatocyte. (F) The tubule contains Sertoli cells only and shows fibrillary degeneration in the cytoplasm of Sertoli cells. (G) Some cells in the tubule contain condensed and pyknotic nuclei. They may represent apoptotic bodies. (H) Leydig cells located in interstitial space are hypertrophic.

**Fig. 5.** Phenotype of 8-week-old male ARKO mice. Six 8-week-old male ARKO mice were killed. The results were always compared among siblings. (A) The external genitalia of male ARKO, wt male, and wt female. (B and C) The internal genitalia of male ARKO and wt male mice. Arrows in B identify the testis. (D) The testes of wt male and ARKO mice.

**Fig. 4.** Genotyping of ARKO mice. We have applied the primers “select” and “2–9” to identify wt and ARKO male mice in our study. (A) Schematic presentation of the DNA construct and primer location in exon 2 area of wt, KO, and floxed AR genes. (B) List of the sizes of PCR product amplified by designed primer pairs. (C) The identification of wt and ARKO mice, by using select and 2–9. We amplified a DNA fragment with 580 bp, which represents wtAR, and with 238 bp, which represents ARKO exon 2. The expression of Cre and internal control IL-2 were confirmed by PCR (Bottom).
metabolism. Taken together, the osteopenic phenotype of ARKO mice and is consistent with the low T concentrations in the mice. The increased in number in ARKO mice, but that they also live longer than normal because of reduced testosterone concentration.

Body Weight and Adipose Tissue. The body weight of ARKO male mice is similar to wt female mice with an average of 18.8 g at 5 weeks and 20.1 g at 8 weeks. In contrast, the average body weight of wt male mice is 24.3 g at 5 weeks and 26.7 g at 8 weeks. Histologic analysis of adipose tissue indicated that the size and number of adipocytes start to be different between wt male and ARKO male mice at 5 weeks. These differences become more obvious in 8-week-old mice (Fig. 8A). In contrast, infrarenal adipocytes were well-developed in both 5- and 8-week-old mice, and show no obvious difference between wt and ARKO mice (Fig. 8B). These results suggest that AR may play roles in adipogenesis. Early reports also suggest that androgens may play some roles in the site specificity of adipose metabolism (38). Women with abdominal fat distribution may have increased percentage of free T in the peripheral blood. In contrast, obesity in men may be characterized by reduced T. However, the detailed mechanisms of how T-AR influence adipose tissue and obesity remain unclear. Because peroxisome-proliferator-activated receptors play important roles in adipogenesis (39, 40) and the AR coregulator, ARA70, can modulate both AR and peroxisome-proliferator-activated receptors’ transcriptional activities (41, 42), it will be interesting to see whether AR can crosstalk to peroxisome-proliferator-activated receptors’ pathway by sharing a common coregulator, such as ARA70, in adipose tissue.

ARKO Female Mice. Because of the infertility of Tfm male mice, it is difficult to generate ARKO female mice to study the roles of AR in female tissues. With floxed AR male mice, we are now able to mate them with ACTB-Cre ar female to generate ARKO in female mice. By using this strategy, we were able to obtain a few ARKO female mice, and we are in the process of obtaining more ARKO female mice and analyzing their phenotypes with focus on the ovary and breast.

Fig. 8. Histology of fat tissues in ARKO mice. (A) s.c. adipose tissue of 5- and 8-week-old ARKO and wt littermates were stained with hematoxylin and eosin. (Bars = 200 μm.) (B) Infrarenal adipose tissue of 8-week-old ARKO and wt littermates were stained with hematoxylin and eosin. (Bars = 200 μm.)
Tissue-Specific Knockout of AR. One advantage of creating the floxed AR mice is to provide a base to generate tissue-specific ARKO in selective tissues such as breast (mating with MMTV-Cre mice), prostate (mating with PSA-Cre or probasin-Cre), and liver (mating with α-fetal protein-Cre or albumin-Cre). We are in the process of generating these tissue-specific ARKO mice to study the roles of AR in these tissues.

In summary, the generation of ARKO male and female mice provides us with a valuable in vivo model to study androgen functions in selective tissues.

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