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SOX Genes and Their Role in Disorders of Sex Development

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Abstract
SOX genesare master regulatory genes controlling development and are fundamental to the establishment of sex determination in a multitude of organisms. The discovery of the master sex-determining gene *SRY* in 1990 was pivotal for the understanding of how testis development is initiated in mammals. With this discovery, an entire family of SOX factors were uncovered that play crucial roles in cell fate decisions during development. The importance of SOX genes in human reproductive development is evident from the various disorders of sex development (DSD) upon loss or overexpression of SOX gene function. Here, we review the roles that SOX genes play in gonad development and their involvement in DSD. We start with an overview of sex determination and differentiation, DSDs, and the SOX gene family and function. We then provide detailed information and discussion on SOX genes that have been implicated in DSDs, both at the gene and regulatory level. These include *SRY*, *SOX9*, *SOX3*, *SOX8*, and *SOX10*. This review provides insights on the crucial balance of SOX gene expression levels needed for gonad development and maintenance and how changes in these levels can lead to DSDs.

Introduction

Sex Determination and Differentiation

Sex determination refers to the mechanisms that drive differentiation of the bipotential gonad in a developing embryo into either a testis or an ovary. In most mammals, sex is normally dictated by chromosomes, whereby males and females carry XY and XX sex chromosomes, respectively. At around week 4 in humans or embryonic day 10 (E10) in mice, the bipotential gonad is formed in both sexes upon expression of steroidogenic factor 1 (*SF-1*; *NR5A1*) and Wilms' tumour 1 (*WT1*) in the genital ridge [Karl and Capel, 1998]. The primordial germ cells (PGCs) are specified prior to gonad formation, proliferate, and migrate from the epiblast to the genital ridge to populate the gonads [McLaren, 2003].

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Expression of the sex-determining region Y (*SRY*) gene at week 6 in humans or E11.5 in mice, in supporting cell precursors, initiates male sex determination [Gubbay et al., 1990; Koopman et al., 1990; Sinclair et al., 1990]. SRY, together with SF-1, activates *SRY* box 9 (*SOX9*) expression, triggering a genetic cascade that drives testis development. An additional factor, GATA binding protein 4 (GATA4), interacts with SF-1 and zinc finger protein FOG family member 2 (FOG2/ZFPM2) to regulate the expression of *SRY* and *SOX9* [Miyamoto et al., 2008; Viger et al., 2008]. *SRY* and *SOX9* expression enables the coelomic epithelial cells to proliferate [Schmahl et al., 2000] and the Sertoli cells to differentiate and encapsulate germ cells to form testis cords [Ross and Capel, 2005]. The steroidogenic foetal Leydig cells then differentiate and reside in the interstitium, between the testis cords.

In developing female mouse ovaries, differentiation of the somatic pregranulosa cells is known to begin as early as E11.5 [Niu and Spradling, 2020], while the germ cells of the ovary start to enter prophase of meiosis between E13.5–E14.5 [Menke et al., 2003; Suzuki et al., 2015; Pannetier et al., 2016]. Differentiation of the primordial follicles occurs just before birth [Ross and Capel, 2005]. Ovarian development is regulated by the expression of Rspondin-1 (*Rspo1*) that activates Wingless-related MMTV integration site 4 (*Wnt4*) signalling [Tomizuka et al., 2008]. Forkhead box L2 gene (*Foxl2*) is also required for granulosa cell function [Uda et al., 2004; Ottolenghi et al., 2005].

Several antagonistic interactions exist between the male and female genetic pathways. For example, both *Wnt* signalling and *Foxl2* repress testicular development in mice [Maatouk et al., 2008; Uhlenhaut et al., 2009]. Disruption of this antagonism, caused by gain- or loss-of-function of the above genes, can lead to sex reversal phenotypes in mice. For example, loss of *Sox9* in mice causes XY sex reversal, leading to ovarian development [Barrionuevo et al., 2006].

Following sex determination, sex differentiation occurs at weeks 6–7 in humans whereby the genital tract and external genitalia progressively acquire male or female structures. Prior to sex differentiation, all embryos have both male and female reproductive ducts. In the developing male embryo, the male-specific wolffian duct develops into the vas deferens, epididymis, and seminal vesicles through the action of testosterone produced by embryonic Leydig cells. The female-specific müllerian duct will degenerate due to the action of anti-müllerian hormone (AMH) produced by Sertoli cells [Kobayashi and Behringer, 2003]. In developing female embryos, lack of testosterone and AMH regresses the wolffian duct and develops the müllerian duct into the oviducts, uterus, and the upper vagina. Up to week 9, the external genitalia remain undifferentiated in humans. The genital tubercle then differentiates into the penis in males and clitoris in females, while the labioscrotal swellings develop into the scrotum in males and the labia and vagina in females [Rey et al., 2000]. Theca cells surrounding follicles produce androgens, which are converted into oestrogens by granulosa cells to ensure development of female phenotypes [Matzuk and Lamb, 2008].

Disorders of Sex Development

Perturbation of the sex determination and differentiation pathways can lead to congenital conditions known as disorders of sex development (DSD) where chromosomal, gonadal, or anatomic sex is atypical [Hughes et al., 2006]. In patients with DSDs, sex determination and differentiation are often incomplete or mixed, whereby phenotypic sex is inconsistent with the genotypic sex [Matzuk and Lamb, 2008]. Disruptions affecting sex determination can perturb early gonad formation, which would subsequently affect internal and/or external genitalia development. In contrast, disruptions to sex differentiation would only affect internal and/or external genitalia development.

DSDs are categorised as follows: (a) sex chromosome DSD (e.g., 45,X Turner syndrome and 47,XXY Klinefelter syndrome); (b) 46,XY DSD which include disorders of testicular development (e.g., complete/partial gonadal dysgenesis, gonadal regression, and ovotesticular DSD), disorders of androgen synthesis or action, and non-classified forms such as hypospadias (abnormal urethral development); (c) 46,XX DSD which include disorders of ovarian development (e.g., ovotesticular DSD, testicular DSD, and gonadal dysgenesis) and disorders of androgen excess [Lee et al., 2006; Mendonca et al., 2009].

DSDs are currently one of the most common birth defects, with 1 in 150 boys presenting with hypospadias, 1 in 4,500 births with ambiguous genitalia, and 1 in 20,000 births with gonadal dysgenesis (complete sex reversal) [Thyen et al., 2006; Nassar et al., 2007].

DSDs are a major paediatric concern and are often misdiagnosed due to limited genetic information. Current genetic diagnostic rates are 43% for 46,XY DSDs and 17% for 46,XX disorders of gonadal development [Eggers et al., 2016]. An accurate genetic diagnosis of DSDs would be highly beneficial for clinicians in order to make informed decisions with regard to clinical management and

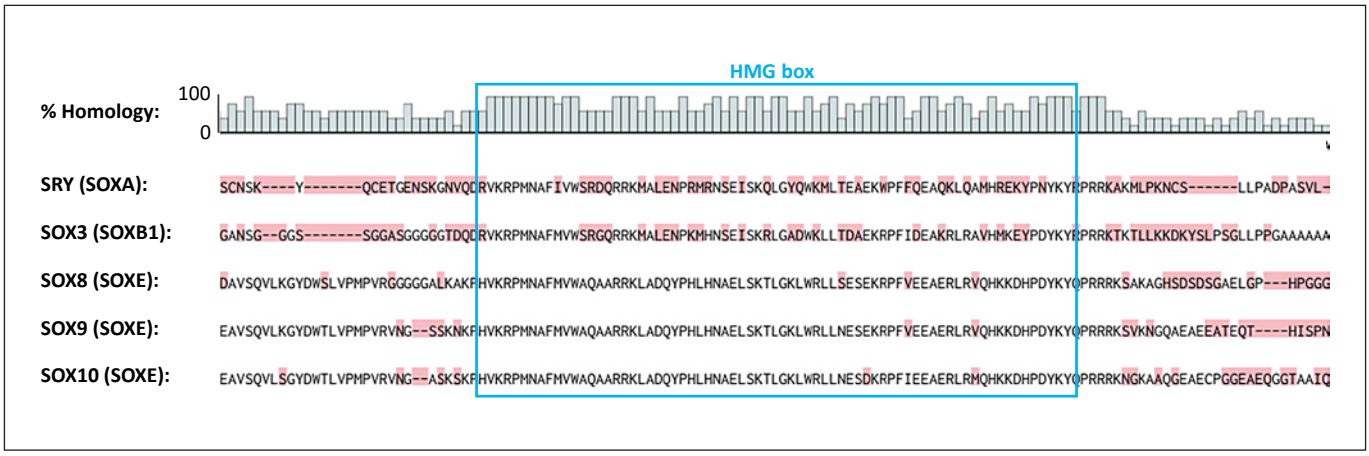

Fig. 1. Amino acid alignment of the HMG box and flanking regions of SRY, SOX3, SOX8, SOX9, and SOX10. Percentage homology among the sequences is shown above the alignment. Corresponding SOX groups are shown for each protein in parentheses. Mismatched amino acids are highlighted in red.

treatment options, thereby improving outcomes for patients. The genetic diagnosis can also offer couples who have a child with DSD to undergo assisted reproduction followed by preimplantation genetic diagnosis (PGD) in order to screen embryos and transplant only embryos that do not carry the DSD-causing variant.

SOX Gene Family and Function

With the seminal discovery of the mammalian testis determining factor, *SRY* [Gubbay et al., 1990; Koopman et al., 1990; Sinclair et al., 1990], research on the SOX gene family began. *SRY* contains a high mobility group (HMG) domain that binds DNA in a sequence-specific manner. Proteins that contain a HMG domain with at least 50% amino acid similarity to the HMG domain of *SRY* are called SOX proteins (*Sry*-related HMG box). Twenty different SOX genes with highly variable developmental functions have been discovered in mice and humans, with most playing crucial roles as transcription factors during cell fate decisions [Schepers et al., 2002].

SOX proteins bind to the consensus sequence ATTGTT or similar sequence motifs via their HMG domain [Badis et al., 2009; Kondoh and Kamachi, 2010]. The interaction of the HMG domain with the minor groove of DNA facilitates the widening of the minor groove and bending of the DNA towards the major groove [Remenyi et al., 2003].

SOX proteins that share an HMG domain with more than 80% sequence identity are categorised into 10 groups (A–J) [Bowles et al., 2000]. Within each group, SOX proteins share similar biochemical properties and functions [Wegner, 2010]. Despite recognising a similar consensus DNA sequence, SOX proteins from different groups and sub-groups have distinct biological functions. Specific target gene selectivity of SOX proteins is achieved via a differential affinity for sequences flanking the consensus SOX sites, homo- or heterodimerisation between SOX proteins, as well as post-translational modifications or interaction with other co-factors [Wegner, 2010]. This molecular versatility enables the same SOX protein to have varying functions within different biological contexts and tissues.

In this review, we will focus on SOX factors that have been implicated in DSDs. These include *SRY*, *SOX9*, *SOX3, SOX8,* and *SOX10*. The *SRY* gene is the only member of the SOXA group. Although the HMG domain is highly conserved among the *SRY* gene of different mammalian species, sequences outside this domain are highly divergent [Sekido, 2010; Miyawaki et al., 2020; Thomson et al., 2022]. The SOXB group comprises 2 sub-groups, with *SOX3* falling into the SOXB1 group. These family members have HMG domains flanked by short N-terminal and long C-terminal sequences, with a transcriptional activation domain located in the C-terminal [Kamachi et al., 1998]. *SOX8, SOX9,* and *SOX10* are categorised into the SOXE group [Stolt and Wegner, 2010]. The SOXC, SOXE, and SOXF groups have similar protein structures to SOXB1 except for unique amino acid sequences outside the HMG domain for each group [Kamachi and Kondoh, 2013]. An alignment of the HMG box and flanking regions of SRY, SOX3, SOX8, SOX9, and SOX10 is shown in Figure 1.

SOX Genes Implicated in DSDs

Through genomic analysis of DSD patients, several gene variants linked to sex reversal have been identified. The role of these genes in the control of gonad development have been demonstrated through the use of gain- and loss-of-function studies in mouse models. SOX genes that have been implicated in DSDs are described here.

SRY (Sex Determining Region Y)

The Role of the *SRY* Gene in DSD

The *SRY* gene, located on the Y chromosome, encodes a transcription factor that initiates the genetic cascade of testicular differentiation [Sinclair et al., 1990; Koopman et al., 1991]. It is required for the activation of *SOX9* expression, resulting in Sertoli cell differentiation from somatic precursor cells of the bipotential embryonic gonad. XY mice lacking *Sry* as well as XX mice with *Sry* translocations show complete sex reversal [Gubbay et al., 1990; Lovell-Badge and Robertson, 1990; Koopman et al., 1991]. Both 46,XY and 46,XX DSDs can result from variations in *SRY*. In 46,XY individuals, *SRY* variants result in Swyer syndrome with phenotypes such as testicular dysgenesis and ovotestis, with most patients presenting with a female appearance while some have ambiguous external genitalia [Berta et al., 1990; Sinclair et al., 1990; Hawkins et al., 1992]. 46,XX DSD patients with *SRY* translocations show female-to-male sex reversal with male or ambiguous genitalia [Goodfellow and Lovell-Badge, 1993]. Approximately 15% of all cases of 46, XY gonadal dysgenesis harbour a variation within *SRY* [McElreavey and Fellous, 1999].

The Role of *SRY* Regulatory Elements in DSD

The majority of DSD cases caused by variations in *SRY* result from aberrations affecting the coding region, and mostly the HMG domain. Several 46,XY DSD patients have been found with variations affecting the non-coding regions flanking *SRY* (Fig. 2a) [reviewed in Larney et al., 2014]. A patient with streak gonads and external female genitalia was found to have a large deletion that spanned from ~1.8 kb to between 23 kb and 50 kb upstream of *SRY* [McElreavy et al., 1992]. A 2.5–7 kb deletion beginning between 2 kb and 3 kb 3′ of *SRY* that extended into the pseudoautosomal region was discovered in a patient with testicular dysgenesis and external female genitalia [McElreavey et al., 1996]. In both cases, the variations were de novo. These cases point towards the possible existence of regulatory elements that drive *SRY* expression both in the genomic region between *SRY* and its neighbouring gene ribosomal protein S4, Y-linked 1 (*RPS4Y1*), as well as in the region downstream of *SRY*.

In addition to the above cases, a 3-bp deletion upstream of *SRY* within the promoter was discovered in a patient with 46,XY complete gonadal dysgenesis (Fig. 2a). The deletion altered a Sp1 transcription factor binding site which abolished Sp1 binding in vitro. The variant was inherited from the father who had severe hypospadias [Assumpcao et al., 2005].

SOX9 (SRY-Box 9)

The Role of the *SOX9* Gene in DSD

SOX9 is a transcription factor that contains an HMG box DNA-binding domain similar to that of *SRY*. *Sox9* is expressed in a broad variety of tissues including the cartilage, testes, craniofacial regions, brain, neural crest, pituitary, lung, heart, pancreas, hair follicles, ear, and eye [Wright et al., 1995; Kent et al., 1996; Ng et al., 1997; Sarkar and Hochedlinger, 2013; Jo et al., 2014]. In humans, *SOX9* is expressed in the cartilage, testis, colon, small intestine, stomach, oesophagus, gall bladder, pancreas, thyroid, heart, kidney, prostate, cerebral cortex, and salivary gland [Haag et al., 2008; Djureinovic et al., 2014]. This broad expression pattern is reflective of the critical role that SOX9 plays in regulating the formation of multiple tissues and organs.

Heterozygous mutations within and around *SOX9* cause campomelic dysplasia (CD, OMIM #114290), a human skeletal disorder characterised by shortening and bowing of the long bones. CD is usually lethal in the first year of life and 75% of 46,XY patients have complete or partial male-to-female sex reversal [Foster et al., 1994; Wagner et al., 1994; Mansour et al., 1995]. Conversely, duplication of *SOX9* has been linked with testicular development in *SRY*-negative 46,XX individuals [Huang et al., 1999]. Therefore, *SOX9* is necessary and sufficient for male sex determination.

Analysis of homozygous *Sox9* knockout mice revealed that *Sox9* is required for chondrogenesis and development of the testes, heart, trunk neural crest, spinal cord, notochord, perinotochordal sclerotome, pancreas, prostate, intestine, liver, and hair [Bi et al., 1999, 2001; Akiyama et al., 2002, 2004a, b; Stolt et al., 2003; Chaboissier et al., 2004; Cheung et al., 2005; Vidal et al., 2005; Barrionuevo et al., 2006, 2008; Bastide et al., 2007; Seymour et al., 2007; Antoniou et al., 2009; Huang et al., 2012]. SOX9 is expressed in Sertoli cells of the testes, and homozygous *Sox9* knockout mice show XY sex reversal [Barrionuevo et al., 2006]. Conversely, ectopic expression of

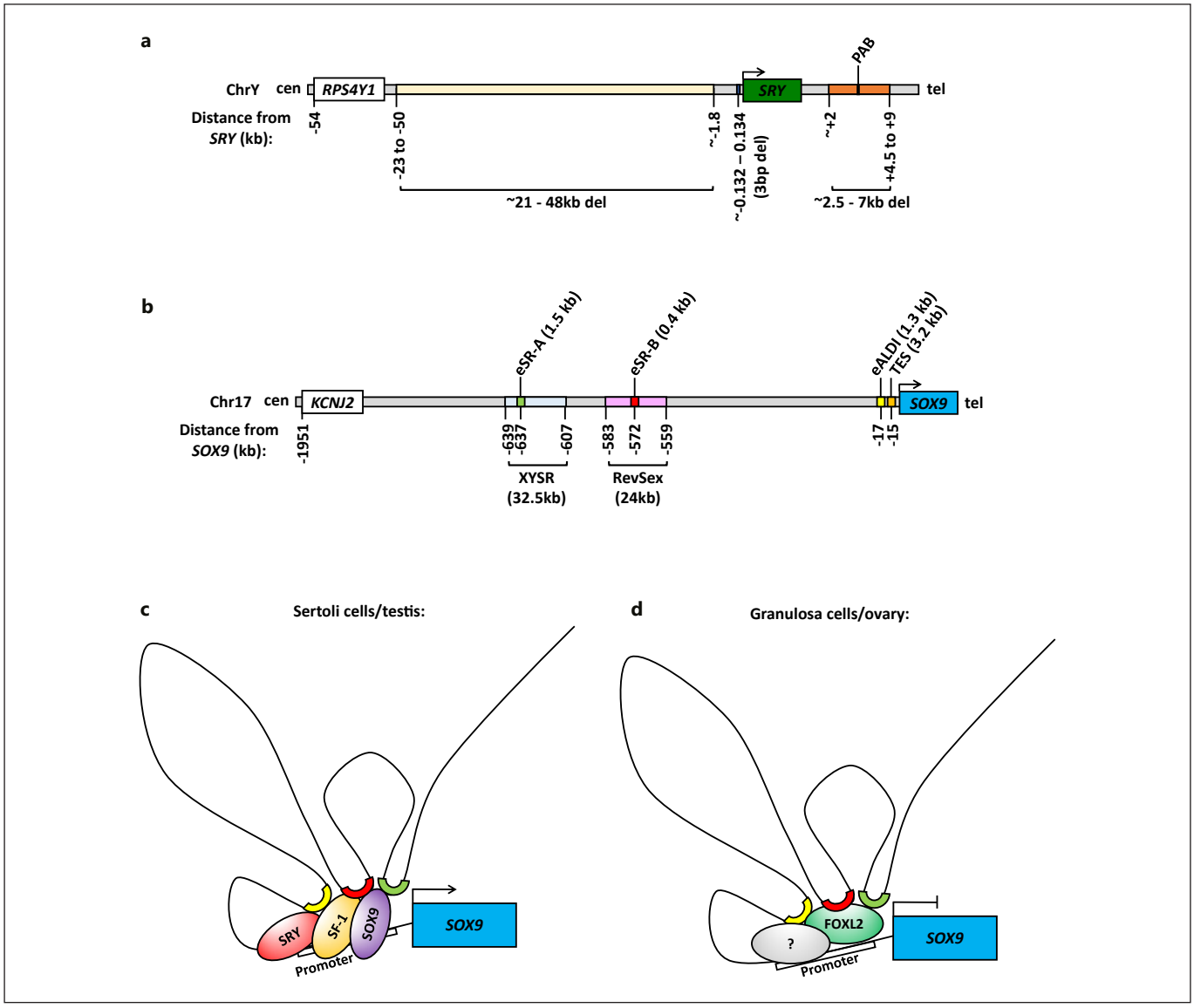

Fig. 2. Schematic diagram of the *SRY* and *SOX9* regulatory regions. **a** Regulatory region flanking *SRY* showing deletions (del) found in 3 individuals with 46,XY DSD. The location of the deletions relative to *SRY* and the nearest upstream gene *RPS4Y1* are indicated. cen, centromeric end; tel, telomeric end; PAB, pseudoautosomal boundary. **b** Regulatory region upstream of *SOX9*. The sizes and location of *SOX9 t*estis enhancers eSR-A, eSR-B, eALDI, and TES upstream of the *SOX9* transcription start site are indicated. Genomic regions associated with sex reversal (RevSex and XYSR) and the nearest upstream gene *KCNJ2* are also shown. **c** Proposed mechanism of *SOX9* activation via looping of the eSR-A, eSR-B, and eALDI enhancers onto the *SOX9* promoter, facilitated by binding of transcription factors SRY, SF-1, and SOX9 itself. **d** Proposed mechanism of inhibition of *SOX9* expression via looping of the eSR-A, eSR-B, and eALDI enhancers onto the *SOX9* promoter, mediated by binding of FOXL2 and an unknown factor. Yellow: eALDI, red: eSR-B, green: eSR-A. Figures are not drawn to scale.

Sox9 results in testicular development in XX mice despite the absence of *SRY* [Vidal et al., 2001]. Thus, as in humans, *Sox9* alone is needed and sufficient to induce testis development in mice.

Sox9 acts as a "central hub" gene as it regulates and is being regulated by several genes in the gonad development pathway including *Sf-1*, *Sry*, *Sox9* itself, *Amh*, *Fgf9*, *Fgfr2*, *Ptgds*, *Foxl2*, *Ctnnb1*, and *Wnt4* [Sekido and Lovell-Badge, 2013; Windley and Wilhelm, 2015]. Therefore, apart from mutations directly affecting the *SOX9* coding region, misregulation or disruption of the regulatory region of *SOX9* can also result in DSDs.

The Role of *SOX9* Regulatory Elements in DSD

SOX9 has a long and complex regulatory region (~2 Mb upstream and 0.5 Mb downstream) containing multiple cis-acting transcriptional regulatory elements responsible for its expression in specific tissues (Fig. 2b). Many translocations, deletions, duplications, and inversions leading to CD with or without DSD have been discovered in the *SOX9* regulatory region [Leipoldt et al., 2007; Baetens et al., 2017; Gonen and Lovell-Badge, 2019]. While variants within the coding region of *SOX9* result in the full CD syndrome, copy number variants (CNVs) affecting individual tissue-specific regulatory elements can lead to isolated DSD [Gordon and Lyonnet, 2014].

A first attempt to identify testis-specific enhancers of *Sox9* was undertaken in mice, and a 3.2-kb element with a core of 1.4 kb was identified and termed TES/TESCO (for Testis-specific Enhancer of *Sox9*/TES Core) [Sekido and Lovell-Badge, 2008]. This regulatory element is located 13 kb upstream of the *Sox9* start site. Transgenic reporter mice carrying the TES/TESCO enhancer exhibit testis-specific expression only in Sertoli cells. Chromatin immunoprecipitation experiments indicated that both SRY and SF-1 bind the TES enhancer. Deletion of the TES or TESCO element in mice led to 55 or 40% reduction in *Sox9* levels compared to wildtype gonads, respectively. This did not result in XY male-to-female sex reversal in mice. By contrast, a 50% decrease in *SOX9* levels in human patients will cause, in the majority of cases, XY male-to-female sex reversal in CD patients with DSD [Gonen et al., 2017]. It has been demonstrated that FOXL2 represses *Sox9* expression via TESCO postnatally and that the repression is even stronger when FOXL2 and ERα act synergistically. This antagonism of *Sox9* expression by FOXL2 actively prevents Sertoli cell differentiation and masculinisation of the ovary in adulthood [Uhlenhaut et al., 2009].

Using cell lines, the human TES homologue was shown to be regulated by SF-1, SRY, and SOX9, similarly to what was observed in mice [Knower et al., 2011]. Introducing pathogenic variants identified in human DSD patients into recombinant SF-1, SRY, and SOX9 proteins and examining their ability to activate the hTES enhancer showed that the majority of variants failed to activate the TES enhancer in vitro [Knower et al., 2011]. However, in a screen of 66 patients with 46,XY DSD, no mutations were discovered within TESCO [Georg et al., 2010]. In addition, breakpoints in CD patients with XY sex reversal range from 50 kb to up to several hundred kb upstream of *SOX9*, leaving the TES enhancer intact (Fig. 2b) [Leipoldt et al., 2007]. Several CD patients with XY sex reversal also have breakpoints much further upstream from TESCO [Pop et al., 2004; Leipoldt et al., 2007; Lecointre et al., 2009]. These findings suggested that although TES is important in maintaining *Sox9* expression levels, at least in the mouse, additional testis-specific enhancers besides TESCO may be functioning elsewhere in the *Sox9*/*SOX9* regulatory region and may be needed for the initial activation of *Sox9*/*SOX9* by SRY.

Several putative gonadal enhancer regions upstream of *SOX9* have been identified recently in humans. One study identified a 178-kb duplication 600 kb upstream of the *SOX9* transcription start site that resulted in isolated DSD (complete sex reversal) in a family with 46,XX males [Cox et al., 2011]. An overlapping 96-kb triplication was subsequently identified in 2 brothers with isolated 46,XX DSD [Vetro et al., 2011]. An analysis of 4 families with overlapping duplications in isolated 46,XX individuals and deletions in isolated 46,XY individuals in the *SOX9* regulatory region, together with previously reported genomic alterations at the *SOX9* locus associated with DSDs [Pop et al., 2004; Lecointre et al., 2009; Cox et al., 2011; Vetro et al., 2011; White et al., 2011], facilitated the discovery of a 78-kb sex-determining region named RevSex, located 517–595 kb upstream of *SOX9* [Benko et al., 2011]. It was predicted that a gonad-specific enhancer(s) existed within RevSex, and a gain or loss of this enhancer might explain the development of 46,XX and 46,XY DSDs in these patients due to increased or decreased gonadal SOX9 levels, respectively. A study by Kim et al. [2015] that identified 7 DSD individuals who were either 46,XY carrying deletions or 46,XX carrying duplications in this upstream region allowed to split the original large genomic element into 2: a 68-kb region called XXSR (−584 to −516 kb upstream of *SOX9*) found duplicated in 46,XX DSD patients and a new 32.5-kb XYSR element (−639.6 to −607.1 kb upstream of *SOX9*) found deleted in 46,XY DSD patients. Several groups have since further refined the XXSR region from 68 kb [Kim et al., 2015] to 41 kb [Hyon et al., 2015] and most recently to 24 kb [Croft et al., 2018].

Several recent studies explored the complex regulation of *SOX9*/*Sox9* gene expression in both human and mouse. In the human, Ohnesorg et al. [2016] examined publicly available DNaseI hypersensitivity data from foetal human testes and ovaries. Looking at the regulatory region of *SOX9,* they identified 13 putative enhancers, many of which showed enhancer activity in luciferase assays in vitro. In a later study, Croft et al. [2018] described 2 novel *SRY* negative, 46,XX DSD patients carrying micro-dupli-

cations located in the XYSR region with an overlapping minimal region of 5.2 kb. Using DNaseI hypersensitivity data and in vitro luciferase assays on sub-fragments of the 5.2-kb region, we identified a 1,514-bp enhancer which we termed Sex Reversal Enhancer-A (eSR-A, Fig. 2b). This element was strongly activated in the presence of hSF-1 and hSOX9 in various cell lines; however, no activation was observed in the presence of hSF-1 and hSRY. Variants in the putative SRY/SOX9 binding site and 1 of the 2 SF-1 putative binding sites markedly decreased reporter activation.

A parallel study performed in the mouse explored the 2-Mb gene desert upstream of *Sox9*. By analysing DNaseI hypersensitivity data [Maatouk et al., 2017] along with ATAC-Seq and H3K27Ac ChIP-Seq data performed on sorted embryonic Sertoli and granulosa cells, Gonen et al. [2018] identified 33 putative enhancers. Sixteen of these were screened in vivo using transgenic reporter mice and 4 showed gonad-specific expression. One of these enhancers, termed Enh13 (557 bp long), localised within the 5′ side of the XYSR region, previously was found deleted in 46,XY DSD human patients [Kim et al., 2015]. Enh13 is fully embedded within the 1,514-bp eSR-A enhancer, and both enhancers harbour 80% sequence conservation. Deletion of Enh13 in both C57BL6/J and mixed genetic background mice led to XY male-to-female sex reversal.

Another study which explored the XYSR region in mice demonstrated that deletion of a 783-bp fragment which fully contains the 557-bp Enh13 leads to XY sex reversal [Ogawa et al., 2018]. Interestingly, ChIP-Seq experiments demonstrated that Enh13 is bound by SRY and later by SOX9 [Gonen et al., 2018]. Based on the above and the sex reversal phenotype of deleting Enh13, it is likely that Enh13 serves as the critical element to which SRY binds in order to activate *Sox9* expression to a level above the required threshold, hence leading to testis development in mice. Surprisingly, luciferase reporter assays with the human eSR-A enhancer showed activation with hSOX9 and hSF-1 but failed to be activated with hSRY and hSF-1 [Croft et al., 2018]. Similar experiments performed with the mouse Enh13 showed that Enh13 is strongly activated by both mSRY and hSF-1 as well as mSOX9 and hSF-1 [Croft et al., 2018]. It may be that this enhancer functions differently in mouse and human or that this enhancer functions similarly in activating early expression of *SOX9* in response to SRY, yet the work with in vitro cells, which are not gonadal cells, is limited and these cells may not contain all the required co-factors that are normally present in gonadal cells.

It is striking that XX patients carrying extra copies of a regulatory element, eSR-A/Enh13, in the absence of *SRY* develop testis/ovotestis. We speculate that this is due to the presence of basal levels of *SOX9* normally present in both XX and XY early gonads [Kent et al., 1996; Morais da Silva et al., 1996]. SOX9 can bind to eSR-A/Enh13 and probably together with SF-1 activate its own expression above the threshold needed in order to initiate testis development. The fact that 46,XY patients with deletions in XYSR (which contains the eSR-A/Enh13) develop a female phenotype further support the role of eSR-A in activating early *SOX9* expression in response to SRY [Kim et al., 2015], as was also demonstrated in the mouse [Gonen et al., 2018; Ogawa et al., 2018].

Two additional human *SOX9* enhancers were identified. One of these is located within the 24-kb RexSex/XXSR region described above. Unbiased screening using in vitro reporter assays of 16 overlapping ~2-kb fragments covering this 24-kb region led to the identification of the 416-bp eSR-B enhancer which contains a single SOX9 binding site (Fig. 2b). Mutations in this site resulted in 60% reduction in reporter activity. Transgenic reporter mice carrying the human eSR-B sequence exhibited equivalent expression in both testis and ovaries at E12.5. eSR-B shares 75% conservation with the mouse sequence, yet deletion of eSR-B in the mouse did not present any gonadal phenotype or reduced *Sox9* expression, suggesting that eSR-B may function as a human-specific *SOX9* testis enhancer [Croft et al., 2018].

The third human *SOX9* enhancer identified, termed eALDI (Alternate Long-Distance Initiator) is a 1,259-bp enhancer located 1.4 kb upstream of the human TESCO (Fig. 2b). This enhancer was strongly activated upon co-transfection of hSRY and hSF-1 as well as hSOX9 and hSF-1. In contrast, hTESCO was not significantly activated by SF-1 and SRY or SF-1 and SOX9. In addition, bioinformatic analysis using enhancer databases and DNaseI hypersensitivity data from human foetal testis and ovary revealed that hTESCO had less enhancer potential and lower levels of conservation compared to eALDI [Croft et al., 2018].The eALDI DNA sequence shares 78% conservation with the mouse sequence, and deletion of the mouse homologue led to ~50% reduction in *Sox9* mRNA levels at E11.5 and E14.5 gonads [Croft et al., 2018]. This is comparable to the ~50% reduction in *Sox9* expression seen upon TES/TESCO deletion [Gonen et al., 2017]; hence it may be that eALDI is the TES homologue in human.

Examination of the synergistic activity of all 3 human enhancers demonstrated that only eALDI is responsive

to hSRY, and hence we speculate that eALDI is responsible for the initial activation of *SOX9* and that later on SOX9 and SF-1 maintain *SOX9* expression via binding and activation to eSR-A and eSR-B. Yet, in the absence of a human in vitro gonadal cell line, we are unable to rule out that eSR-A/Enh13 also have earlier roles in binding SRY and activating the early *SOX9* expression in humans. It will be interesting to explore whether *SOX9* is activated within the developing testis via looping of the eSR-A, eSR-B, and eALDI enhancers onto the *SOX9* promoter, facilitated by binding of SRY, SF-1, and SOX9 itself (Fig. 2c). Given that eALDI, eSR-A, and eSR-B enhancer activities are repressed by FOXL2 in vitro [Croft et al., 2018], it is possible that within the ovary, *SOX9* expression could be inhibited via looping of the eSR-A, eSR-B, and eALDI enhancers onto the *SOX9* promoter, mediated by binding of FOXL2 and an unknown factor (Fig. 2d).

Apart from its role in regulating testis development, SOX9 may also function in the development of the external genitalia. In mice, SOX9 was found to be expressed in the genital tubercle, which is the primordia of the penis and clitoris, with specific expression being detected in the urethral plate epithelium, preputial glands, ventral surface ectoderm, and corpus cavernosa [Sreenivasan et al., 2017]. It was also demonstrated that a putative enhancer of *SOX9*, named SR4, was disrupted in DSD patients who had CNVs affecting the RevSex region [Benko et al., 2011; Sreenivasan et al., 2017]. The SR4 enhancer, which spans 554 bp and is located 575 kb upstream of *SOX9*, was found to be active in the genital tubercle of mice. It was therefore proposed that SR4 may function as a *Sox9* genital tubercle enhancer and that variations affecting SR4 could potentially lead to hypospadias, which was observed in the DSD patients in the RevSex study [Sreenivasan et al., 2017].

SOX3 (SRY-Box 3)

SOX3 is an X-linked transcription factor involved in development of the pituitary and central nervous system and does not normally play a role in sex differentiation or development. Conditions associated with *SOX3* variations include X-linked mental retardation with isolated growth hormone deficiency and panhypopituitarism. Variants in *SOX3* have not been implicated in DSDs. It is believed, however, that *SRY* evolved from the *SOX3* gene located on the X chromosome [Sutton et al., 2011]. Both genes have high sequence similarity and *SOX3* can substitute for *SRY* function. This was demonstrated when ectopic expression of *Sox3* was induced in the developing gonads of transgenic XX mice [Sutton et al., 2011]. This resulted in complete female-to-male sex reversal because *Sox3* was able to function and drive testis development in the absence of *Sry*. Similarly, in humans, duplication of the *SOX3* gene itself [Moalem et al., 2012; Vetro et al., 2015; Grinspon et al., 2016; Tasic et al., 2019] or rearrangements affecting *SOX3* regulatory regions [Sutton et al., 2011; Haines et al., 2015] can cause it to be ectopically expressed in the developing gonad and substitute for *SRY*. This resulted in 46,XX (SRY negative) female-to-male sex reversal, where patients develop small testes with abnormal spermatogenesis and normal male external genitalia [Sutton et al., 2011].

SOX8 (SRY-Box 8)

SOX8 is a member of the SOXE family of transcription factors along with *SOX10* and *SOX9*. *Sox8* mutant mice display progressive male infertility along with idiopathic weight loss [Sock et al., 2001; O'Bryan et al., 2008]. Recently, three 46,XY DSD patients were identified with variants involving the *SOX8* gene [Portnoi et al., 2018]. Two 46,XY DSD individuals carried genomic rearrangements covering the *SOX8* locus, and a third individual carried a variant within the HMG box of the *SOX8* gene. This highlights that despite the predicted redundancy in the function of *SOX9* and *SOX8* during gonadal differentiation [Chaboissier et al., 2004], it is possible that each also harbours unique functions, and hence, we observe lack of proper gonad differentiation in the absence of each alone. *SOX8* variants have been found at increased frequencies in oligozoospermic men and women with primary ovarian insufficiency (POI) when compared to fertile controls [Portnoi et al., 2018], supporting the progressive male infertility observed in *Sox8* null mice.

SOX10 (SRY-Box 10)

SOX10, another member of the SOXE family, encodes a transcription factor that is closely related to *SOX9* and is involved in enteric neural crest and glial development. *Sox10* overexpression in XX mice leads to female-to-male sex reversal as it was shown that SOX10 can activate some of the downstream targets of *Sox9* in the testis, such as *Amh* [Polanco et al., 2010]. In human, partial or complete duplications of chromosome 22, involving *SOX10*, were described in several 46,XX DSD patients, leading to an almost complete masculinization of the external genitalia or ambiguous genitalia, along with partial sex reversal of the gonads [Cantu et al., 1981; Nicholl et al., 1994; Aleck et al., 1999; Seeherunvong et al., 2004].

Conclusion

SRY and the other SOX proteins, which all contain an HMG-box DNA-binding domain, share a high level of similarity and can probably compensate and replace each other in certain situations. While *SRY* is the sex-determining gene that initiates differentiation of the bipotential gonads towards testis and the male pathway, it is currently unclear whether it has additional roles and downstream targets apart from activating *SOX9* expression. This is partly because there are very few good antibodies for SRY and the short period of time it is expressed in the mouse gonad. It is also unclear whether SRY has a role in creating sexual dimorphism in tissues and organs other than the gonads. As evident from both mouse and human studies, SOX9 is the key player that activates most of the downstream targets needed in order to form a testis and it is both necessary and sufficient for male differentiation [reviewed in Gonen and Lovell-Badge, 2019].

Other members of the SOXE family, SOX8 and SOX10, are known to also be expressed in Sertoli cells [Guo et al., 2017; Li et al., 2017] and are thought to work redundantly with SOX9. They may possess unique roles that are different from SOX9 as indicated by *Sox8* knockout mice which develop progressive infertility even though they do express *Sox9* at normal levels [Sock et al., 2001; O'Bryan et al., 2008]. Similar phenotypes are present in DSD patients with variants in *SOX8* [Portnoi et al., 2018]. Loss of *Sox9*/*SOX9* in both mouse and human leads to XY sex reversal without the need to mutate *Sox8* and *Sox10*, indicating that although all 3 proteins are co-expressed in Sertoli cells, *Sox9* is the critical gene for testis development. A possible reason for this is that *Sox8* starts to be expressed only after the onset of *Sox9* expression and hence it is believed to be a downstream target of SOX9 [Sock et al., 2001; Chaboissier et al., 2004; O'Bryan et al., 2008]. However, once *Sox8* is expressed, both genes are predicted to work in a redundant manner. This was supported by late deletion of *Sox9* in the mouse (using Amh-Cre which starts to be expressed at E14.0) that showed no apparent phenotype. This can be explained by the fact that SOX8 was able to compensate for most of SOX9 activities. Yet, upon crossing of these mice to a *Sox8* null background, the mice became infertile [Barrionuevo et al., 2009]. Overexpression of SOX genes in the mouse or duplications of SOX genes in 46,XX DSD patients all strongly support the hypothesis that other SOX genes can replace SRY/SOX9 function and activate the male/testis cascade.

Many genetic studies have highlighted that the decision of the bipotential gonad to adopt either a testicular or ovarian fate relies on a delicate balance of the expression levels of several pro-male versus pro-female factors, among them is *Sox9*. Many cases of CD patients support the idea that a threshold of *SOX9* expression exists below which testes will not form and ovaries may develop instead. This is evident in that only 70% of 46,XY DSD patients with CD will present with sex reversal, while 30% will still manage to develop as males. The current hypothesis is that due to the genetic background, some individuals start with slightly higher basal levels of SOX9, and hence with heterozygous variants in *SOX9*, the residual SOX9 levels being expressed from the unaffected allele are enough to induce testis differentiation. Interestingly, it seems that this 50% threshold levels of SOX9 seen in humans is different from the threshold levels needed for testis differentiation in the mouse. Heterozygous mice carrying a mutation in one allele of *Sox9* develop as males [Bi et al., 2001]. Using mice carrying one allele with *Sox9* mutation and another allele carrying a TES deletion we were able to show that the threshold below which testis will not develop in the mice is around 25% of normal *Sox9* levels which corresponds to the 50% levels seen in humans [Gonen et al., 2017].

The critical and multifaceted roles of SOX genes in sex determination are well conserved through evolution, and it will be interesting to see if other SOX genes have the capacity to replace SOXE proteins and affect gonad development. It will also be important to better understand the direct and indirect downstream targets of SOX genes in the testis, the partners they work with, and how SOX genes are repressed during ovarian differentiation.

Ultimately, knowledge on the SOX family can provide valuable diagnostic information on DSDs for clinicians, patients, and their families. A greater understanding of DSDs, derived from the elucidation of the role that SOX factors play in gonad development and function, would lead to improved clinical management and health outcomes for individuals with DSD.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

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