The Ad4BP/SF1 Function of Testes BACAd4BPtTAZ Transgenic-Knockout Mice normally regulated during Development.*)

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Abstract

To address the function of Ad4BP/SF1 in developing gonad on Ad4BP/SF1 knockout mouse, we have modified the BACAd4BP endogenous by homologous recombinant with modification cassette-containing Tet-Off system and LacZ reporter genes to allow the expression of Ad4BP/SF1 in developing tissues and organ differentiation, regulate of Ad4BP/SF1 mechanism, and reveal the defect due to Ad4BP/SF1 deficiency. The BACAd4BPtTAZ recombinant has a potential to express a lacZ reporter gene and Ad4BP/SF-1 in the tissues where the endogenous Ad4BP/SF-1 gene is expressed. Expectedly, the lacZ expressions in the BAC transgenic mice mostly recapitulated the endogenous gene expressions. However, protein level of upregulated Ad4BP/SF-1 varied among the transgenic mice. Showing a good correlation with the expression levels, the transgene differentially affected the target tissues. We further applied the BAC-transgenic mice to rescue Ad4BP/SF-1 gene disrupted mouse. Interestingly, although the Ad4BP/SF1 protein expression levels are slightly high in testes of BACd4BPtTAZ-Tg mice, the BAC recombinants successfully rescued and normally developed the gonad of the KO mouse.

Keywords: *Ad4BP, SF1, BAC transgenic, Steroidogenesis, Testis*.

I. Introduction

The orphan nuclear receptor, Ad4BP (adrenal-4-binding protein; NR5A1) or SF-1 (steroidogenic factor-1), has emerged as a key regulator of the hypothalamus-pituitary-gonadal (HPG) and -adrenal (HPA) axes, and it also as a transcription factor essential for the development of the tissues comprising the axes. Ad4/SF-1 site, the recognition sequence of Ad4BP/SF-1, was initially identified as a conserved regulatory motif in the promoter regions of genes encoding steroid hydroxylase P-450s and thereafter found in other genes involved in the distinctive functions of the tissues [1-3]. Concerning 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 [4-6]. In addition to these tissues, it has been demonstrated that the factor is expressed in the ventromedial hypothalamus (VMH), anterior pituitary gonadotrophs [7-9], and spleen [10].

Gene disruption studies were performed to investigate the functions of Ad4BP/SF-1 during the tissue development. The gene knockout mice displayed agenesis of the adrenal gland and gonads at birth, reflecting increased apoptic 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 probably due to the absence of the adrenal gland, they died shortly after birth [8, 11, 12]. According to these phenotypes, it has been established that Ad4BP/SF1 is essential for the structural and functional development of those tissues probably mediating downstream gene expression.

To address the function of Ad4BP/SF1 in developing gonad and tissues-related Ad4BP/SF1 regulation on FtzF1 knockout mouse, we have modified the BACAd4BP endogenous by homologous recombinant with modification cassette-containing tetracycline transactivator system and LacZ reporter genes to allow the expression of Ad4BP/SF1 in developing tissues and organ differentiation, regulate of Ad4BP/SF1 mechanism, and reveal the defect due to Ad4BP/SF1 deficiency.

II. Materials & Methods

Experimental Animal

Ad4BP/SF1 KO mice were generated as described previously [8]. Background animals of

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BAC transgenic were F1 of C3H and and C57BL/6 female and male mouse (Japan Clea, Tokyo, Japan). The BACAd4BP- tTAZ-Tg mice were generated as described previously [13]. All animals were performed at the specific pathogen-free of Transgenic Animal Care Facilities of National Institute for Basic Biology, National Institute of Natural Science, Okazaki, Japan. All protocols were approved by the Institutional Animal Care and Use Committee of the National Institute for Basic Biology.

Whole-mount lacZ staining

Detection of lacZ enzymatic activity with whole-mount samples were performed as described [14] with minor modification.

Southern blot analyses

Southern blot analysis was performed basically as described [15]. Ten μg genomic and BAC DNAs were digested with *EcoR*I at 37ºC overnight, and the digested products were separated on 0.8% agarose. They were transferred to a nylon filter (Hybond-N+, Ammersham Biosciences) using a base solution (0.5M NaOH and 0.5M NaCl) followed by neutralization with 0.5 M Tris-HCl (pH8) and 1.5 M NaCl. After UV-crosslinking, the filter was hybridized with ^{32}P-labeled probes, washed, and finally exposed to X-ray film and BAS1800 system.

Immunohistochemistry

Embryos were fixed in 4% PFA and embedded in paraffin. Five μm sections were prepared for immunohistochemistry with rabbit polyclonal antibodies to Ad4BP/SF-1, 3β-Hsd and β-galactosidase (supplied by Dr. K Mihara and M. Sakaguchi (Kyushu University)), and goat polyclonal antibody to Mis (Müllerian inhibiting substance) (Santa Cruz Biotechnology, Santa Cruz, CA). Antisera to 3 β-□Hsd were prepared by immunizing rabbits with mouse 3β-Hsd proteins, which was expressed in E. Coli and purified. Either biotin-conjugated goat anti rabbit IgG or biotin-conjugated rabbit anti goat IgG (Jackson Immunoresearch, West Grove, PA) were used as the secondary antibody.

III. Result & Discussion

In the present study, we generated mouse lines to express a lacZ reporter gene and Ad4BP/SF-1 by a dual reporter/Tet-off system integrated in mouse BAC clones. Since the BAC constructs carried a lacZ reporter gene, we expected that the exogenous Ad4BP/SF-1 gene expression is evaluated indirectly by the transgene expression. Expectedly, lacZ expression pattern in the Tg mouse mostly, if not all, reproduced the endogenous expression of Ad4BP/SF-1. Thus, we applied the BAC-transgenic (BAC-Tg) mice to rescue Ad4BP/SF-1 KO mouse, and found interestingly that the tissues were rescued with varied levels.

The gonad of BACAd4BPtTAZ-FtzF1 knockout mice can rescued normally

Previously, some studies have reported

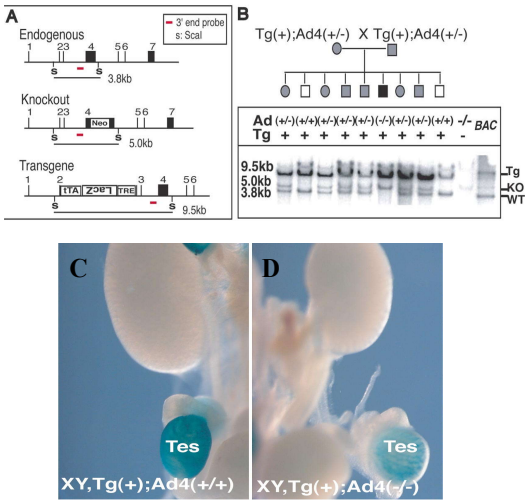

Fig1. Rescue of Ad4BP/SF-1 KO mice by B1L1 or B5L2. A, Genotyping of the endogenous Ad4BP/SF-1, knockout, and BAC-Tg is schematically presented. B. Identification of Tg-KO by Southern blot. C & D, Whole-mount lacZ staining of Embryo 14.5dpc.

that Ad4BP/SF-1 knockout mice have development and endocrine function abnormalities [8, 11]. To address the rescue Ad4BP/SF-1 knockout mice by BACAd4BPtTAZ transgenic mice from development abnormalities, recently studies has generated progeny of transgenic-Ad4BP heterozygote (BACTg-Ad4BP+/-) by natural mating. The mating is resulting the BACTg-Ad4BP+/- and BAC-Tg Ad4BP knockout mice (BACTg-Ad4BP-/-). Embryo mice were determined with Southern blot analysis by 5'-probe of Ad4BP gene (fig 1A and B). The BACTg-Ad4BP-/- embryo mouse has lack WT band (fig 1B). The profile LacZ activity expression could detected clearly in fetus at E12.5, 14.5 and 16.5dpc by whole mount and frozen section of LacZ-staining. The LacZ expression in adrenal and testis are predominant (fig 1C and D). Even though in XX-gonad is faint LacZ expression, the gonad in both sex of BAC transgenic-Ad4BP knockout mice are arrested and developed normally as wild type feature (data not shown). By using in situ hybridization and confirmed by immunohistochemistry analysis, the mRNA of lacZ, was expressed in gonad E11.5dpc strongly in BACTg,Ad4BP-/- fetus that compared with BACTg-Ad4BP+/+ fetus lacZ only expressed in

few cell in gonad. Thus, the 3β-HSD transcripts also detected in few cells of gonad BACTg-Ad4BP+/+ at E11.5dpc, perhaps some of these cells are precursor of Leydig cells. Ad4BP transcripts are expressed strongly in BACTg-Ad4BP-/- fetus. The gonad of BACTg-Ad4BP-/- feature is thinner than normal gonad at E11.5dpc (data not shown), it seems the gonad could not developed well. But at E14.5dpc stage (fig2 A to H), the gonad of BACTg-Ad4BP-/- fetus have the feature as same as normally gonad.

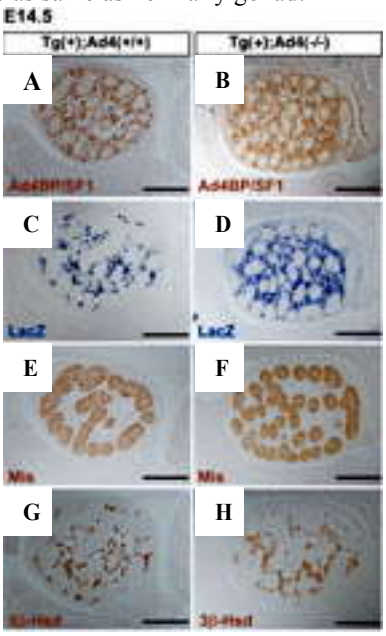

Fig2. Characterization of genes is expressed on gonad E14.5 by immunohistochemistry with specific antisera. Wild type embryos are A, C, E, G; and BACTg-Ad4BP embryos are B, D, F, & H.

From immunohistochemistry analysis showed some gene related with gonad development are expressed respectively. The Ad4BP and LacZ are expressed in Leydig and Sertoli cells. The 3β-HSD, as Leydig marker, and MIS, as Sertoli marker, are also similar between animal groups. This study has show the gonad of Ad4BP/SF-1 knockout arrested as normal as wild type embryos.

Analyses of these Ad4BP/SF-1 knockout mice revealed an absence of adrenal glands and gonads, because of apoptosis in the primordial organs; just as both adrenal and gonad differentiation normally takes place [11]. However the molecular basis of Ad4BP/SF1 regulation is remains unclear. Proliferation increases in XY-gonads by 11.5dpc and concentrated in coelomic epithelium, which is a zone of multi-potent precursor for somatic cell in both gonads. During the early phase of proliferation (11-11.5dpc) both Sertoli and interstitial Leydig cells derive from the division of SF1-expressing cell in this layer [16]. At E11.5, Expression of Ad4BP/SF1 marks in both the pre-Sertoli and pre-Leydig cell population. Subsequently, Ad4BP/SF1 expression decreased in Sertoli cells and increased in differentiating Leydig cells at the time when begin to express to enzymes that are essential for testosterone production [17]. By BAC-Ad4BP studies, the result showed that the Ad4BP/SF1 can regulate precisely the gonad differentiation and development in Ad4BP/SF1 deficiency. The precursor of Leydig and Sertoli cells are developed at embryonic day11.5 respectively between wild type BAC transgenic and BACTg(+) Ad4BP KO. Even though the gonad BACTg-Ad4BP-/- (Tg(+);Ad4(-/-)) less developed than BACTg-Ad4BP+/+ (Tg(+);Ad4(+/+)) the expression some gene related with gonad development such as 3β-HSD transcripts is not expressed yet in fetal Leydig cell of BACTg(+) Ad4BP KO in this stage. By E14.5, these gonads can differentiate normally and gene-associated with steroidogenic tissues development completely have same pattern with wild type animal. The regression gonad in Ad4BP/SF-1 deficiency in earlier stage was failed by BAC-Ad4BP transgene. This result suggesting that at 11.5dpc Ad4BP/SF1 is co-localizes with proliferating cells to direct and/or indirect stimulation cell proliferation, and also necessary for survival of early progenitors of gonads, and at sufficient level can induce organ formation.

Study of SF1/eGFP-BAC transgenic mouse showed that 50kb-SF1in upstream of exon2 Ad4BP/SF1 give strong signal in Leydig cell, but not in Sertoli cell [18]. This observation have good correlating with our BAC-Ad4BP transgene analysis that upstream of exon 2 of Ad4BP/SF1 gene has fulfill established the Ad4BP function in precursor, fetal and adult Leydig cells since early to late stage embryogenesis and adult development. It may indicate that this region is strong candidate of Ad4BP/SF1 regulatory elements of Leydig and has promising foundation to serve the future observation.

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