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SOX9 regulates expression of the male fertility gene Ets variant factor 5 (*ETV5*) during mammalian sex development☆

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ABSTRACT

In humans, dysregulation of the sex determining gene SRY-box 9 (*SOX9*) leads to disorders of sex development (DSD). In mice, knock-out of *Sox9* prior to sex determination leads to XY sex reversal, while *Sox9* inactivation after sex determination leads to spermatogenesis defects. SOX9 specifies the differentiation and function of Sertoli cells from somatic cell precursors, which then orchestrate the development and maintenance of other testicular cell types, largely through unknown mechanisms. Here, we describe a novel testicular target gene of SOX9, Ets variant factor 5 (*ETV5*), a transcription factor responsible for maintaining the spermatogonial stem cell niche. *Etv5* was highly expressed in wild-type XY but not XX mouse fetal gonads, with ETV5 protein localized in the Sertoli cells, interstitial cells and germ cells of the testis. In XY *Sox9* knock-out gonads, *Etv5* expression was strongly down-regulated. Similarly, knock-down of *SOX9* in the human Sertoli-like cell line NT2/D1 caused a decrease in *ETV5* gene expression. Transcriptomic analysis of NT2/D1 cells over-expressing SOX9 showed that *ETV5* expression was increased in response to SOX9. Moreover, chromatin immunoprecipitation of these cells, as well as of embryonic mouse gonads, showed direct binding of SOX9 to *ETV5* regulatory regions. We demonstrate that SOX9 was able to activate ETV5 expression via a conserved SOX site in the 5′ regulatory region, mutation of which led to loss of activation. In conclusion, we present a novel target gene of SOX9 in the testis, and suggest that SOX9 regulation of *ETV5* contributes to the control of male fertility.

Abbreviations: AGRF, Australian genomic research facility; Amh, anti Müllerian hormone; BAC, bacterial artificial chromosome; CCL9, chemokine (C-C motif) ligand 9; CSLM, confocal laser scanning microscopy; CYP17A1, cytochrome p450 family 17, subfamily A, protein 1; Cyp26b1, cytochrome p450 family 26, subfamily B, protein 1; CXCL12, chemokine (C-X-C motif) ligand 12; DAPI, 4′,6-diamidino-2-phenylindole; Dgkg, diacylglycerol kinase, gamma; Dhh, desert hedgehog; Dmrt1, doublesex and mab-3 related transcription factor 1; DSD, disorders of sex development; Ets, E-twenty-six; Etv1, Ets variant 1; Etv4, Ets variant 4; Etv5, Ets variant 5; FACS, fluorescence activated cell sorting; Fgfr2, fibroblast growth factor receptor 2; Fgf9, fibroblast growth factor 9; Gdnf, glial cell derived neurotrophic factor; Gstm6, glutathione S-transferase mu 6; HA, hemagglutinin; KOH, potassium hydroxide; Luc, luciferase; NCBI, National Center for Biotechnology Information; NOA, non-obstructive azoospermia; Pea3, polyomavirus enhancer activator-3; Ptgds, prostaglandin D synthase; Ret, Ret proto-oncogene; RIPA, radioimmunoprecipitation buffer; SCO, sertoli cell only; Sdha, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); Sf1, steroidogenic factor 1; Sox9, SRY-box-9; Sry, sex determining region on Y; SSC, spermatogonial stem cell; Tra2b, transformer 2 beta homolog; Vnn1, Vanin 1.

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1. Introduction

In mammals, several core transcription factors coordinate the molecular and cellular processes that occur during gonad development. In males, sex determination is triggered by sex determining region on the Y chromosome (SRY), which initiates the differentiation of the male somatic Sertoli cells from bipotential precursor cells (Albrecht and Eicher, 2001; Koopman et al., 1990; Sekido et al., 2004). The central function of SRY is to up-regulate expression of the related gene *SOX9*, which begins to direct downstream events in testicular development from embryonic day 11.5 (E11.5) in mouse embryogenesis (Sekido and Lovell-Badge, 2008). Under the control of SOX9, Sertoli cells differentiate and initiate the differentiation of other testicular cell types including the Leydig and germ cells (reviewed in Svingen and Koopman, 2013).

SOX9 mutations in humans cause campomelic dysplasia (CD, OMIM: 114290), an autosomal dominant disorder of the bone frequently accompanied by abnormal gonad development, including complete XY sex reversal (Foster et al., 1994; Wagner et al., 1994). Conversely, the gain of SOX9 function leads to inhibition of female development and initiation of male sex development in XX individuals (Cox et al., 2011). In XY mice, sex reversal is observed when both *Sox9* alleles are lost prior to sex determination (Barrionuevo et al., 2006; Lavery et al., 2011). The mice develop as infertile phenotypic females with typical female behavior. XX mice transgenic for *Sox9* develop testes and secondary male phenotype (Vidal et al., 2001).

The spectrum of phenotypes caused by the misexpression of *SOX9* suggests that there are many genes that lie downstream of SOX9 during the crucial sex developmental stages. For example, *Sox9* loss at E13.5, that is, after sex determination, leads to male infertility postnatally suggesting a paracrine role for SOX9 in the control of spermatogenesis (Barrionuevo et al., 2009). This is of importance as only ~20% of 46, XY DSD cases and 60% of male idiopathic infertility can be explained by mutations in known genes (Hughes et al., 2006; Krausz et al., 2015).

Currently, SOX9 is known to regulate several target genes with diverse roles within the testis. These include the repression of female development (*Amh, Fgf9*) and maintenance of SOX9 expression in the testis (*Sox9, Fgf9*, *Ptgds*) (de Santa Barbara et al., 2001; Jameson et al., 2012; Kim et al., 2006; Moniot et al., 2009; Wilhelm et al., 2007). SOX9 also regulates the expression of *Sf1*, *Vnn1*, *Cyp26b1, Cbln4, VprBP* and *Gstm6* during sex development and controls key testicular processes such as cell migration, degradation of retinoic acid and protection of the testis against toxicants (reviewed in Sekido and Lovell-Badge, 2013; Beverdam et al., 2009; Wilson et al., 2005).

Here, we show that SOX9 directly regulates the expression of *ETV5* during testis development. ETV5 belongs to the polyomavirus enhancer activator −3 (PEA3) group of transcription factors, which is a part of the Ets family, and also includes ets variant factor 1 (ETV1) and ets variant factor 4 (ETV4) (Chotteau-Lelievre et al., 1997). Previous studies in humans and mice have shown that ETV5 is an important reproductive factor. In a cohort of infertile Australian men, a variant of *ETV5* (+48845 G > T) was associated with higher risk of non-obstructive azoospermia (NOA) and Sertoli cell only (SCO) phenotype (O'Bryan et al., 2012). Prior to the human study, loss of *Etv5* was analysed in mice, showing that it is essential for the maintenance of the spermatogonial stem cell (SSC) niche via ret proto-oncogene (RET) signalling as well as contributing to the essential Sertoli cell functions such as the maintenance of the blood-testis barrier and secretion of chemokines required to maintain SSC integrity (Chen et al., 2005; Morrow et al., 2009; Simon et al., 2010; Tyagi et al., 2009). The association of ETV5 with fertility in humans and mice presents this gene as an important player in mammalian reproduction.

Our aims were to measure *ETV5*/*Etv5* expression in response to perturbations in *SOX9*/*Sox9* levels using in vivo and in vitro models and define the molecular mechanism involved. Our findings demonstrate that SOX9 is required for *ETV5*/*Etv5* expression and therefore, for the maintenance of male fertility.

2. Materials and methods

All reagents were purchased from Sigma Aldrich unless otherwise specified.

2.1. Wild-type and knock-out mice

Breeding pairs were set up and mouse embryos dissected at E11.5, E12.5, E13.5, and E.15.5 with E0.5 being the day of vaginal plugging. The exact age of embryonic gonads was determined by counting tail somite number (Hacker et al., 1995). All animal work was performed under Monash Medical Centre Animal Ethics approval (MMCB/2012/03). *CK19-Cre/Cre*; *Sox9:flox/flox* embryos on a 129P2/OlaHsdxC57BL/6 mixed background were generated, sexed and genotyped as described previously (Barrionuevo et al., 2006).

2.2. Whole mount in situ hybridization (WISH)

Embryos were fixed with 4% paraformaldehyde in 1 × PBS at 4 °C overnight. Further processing of embryos and in situ hybridization were carried out as described in (Wilkinson, 1992). *Etv5* riboprobe was synthesized according to (Lu et al., 2009). In situ hybridization with digoxigenin-labelled probes was performed as per (Vernet et al., 2006). Each experiment was repeated on 4 gonads. Post-hybridization washes were done in 100 mM maleic acid pH7.5, 150 mM NaCl, 0.1% (v/v) tween-20 (MABT). After staining with BM purple AP substrate (Boehringer), embryos were dissected under the stereomicroscope, photographed and stored in 4% PFA/PBS.

2.3. Expression plasmids

All mammalian expression plasmids were of pcDNA3 origin. HA-*SOX9* was described in (McDowall et al., 1999).

2.4. Cell culture and transfections

NT2/D1 (ATCC CRL-1973) cells were cultured, seeded and transfected in 6-well plates as previously shown (Bernard et al., 2011). Briefly, cells were transfected with SOX9 expression vector in a 3:1 ratio with pEF-GFP in order to maximize selection for SOX9 transfected cells during fluorescence activated cell sorting (FACS) (Knower et al., 2011), 24 h after seeding and harvested after 48 h. Cells were seeded for knock-down studies as per above, and transfected with *siSOX9* or control siRNA (Santa Cruz Biotechnology: *siSOX9* sc36533) using Lipofectamine 2000 (Invitrogen), and fixed for immunocytochemistry or harvested for RNA after 48 h. Transfection experiments were performed three to five times in independent biological experiments.

2.5. Immunocytochemistry and immunofluorescence

NT2/D1 cells used for immunocytochemistry were seeded on coverslips placed into 6-well plates and transfected as described above. The primary antibodies used were rabbit anti-Hemagglutinin (HA) (1:400) (Ab9110, Abcam) and rabbit anti-SOX9 (1:1000) (Ab5535, Merck Millipore). The secondary antibodies were donkey anti-rabbit Alexa 594-conjugated IgG or donkey anti-rabbit Alexa 488-conjugated IgG from Molecular Probes (1:1000). Coverslips were mounted onto slides with DAKO fluorescence