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Differential gene dosage effects of Ad4BP/SF-1 on target tissue development

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Abstract

Ad4BP/SF-1 (NR5A1) was identified as a key regulator of the hypothalamus–pituitary–gonadal and –adrenal axes. Loss-of-function studies revealed that *Ad4BP/SF-1* is essential for the development of these tissues and spleen. Here, we generated transgenic mouse with BAC recombinants carrying a dual promoter and Tet-off system. These recombinants have a potential to express lacZ and Ad4BP/SF-1 in the tissues where endogenous *Ad4BP/SF-1* is expressed. However, protein level of Ad4BP/SF-1 varied among the tissues of the transgenic mice and probably thereby the target tissues are affected differentially. The BAC-transgenic mice were applied to rescue *Ad4BP/SF-1* KO mouse. Interestingly, the mice successfully rescued the gonad and spleen but failed to rescue the adrenal gland. This variation might be dependent on in part the protein expression levels among the tissues and in part on differential sensitivities to the gene dosage.

Keywords: BAC transgenic; Ad4BP; SF-1; Steroidogenesis; Testis; Adrenal; Gonad; Spleen; Gene dosage

The orphan nuclear receptor, Ad4BP (adrenal-4-binding protein; NR5A1 [1]) or SF-1 (steroidogenic factor-1), has emerged as a key regulator of the hypothalamus–pituitary–gonadal and hypothalamus–pituitary–adrenal axes through regulating genes essential for the development of the tissues [2–4]. With regard to the expression of the factor, early studies demonstrated that particular cell types in the testis, ovary, and adrenal cortex are the predominant expression domains of Ad4BP/SF-1 [5–7]. In addition to these tissues, the factor has been demonstrated to be expressed in the ventromedial hypothalamus (VMH), anterior pituitary gonadotrophs [8–10], and spleen [11,12].

Gene disruption studies were performed to investigate the functions of Ad4BP/SF-1 during tissue development. The gene knockout mice displayed agenesis of the adrenal gland and gonads at birth, reflecting increased apoptotic cell death in the particular cell population comprising the tissue primordia. Due to the absence of the gonad in the fetal stage, the gene-disrupted mice exhibited male to female sex reversal, while they died shortly after birth because of the lack of the adrenal gland [9,13,14]. These mice also exhibited abnormally organized VMH and functionally impaired pituitary gonadotroph [8,9]. Moreover, the splenic vascular architecture was impaired and thus the spleen of the knockout mouse was structurally and functionally hypoplastic [11]. Based on these phenotypes, Ad4BP/SF-1 is considered essential for the structural and functional development of the tissues. However, since the knockout mice died shortly after birth, the function of Ad4BP/SF-1 in the adult tissues could not be evaluated by the gene knockout studies.

The mechanisms underlying *Ad4BP/SF-1* gene regulation have been investigated using reporter gene assays. Studies with steroidogenic cultured cells revealed that E and CCAAT boxes localized at the proximal upstream of

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the gene are crucial for transcription [15–17]. In support of these observations, Pod1/Capsulin, an E box-binding protein, was demonstrated to suppress *Ad4BP/SF-1* gene transcription [18]. Moreover, a loss-of-function study of the *Pod1/Capsulin* gene strongly supported the observation given above [19]. However, these assays with cultured cells are highly dependent on whether the cells reflect the intrinsic state of gene regulation. In addition, the construct for the reporter gene assay usually contains solely 5′ upstream of gene of interest. Therefore, reporter gene studies could easily overlook regulatory elements when they reside at the intronic or 3′ downstream regions. In order to characterize the whole genomic region of*Ad4BP/SF-1*, transgenic (Tg) studies have been performed with long DNA fragments. Studies with a mouse BAC clone [20], a rat YAC clone [21], and mouse Cosmid clones [22] recapitulated the expression in most, if not all, cell types where the endogenous *Ad4BP/SF-1* gene is expressed.

In the present study, we attempted to investigate the function of Ad4BP/SF-1 in the adult tissues of *Ad4BP/SF-1* KO mouse rescued by Tg mice harboring a dual reporter/Tet-off system. Since the construct is able to arrest the expression of Ad4BP/SF-1 by chemical treatment, we expected to examine effects of disappearance of Ad4BP/SF-1 from the rescued tissues. Unfortunately, the expression levels of the exogenous Ad4BP/SF-1 varied among tissues, and possibly thereby the target tissues were rescued at differential levels. Indeed, the adrenal gland could not be rescued, and therefore the mice failed to grow. Interestingly, however, our study clearly revealed that gene dosage of *Ad4BP/SF-1* affected differentially the development of the target tissues.

Materials and methods

Construction of modified-cassette and preparation of BAC recombinant. DNA fragments encoding tetracycline transactivator-VP16 (tTA) prepared from pTet-off (Clontech Laboratories, Palo Alto, CA) and TRE (tetracycline responsive element) from pBI (Clontech) were ligated with lacZ gene to generate tTA-lacZ-TRE (tTAZT), and the resultant fragment was inserted into *Spl*I site in the second exon of *Ad4BP/SF-1*. This recombinant gene was digested by *Xba*I and *Eco*RI to prepare a modification cassette containing tTAZT with 500 bp upstream region from the *Spl*I site (A-arm) and 1.5 kb downstream region from the*Spl*I site (B-arm) (Fig. 1A). To generate pSV1-RecA-A-tTAZT-B, the modification cassette was cloned into pSV1-RecA to facilitate homologous recombination in RecA$^-$ *Escherichia coli*.

Two distinct BAC clones, BAC1-Ad4BP carrying 202,510 bp and BAC5-Ad4BP carrying 110,950 bp (Incyte Genomics, CA), were used (Fig. 1A). DH5α (RecA$^-$) carrying the original BAC clone was transformed with pSV1-RecA-A-tTAZT-B, and thereafter selected with 10 mg/ml tetracycline and 12.5 mg/ml chloramphenicol. The drug-resistant clones were subjected to PCR analyses to confirm homologous recombination [23] in the A-arm with Ad4BP/SF-1 intron 1/s (5′-GTCGTTGGC ACCGCATTCCTG-3′) and pTet-off/as (5′-CATTAAGCAGCTCTAAT GCGCGT-3′), while in the B-arm with p1PBI/s (5′-GTACCCGGGGAT CCTCTAGTC-3′) and Ad4BP-intron 3/as (5′-CCAGTCCAATGTTGCC ACCTC-3′).

To confirm that neither artificial recombination nor deletion occurred throughout the process of recombination, the BAC recombinants were examined by PCR of *Gcnf* exon 5 (region a in Fig. 1A) with 5′-GGAGC CACATTACCACGTTTC-3′ and 5′-GCTGTCCTGGAATTCACTAT G-3′, *Gcnf* exon 9 (region b) with 5′-GACCTGGGAACCGGAACTTAC-3′ and 5′-GCTCTTGCCACCACCTACTCA-3′, *Ad4BP/SF-1* exon 1a (region c) with 5′-CCGCTGCTGGGTGAAGAAGTT-3′ and 5′-GAGC AAGGCACTGAAGAGG-3′, *Ad4BP/SF-1* exon 4 (region f) with 5′-TG GCTGGCTACCTCTATCCTG-3′ and 5′-AAAGACCATGCACCTTC GTGC-3′, and *Ad4BP/SF-1* exon 7 (region g) with 5′-ATGCCACTGCCT CCAAAAGAC-3′ and 5′-GGGTTAGGGCAGGAATGTTGG-3′, 17.1 kb downstream from exon 7 of *Ad4BP/SF-1* (region h) with 5′-CCGC TGCTGGGTGAAGAAGTT-3′ and 5′CACCCTTATCCGGCTGAGA AT-3′, and *Psmb7* exon 4 (region i) with 5′-TTGCAGACTACTCAGA ACACC-3′ and 5′-CACACCCTGGAAACCTTACCT-3′. Original and recombinant BAC DNAs were digested by *Eco*RI and *Bam*HI, and subjected to Southern blot using either 5′-probe (0.5 kb between *Eco*RI and *Xho*I) or 3′-probe (1.5 kb between *Bam*HI and *Eco*RI).

Production of BAC Tg mouse lines. The BAC1-Ad4BP-tTAZT and BAC5-Ad4BP-tTAZT DNAs (1 ng/μl) were subjected to Tg mouse generation [24]. The Tg offsprings were genotyped by PCR with p1-LacZ (5′-GCCGAAATCCCGAATCTCTATC-3′) and p2-lacZ (5′-GATTCA TTCAGCGACCAG-3′) to detect lacZ gene. To generate Ad4BP/SF-1-null mice carrying the BAC transgene, the BAC Tg (Tg(+)) mice were crossed with Ad4BP/SF-1(+/−), and thereafter the progeny of Ad4BP/SF-1(+/−) carrying the BAC transgene (Tg(+);Ad4BP(+/−)) was mated. The offsprings were genotyped with PCR using two sets of primers, p1PBI/s and Ad4BP exon 3/as (5′-TCTCGGTGCACGTGTAATG-3′) and Neo primer set (the Jackson Laboratory) Neo-oIMR0013 (5′-CTTG GGTGGAGAGGCTATTC-3′) and Neo-oIMR0014 (5′-AGGTGAGAT GACAGGAGATC-3′). Since the two PCR gave the same fragments between Tg(+);Ad4BP(+/−) and Tg(+);Ad4BP(−/−), further genotyping was performed with Southern blot using the 0.5 kb 5′-probe and 1.5 kb 3′-probe described above (Fig. 1A). Detection of lacZ activity with whole-mount samples was performed as described [25].

RT-PCR and Northern blot analyses. Total RNAs were subjected to RT-PCR analyses to detect Ad4BP/SF-1 mRNA transcribed from the endogenous gene which were performed with primers, MAP1A (5′-CCGC TGCTGGGTGAAGAAGTT-3′) and pAd4BP-Ex4/as (5′-CGCATTCG ATCAGCACGCAC-3′). For transgene transcripts, p1PBI/s and pAd4BP-Ex4/as were used. Ten micrograms of total RNAs was used for Northern blot analyses probed with Ad4BP/SF-1 (814 bp from *Sac*I to *Sac*I site) or Gapdh (791 bp of PCR product from 53 to 842).

Relative quantification of transgene by PCR. After DNAs isolated from the BAC-Tg mice were digested with *Eco*RI, 50 ng of the DNAs was used for relative quantification of the transgene with Applied Biosystems 7300/7500 real time PCR system (Applied Biosystems, Foster City, CA). Gaphd was used as a control. PCR primers were as follows (Fig. 4A). The 5′-end primers for BAC1 to amplify fragment a were 5′-TGGAGGTGGTTAA AATGCTAGAGTAG-3′ and 5′-AAAGGGCAGCACGGAGATC-3′, while the 3′-end primers for BAC1 to amplify fragment e were 5′-GCA TTGTTGGCCCTGCATCT-3′ and 5′-TGCCTCGCCAAGAAGCA-3′. The 5′-end primers for BAC5 to amplify fragment b were 5′-GGGCAG AGGCAGGCAAAT-3′ and 5′-TGTCCTGGAACTCGCTCTGTAG-3′, while the 3′-end primers for BAC5 to amplify fragment d were 5′-CAGC CCAAACCTCAAATAATGG-3′ and 5′-CACAACCCAGGAGAACA TTACACT-3′. The 5′-end probe for BAC5 is 5′-FAM (5′-reporter dye 6-carboxyfluorescein quenchers) [26] -CTGAGTTCAAGGTTAGCC-MGB (minor groove binder, fluorogenic probe) while the 3′-end probe for BAC5 is 5′-FAM-ACAAGTGAGGGCCATC-MGB. The 5′-end probe for BAC1 is 5′-FAM-CTTAACCATGGCATCTC-MGB while the 3′-end probe for BAC1 is 5′-FAM-CTTCAGGCCTTTCTTGTT-MGB. Copy number of the *Ad4BP/SF-1* gene was determined with Ad4BP primer (5′-T CCAACCTGGCTCTCCCTTT-3′ and 5′-CTCAGTGCTGCCACCTA GCA-3′) to amplify fragment c. Ad4BP probe was 5′-FAM-CCTCAGTC CCCACCCTTGCCG-TAMRA (3′-quencher dye tetramethylrhodamine). Quantitative PCR was performed in triplicate.

Southern and Western blots, in situ hybridization, and immunohistochemistry. Southern blot analysis was performed as described [27]. Ten micrograms of genomic and BAC DNAs was used. Western blot analysis with rabbit antibodies to Ad4BP/SF-1 and α-tubulin (Sigma Chemical,