Journal of Steroid Biochemistry & Molecular Biology 170 (2017) 28–38

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Original research article

Altered *SOX9* genital tubercle enhancer region in hypospadias

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ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 17 October 2016
Accepted 24 October 2016
Available online 29 October 2016

Keywords:
SOX9
Enhancer
Disorder of sex development
Genital tubercle
Hypospadias

ABSTRACT

Human mutations in the *SOX9* gene or its regulatory region can disrupt testicular development, leading to disorders of sex development (DSDs). Our previous work involving the genomic analysis of isolated DSD patients revealed a 78 kb minimal sex determining region (RevSex) far upstream of *SOX9* that was duplicated in 46,XX and deleted in 46,XY DSDs. It was postulated that RevSex contains a gonadal enhancer. However, the most highly conserved sub-region within RevSex, called SR4, was neither responsive to sex determining factors *in vitro* nor active in the gonads of transgenic mice, suggesting that SR4 may not be functioning as a testicular enhancer. Interestingly, SR4 transgenic mice showed reporter activity in the genital tubercle, the primordium of the penis and clitoris, a previously unreported domain of *Sox9* expression. SOX9 protein was detected in the genital tubercle, notably in the urethral plate epithelium, preputial glands, ventral surface ectoderm and corpus cavernosa. SR4 may therefore function as a *Sox9* genital tubercle enhancer, mutations of which could possibly lead to hypospadias, a birth defect seen in the DSD patients in the RevSex study. SR4 activity and the observed SOX9 expression pattern suggest that SR4 may function as a *Sox9* genital tubercle enhancer. However, conditional ablation of *Sox9* in the genital tubercle using *Shh-Cre/+;Sox9*$^{flox/flox}$ mice revealed no genital tubercle abnormalities, possibly due to compensation by similar Sox factors. To conclude, we have identified a novel regulatory enhancer driving *Sox9* expression during external genitalia development.

1. Introduction

Human sex determination involves crucial cell fate decisions in which the bipotential gonad either develops into testes in 46,XY individuals or ovaries in 46,XX individuals. The testicular fate is determined by the expression of the Sex-determining region Y (*SRY*) gene on the Y chromosome, which activates *SRY*-related HMG box gene 9 (*SOX9*) to initiate a cascade of genetic expression leading to testicular development. Heterozygous mutations in *SOX9* lead to a skeletal malformation syndrome called campomelic dysplasia (CD) where 75% of XY individuals present with disorders of sex development (DSDs) [1,2]. DSDs are congenital conditions in which chromosomal, gonadal or anatomic sex is atypical. Around 70% of 46, XY DSDs remain unexplained genetically. In XX humans, ectopic expression of SOX9 induces testicular development despite the absence of SRY [3,4]. *SOX9* is therefore necessary and sufficient for testis development.

Multiple clinical phenotypes manifest in CD and this is reflective of the broad expression pattern of *SOX9* in various tissues including the cartilage, testes, notochord, neural crest and central nervous system [5]. The precise spatiotemporal expression pattern of *SOX9* is controlled by its complex regulatory region, containing multiple tissue-specific transcriptional regulatory elements, and spanning ~1.5 Mb upstream and downstream of the coding sequence [6,7]. Long distance genomic alterations at the *SOX9* locus disrupting such tissue-specific regulatory elements can result in isolated clinical phenotypes. For example, disruption of a mandibular mesenchyme enhancer ~1.4 Mb upstream of *SOX9* can cause isolated Pierre Robin sequence [PRS; [6]], a craniofacial anomaly typically observed in CD patients.

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http://dx.doi.org/10.1016/j.jsbmb.2016.10.009
0960-0760/

A major discovery in the genetic regulation of *Sox9* expression in the early mouse testis was the identification of a testis-specific enhancer (TES; core region known as TESCO), located 13 kb upstream of *Sox9* [8]. Through the TES enhancer, *Sox9*/*SOX9* expression is initiated by Steroidogenic factor 1 (SF-1), upregulated by SF-1-SRY synergy and maintained by SF-1-SOX9 synergy [8,9]. However, in a screen of 66 46,XY DSD patients performed by our laboratory, no mutations were discovered within TESCO [10]. In addition, breakpoints in CD translocation cases with XY sex reversal range from 50 kb to up to several hundred kb upstream of *SOX9*, thereby leaving the TES enhancer intact [11]. These findings indicate that one or more testis-specific enhancers may be functioning elsewhere in the *SOX9* regulatory region of humans.

In our previous study [12], analysis of genomic DNA from isolated 46,XX and 46,XY DSD patients revealed four families that had overlapping duplications in 46,XX individuals and deletions in 46,XY individuals in the *SOX9* regulatory region. The region of overlap between these genomic alterations, and previously reported deletions and duplications at the *SOX9* locus associated with syndromic and isolated cases of 46,XX and 46,XY DSD [13–17], defined a 78 kb sex determining region named 'RevSex' (or 'Reversal of Sex') located in a gene desert 517–595 kb upstream of the *SOX9* gene [12]. It was predicted that the RevSex region contains a gonad-specific enhancer(s), gain or loss of which results in activation or inactivation of gonadal *SOX9* expression, respectively. Further studies analysing additional 46,XX and 46,XY DSD cases have now refined this candidate sex determining region to 40.7–41.9 kb [18–21].

Apart from gonadal abnormalities, it was interesting to note that all four families analysed in the RexSex study presented with hypospadias [12], a form of DSD in which the urethra opens at an abnormal site along the penis. Hypospadias, affecting one in 200–300 boys [22], is currently the second most common congenital defect and has doubled in frequency in the past 30 years [23,24]. A disruption in external genitalia development can lead to hypospadias.

There are two distinct phases of external genitalia development in vertebrates. In the first hormone-independent phase (5–8 weeks of gestation in humans; E10.5–E15.5 in mice), the primordium of the penis and clitoris, known as the genital tubercle, undergoes an initial outgrowth and patterning in both male and female embryos [25–27]. During this ambisexual phase, male and female external genitalia are morphologically indistinguishable. The second phase (8–20 weeks in human; E16 to postnatally in mice), which is hormone-dependent, involves either the continued growth and differentiation of the penis in males or the arrest of outgrowth and differentiation of the clitoris in females. Continued growth of the genital tubercle is coordinated by three dimensional tissue patterning and urethral tube formation wherein the urethral plate epithelium extends distally and canalises to form a urethral tube in the penis. Much remains to be discovered on the genetic mechanisms regulating the development of early external genitalia. Consequently, the aetiology of hypospadias remains largely unknown.

In the present study, we hypothesised that RevSex contains a novel enhancer of *SOX9* required for gonadal development in humans. A sequence conservation analysis was performed and the most highly conserved sub-region within RevSex, named SR4, was tested for enhancer activity using *in vitro* transactivation assays with known sex determining factors. Unexpectedly, analysis of mice transgenic for the *LacZ* reporter gene driven by SR4 revealed SR4 enhancer activity in the genital tubercle and not the gonads. While the role of *Sox9* in testicular development, and consequently, its indirect role in masculinisation of the external genitalia, is well known, SR4 activity in the genital tubercle suggests a previously unknown function of *SOX9* in the direct regulation of external genitalia development. The expression pattern of SOX9 protein was then examined in the genital tubercle and a preliminary investigation of a putative direct role of *Sox9* in external genitalia development was undertaken by performing a conditional knockout of *Sox9* in the genital tubercle of mice.

2. Materials and methods

2.1. Clinical data

A 78 kb sex determining region (RevSex) was identified in our previous study through array comparative genomic hybridisation analysis of four families (DSD1–DSD4) with isolated 46,XX and 46,XY DSDs [12]. Gonadal and genital phenotypes of the patients are summarised in Table 1.

2.2. Comparative genomic analysis

The human genomic region chr17:69,522,161–69,600,161 (DSD2-CD1; GRCh37/hg19 coordinates) was used for genomic comparative analysis using PhastCons and rVista [12,28,29]. Elements over 100 bp that had greater than 70% conservation between human, mouse and lizard were selected for further analysis.

2.3. Transcription factor binding site analysis

The RevSex DNA sequence was analysed for binding sites of transcription factors SF-1, SRY, SOX9, GATA binding protein 4 (GATA4) and Forkhead box L2 (FOXL2) that are known to be involved in sex determination. Both manual sequence inspection and the program Matinspector (www.genomatix.de; Genomatix, Munich, Germany) were used, with core and matrix similarity settings at 1.00.

2.4. In vitro *basal enhancer activity assays*

To generate luciferase reporter constructs, the SR4, SR5, SR6 and SR7 putative enhancers were PCR-amplified from human DNA

Table 1
Clinical phenotypes of four families (DSD1–DSD4) with isolated DSDs from which the RevSex sex determining region was identified [detailed clinical reports in [12]].

Family	Genotype	Gonadal phenotype	Genital phenotype
DSD1	46,XX male	Ovotestes	Hypospadias, bifid scrotum
DSD2	46,XX male	Right scrotum: normal testis, left scrotum: dysgenic ovary	Perineal hypospadias, rudimentary vagina and uterus
DSD3	46,XX male	Right scrotum: streak gonad (partial dysgenic ovary), left scrotum: ovotestes	Perineal hypospadias, vaginal pouch, uterus
DSD4	Case 1: 46,XY female	Case 1: right gonad: small testis, left gonad: streak gonad	Case 1: severe hypospadias, ambiguous asymmetric external genitalia, fallopian tube, hemiuterus
	Case 2: 46,XY female	Case 2: Left gonad: ovary, right gonad: streak gonad with gonadoblastoma	Case 2: normal external female phenotype, uterus