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Novel role for the orphan nuclear receptor Dax1 in embryogenesis, different from steroidogenesis

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Abstract

Cytomegalic adrenal hypoplasia congenita (AHC) is an X-linked disease caused by mutations in DAX1-encoding gene *NR0B1*, previously thought to function primarily in steroidogenesis. We sought to determine the expression pattern for Dax1 along with known network partners in early embryogenesis and to determine a steroidogenic capacity for the embryo prior to the establishment of the urogenital ridge at embryonic day 9 (E9). Here, we report that murine Dax1 is a unique marker in early embryonic development, distinguishing the extraembryonic (proximal) endoderm from the remainder of the developing embryo. We showed that Wilms tumor 1, steroidogenic factor 1, and estrogen receptor β were expressed throughout the embryo, but the progesterone, estrogen α and androgen receptors, cytochrome P450 (Cyp11a1) and Nur77 were not observed in any of the embryonic layers. Lack of Cyp11A1 expression at this stage confirmed an absence of inherent steroidogenic capacity for the early embryo. The role of *Nr0b1* in embryonic stem (ES) cells was investigated using siRNA knockdown, resulting in differentiation toward endoderm-like fate. *Nr0b1* conditional knockout in ES cells led to differentiation, confirming our knockdown results. Our investigations suggest that *Nr0b1* functions in a novel role in the maintenance of a relatively undifferentiated state. Our results further suggest that the failure of conventional murine *Nr0b1* knockout attempts may be due to disregulated differentiation.

Keywords: DAX1; *NR0B1*; Embryogenesis; Steroidogenesis; Orphan nuclear receptor; Embryonic stem cells

Introduction

The *NR0B1* gene encodes an orphan member of the nuclear receptor superfamily that maps to Xp21 [1]. The human protein is designated DAX1, which stands for dosage sensitive sex-reversal (DSS), adrenal hypoplasia congenita (AHC) locus on the X-chromosome, gene 1 [2]. Duplications of the 160 kb DSS region of the X chromosome, containing the *NR0B1* gene, results in an XY sex-reversed female phenotype [3]. Thus, it appears that *NR0B1* gene dosage is critical for normal human development.

DAX1 has a known role in the establishment and maintenance of steroid producing tissues (reviewed in [4]). Among the proteins known to function in a network with DAX1, the best understood are Wilms' tumor 1 (WT1) and steroidogenic factor 1 (SF1, *NR5A1A*) (reviewed in [5]). It has been proposed that DAX1 opposes testis development by binding to SF1 and antagonizing the SF1 and WT1 synergistic activation [6]. In addition, DAX1 has been proposed to repress the transcriptional activity of Nur77 (*Nr4a1*) and therefore presumably to inhibit the development of the testis [7].

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During the development of the adrenal cortex, DAX1 is thought to be downregulated as this tissue develops from the fetal to the mature post-infancy gland with the regression of the fetal zone and the establishment of normal mature zonation [8]. The functional adrenal cortex is the centre for synthesis of steroid hormones, where cholesterol is initially converted to pregnenolone, which is catalyzed by cytochrome P450scc, the cholesterol side-chain cleavage enzymes, CYP11A1 and CYP11B1 [9,10]. There are three classes of steroid hormones: mineralocorticoids (e.g., aldosterone), glucocorticoids (e.g., cortisol), and sex steroids (e.g., androgens, estrogens, and progestogens). Androgen (AR, *NR3C4*), estrogen (ER, *NR3A1–2*), and progesterone (PR, *NR5A2*) receptors, for which the sex steroids are ligands, are required for production of steroid hormone synthesis [11,12].

Dax1 function in steroidogenesis is well established, however, failure of a true murine *Nr0b1*/*Ahch*/Dax1 knockout model for AHC suggests an earlier role in embryogenesis [13–15]. A hypomorphic animal model with controlled excision of exon two has been reported and only spermatogenic and testis cord defects were observed [14,16,17]. Null or partial *Nr0b1* deletions have been hypothesized to be embryonic stem (ES) cell lethal [14,15,18].

We previously reported *Nr0b1*/Dax1 expression throughout harvested preimplantation blastocysts at embryonic day 4.5 (E4.5) and in cultured ES cells with differentiation coincident with a loss of *Nr0b1* RNA [18]. In the formation of the preimplanted murine embryo, two distinct cell types are present at E3.5–4.5, the trophectoderm (TE) and the inner cell mass (ICM). The cells of the ICM lining the blastocoel cavity of the preimplanted embryo are called the primitive endoderm (PE). Upon implantation at about E5, the TE contributes to the trophoblast and extraembryonic ectoderm (ExEc); the ICM gives rise to the epiblast and ultimately the embryonic ectoderm (EEc) and the embryonic mesoderm (EM); and the PE will cover the egg cylinder and the epiblast to become the extraembryonic parietal and visceral endoderm (VE) layers [19]. The proximal VE ultimately forms the yolk sac endoderm surrounding the developing embryo and gives rise to cells resembling hematopoietic stem cells [20,21]. It is thought that cells of distal VE are eventually replaced by definitive endoderm (DE) cells originating from the epiblast [22,23]. In the developing epiblast, a cavity can be seen, which is hypothesized to be formed due to a mix of apoptotic and cell survival signals from the VE [24]. It is thought that signals arising from the VE allow epiblast cells to differentiate into embryonic ectoderm and mesoderm [25].

Here, we sought to determine the true expression pattern of *Nr0b1*/Dax1 in early embryonic development, prior to steroidogenesis, and to show that it serves as a unique and useful marker in predicting early axis development. We investigated other known Dax1 developmental markers to elucidate a functional role at this early developmental stage. Our failure to observe expression of the rate-limiting enzyme for steroid-hormone production, Cyp11a1 in the embryo at this stage indicates a lack of steroidogenic capacity of its own and suggests a pleiotropic function for known steroidogenic genes during early embryogenesis. To investigate further the possible involvement of *Nr0b1*/Dax1 in murine ES cells, we performed knockdown and true conditional knockout (KO) experiments, resulting not in lethality as has been previously predicted, but rather, in a coincident loss of ES cell pluripotency.

Materials and methods

All experiments involving mice were carried out according to protocols approved by the UCLA Animal Research Committee (ARC).

Immunofluorescence and imaging

Immunofluorescence and imaging were performed as previously described [18] with DAX1 antibody (sc841, Santa Cruz Biotechnology, Santa Cruz, CA) and Texas red secondary antibody (Invitrogen, Carlsbad, CA).

Immunohistochemistry

E5–E12 embryos were harvested from C57BL/6 wildtype females (Charles Rivers Labs, Wilmington, MA). Antibodies (see below) were applied to 4% paraformaldehyde-fixed paraffin embedded sections. All labelling was done at 4 °C overnight. Antibodies were detected using steam antigen retrieval [26], with a vegetable steamer as the steam source and a commercially available kit (Antigen Retrieval Citra, BioGenex). Staining was performed using biotin/avidin immunoperoxidase reaction (Vector Lab.), 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Innovex, Pinole, CA), and hematoxylin (Biomeda, Foster City, CA) counterstain.

Antibodies and dilutions used for immunohistochemistry (IHC) were as follows: rabbit anti-DAX1 (sc841, dilution 1:2000); rabbit anti-Wilms' tumor-1 (sc192, dilution 1:2500); rabbit anti-estrogen receptor α (sc542, dilution 1:2000); rabbit anti-estrogen receptor β (sc8974, dilution 1:1000); rabbit anti-progesterone receptor (sc538, dilution 1:3000); rabbit anti-androgen receptor (sc816, dilution 1:1000); rabbit anti-Nur77 (sc5569, dilution 1:1000); goat anti-cytochrome P450scc (sc18043, dilution 1:1000) (Santa Cruz Biotechnology); rabbit anti-steroidogenic factor-1 (dilution 1:1000, Upstate Biotechnology, Waltham, MA).

In situ hybridization

In situ hybridizations were performed as described [27]. *Nr0b1* cDNA was obtained by probing a mouse adrenal cDNA library and subcloned in pBluescript (Stratagene, La Jolla, CA). Anti-sense RNA probe was generated from an *Xho*I site and full-length *Nr0b1* was amplified with T3 RNA polymerase (Promega, Madison, MI). Sense RNA probe was generated from a *Not*I site and full-length *Nr0b1* was amplified with T7 RNA polymerase (Promega). Probes were labelled with digoxigenin-11-UTP (Roche, Indianapolis, IN). Harvested E6–E7.5 C57BL/6 embryos (Charles Rivers Labs) were separately incubated with the probes for whole mount technique, and Fast Red Detection reagent (Roche) was used to visualize signal.

Total RNA isolation

E6–E7.5 embryos harvested from C57BL/6 mice (Charles Rivers Labs) were immediately placed in RNAlater solution (Qiagen, Valencia, CA). Embryos were homogenized with a Polytron homogenizer