Failure of SOX9 Regulation in 46XY Disorders of Sex Development with SRY, SOX9 and SF1 Mutations

Kevin C. Knower1*, Sabine Kelly1, Louisa M. Ludbrook1, Stefan Bagheri-Fam1,2, Helena Sim1, Pascal Bernard1, Ryoho Sekido4, Robin Lovell-Badge4, Vincent R. Harley1,3

1 Molecular Genetics and Development, Prince Henry’s Institute, Melbourne, Victoria, Australia, 2 Department of Anatomy and Developmental Biology, Monash University, Clayton, Melbourne, Australia, 3 Department of Molecular Biology and Biochemistry, Monash University, Clayton, Melbourne, Australia, 4 Division of Developmental Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London, United Kingdom

Abstract

Background: In human embryogenesis, loss of SRY (sex determining region on Y), SOX9 (SRY-related HMG box 9) or SF1 (steroidogenic factor 1) function causes disorders of sex development (DSD). A defining event of vertebrate sex determination is male-specific upregulation and maintenance of SOX9 expression in gonadal pre-Sertoli cells, which is preceded by transient SRY expression in mammals. In mice, Sox9 regulation is under the transcriptional control of SRY, SF1 and SOX9 via a conserved testis-specific enhancer of Sox9 (TES). Regulation of SOX9 in human sex determination is however poorly understood.

Methodology/Principal Findings: We show that a human embryonal carcinoma cell line (NT2/D1) can model events in presumptive Sertoli cells that initiate human sex determination. SRY associates with transcriptionally active chromatin in NT2/D1 cells and over-expression increases endogenous SOX9 expression. SRY and SF1 co-operate to activate the human SOX9 homologous TES (hTES), a process dependent on phosphorylated SF1. SOX9 also activates hTES, augmented by SF1, suggesting a mechanism for maintenance of SOX9 expression by auto-regulation. Analysis of mutant SRY, SF1 and SOX9 proteins encoded by thirteen separate 46,XY DSD gonadal dysgenesis individuals reveals a reduced ability to activate hTES.

Conclusions/Significance: We demonstrate how three human sex-determining factors are likely to function during gonadal development around SOX9 as a hub gene, with different genetic causes of 46,XY DSD due a common failure to upregulate SOX9 transcription.

Citation: Knower KC, Kelly S, Ludbrook LM, Bagheri-Fam S, Sim H, et al. (2011) Failure of SOX9 Regulation in 46XY Disorders of Sex Development with SRY, SOX9 and SF1 Mutations. PLoS ONE 6(3): e17751. doi:10.1371/journal.pone.0017751

Introduction

DSDs are among the most common genetic diseases in humans referring to a group of congenital conditions in which the development of the chromosomal, gonadal or anatomical sex has been abnormal [1]. Mutations in the key testis-determining factor SRY result in 46,XY DSD. Significantly, almost all 46,XY female patients with SRY mutations show complete gonadal dysgenesis [2,3], consistent with the function of SRY acting early in the development of the embryonic testis. The incidence of SRY mutations in 46,XY DSD is however quite small (10–15%) and does support the notion that genes other than SRY are essential for proper testis development. Despite the ongoing identification of a number of these key testis-determining genes [4], most of which are transcription factors, the actions, co-factors and downstream targets of human SRY have proven difficult to ascertain.

SRY which is expressed in Sertoli cells plays key cellular roles in the developing gonad including the differentiation of Sertoli cells [5]; inducing migration of cells from the mesonephros into the gonad [6]; inducing proliferation of cells within the gonad [7]; inducing the development of the vasculature patterning of the XY gonad [8]; and glycogen accumulation in pre-Sertoli cells [9]. Each role may be mediated by a direct interaction between SRY and one or more partner proteins on one or more independent target genes. Hence, one question arising is whether the various and multiple roles played by SRY are direct or indirect?

The human SOX9 gene when mutated causes CD/SRA1 (Campomelic Dysplasia/Autosomal Sex Reversal), and has become known as a pivotal sex-determining gene [10,11]. The upregulation, sexual dimorphic expression pattern and conserved protein structure of SOX9 are consistent across all vertebrate species, regardless of the switch mechanism controlling sex determination, being SRY in mammals (except for the mole vole, [12]), ZW chromosome gene(s) in birds [13] and temperature sensitivity of egg incubation in turtles and crocodiles [14,15]. In XX Sox9 transgenic mice, and probably also in human XX males with SOX9 duplications or translocations, the increased levels of SOX9 are sufficient to initiate testis formation in the absence of Sry/SRY [16,17,18,19]. This raised the possibility that Sox9/SOX9 might be a direct and potentially only target for SRY. In
agreement, the recent identification of a conserved testis-specific enhancer of Sox9 (TES) in the mouse has revealed a co-transcriptional network of SRY, SF1, and SOX9 involved in the direct initiation, upregulation and maintenance of Sox9 expression in the mouse XY gonad [20]. Like SRY and SOX9, mutations in human SF1 lead to 46,XY gonadal dysgenesis [21,22,23].

While in the mouse, SRY directly up-regulates Sox9 expression to induce testis development [20], the relationship between human SRY and SOX9 is less clear. The understanding of human SRY protein function is hampered by its lack of protein sequence conservation across mammalian species. Protein structural domains of SRY are poorly conserved, the only conserved domain between human and mouse is the mobility group (HMG) domain [24], yet human SRY under the control of mouse regulatory sequences can still induce testis development in XX transgenic mice [25]. The importance of the HMG domain in the function of the human SRY protein is also highlighted by the fact that most 46,XY gonadal dysgenesis mutations cluster within this domain. It has thus been proposed that human SRY instigates testis-determination by potentially (i) activating gene expression through its consensus binding site (A/T)AACAAT [26], (ii) functioning as an architectural factor by bending DNA [27], (iii) repressing a putative suppressor of a testis-promoting factor [28], and (iv) being involved in pre-mRNA splicing [29]. The lack of a suitable in vitro model system, including a bone fide SRY testis-determining target gene, has hindered the ability to test such hypotheses of human SRY.

In the current study, we aimed to develop an in vitro assay to understand the molecular actions of human SRY in sex determination. We demonstrate that endogenous SOX9 is upregulated in the human embryonal carcinoma cell line NT2/D1 over-expressing SRY, a model of presumptive Sertoli cells [30]. This upregulation is associated with SRY localisation to actively transcribed chromatin and not pre-mRNA splicing complexes. Furthermore, we reveal that the human homologous Sox9 testis-specific enhancer (hTES) is responsive to human SRY, SF1 and SOX9 co-transcriptional activation. However, mutant SRY, SF1 and SOX9 proteins encoded by 46,XY DSD individuals exhibit a reduced ability to activate hTES. As a central hub gene, SOX9 regulation is an important event in mammalian sex determination. This study provides an important insight into the molecular actions of human SRY in this process. Furthermore, by assaying SRY, SF1 and SOX9 from 46,XY DSD individuals we have provided functional evidence of mutations that result in reduced SOX9 expression.

Results

SRY up-regulates SOX9/SOX9 expression in the human NT2/D1 cell line

To test whether human SRY can activate SOX9 transcription we evaluated the human embryonal carcinoma cell line, NT2/D1, as a model of presumptive Sertoli cells. NT2/D1 cells express many of the genes implicated in testis differentiation, including SRY, SOX9 and SF1 [30]. To simulate the onset of SRY expression, we transiently transfected SRY into NT2/D1 cells. A significant 2.2-fold increase in SOX9 mRNA was measured by quantitative RT-PCR (QRT-PCR) (Fig. 1A). SOX9 protein levels increased accordingly, as represented by the positive correlation between exogenous SRY and endogenous SOX9 immunofluorescence (Fig. 1B). In vivo, SRY can act as an architectural transcription factor, binding and bending specific DNA sequences or structures [31]. We co-immunostained NT2/D1 cells to detect both SRY and tri-methylated histone 3 lysine 4 (H3 Me3K4), which is exclusively associated with actively transcribed chromatin regions [32]. The co-localization of SRY and H3 Me3K4 proteins within the nucleus of NT2/D1 cells suggests that, when upregulating SOX9, SRY functions in a transcriptional complex (Fig. 1C, upper panel). It has been proposed that SRY also acts in pre-mRNA processing [29], however, in our study, and in agreement with others [33], Flag-SRY protein does not co-localize with splicing factor SC-35 (Fig. 1C, lower panel).

SRY, SF1 and SOX9 activate the human homologue of the testis-specific enhancer of Sox9 (hTES)

SRY/Sox9 and SF1 synergistically activate a ~3.2 kb, testis-specific enhancer of the mouse Sox9 gene, 13.2–10.1 kb upstream of the transcription start site (termed mTES) [20]. mTES is highly conserved across species including human [20,34]. What we believe to be the equivalent human enhancer element (termed hTES) is positioned 14.7–11.6 kb upstream of the transcriptional start site of the human SOX9 gene. To test whether SRY could activate the human enhancer, hTES and mTES (as control) sequences were cloned into a reporter construct and each was co-

![Figure 1. Endogenous SOX9 is up-regulated in NT2/D1 cells in response to SRY.](image-url)

(A) SOX9 is significantly upregulated in NT2/D1 cells transiently overexpressing SRY (1 μg), as measured by QRT-PCR and compared to empty vector (n = 3). (B) Immunostaining for endogenous SOX9 and exogenous SRY (Anti-Flag) reveals a positive correlation between SOX9 fluorescence and exogenous SRY fluorescence (R² = 0.835, n = 50). Scale bar represents 10 μm. Each point averages three fluorescence readings per NT2/D1 cell (Blue Diamond - SRY-transfected cells; Pink triangle - non-Flag cells). (C) (Upper panel) H3 Me3K4 (red) is exclusively nuclear in NT2/D1 cells. Overlap of SRY (green) and H3 Me3K4 in NT2/D1 cells reveals co-localization (yellow) within nuclear compartments. (Lower panel) SC-35 (red) is localized to alternate nuclear compartments, as overlap images of Flag-SRY and SC-35 staining do not show co-localization. DAPI (blue) stains nuclear DNA. Scale bar represents 10 μm. Error bars represent the standard error of mean values. Two-tail t-Test of paired sample means was performed. * P<0.05.

doi:10.1371/journal.pone.0017751.g001
SRY transcriptional activity [35]. We also obtained similar results using HEK293 cells, derived from a human embryonic kidney (HEK293) cell line, which lack endogenous Sry but confer SRY transcriptional activity [35]. We also obtained similar results transfecting human CHO cells with exogenous human SRY (100 ng) compared to vector alone. (n = 3–5) SRY fusion to VP16 (SRY-VP16, 104 ng) increased activation of hTES, whereas SRY fusion to EnR (SRY-EnR, 120 ng) reduced activation compared to SRY (n = 2). All reporter assays were conducted in duplicate. Error bars represent the standard error of mean values. Two-tail t-test of paired sample means was performed. *** P < 0.001, ** P < 0.01. (C) In vitro translated SRY-VP16 and SRY-EnR fusion proteins migrate at their expected product size compared to SRY WT. (D) Electrophoretic mobility shift assay demonstrates that in vitro translated SRY-VP16 and SRY-EnR fusion proteins bind to a 32P-labelled DNA oligonucleotide containing the SOX DNA binding consensus sequence AACAAT. SRY WT also binds this oligonucleotide whereas the luciferase control protein cannot.

doi:10.1371/journal.pone.0017751.g002

Figure 2. SRY acts as a transcriptional activator to stimulate the human homologous testis-specific SOX9 enhancer (hTES). (A) mTES and hTES reporter constructs (1.6 μg) are activated ~6-fold in CHO cells by exogenous human SRY (100 ng) compared to vector alone. (n = 3–5) SRY fusion to VP16 (SRY-VP16, 164 ng) increased activation of hTES, whereas SRY fusion to EnR (SRY-EnR, 120 ng) reduced activation compared to SRY (n = 2). All reporter assays were conducted in duplicate. Error bars represent the standard error of mean values. Two-tail t-test of paired sample means was performed. *** P < 0.001, ** P < 0.01. (C) In vitro translated SRY-VP16 and SRY-EnR fusion proteins migrate at their expected product size compared to SRY WT. (D) Electrophoretic mobility shift assay demonstrates that in vitro translated SRY-VP16 and SRY-EnR fusion proteins bind to a 32P-labelled DNA oligonucleotide containing the SOX DNA binding consensus sequence AACAAT. SRY WT also binds this oligonucleotide whereas the luciferase control protein cannot.

doi:10.1371/journal.pone.0017751.g002

transfected with a human SRY expression vector into Chinese Hamster Ovary (CHO) cells that lack endogenous Sry but confer SRY transcriptional activity [35]. We also obtained similar results using HEK293 cells, derived from a human embryonic kidney (HEK293) cell line, which lack endogenous Sry but confer SRY transcriptional activity [35]. Both enhancers were stimulated by human SRY to a similar extent (Fig. 2A), whereas SRY did not activate the empty E1b-luc reporter vector (data not shown). This indicates that SRY-mediated activity was via the human and mouse SOX9/SOX9 enhancer sequences. Within the transcriptional complex, SRY has been proposed to function either as a transcriptional activator [26] or as a repressor of a transcriptional repressor [28]. We fused SRY to the activation domain of the viral protein VP16 [36] and to the repressor domain of the Drosophila melanogaster Engrailed (EnR) [37]. Both SRY fusion proteins were stably translated and bound SRY consensus DNA sequences with wild-type affinities (Fig. 2C and 2D respectively). If SRY were a repressor of a repressor of SOX9, SRY-VP16 would decrease, whereas SRY-EnR would increase hTES activity in comparison to wild-type SRY. However, fusion to VP16 significantly increased SRY-mediated hTES activation 2-fold, whereas SRY-EnR decreased hTES activation 3-fold (Fig. 2B). Taken together, these data support a model where SRY functions as a transcriptional activator.

We next tested the effects of SF1 on SRY-mediated hTES activity. Transfection of exogenous SF1 into CHO cells, which contain low levels of endogenous SF1 [22], activated the hTES enhancer 1.8-fold, while SRY activated hTES 8-fold. Together, SRY and SF1 stimulate hTES activity 16-fold, suggesting cooperative regulation of the SOX9 enhancer is occurring (Fig. 3A). Phosphorylation of SF1 appears to be essential for this cooperation with SRY, as co- operativity was abolished when the SF1-S203A phosphorylation mutant was used (Fig. 3B). This mutant is known to disrupt co-factor recruitment and, as a consequence, SF1 transcriptional activity [38].

Previously, it has been shown that in the presence of SF1, SOX9 can synergistically activate mTES [20]. To investigate whether SOX9 could help maintain its own expression also on the human enhancer, a SOX9 expression plasmid was co-transfected with the hTES reporter construct. SOX9 significantly stimulated hTES reporter activity 2-fold (Fig. 3A). In the presence of SF1, SOX9-mediated stimulation increased 3-fold. SOX9 mutations caused 46,XY gonadal dysgenesis frequently truncate the C-terminus leading to loss of PQS and PQA transactivation domains [2]. Deletion analysis indicated that the PQS transactivation domain was essential for the hTES enhancer activation that we observed, whereas the PQA domain was not (Fig. 3C). The data suggest that DNA binding and the PQS-region, but not dimerization, are required for normal SOX9 activity.

Taken together, these data support a model of human gonadal development whereby SF1 participates firstly with SRY to up-regulate SOX9 expression, and then with SRY, SF1 and SOX9 to maintain its own expression. These findings agree with data obtained with mTES in COS cells [20], although here we observe a co-operativity rather than a synergy between SF1 and SOX9.

SRY, SF1 and SOX9 mutant proteins fail to activate the hTES enhancer

Patients with 46,XY gonadal dysgenesis carry mutations in testis-determining genes including SRY, SF1 and SOX9. We used hTES as in vitro assay to study the impact of SRY, SF1 and SOX9 mutations on SOX9 gene regulation. We first assayed the transcriptional activity of SRY from eight 46,XY females using the hTES enhancer. The SRY mutants varied in the location of SRY mutation, mode of inheritance (Fig. 4A) and biochemical defects (Table 1). Six of the eight SRY mutants tested showed a significant reduction in transcriptional activity (Fig. 4A). Of the de novo mutants with DNA binding defects, SRY-R62G, -R75N and -R76P showed ~0–5% of wild-type SRY transcriptional activity, while the nuclear import mutant SRY-R133W showed ~50% activity. Interestingly, the familial SRY-I90M mutant showed a significant ~100% increase in hTES activation. This mutation is predicted to alter the hydrophobic isoleucine residue within a nuclear export sequence (Lex1–3Lex2–5Lex3) suggesting correct SRY nuclear export could be important in vivo. Familial mutants inherited from fertile fathers (SRY-S18N, -R30I, -L163X) positioned outside of the HMG domain were investigated. Consistent with the partial penetrance of these mutations, their ability to activate hTES was consistently higher (~50–90%) than that of the de novo mutants, highlighting the potency of this assay.
SF1 mutations cause 46,XY gonadal dysgenesis and adrenal defects in some cases [21,23]. Three SF1 mutations (SF1-G35E, -R92Q and -A88p) localized to different regions of the SF1 protein affect DNA binding and transactivation of adrenal target genes [21,39] (Table 2). We tested the ability of these SF1 mutants to activate hTES (Fig. 4B). The homozygous mutation SF1-R92Q showed ~50% of wild-type transcriptional activity, consistent with the lack of phenotype in the heterozygous parents [21]. In contrast, heterozygous mutants SF1-G35E and SF1-A88p showed no transcriptional activity. All three mutations significantly reduced the co-operative activation, with SRY, of hTES (Fig. 5B).

Heterozygous SOX9 point mutations cause CD/SRA in most 46,XY individuals [2]. The SOX9 mutant, SOX9-A158T causes CD/SRA with 46,XY gonadal dysgenesis, due to defects in SOX9 nuclear import and DNA binding [40] (Table 3). The SOX9-A158T mutant failed to activate the hTES enhancer both alone (Fig. 4C) or in the presence of SF1 (Fig. 5C). In contrast, the SOX9-A76E mutant is encoded by a CD patient without 46,XY gonadal dysgenesis and lacks the ability to dimerize [41]. The SOX9-A76E mutant demonstrated wild-type activation of the hTES enhancer, consistent with dimerization of Sox9 not being required for sex determination.

**Discussion**

Regulation of SOX9 expression in the gonad is tightly controlled. The key event that defines testis determination is the male-specific upregulation of SOX9. Results obtained in this study replicate the earliest developmental step in sex determination, the activation of SOX9 by SRY. Our findings have demonstrated a role for human SRY, SF1 and SOX9 in activating SOX9 expression, endogenously in the NT2/D1 ‘Sertoli’-like cell line and also via a conserved human SOX9 testis-specific enhancer, hTES. These same factors are involved in the initiation, upregulation and maintenance of Sox9 expression in XY gonads of mice [20].

Poor sequence conservation outside of the HMG domain between human and mouse SRY has led to the belief that human SRY may function through differing mechanisms. For example, mouse SRY has a transactivation domain that is absent in human SRY [26]. Furthermore, unlike the narrow window of transient expression of mouse Sry, beginning at E10.5 and ending abruptly at E12.5 [42], human SRY is first observed at E41 and is still present after 18 weeks gestation [43]. SRY function has been difficult to test due to the lack of bone fide target genes and good transcriptional assays. We demonstrate a role for SRY as a transcriptional activator, both on the hTES enhancer and on an endogenous target in NT2/D1 cells over-expressing SRY. In support of this, SRY was found to co-localise with transcriptionally active chromatin in NT2/D1 cells correlating with its proposed architectural property [27], but not pre-mRNA splicing as previously demonstrated [29]. Likewise, fusion of the transcriptional activator domain VP16 and not the repressor domain EnR to SRY resulted in increases in hTES reporter activity, suggesting that SRY is not merely repressing a repressor. While transcriptional activation of SOX9 by SRY is clearly a pivotal process for proper testis development, the repressive actions of human SRY on ovary-determining pathways have also been proposed [44,45].

The variability in transactivating properties of SRY, SF1 and SOX9 DSD mutants used in this study contribute to lower doses of SOX9 and provide important functional information. The transcriptional action of SRY identified here enabled us to investigate why certain SRY mutations always cause gonadal dysgenesis while other mutations do not. Certain sex-reversing SRY mutations show variable reduced DNA binding, DNA bending and nuclear import defects compared to wild type SRY (Table 1), however, studying the transcriptional activity of such mutants has been impossible due to a lack of known targets. Three missense de novo mutants, SRY-R62G, -R75N and -R76P located in the HMG box N-terminal NLS showed large losses in transcriptional activity of hTES. The de novo SRY-R133W mutant localised in the C-terminal NLS of SRY also showed a ~50% loss in transcriptional activity in comparison to wild type SRY. These four mutants demonstrate the relative importance of DNA binding, bending and nuclear import for the activation of hTES. Of the four mutants, SRY-R62G and SRY-R75N have the most severe biochemical defects and this is also reflected by their transcriptional activation properties being the lowest (Table 1). The higher activation of hTES by SRY-R133W compared to SRY-R76P may be accounted for by its relatively normal DNA
binding affinity. This result also reflects the importance of nuclear import to the proper function of SRY [46,47]. SRY-R133W has 50% the nuclear import capacity of wild type SRY due to a loss in importin-b recognition [46]. Therefore, the use of such mutants in the assay developed in this study is somewhat more representative of SRY's action in vivo, where even a subtle mutation such as SRY-R133W may reduce the ability of SRY to up-regulate SOX9.

Intriguingly, the SRY-190M mutation, which shows slightly reduced DNA binding and normal DNA bending by EMSA [27], activated the hTES enhancer, 50% greater than that of wild type SRY. The location of this mutation at residue 90 coincides with a conserved hydrophobic residue present in a number of SOX proteins that forms part of a functional nuclear export signal (NES) [48]. Substitution of the hydrophobic amino acid position in the SOX9 NES (L142A) produces a protein unable to be exported from the nucleus. By analogy, the observed increase in transactivation of the SRY I90M mutant could be a consequence of increased nuclear accumulation. It is possible to speculate that a mutation that increases levels of nuclear SRY could be deleterious and the cause of 46,XY gonadal dysgenesis. This may also relate with the finding that an individual with a XYY karyotype, bearing two copies of the wildtype SRY coding sequence is female [49].

Patients with missense mutations lying outside of the HMG domain present as rare cases of 46,XY individuals with partial gonadal dysgenesis i.e. the two familial mutations SRY-S18N [50].
and SRY-R30I [51]. The decrease in transcriptional activity of the SRY-S18N mutant points to a possible functional role for this N-terminal region. Interestingly, the same SRY-S18N mutation has also been isolated in a separate individual who presented with Turner syndrome and Y chromosome mosaicism (Ulrich Turner Syndrome) [52]. The SRY-R30I mutant was present in six 46,XY siblings where one patient showed complete gonadal dysgenesis, two showed partial gonadal dysgenesis and three were unaffected [51]. The SRY-R30I mutation is located near serine residues that two showed partial gonadal dysgenesis and three were unaffected siblings where one patient showed complete gonadal dysgenesis.

Table 1. Summary of defects in SRY mutants.

| SRY Mutants | Position       | Phenotype        | DNA Binding | DNA Binding | Nuclear Localization | hTES enhancer activation |
|-------------|----------------|------------------|-------------|-------------|----------------------|--------------------------|
| S18N*       | N terminal to HMG | PGD/CGD         | ~90         | ~100        | ND                   | ~50                      |
| R30I*       | N terminal to HMG | PGD/CGD         | ~50         | ND          | ND                   | ~90                      |
| R62G        | N-NLS           | CGD              | <1          | ~67         | ~25                  | <1                       |
| R75N        | N-NLS           | CGD              | <1          | ND          | ~28                  | <1                       |
| R76P        | N-NLS           | CGD              | ~73         | ~95         | ~50                  | ~5                       |
| R90M*       | HMG             | TH/PGD/CGD      | ~95         | ~100        | ND                   | ~200                     |
| R133W       | C-NLS           | CGD              | ~95         | ~100        | ~52                  | ~40                      |
| L163X*      | C-terminal to HMG | CGD          | ND          | ND          | ND                   | ~50                      |

ND – Not determined.
* denotes a familial mutation. N- and C- NLS – terminal nuclear localization signals. HMG – high mobility group domain. CGD – complete gonadal dysgenesis. PGD – partial gonadal dysgenesis. TH – true hermaphrodite. Biochemical defects obtained from respective references [27,46,51,77].

Table 2. Summary of defects in SF1 mutants.

| SF1 Mutants | Position | Adrenal Failure | 46,XY Gonadal Dysgenesis | DNA Binding | Transcriptional activity | hTES enhancer activation |
|-------------|----------|-----------------|--------------------------|-------------|--------------------------|--------------------------|
| G35E/WT     | DBD      | Yes             | Yes                      | <1          | <1                       | <5                       |
| R92Q/R92Q   | FtzF1    | Yes             | Yes                      | ~50         | ~25                      | ~50                      |
| A8bp/WT     | LBD      | No              | Yes                      | ~50         | ~30                      | <1                       |

DBD – DNA binding domain. FtzF1 – FtzF1 box. LBD – Ligand binding domain. Biochemical defects obtained from respective references [21,39]. Transcriptional activity for SF1 was assayed using reporter constructs for the CYP11A1 [21] and CYP17 [39] adrenal genes respectively.

The incomplete penetrance of SRY mutants such as SRY-S18N, -R30I, -L163X with the documentation of some families with fertile fathers being mosaic for both wild type and mutant SRY [57,58,59], does support the notion that dose dependency, genetic background and in extension, SOX9 activation levels, all play important roles in sex determination. With Sry, this can be demonstrated in mice where ectopic SRY activates sox9 in a dose-dependent manner [60]; expression of Sry transgene constructs below a critical threshold level in XX transgenic mice results in partial gonadal dysgenesis [33,61,62, 63,64]; and Sry alleles from some mouse strains will cause gonadal dysgenesis when placed in certain genetic backgrounds [65,66,67,68]. Whereas in humans the disruption to SF1 and SOX9 can result in 46,XY gonadal dysgenesis, haploinsufficiency of these critical factors in mice does not, making the assessment of essential SF1 or SOX9 dosage levels difficult in this context. In agreement to data obtained with the mTES [20], we have shown that both SF1 and SOX9 are also involved in the activation of hTES. In contrast to the mTES, our data does not reveal a synergistic activation between SF1 and SOX9. This result may reflect the sustained expression of human SRY in the XY gonad that is responsible, in addition to SF1 and SOX9, for maintaining SOX9 levels. Indeed, human SRY is able to stimulate hTES on its own, whereas mouse SRY on mTES cannot [20].

Importantly, the SF1 and SOX9 transcriptional properties of mutants and levels of hTES activity can be related to DSD phenotypes. The SOX9 mutant, SOX9-A158T causes CD/SRA with 46,XY gonadal dysgenesis, due to defects in SOX9 nuclear import and DNA binding [40], whereas the SOX9-A76E mutant is encoded by a CD...
SOX9 Regulation in Disorders of Sexual Development

Table 3. Summary of defects in SOX9 mutants.

| SOX9 Mutants | Position | Phenotype | DNA Binding | DNA Bending | Nuclear Localization | hTES enhancer activation |
|--------------|----------|-----------|-------------|-------------|----------------------|-------------------------|
| A76E         | Dimerization | CD alone | ~100 | ND | ~100 | ~100 |
| A135T        | HMG       | CD with CGD | ~17 | ~100 | ~50 | <1 |

HMG – high mobility group domain. CGD – complete gonadal dysgenesis. CD – Campomelic Dysplasia. Biochemical defects obtained from respective references [40,41].

doI:10.1371/journal.pone.0017751.t003

patient without 46,XY gonadal dysgenesis and lacks the ability to dimerize [41]. In fitting with previous observations, SOX9-A135T could not activate hTES, while the SOX9-A76E mutant demonstrated wild-type activation, consistent with dimerization of SOX9 not being required for sex determination. It is noteworthy that identical mutations in SOX9 can cause 46,XY DSD in one instance but not in another [69,70]. While genetic background and SOX9 expression levels may be contributing to these diverse phenotypes, analysis of the transcriptional activities of such mutations in the context of the hTES assay warrant further investigation, as the activity of these mutants might be reduced to a threshold level.

The three SF1 mutants used all present 46,XY gonadal dysgenesis with SF1-G35E and SF1-R92Q mutants also showing adrenal failure (Table 2). The biochemical action of the SF1-G35E and SF1-R92Q mutants has previously been tested only on adrenal targets and not sex-determining genes [21,71,72]. Both SF1-G35E and SF1-R92Q on its own or in combination with SRY have reduced transcriptional activity on hTES, presumably through reduced DNA binding properties (Table 2). It is noteworthy that the homozygous SF1-R92Q mutant is recessive, familial in nature and that the gonadal dysgenesis phenotype only presents on certain genetic backgrounds [21]. The hypomorphic nature of this mutant reflects an ability to activate the SOX9 enhancer to half the extent of SF1-WT. The SF1-A158p mutant lacks half of the ligand binding domain and also the AF2 domain important for facilitating interactions with co-activators to stimulate transcription [73], although not sufficient alone [74]. We show a significant decrease in SF1-A158p transcriptional activity on hTES. The SF1-A158p has also been demonstrated to show a dominant-negative effect on wild type SF1 transcriptional activation of the Cyp17 promoter in human HEK293 (kidney), mouse MA10 (Leydig) and mouse Y1 (adrenal) cell lines [39]. The reduction in hTES activity, both alone and in cooperation with SRY, may therefore be a result of a dominant-negative effect of SF1-A158p on endogenous SF1 or lack of co-factor recruitment in CHO cells. In conclusion, we have developed an in vitro assay that replicates the initial events of mammalian sex determination and puts into context the functional properties of mutations to SRY, SF1 and SOX9 in DSD.

Materials and Methods

Cell Culture and transfections

NT2/D1 (ATCC CRL-1973) cells were grown in Dulbecco’s and Ham’s F12 medium; CHO cells (ATCC CCL-61) were grown in Dulbecco’s medium. Medium was supplemented with 10% fetal bovine serum and L-glutamine in an atmosphere of 5% CO2. Transient transfections were conducted using FuGene6 (Roche) in accordance with the manufacturer’s instructions.

Expression Plasmids

All mammalian expression plasmids were of pcDNA3 origin (Clontech) unless otherwise stated. DNA encoding wild-type Flag-tagged human SRY was previously described [46]. SRY mutants R62G, R75N, R76P and R133W were also previously described [46]. SRY mutants S18N, R30I, R90M and L163X were produced using site-directed Quick change mutagenesis kit (Qiagen) according to the manufacturer’s instructions. pS4x-VP16 and -Engrailed shuttle vectors were a kind gift from Dr. Jonas Muhr as previously described [75] and were used to produce SRY fusion proteins. Briefly, the SRY ORF was amplified by PCR from pcDNA3-SRY using a forward oligonucleotide (containing BamHI, FLAG and KOZAK sequences) and a reverse oligonucleotide that replaces the stop codon with an EcoRI restriction site. The PCR product was then ligated to an EcoRI restriction site that was the beginning of the ORF of either VP16 or Engrailed domains. The fusion proteins were subsequently sub-cloned into pcDNA3. HA-tagged human SF1 and G35E SF1 mutant expression plasmids were kind gifts from Dr. Robert Viger as previously described [72]. All SF1 mutant was a kind gift from Dr. Keith Parker as previously described [39]. The R92Q SF1 mutant was received as a gift from Dr. Larry Jameson as previously described [21]. pCI-neo-mSF1 (mouse cDNA) and the S203A phosphorylation mutant in the same expression plasmid were gifts from Dr. Holly Ingham as previously described [39]. HA-tagged human SOX9 expression plasmid, SOX9-AP/QA and SOX9-1-463 plasmids are previously described [76].

Reporter Plasmids

The hTES sequence was amplified from the BAC contig clone RP11-41E24 (Genbank Accession AC000467), located on chromosome 17, by PCR using a forward oligonucleotide containing an XhoI restriction site (GATCATCCGAGGTTTGGAGTGAAACTGT) and a reverse oligonucleotide containing an AciI restriction site (GATGGCCGCTCAACCCACTTTGGCTCAATCTACAC). The resultant PCR product was cloned into the multiple cloning site of the E1b-CAT reporter construct [26]. Subsequently, the CAT reporter gene was replaced with the open reading frame of the lacI operon reporter gene derived from the pGL3-basic (Promega). The mTES sequence contained within a shuttle vector was also sub-cloned into E1b-lac.

Fluorescence activated cell sorting (FACS) and RNA extraction

NT2/D1 cells were seeded at a density of 2.5×10⁵ cells per well in 6-well plates 24 hours prior to transfection. In each transfection, pEF-GFP expression plasmid was added in a 1:3 molar ratio with other expression plasmids to ensure efficient transfection efficiency. After 48 hours, cells were subjected to FACS. RNA was collected from GFP-positive cells using the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions.

Quantitative RT-PCR

0.5 μg of total RNA was reverse transcribed using the modifying enzyme Superscript III according to the manufacturer’s specifica-
tions (Invitrogen). Real time quantification of mRNA levels was conducted using Lightcycler technology (Roche). Values obtained for each sample were standardized to amplification levels of the housekeeping gene β-2-Microglobulin (β-2-M). Standardized values were divided by vector alone transfectants to obtain total fold differences. Sequences of oligonucleotides used to amplify cDNA are (5’ to 3’ orientation): SOX9 F-AAGACATTTAAGCTAA-AGGCCAATCTGTAC; R- TGTACACAGTTCTCCATCATC- TCCGT; β-M F-TGAATTGCTATGTGTCTGGGT, R- CCT- CCATGATGCTGTTTACAT.

Reporter assays
CHO cells were seeded at a density of 2.3×10^5 cells per well in 6-well plates 24 hours prior to transfection. 48 hours post-transfection the culture media was removed, cell lysate collected (1:1000) (Sigma) and the rabbit Anti-Tri-methylated lysine 4 of FLAG (1:500) (Sigma), mouse Anti-SC35 monoclonal antibody (1:400), affinity-purified mouse monoclonal anti-SOX9 (1:400), affinity purified sheep anti-human SRY (1:400), affinity purified donkey anti-mouse IgG (1:800) (Amersham).

Immunohistochemistry
NT2/D1 cells used for immunohistochemistry were seeded on coverslips placed into 6-well plates. Standard protocols were used for immunohistochemistry. The primary antibodies used include affinity purified sheep anti-human SRY (1:400), affinity purified rabbit anti-SONX9 (1:400), affinity-purified mouse monoclonal anti-FLAG (1:500) (Sigma), mouse Anti-SC35 monoclonal antibody (1:1000) (Sigma) and the rabbit Anti-Tri-methylated lysine 4 of histone 3 [H3-MeK4] polyclonal antibody (1:200) (Upstate Biotech). The secondary antibodies used include Alexa 488-conjugated donkey anti-rabbit IgG (1:1000), Alexa 594-conjugated donkey anti-mouse IgG (1:1000) (Molecular Probes). Coverslips were mounted onto slides with DAKO fluorescence mounting medium containing DAPI (final 0.6 mg/ml). Images were captured using an Olympus FV500 confocal laser scanning microscope. Image analysis was performed using NIH ImageJ (public domain software).

In vitro translation and EMSA analysis of SRY fusion proteins
Proteins were produced using an in vitro coupled transcription and translation process using a TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer’s guidelines. A small proportion of each sample was translated with the incorporation of 35S-Methionine to produce a radio-labeled protein to enable verification that the protein produced was of the correct molecular mass. EMSA analysis was performed using in vitro translated protein combined with 1 μl of 35S-labeled DNA probe (~40,000 cpm) using standard protocols. Oligonucleotide sequence of the SRY consensus probe was (5’ to 3’ orientation) GGGTTAATAGATGATGATGATGGTGA; core binding sequence is underlined. The gel was visualized using the Storm phosphor-imaging system and ImageQuant analysis software (Amersham).

Author Contributions
Conceived and designed the experiments: KK SK HS PB LL VH. Performed the experiments: KK SK HS PB LL. Analyzed the data: KK SK HS PB LL VH. Contributed reagents/materials/analysis tools: KK SK HS PB LL RS. Wrote the paper: KK SK HS LL SBF RS RLB VH.

References
1. Hughes IA, Houk C, Ahmed SF, Lee PA (2006) Consensus statement on the management of urorectal septum defects. J Pediatr Urol 2: 149–152.
2. Cameron FJ, Sinclair AH (1997) Mutations in SRY and SONX9: testis-determining genes. Hum Mutat 9: 388–395.
3. McLaren E, Kell OS (1999) Sex determination and the Y chromosome. Am J Med Genet 89: 176–183.
4. Sekido R, Lovell-Badge R (2006) Sex determination and SRY: down to a wink and a nudge? Trends Genet 22: 19–29.
5. Rossi P, Doki S, Albaranes C, Grimaili P, Geremia R (1993) Direct evidence that the mouse sex-determining gene Sry is expressed in the somatic cells of male-letal gonads and in the germ cell line in the adult testis. Mol Reprod Dev 34: 369–373.
6. Capel B, Albrecht KH, Washburn LL, Eicher EM (1999) Migration of mesonephric cells into the mammalian gonad depends on Sry. Mech Dev 84: 127–131.
7. Schmahl J, Eicher EM, Washburn LL, Capel B (2000) Sry induces cell proliferation in the mouse gonad. Development 127: 63–73.
8. Brennan J, Karl J, Capel B (2002) Divergent vascular mechanisms downstream of Sry establish the arterial system in the XY gonad. Dev Biol 244: 418–428.
9. Matoba S, Kanai Y, Kidokoro T, Kanai-Azuma M, Kawakami H, et al. (2005) A novel Sry-d MSTAT downstream cellular event which preserves the readily available energy source of glycogen in mouse sex determination. J Cell Sci 118: 1449–1459.
10. Foster JW, Dominguez-Steglich MA, Guidoni S, Kosik K, Weller PA, et al. (1999) Camptocyclin dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 372: 525–530.
11. Wagner T, Wirth J, Meyer J, Zabel B, Held M, et al. (1994) Autosomal sex reversal and camptocyclin dysplasia are caused by mutations in and around the SRY-related gene SONX9. Cell 79: 1111–1120.
12. Just W, Lau W, Vogel W, Akhverdian M, Fredga K, et al. (1995) Absence of Sry in species of the vole Elllobius. Nat Genet 11: 117–118.
13. Oreal E, Picais C, Mateu MG, Jossos N, Picard JY, et al. (1998) Early expression of AMH in chicken embryonic gonads precedes testicular SONX9 expression. Dev Dyn 212: 522–532.
14. Moreno-Mendoza N, Harley VR, Merchant-Larios H (1999) Differential expression of SONX9 in gonads of the sea turtle Lepidochelys olivacea at male- or female-promoting temperatures. J Exp Zool 284: 705–710.
15. Western PS, Harry JL, Graves JA, Sinclair AH (1999) Temperature-dependent sex determination: upregulation of SONX9 expression after commitment to male development. Dev Dyn 214: 171–177.
16. Huang B, Wang S, Ning Y, Lamb AN, Bartley J (1999) Autosomal XX sex reversal caused by duplication of SONX9. Am J Med Genet 87: 349–353.
17. Bishop CE, Whitworth DJ, Qin Y, Ageroulis AI, Agouliluc IU, et al. (2000) A transcriptional insertion upstream of SRF is associated with dominant XX sex reversal in the mouse. Nat Genet 26: 490–494.
18. Vidal VP, Chaboisier MC, de Rosoi DG, Scheffl A (2001) Sox9 induces testis development in XX transgenic mice. Nat Genet 28: 216–217.
19. Refai O, Friedman A, Terry L, Jezewt T, Pearlman A, et al. (2010) De novo 12:17 translocation upstream of SONX9 resulting in 46,XX testicular disorder of sex development. Am J Med Genet A 152A: 422–426.
20. Sekido R, Lovell-Badge R (2008) Sex determination involves synergistic action of SRY and Sox9 on a specific Sox9 enhancer. Nature 453: 930–934.
21. Achermann JC, Oezi G, Ito M, Onan UA, Harmanic K, et al. (2002) Gonadal determination and adrenal development are regulated by the orphan nuclear receptor steroidogenic factor-1, in a dose-dependent manner. J Clin Endocrinol Metab 87: 1829–1833.
22. Lin L, Palibeni P, Ferraz-de-Souza B, Kelberman D, Homfray T, et al. (2007) Heterozygous missense mutations in steroidogenic factor 1 (SF1/AdhBp, NR3A1) are associated with 46,XY disorders of sex development with normal adrenal function. J Clin Endocrinol Metab 92: 991–999.
23. Kohen D, Lin L, Ferraz-de-Souza B, Wiecek P, Heidemann P, et al. (2008) Five novel mutations in steroidogenic factor 1 (SF1/NR3A1) in 46,XY patients with severe underandrogenization but without adrenal insufficiency. Hum Mutat 29: 59–64.
24. Whitting LS, Lovell-Badge R, Goodfellow PN (1993) Rapid sequence evolution of the mammalian sex-determining gene SRY. Nature 364: 713–715.
25. Lovell-Badge R, Canning G, Sekido R (2002) Sex-determining genes in mice: building pathways. Novartis Found Symp 244: 4–18; discussion 18–22, 35–42, 253–257.
26. Dubin RS, Ouster H (1994) Sry is a transcriptional activator. Mol Endocrinol 8: 1102–1119.
27. Pontiggia A, Rimini R, Harley VR, Goodfellow PN, Lovell-Badge R, et al. (1994) Sex-reversing mutations affect the architecture of SRY-DNA complexes. EMBO J 13: 6115–6124.
author/s:
Knower, KC; Kelly, S; Ludbrook, LM; Bagheri-Fam, S; Sim, H; Bernard, P; Sekido, R; Lovell-Badge, R; Harley, VR

title:
Failure of SOX9 Regulation in 46XY Disorders of Sex Development with SRY, SOX9 and SF1 Mutations

date:
2011-03-11

citation:
Knower, K. C., Kelly, S., Ludbrook, L. M., Bagheri-Fam, S., Sim, H., Bernard, P., Sekido, R., Lovell-Badge, R. & Harley, V. R. (2011). Failure of SOX9 Regulation in 46XY Disorders of Sex Development with SRY, SOX9 and SF1 Mutations. PLOS ONE, 6 (3), https://doi.org/10.1371/journal.pone.0017751.

persistent link:
http://hdl.handle.net/11343/257320

file description:
published version

license:
CC BY