Cytogenet Genome Res 101:185–198 (2003)
DOI: 10.1159/000074336

Cytogenetic and Genome Research

Turning on the male – SRY, SOX9 and sex determination in mammals

K.C. Knower,[a,b] S. Kelly[a,b] and V.R. Harley[a]

[a] Human Molecular Genetics Laboratory, Prince Henry's Institute of Medical Research, Clayton, Victoria
[b] Department of Biochemistry and Molecular Biology, Monash University, Clayton Campus, Victoria (Australia)

Abstract. The decision of the bi-potential gonad to develop into either a testis or ovary is determined by the presence or absence of the Sex-determining Region gene on the Y chromosome (SRY). Since its discovery, almost 13 years ago, the molecular role that SRY plays in initiating the male sexual development cascade has proven difficult to ascertain. While biochemical studies of clinical mutants and mouse genetic models have helped in our understanding of SRY function, no direct downstream targets of SRY have yet been identified. There are, however, a number of other genes of equal importance in determining sexual phenotype, expressed before and after expression of SRY. Of these, one has proven of central importance to mammals and vertebrates, SOX9. This review describes our current knowledge of SRY and SOX9 structure and function in the light of recent key developments.

The unraveling of the molecular genetic interactions during the initiation of mammalian sex determination has been slow, despite intense investigation following the identification of SRY as the Y-linked testis-determining gene (Sinclair et al., 1990). The study of sex-reversed patients (XX males and XY females) and the experimental induction of a variety of sex-reversing situations in the mouse have nonetheless identified a number of new genes involved in sex determination. The interactions of these genes have shed some light on possible mechanisms. However, complete characterization of what seems to be a large network of genes and intercalating pathways will not be fully resolved until the triggering role of SRY has been clearly established.

Our understanding of SRY is further hampered by its lack of protein sequence conservation across mammalian species. For example, most protein structural domains of SRY are poorly conserved, the only conserved domain between human and mouse being the high mobility group (HMG) domain (Whitfield et al., 1993). Furthermore, within domestic mice there are both elongated and truncated forms of SRY (Coward et al., 1994), while in some Old World rodent species multiple copies of SRY are present (Nagamine, 1994). In comparison, some species of vole do not have SRY sequences at all (Just et al., 1995). This review discusses current knowledge of SRY function in the light of recent developments such as the relationship between SRY and another key sex-determining gene, SOX9. The mechanism through which they act, the proteins with which they interact, the genes they may act upon, and the factors that regulate their expression will be discussed.

Expression and regulation of the SRY gene

In most mammals, the fate of the bi-potential gonad to develop into a testis or an ovary is based on the presence or absence of SRY. When present in the XY gonad, SRY induces a cascade of sexual development leading to testis formation. Consistent with this role, SRY is expressed in pre-Sertoli cells

Supported by NHMRC (Australia) grant no. 198713.

Received 16 June 2003; manuscript accepted 2 July 2003.

Request reprints from Vincent R. Harley
Prince Henry's Institute of Medical Research
Monash Medical Centre, PO Box 5152
Clayton 3168 Victoria (Australia)
telephone: +61 395943244; fax: +61 395946125
e-mail: Vincent.Harley@med.monash.edu.au

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(Albrecht and Eicher, 2001; Bullejos and Koopman, 2001) as the first evidence of sexually differentiated gene expression of the mammalian gonad. In the mouse urogenital ridge, the window of Sry expression is narrow, beginning at 10.5 days post coitum (dpc), reaching a peak at 11.5 dpc and ending abruptly at 12.5 dpc (Hacker et al., 1995; Albrecht and Eicher, 2001); Bullejos and Koopman, 2001. Expression is seen as a dynamic wave originating from the centre of the urogenital ridge within the somatic cell component (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001), although circular non-translatable transcripts, a result of a second transcriptional start site within the mouse Sry promoter, are also detected in germ cells of the adult testis (Koopman et al., 1990; Capel et al., 1993).

Urogenital ridge expression patterns for SRY have now been described in a number of mammalian species including humans, pig, sheep and rat. In each case the onset of SRY expression coincides with the first overt differentiation of the gonad, however SRY expression seems prolonged and without the abrupt termination observed in the mouse genital ridge. For example the sheep SRY transcript is first detected at 23 dpc and can still be detected at the time of birth, 21 weeks after conception (Payen et al., 1996). In the pig, SRY transcripts are first detected at 21 dpc and are still present at 51 dpc (Parma et al., 1999) and in the human SRY is first observed at embryonic day 41 and is still present after 18 weeks of gestation (Hanley et al., 2000). In addition to differences in temporal expression, analysis of different human and marsupial fetal and adult tissues has revealed that SRY expression is not as tightly regulated spatially as it is in the mouse, being expressed in tissues such as the brain, pancreas or heart (Clepet et al., 1993, Harry et al., 1995). The biological significance of these non-gonadal expression profiles is still undefined, and a role for brain SRY in sexual behavior is speculated (De Vries et al., 2002). Intriguingly, linear mouse Sry transcripts are also present in the brain (Mayer et al., 2000).

Regulation of the SRY gene and its promoter and regulatory elements are poorly understood. Variations in SRY expression patterns between species and the fact that SRY is poorly protected from mutational change during evolution (Graves, 1998) have made the task of identifying conserved regulatory elements difficult. Comparisons of 5′ SRY sequences of ten different species of mammals (including primates, bovine and rodents) reveal that homologies are maintained within the first 400–600 base pairs (bp) upstream from the translational start site within related mammalian groups but not between mammalian groups (Margarit et al., 1998). Promoter studies have been performed in vitro for human SRY sequences and have revealed that the minimal region required for SRY promoter activity spans from 310 bp upstream to the translational start site (Su and Lau, 1993). This region also contains two defined SP1 binding sites that are capable of acting as basal elements for the regulation of SRY expression (Desclozeaux et al., 1998a). However, SP1 is a ubiquitously expressed transcription factor that regulates tissue-specific genes by acting in a combinatorial way with other transcription factors (Perkins et al., 1993). Two such factors that may contribute to SP1 regulation of SRY are Wilms' tumor 1 (WT1) and NR5A1 (commonly referred to as steroidogenic factor-1; SF-1).

Transfected cell studies show that SF-1 and WT1 (when dephosphorylated) are capable of binding and activating the human SRY promoter (Shimamaru et al., 1997; De Santa Barbara et al., 2001; Hossain and Saunders, 2001). These factors may contribute to the gonad-specific expression of human SRY as they are both required early (10.5 dpc) for the formation of the mouse urogenital ridge (Kreidberg et al., 1993; Luo et al., 1994; Patek et al., 1999) and later (12.5 dpc) for regulation of the gene encoding the Anti-Müllerian hormone (AMH) (De Santa Barbara et al., 1998). Furthermore, pig SF-1 is also capable of activating the pig SRY gene (Pilon et al., 2003). However, SF-1 binding site sequences in the pig SRY promoter are not equivalent to those in the human SRY promoter suggesting a different conserved site (Pilon et al., 2003). Interestingly, in vitro studies on the NR0B1 gene (commonly referred to as DAX1: dosage sensitive sex-reversal-adrenal hypoplasia congenital-critical region of the X chromosome, gene 1), which when duplicated results in XY sex reversal (Bardoni et al., 1994), support a role for DAX1 protein in the inhibition of SF-1-mediated transcriptional activation (Ito et al., 1997, Zazopoulos et al., 1997, Crawford et al., 1998, Nachtigal et al., 1998). As a consequence, it is proposed that DAX1 is an antagonist of SRY regulation, as their temporal and spatial expression patterns also overlap within the gonad (Swain et al., 1998). Furthermore, male mice over expressing *Dax1/Nr0b1* can develop as females, but only within the mouse strain B6XY^{POS} which displays a late acting or "weak" *Sry* allele (Swain et al., 1998).

Also, GATA4 and Friend of GATA2 (FOG2/ZFPM2) genes are also involved in the regulation of SRY. When FOG2 is knocked out in mice, there is a 25% decrease in *Sry* transcripts (Tevosian et al., 2002). However, it was not established whether interactions were direct or indirect on the Sry promoter. These mice also show defects in Sertoli cell differentiation when either GATA4 or FOG2 genes are knocked out, a process governed by *Sry* (see later). The authors suggest that regulation of *Sry* may be in a separate pathway from either WT1 or SF-1 as the expression level of these genes is not affected in the *Gata4* or *Fog2* knockout mice. Presumably *Sry* is not the only target for *Gata4/Fog2*, as they are also required for normal ovarian development (Anttonen et al., 2003).

Structurally, the mouse *Sry* locus is flanked by a large inverted repeat region (approximately 15 kbp) not observed in other species, a genomic feature that has confounded promoter studies in this species. Reports into the regulation of the mouse *Sry* gene have defined how little 5′ or 3′ region of *Sry* is required to initiate testis formation in XX transgenic mice (Koopman et al., 1991, Bowles et al., 1999). Constructs with deletions of 5′ sequence to within 57 bp of the *Sry* transcriptional start site containing normal UTR 3′ region do not prevent the development of testis (Koopman et al., 2001). However, these constructs also gave rise to widespread expression in non-gonadal tissues, a phenomenon not observed in wild-type mice (Koopman, 1999). There is, however, a five-fold excess in exogenous *Sry* expression in the genital ridge compared to other tissues, indicating that these constructs must contain gonad-specific regulatory elements.

A recent study which aimed to identify gonad and stage-specific *cis*-acting elements responsible for *Sry* expression has described possible elements required for *Sry* regulation (Yokouchi et al., 2003). 2.5 kbp of the 5′ upstream regulatory region of *Sry* was separated into DNA fragments and allowed to form DNA-protein complexes with nuclear extracts from 11.5, 12.5 and 13.5 dpc gonads. DNAse 1 footprinting analysis identified a 52-bp stage-specific element bound to nuclear extracts from 11.5 dpc. This element, however, was positioned upstream of both the transcription start point of the circular transcript and the linear transcript of *Sry*. As a result, its role as a *cis*-acting element to these individual transcripts was not defined.

Fig. 1. Schematic comparison of the human and *Mus musculus* SRY proteins showing the conservation of the HMG domain and the presence of the glutamine-rich region of the mouse Sry protein. Relative amino acid numbers are indicated above regions.

SRY protein structure and function

The only structural motif of the SRY protein conserved across mammalian species is the HMG domain (Whitfield et al., 1993) (Fig. 1). Comprised of 80 amino acids, this motif is present in many DNA-binding proteins, including several DNA bending transcription factors (Grosschedl et al., 1994). Such, it is thought that SRY acts as an architectural transcription factor when instigating male development. In vitro studies have demonstrated that recombinant SRY has the ability to recognize the DNA binding motif A/TAACAAT/A with highest affinity, enhanced through phosphorylation (Harley et al., 1994, Desclozeaux et al., 1998b). This high affinity has also been demonstrated in vivo (Bergstrom et al., 2000), and highlights the variability in the sequence-specific binding properties of SOX (Sry-related HMG BOX) proteins. When the Sry HMG domain is substituted with the Sox9 or Sox3 HMG domains, these domains are capable of causing sex reversal in XX transgenic mice but only when over expressed (Bergstrom et al., 2000), presumably to compensate for the sub-optimal binding affinity. It is also possible to cause sex reversal in XX transgenic mice with the open reading frame (ORF) of human SRY (Lovell-Badge et al., 2002), demonstrating that perhaps the HMG domain is the only property of human SRY required for sex determination but the regions outside of the HMG domain somehow attenuate the HMG domain function.

The SRY HMG domain induces a bend in DNA (Giese et al., 1994). The role of this protein-directed DNA bending is possibly to rearrange chromatin to facilitate binding of transcriptional machinery, a process that has been demonstrated on nucleosomes at the fra-2 promoter (Ng KW et al., 1997). The degree to which this bend occurs depends on flanking sequences and also differs between species (Giese et al., 1994). Positioned at the N- and C-termini of the HMG domain are two nuclear localization signals (NLSs) (Poulat et al., 1995, Sudbeck and Scherer, 1997). The N-terminal NLS forms part of a calmodulin binding domain (Harley et al., 1996) and suggests that nuclear import is mediated by calmodulin, as seen in other calmodulin binding nuclear proteins (Sweitzer and Hanover, 1996). The C-terminal NLS, on the other hand, interacts with the nuclear import factor importin-β (Forwood et al., 2001). The presence of these NLSs is consistent with the observed localization of the SRY protein in the nucleus of pre-Sertoli cells (Hanley et al., 2000) and a transcriptional function. Furthermore, SRY mutations in either of the NLSs occur in XY male-to-female sex reversal (see later).

Outside of the HMG domain, there is very little sequence conservation between mammals. The last seven amino acids of the human SRY protein contains a domain shown to interact with SRY interacting protein 1 (SIP-1) (Poulat et al., 1997), a PDZ domain protein, that may provide an interface for interactions with other proteins. This C-terminal is however not present in mouse Sry. Instead, the C-terminal end of the mouse Sry protein harbors a large glutamine-rich repeat region that occupies over half of the protein (Fig. 1). The functional significance of this domain for the regulation of male sex-determining genes can be demonstrated when looking at XX transgenic mice lacking this domain. Truncated forms of the Sry protein missing the C-terminal domain are incapable of causing sex reversal in XX transgenic mice suggesting some biological function for this domain (Bowles et al., 1999). Furthermore, it has also been demonstrated in reporter gene assays that this repeat-region can act as a transcriptional activation domain in transfected cells (Dubin and Ostrer, 1994) and has also been shown to interact with another Sry interacting protein (also termed Sip1, not to be confused with the SIP-1 PDZ protein) (Zhang et al., 1999). Therefore, it seems that the functional differences observed between mouse and other mammalian SRY proteins have most likely arisen due to additional biochemical roles required in the mouse which are not required in other mammals.

Clinical mutations of SRY

46,XY gonadal dysgenesis can be subdivided into complete gonadal dysgenesis (Swyer Syndrome) and partial gonadal dysgenesis (see Ostrer, 2001 for review). Swyer syndrome is characterized by completely female external genitalia, as well as developed Müllerian structures, and a gonad composed of a streak fibrous tissue. The phenotype of partial gonadal dysgenesis is characterized by partial testis formation, usually a mixture of Wolffian and Müllerian ducts, and varying degrees of masculinization of external genitalia. Mutations in SRY result in male-to-female sex reversal in XY individuals. The incidence of SRY mutations in XY females (15%) is quite low and supports the idea that genes other than SRY are also required for the development of the male phenotype. Significantly, almost all XY female patients with SRY mutations show com-

Table 1. Clinical mutations of the SRY ORF

Codon	Nucleotide	Base change	Amino acid change	Phenotype[a]	Reference
2[b]	4	C→T	Q2X	POF	Brown et al., 1998
4[b]	12	T→A	Y4X	CGD	Veitia et al., 1997
4[b]	12	T deletion	Y4X	UTS	Takagi et al., 1999
18[b]	53	G→A	S18N	PGD/UTS[c]	Domenice et al., 1998 Canto et al., 2000
30[b]	89	G→T	R301	CGD/PGD[c]	Assumpção et al., 2002
43[b]	127	T insertion	K43X	CGD	Scherer et al., 1998
59	175	A→G	R59G	CGD	Fernandez et al., 2002
60	178	G→C	V60L	CGD[c]	Berta et al., 1990
60	179	T→C	V60A	CGD	Hiort and Klauber, 1995
62	184	C→G	R62G	CGD	Affara et al., 1993
64	191	T→G	M64R	CGD	Scherer et al., 1998
64	192	G→A	M64I	CGD	Berta et al., 1990
65	193	A→C	N65H	CGD	Assumpção et al., 2002
67	199	T→G	F67V	CGD[c]	Hines et al., 1997
68	203	T→C	168T	CGD	McElreavey et al., 1992a
70	209	G→A	W70X	CGD	Hawkins et al., 1992 Graves et al., 1999 Assumpção et al., 1999
74	220	C→T	E74X	CGD[c]	Affara et al., 1993
75	224–225	GG→AT	R75N	CGD	Battiloro et al., 1997
76	226	C→A	R76S	CGD[c]	Imai et al., 1999
78	233	T→C	M78T	CGD	Affara et al., 1993
79	237	T→C	A79A	TH	Braun et al., 1993
86	256	C→T	R86X	CGD	Cameron et al., 1998
87	259	A→T	N87T	CGD	Okuhara et al., 2000
90	270	C→G	190M	CGD[c]	Hawkins et al., 1992 Dörk et al., 1998
91	271	A→G	S91G	CGD[c]	Schmitt-Ney et al., 1995
92	274	A→T	L92X	CGD	Muller et al., 1992
93	277	C→T	Q93X	CGD	McElreavey et al., 1992c
94	281	T→C	L94P	N/A	Zhi et al., 1996
95	283	G→C	G95R	CGD	Hawkins et al., 1992
95	284	G→A	G95E	CGD	Schäffler et al., 2000
97	289	C→T	G97X	CGD[c]	Bilbao et al., 1996
101	302	T→A	L101H	TH	Imai et al., 1999
106	317	A→T	K1061	CGD	Hawkins et al., 1992
107	320	G→A	W107X	CGD	Iida et al., 1994
108	323	C→G	P108R	CGD	Jakubiczka et al., 1998
108	324	A deletion	Frameshift	CGD	Hawkins et al., 1992
109	326	T→C	F109S	CGD[c]	Jäger et al., 1992
113	337	G→A	A113T	CGD	Zeng et al., 1993
121–22	363–66	AGAG del	Frameshift	CGD	Jäger et al., 1990
125	374	C→T	P125L	CGD[c]	Schmitt-Ney et al., 1995
127	380	A→T	Y127F	CGD[c]	Jordan et al., 2002
127	380	A→G	Y127C	CGD	Poulat et al., 1994
127	381	T→A	Y127X	CGD	McElreavey et al., 1992a
129	385	T→A	T129N	PGD	Baud et al., 2002
131	392	C→G	P131R	CGD	Lundberg et al., 1998
133	397	C→T	R133W	CGD	Veitia et al., 1997 Affara et al., 1992
136[b]	407	A→G	L136S	CGD	Uehara et al., 1999
163[b]	488	T→A	L163X	CGD[c]	Tajima et al., 1994

[a] POF – Premature Ovarian Failure; CGD – Complete Gonadal Dysgenesis; PGD – Partial Gonadal Dysgenesis; UTS – Ullrich–Turner syndrome; TH – True Hermaphroditism.
[b] Mutations positioned outside of the HMG domain.
[c] Familial mutations.

plete gonadal dysgenesis (Table 1) (Cameron and Sinclair, 1997; McElreavey and Fellous, 1999), consistent with the onset of SRY early in the development of the embryonic testis.

Most of the sex-reversing mutations described in the SRY ORF cluster within the region encoding the HMG domain (Table 1), highlighting the importance of this domain in the function of the protein. In vitro studies have shown that variations in both DNA binding and/or bending properties of SRY occur in SRY from XY females with mutations residing within the HMG domain (Harley et al., 1992; Jäger et al., 1992, Giese et al., 1994, Pontiggia et al., 1994, Poulat et al., 1994, Schmitt-Ney et al., 1995, Mitchell et al., 2002). SRY protein missense mutants display an assortment of biochemical properties, ranging from complete absence of DNA binding or bending activity (e.g. R62G and M78T); reduced DNA binding but relatively normal bending (e.g. R75N and L94P); abnormal bending (e.g.

M64I and M64R); and near wild-type affinity (e.g. F67V and R133W). Also, the nuclear import properties of some SRY mutants are also affected (Li et al, 2001, Harley et al., 2003) arising in some cases (R62G and R133W) by defective importin-β recognition (Harley et al., 2003). Together, the variations in biochemical properties observed between different mutants have made the drawing of correlations with severity of phenotype or mode of inheritance difficult.

As some of these complete XY gonadal dysgenesis patients show residual activity of SRY, it has been speculated that SRY acts at a threshold activity level in the XY gonad (Harley et al., 1992). Although about 85% of these mutations observed are de novo, some are inherited from a normal fertile father (Table 1). The incomplete penetrance of these mutations may also be caused by a decrease in activity to a certain threshold level (Harley et al., 1992). This theory seems plausible as it is becoming increasingly evident that gene dosage plays a large part in deciding the sexual fate of the gonad at the time of sex determination. Furthermore, in some families, the fertile fathers were shown to be gonadal mosaic for both wild-type and mutant SRY alleles which would explain the variability in dosage levels (Barbosa et al., 1995; Bilbao et al., 1996; Hines et al., 1997). It has also been suggested that these SRY variants may be interacting with other sex-determining genes that are segregating within the family (Vilain and McCabe, 1998; McElreavey and Fellous, 1999). This could be somewhat similar to what is observed in the mouse, where Sry alleles from some strains will cause sex reversal when placed in certain genetic backgrounds (Eicher et al., 1996; Lee and Taketo, 2001; Albrecht et al., 2003).

Outside of the HMG domain, there are a handful of SRY mutations which produce a sex-reversed phenotype due to truncated non-functional forms of the protein (Table 1). There are mutations which lie outside of the SRY ORF, both 5′ (McElreavey et al., 1992a; Kwok et al., 1996a) and 3′ (McElreavey et al., 1996), which also show variable degrees of XY gonadal dysgenesis presumably through reduced expression of SRY. Also, the rare case of XY individuals with partial gonadal dysgenesis arising from mutations to SRY (with the exception of one, Y129N) show missense mutations lying outside of the HMG domain, e.g. S18N (Domenice et al., 1998) and R30I (Assumpção et al., 2002). Interestingly, both of these mutations are familial, the latter presenting as an unusual case. The family pedigree shows that the mutation was present in seven XY siblings, one of these siblings displayed XY complete gonadal dysgenesis and two displayed partial gonadal dysgenesis. This mutation lies within serine residues that when phosphorylated show an increase in wild-type SRY DNA binding (Desclozeaux et al., 1998b). Therefore, it is speculated that the mutation to these residues could affect the ability of SRY to function in vivo (Assumpção et al., 2002). Furthermore, it seems that in contrast to the effects of de novo mutations, inherited variants produce proteins with near wild-type activities. The variability in function and penetrance, together with a possible decrease in DNA binding affinity due to altered phosphorylation, may affect SRY interactions with downstream targets and can somewhat explain the three different phenotypes observed in this family.

True hermaphroditism (TH) is also a rare form of sex reversal characterized by the presence of both testicular and ovarian tissue in the same individual, either in a single gonad (ovotestis), or in opposite gonads with one testis and one ovary on each side (Van Niekerk and Retief, 1981). These patients have ambiguous external genitalia and variable development of Wolffian and Müllerian ducts (Torres et al., 1996). Molecular analysis has demonstrated that SRY is present in only 10% of TH cases with a 46,XX karyotype (Berkovitz et al., 1992; McElreavey et al., 1992b; Boucekkine et al., 1994; Damiani et al., 1997) therefore, leaving 90% of cases having mutations in X-linked or autosomal genes required for testis development. Two point mutations in the SRY ORF have resulted in individuals with TH (Table 1) (Braun et al., 1993; Hiort et al., 1995), however, gonadal SRY mosacism in 46,XX patients is more frequent within TH patients (Hadjiathanasiou et al., 1994; Inoue et al., 1998; Ortenberg et al., 2002; Queipo et al., 2002; Modan-Moses et al., 2003). In these TH individuals, SRY is most often negative in peripheral blood leukocytes, but positive in gonadal tissues, as well as in DNA obtained from the ovotestis.

Cellular pathways initiated by SRY

The discovery of a number of new genes involved in testis formation has shed some light on possible genetic pathways activated in the gonad, however, direct targets of SRY in these pathways have yet to be identified. The cellular changes observed in the mouse XY gonad between the time of 11.5 and 12.5 dpc include rapid patterning of gonadal cells into testis cords and a male-specific pattern of vasculature. Sertoli cells play a vital role in the development of the XY gonad and are capable of directing other cell types within the gonad into their respective cell lineages (Capel et al., 1999). The origin of these cells is thought to arise from the coelomic epithelium as well as the mesonephros. Experiments have demonstrated that before 11.5 dpc, the cells of the coelomic epithelium contribute to the Sertoli lineage (Karl and Capel, 1998). These cells are in a high proliferative state by 12 dpc and express high levels of *Sf-1* (Schmahl et al., 2000). XX mice transgenic for *Sry* show a proliferation pattern within the coelomic epithelium in their gonads identical to that of wild-type XY gonads (Schmahl et al., 2000). Consistent with this result, the B6XY^{POS} mouse strain, which shows weak expression of *Sry*, also displays levels of proliferation in the coelomic epithelium similar to those observed in wild-type XX gonads (Schmahl et al., 2000). These data show that *Sry* is capable of inducing the proliferative state of pre-Sertoli cells within the coelomic epithelium, and as *Sf-1* is expressed at high levels in these cells, it is possible that *Sf-1* may be regulating *Sry*. In contrast, data from other groups suggest that *Sry* is not being expressed in the coelomic epithelium (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). Migrating cells from the coelomic epithelium do not express *Sry* until localized within the genital ridge. This could give rise to an accumulation of Sertoli cells through recruitment of non-*Sry*-expressing cells in a non cell-autonomous manner (Tilmann and Capel, 2002).

The observed expression of *Sry* within pre-Sertoli cells in the genital ridge (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001) also supports previous evidence suggesting that *Sry* induces the differentiation of Sertoli cells (Burgoyne et al., 1988; Rossi et al., 1993; Jamieson et al., 1998). It was noted however that expression of *Sry* was not present in all pre-Sertoli cells (Albrecht and Eicher, 2001). The significance of this is unclear. The authors suggest that perhaps the continual expression of *Sry* is not required once differentiation into a Sertoli cell has taken place. This expression may also be for a very short time and the expression of *Sry* may not be synchronized between pre-Sertoli cells. There may also be an *Sry*-independent period in which *Sry* is not required for the differentiation of Sertoli cells. Mouse chimeras display a majority of XY Sertoli cells, however, they also contain XX Sertoli cells (Palmer and Burgoyne, 1991, Patek et al., 1991) suggesting that a paracrine factor can induce testis differentiation in the developing testis. Recent studies have described such a possible factor responsible for the differentiation of pre-Sertoli cells. The addition of exogenous prostaglandin D2 to cultures of 11.5 dpc female urogenital ridges results in varying levels of masculinization (Adams and McLaren, 2002). One of the evident features of these genital ridges is the presence of prospermatogonia which is indicative of functional Sertoli cells. Therefore, the induction of pre-Sertoli cell differentiation is a direct effect of prostaglandin D2 and may be a direct consequence of Sry regulation of prostaglandin D2 synthase (Ptgds), an enzyme required for the final step of prostaglandin D2 synthesis. Further investigation into this relationship is required.

Along with the coelomic epithelium, the mesonephros also contributes cells to the XY gonad, and when cultured without their adjoining mesonephroi, results in the failure of testis cord formation (Buehr et al., 1993, Merchant-Larios et al., 1993). The migration of at least three cell populations (peritubular myoid cells, endothelial cells and cells associated with the endothelium) occurs between 11.5 and 16 dpc (Martineu et al., 1997). This migration does not however take place in XY mice lacking *Sry* nor in XY gonads with weak alleles of *Sry* (Albrecht et al., 2000). In contrast, XX mice containing an *Sry* transgene known to cause sex reversal display a recruitment of cells from within the mesonephros into the gonad (Capel et al., 1999). Therefore, along with the expression in and differentiation of Sertoli cells, one of the earliest events initiated by Sry is the migration of cells from the mesonephros required for the formation of testicular cords. Recent experiments have demonstrated that the expression of neurotrophin-3 *(Ntf3)* from within Sertoli cells of the gonad can act on high affinity trkC receptors expressed on migrating mesonephros cells (Cupp et al., 2003). Mesonephric cell migration in response to *Ntf3* expression from Sertoli cells may be a direct consequence of an up-regulation of *Ntf3* by *Sry*. When *Ntf3* is inhibited, there is a subsequent decrease in expression of *Sox9*, a gene downstream of *Sry* (see later). The relationship between *Ntf3* and *Sry* warrants further investigation.

A noticeable feature that arises in the XY gonad by 12.5 dpc is a large blood vessel just under the coelomic epithelium, termed the coelomic vessel. This primitive vasculature is identical between XX and XY genital ridges until 11.5 dpc when it shows XY genital ridge specificity which correlates with the onset of *Sry* expression. The early specification of XY vascularisation is important for both developmental patterning and for the formation of testis cords. A recent study has demonstrated that as well as inducing migration of cells from the mesonephros into the genital ridge, Sry is also responsible for the migration of endothelial cells contributing to the development of the arterial system of the XY gonad (Brennan et al., 2002). This newly-formed arterial system establishes a new pattern of blood flow in the XY gonad, which is speculated to have an important role in export of testosterone to masculinize the XY embryo.

The characterization of possible signal(s) downstream of *Sry* responsible for the cell migration observed in the genital ridge has been aided by the development of in vitro gonad culturing systems. Culturing of an XX gonad with an XY gonad at its coelomic surface can induce the migration of cells from the mesonephros into the XX tissue (Martineu et al., 1997). Furthermore, culture of XX gonads with beads coated with proteins from XY gonads leads to cell migration into the XX gonad. In these experiments, cells from the XX gonad organized into structures similar to testis cords and demonstrated that mesonephric cell migration is capable of inducing Sertoli cell differentiation in the absence of *Sry* (Tilmann and Capel, 1999). Recent experimental data displays how the gonad culture approach is revealing possible genes downstream of *Sry*. The role of platelet-derived growth factor (PDGF) family of ligand and receptors has been established as having a role in migration, proliferation and differentiation of cells in various organ systems (Betsholtz et al., 2001). Mouse knockouts of Pdgf-alpha receptor *(Pdgfra)* display disruption in the organization of the vasculature and partitioning of interstitial and testis cord compartments (Brennan et al., 2003). Gonads from these mice also show severe reductions in characteristic XY cell proliferation, mesonephric cell migration, and fetal Leydig cell differentiation. When XX gonads are incubated in culture with exogenous purified PDGF, cells are able to migrate into the XX gonad from the mesonephros. This response is generated by *Pdgfra* signaling acting indirectly in the cells of the gonad. The in vivo requirement of this gene therefore seems extremely important for testis formation and shows similar aberrations to those of fibroblast growth factor 9 *(Fgf9)* knockout mice where migration, differentiation and proliferation of cells are also affected (Colvin et al., 2001). As a consequence, *Pdgfra* and *Fgf9* genes have been placed downstream of *Sry* in testis organogenesis and it would be of great interest to see if any in vivo relationship takes place between *Sry* and *Pdgfra* and if there is an overlapping pathway with *Fgf9*.

It seems, therefore, that within the XY gonad, SRY acts on numerous cellular pathways leading to eventual testis formation. In light of recent experimental evidence, it may also be possible that SRY regulates a number of downstream genes responsible for these cellular changes. One gene that is up-regulated shortly after the onset of SRY in the XY gonad is SOX9. As a consequence, SOX9 has long been referred to as the gene most likely regulated by SRY, and in fact, may be the only gene required for the initiation of male sex determination.

The sex-determining gene, SOX9

Since the discovery of SOX9 (SRY related HMG box) as the cause of CD/SRA1 (Campomelic Dysplasia/Autosomal Sex Reversal) when mutated, SOX9 has become known as a pivotal sex-determining gene due to its high conservation among vertebrates and ability to cause XY male-to-female sex reversal when mutated. Skeletal malformations associated with XY gonadal dysgenesis in 75% of patients are the major phenotypic features of CD/SRA1 (Houston et al., 1983; Mansour et al., 1995). Together with the expression pattern of SOX9 in developing bone and testis, SOX9 is proven to be essential for normal development of the testis in males, and of bone in both males and females.

The SOX9 gene and protein structure

SOX9 belongs to the SOX family of proteins which all share homology with the HMG box of SRY. In humans, the SOX9 gene located on chromosome 17q24.3→q25.1, encodes a 509 amino acid polypeptide which contains several domains that include an SRY-like HMG domain, a proline, glutamine and alanine-rich domain (PQA), a proline, glutamine and serine-rich domain (PQS) and a C-terminal transactivation domain. The presence of an HMG-domain enables SOX9 to act as an architectural transcription factor, making it capable of binding and bending DNA with a specific sequence (Mertin et al., 1999). The transactivation domain of SOX9 is necessary for transactivation of target sequences (Sudbeck et al., 1996; Ng LJ et al., 1997; McDowall et al., 1999). Another feature of SOX9 is its nuclear/cytoplasm shuttling capability (Gasca et al., 2002). SOX9 also contains a number of putative phosphorylation sites, which like in SRY, may be involved in regulating SOX9 DNA binding, nuclear import and transcriptional activity (Lee and Chuong, 1997; Zehentner et al., 1999; Huang et al., 2000; Murakami et al., 2000). Despite minor variations in protein length, SOX9 in alligator, mouse, frog and fish also contain these domains, indicating that SOX9 is highly conserved throughout evolution.

Mutations in SOX9

Patients with CD/SRA1 associated with gonadal dysgenesis are important in the characterisation of the function of SOX9 in sex determination. Mutations in SOX9 include splice acceptor/donor changes, missense, nonsense, translocation and frameshift mutations (Foster et al., 1994; Wagner et al., 1994; Kwok et al., 1995; Meyer et al., 1997; Hageman et al., 1998; McDowall et al., 1999). In comparison to mutations in SRY which generally cluster within the HMG box, SOX9 mutations occur throughout the ORF. Mutations in the SOX9 ORF detected outside the HMG box are nonsense and frameshift mutations that disrupt the C-terminal domain of the protein affecting the ability of SOX9 to efficiently activate transcription of target genes (Sudbeck et al., 1996; Ng LJ et al., 1997; McDowall et al., 1999). Mutations flanking an intact SOX9 gene have also been important in the analysis of SOX9, particularly in the study of translocation breakpoints in CD/SRA1 patients and transgenic mice. Analysis of these mutations and breakpoints have revealed regions possibly containing *cis*-regulatory elements for transcription or silencing of SOX9 (Wirth et al., 1996; Wunderle et al., 1998; Pfeifer et al., 1999). CD/SRA1 is an autosomal dominant disorder where most mutations occur in one allele of SOX9, haploinsufficiency for SOX9 results in insufficient normal SOX9 protein production. Interestingly, a compound heterozygosity mutation of SOX9 has been detected in a CD patient who has a different mutation in each allele of SOX9 (Wagner et al., 1994). Also, an SRY-negative female-to-male sex reversal patient with a duplication of chromosome band 17q23→q24 including the SOX9 gene has also been characterized (Huang et al., 1999), indicating that an extra dose of SOX9 may be sufficient to initiate testis differentiation in the absence of SRY. No cases of sex reversal without CD, due to mutations in SOX9, have been detected (Kwok et al., 1996b).

With such a large number of mutations characterized, no correlation between mutation type or position and disease severity or associated sex reversal has been established. However, a CD patient without sex reversal has recently been characterized and found to have a mutation which affects the ability of SOX9 to activate gene targets in bone, but not testis-specific target genes. The SOX9 mutation was found to affect the ability of SOX9 to dimerise, implying that SOX9 dimerisation is necessary for normal bone formation but not for testis formation (Bernard et al., 2003). The task of correlating mutation with disease phenotype has been made even more difficult by the existence of patients who have the same mutation but have varying phenotypes and degrees of gonadal dysgenesis (Cameron and Sinclair 1997; Meyer et al., 1997; Hageman et al., 1998). One explanation for this observation is that the differences in phenotype may be due to the degree of expressivity of the SOX9 mutation as well as differences in genetic background rather than the specific type of mutation.

Where does SOX9 fit into the sex-determining pathway?

In the undifferentiated gonad of XX and XY embryos, SOX9 protein is located in the cytoplasm (Morais da Silva et al., 1996). Soon after the expression of SRY, SOX9 expression is up-regulated in the male XY embryonic urogenital ridge, while expression is down regulated in the female urogenital ridge. Up-regulation of *Sox9* in the male gonad is associated with the movement of Sox9 from the cytoplasm to the nucleus of Sertoli cells (Morais da Silva et al., 1996). Movement to the nucleus is possibly via either of two NLSs within the HMG box of SOX9 (Sudbeck and Scherer 1997; Preiss et al., 2001). Once in the nucleus, SOX9 is capable of activating other genes and shuttling back to the cytoplasm via a conserved nuclear export signal (NES) within the HMG box of SOX9 (Gasca et al., 2002). It is likely that this highly specific mechanism of nuclear import/export mechanism acts to regulate SOX9 activity. An accumulation of SOX9 in the nucleus may be all that is required to trigger differentiation of the gonad into a testis (Mo-

rais da Silva et al., 1996) since XX female gonad cultured in leptomycin B, an inhibitor of nuclear export, developed into male gonads (Gasca et al., 2002). This was characterised by the differentiation of female somatic cells into Sertoli-like cells which became organised within cord-like structures and expressed both SOX9 and the male specific protein, AMH. Male gonads cultured in the inhibitor were not affected (Gasca et al., 2002). In the male, SOX9 continues to be expressed by Sertoli cells into adulthood (Morais da Silva et al., 1996; De Santa Barbara et al., 2000).

To address the precise function of SOX9 in the gonad, transgenic mice ectopically expressing *Sox9* driven by the *Wt1* promoter have been produced (Vidal et al., 2001). XX transgenic mice developed testes with apparently normal Sertoli cells and Leydig cells. This study supports the human XX male with SOX9 duplication (Huang et al., 1999) and suggests that SOX9 can replace SRY in triggering Sertoli cell differentiation and implies that SRY's only function is to up-regulate SOX9. Confirmation that SOX9 is sufficient to trigger Sertoli cell differentiation has been shown in XX mice which lack estrogen receptors ERα and ERβ (Dupont et al., 2003). In the ovaries of these mice, *Sox9* expression was detected in granulosa cells just prior to their trans-differentiation into Sertoli cells, indicating that Sox9 can substitute for Sry in Sertoli cell differentiation.

The up-regulation, sexual dimorphic expression and conserved protein structure of SOX9 are three features shared among all vertebrates, regardless of the switch mechanism controlling sex determination, being SRY in mammals (except for the mole vole; Just et al., 1995), ZW chromosome gene/s in birds (Oreal et al., 1998) and temperature sensitivity of egg incubation in turtles and crocodiles (Moreno-Mendoza et al., 1999; Western et al., 1999). With the SOX9 protein highly conserved through vertebrate evolution, unlike SRY, these observations support the idea that SOX9 is a pivotal sex-determining gene which is possibly the next gene (after SRY) in the sex determination pathway.

Regulation of the SOX9 gene

At present, it is not known what factor/s activate the *Sox9* promoter in Sertoli cells. The up-regulation of *Sox9* in Sertoli cells upon expression of *Sry* is consistent however, with a role for Sry in the activation of *Sox9* expression (Morais da Silva et al., 1996; De Santa Barbara et al., 2000). Identification of the region/s in the SOX9 gene that are essential for SOX9 transcriptional activation is of marked interest within the sex determination field, due to the pivotal role of SOX9 in sex determination. A conserved minimal interval located between –193 and –73 bp from the transcription start site of *Sox9* was discovered a number of years ago, which contributed to (but was not sufficient for) testis-specific *Sox9* expression (Kanai and Koopman, 1999). Since then some progress has been made in identifying the gonad specific elements of the SOX9 promoter by the use of comparative genomics. By comparing the SOX9 promoters in mouse, human and the evolutionarily distanced Japanese puffer fish *(Fugu rubripes)*, eight conserved elements (E1–E8) have been identified (Bagheri-Fam et al., 2001). Five of the elements were scattered through 290 kbp, 5′ to SOX9 in the region where most of the translocation breakpoints responsible for CD are found. Three elements were found 3′ to SOX9, with the most distal being 452 kbp downstream. In addition, two conserved elements within the SOX9 3′ UTR were identified.

Analysis of the conserved 5′ elements revealed several consensus binding sites, mostly in E3 – a SOX consensus binding site (E3 and E5), a WT1 consensus binding site (E3), a Paired related homeobox protein 2 (PRRX2) consensus binding site (E3) and a Hepatocyte Nuclear Factor 3β (HNF3β) consensus binding site (E1) (Bagheri-Fam et al., 2001). The presence of SOX sites in these elements suggest that SRY and/or other SOX proteins may regulate SOX9. It is also possible that SOX9 may regulate its own expression. WT1 may be involved in SOX9 regulation since WT1 is essential for gonad and kidney development and is expressed before *Sox9* in the undifferentiated gonad (Parker, 1998). PRRX2 and HNF3β may be involved in SOX9 regulation, particularly in the non-gonadal expression of SOX9. This is because PRRX2 and HNF3β are expressed in skeletal precursors and the notochord, respectively, and are essential for the development of these tissues (Ang and Rossant, 1994; Martin et al., 1995; Lu et al., 1999). These are also sites at which SOX9 is expressed (Ng LJ et al., 1997).

In vivo studies of the conserved 5′ elements using a mouse line carrying a transgene reporter construct with the E3, E4 and E5 elements in front of a 200-bp proximal promoter driving *lacZ* revealed these three elements to be active in the cords of testis at 13.5d.p.c. (Scherer, 2002). In comparison, a reporter that included E1 and E2 was not expressed in testis (Scherer, 2002). In other studies, 70 kbp of 5′ and 30 kbp of 3′ sequence flanking SOX9 fused to a β-galactosidase reporter gene was found to give Sertoli-cell specific expression in the testes of transgenic mice (Lovell-Badge et al., 2002). However, no expression was noted in the other sites of SOX9 expression, namely developing bone. In comparison to the earlier findings of Bagheri-Fam and colleagues, this active 70 kbp 5′ region corresponds to and contains the mouse, human and Fugu conserved E1 element which was found to not direct gonad expression when in association with E2 (Scherer, 2002). These different observations suggest that activator sequences for gonad specific SOX9 expression in mammals must be located within the 100 kbp of sequence flanking SOX9 but not in the conserved E1 element. Thus elements required for mammalian sex determination are not conserved and/or are necessary for gonad specific expression of SOX9 in Fugu. This is likely as Fugu lack SRY but express SOX9 in Sertoli cells (Takamatsu et al., 1997; Chiang et al., 2001) suggesting that the sex determination pathway in Fugu is not the same as in mammals. Further characterisation of the SOX9 promoter needs to be carried out and is likely to be quite complex, considering the different sites of SOX9 expression.

Another way in which SRY may function in mammalian sex determination is by acting as a repressor. In accordance with the repressor model (McElreavey et al., 1993), SRY may up-regulate SOX9 by acting as a repressor of a protein which has the function of inhibiting SOX9 transcription. Experimental evidence for this comes from the creation of the "*Odsex*" (ocular degeneration with sex reversal) mouse where XX mice

develop as sterile males with normal *Sox9* expression in cartilage and bone, due to the insertion site of a transgene upstream of *Sox9* which causes a 150 kbp deletion (Bishop et al., 2000).

The regulation of SOX9 expression in the testis is likely to be complex and involve not only SRY but also other sex-determining proteins and possibly a number of other factors, which bind at distant regulatory elements in the SOX9 promoter and/or enhancer. This is likely as testis development in sex reversal can occur in XX transgenic mice when Sry is absent (Vidal et al., 2001). Other factors which have been reported to influence SOX9 expression, particularly in bone include dexamethasone (Sekiya et al., 2001), bone morphogenetic proteins (BMPs) (Uusitalo et al., 2001), growth factors and cytokines (Schaefer et al., 2003). Although involved in influencing SOX9 expression during bone development, some of these factors may influence SOX9 expression during gonadal differentiation.

SOX9 targets in the sex determination pathway

Several in vitro and in vivo studies have shown that the SOX9 protein acts as a potent transcriptional activator during sex determination. To date, SOX9 protein is known to contribute to the activation of transcription of two genes expressed in the gonad: SF-1/NR5A1 and AMH.

SF-1/NR5A1 is a member of the orphan nuclear receptor family, a family of proteins that mediate transcription by regulating the transcription of genes which encode steroid hormones involved in steroidogenesis (Parker, 1998). Heterozygote mutation in the SF-1 gene have been associated with complete XY sex reversal and adrenal failure in humans suggesting that SF-1 regulates the regression of the Müllerian ducts (Achermann et al., 1999, 2002). Like *Sox9*, *Sf-1/Nr5a1* transcripts are initially present in the undifferentiated urogenital ridge of both male and female mouse gonads. After *Sox9* up-regulation, *Sf-1* expression is up-regulated in the male gonad and down regulated in the female ovaries. *Sox9* has been shown to bind with high affinity to a SOX-like sequence in the *Sf-1* promoter and to transactivate *Sf-1* expression from this element (Shen and Ingraham, 2002). This Sox9 binding site, located –110 to 104 bp upstream of the translation start site of *Sf-1* could be bound but not activated by other Sox proteins which are also expressed in the developing gonad and adult testis (Shen and Ingraham, 2002).

SOX9 directly regulates the expression of AMH which is also expressed in the Sertoli cell lineage. AMH has a major role in the male sexual differentiation pathway by causing the regression of the female (Müllerian) reproductive tract (Shen et al., 1994; De Santa Barbara et al., 1998). DNAse 1 footprint analysis shows that Sox9 binds to a conserved Sox-like site in the *Amh* proximal promoter, adjacent to the Sf-1 binding site, MISRE1 (De Santa Barbara et al., 1998; Arango et al., 1999). When this Sox-like site is mutated, transgenic mice develop with complete retention of Müllerian duct derived organs and an absence of *Amh* transcript (Arango et al., 1999).

The activation of AMH by SOX9 also involves a number of co-factors that are also expressed in Sertoli cells during testis formation. These are SF-1 which SOX9 up-regulates earlier, WT1 and GATA-4 (De Santa Barbara et al., 1998; Nachtigal et al., 1998; Viger et al., 1998; Watanabe et al., 2000; Tremblay and Viger, 2001). Together with SOX9, these proteins form a multi-protein complex on the AMH promoter.

Several key experiments have shown that regulation of AMH requires co-operative interaction between SOX9 and SF-1 and that SOX9 is essential as reporter gene assay experiments have shown a co-operative increase in transcriptional activity with the co-transfection of SF-1 and SOX9 compared to SF-1 alone. Also, in comparison to the effect of SOX9 binding site being mutated, mutations in the Sf-1 binding site (MISRE1) cause only a partial decrease in Amh levels and partial regression of the Müllerian ducts (Arango et al., 1999). The other co-factors involved in the activation of AMH are thought to be involved via protein-protein interactions, such as GATA-4 which interacts through its zinc finger domain with SF-1 to activate AMH transcription (Tremblay and Viger, 2001). WT1, also required for male gonad development, interacts weakly with SF-1 (Nachtigal et al., 1998) which is possibly, further stabilised through HSP70-WT1 (Maheswaran et al., 1998) and HSP70-SOX9 interactions (Marshall and Harley, 2001). Clearly numerous protein-protein interactions exist at the AMH promoter and with the ability of the SOX9 HMG box to bend DNA, this may bring SF-1 and GATA-4 into close proximity. Along with WT1 and HSP70, a tightly associated protein complex forms which activates transcription of the AMH gene. Each of these co-factor proteins has important roles in the sex determination pathway. Their expression is vital for normal testis formation, however AMH is not the only target of SOX9 in testis formation as null AMH mice are not sex-reversed (Behringer et al., 1994).

Recent studies of the pig SRY gene have shown that SOX9 may be involved in the initiation of SRY transcription and possible feedback amplification of the SRY gene (Daneau et al., 2002). This interaction was found to occur via a motif conserved in humans and bovine, which is present 205 bp upstream of the translational start site of SRY in the pig (Daneau et al., 2002). In these studies, *Odsex* XX embryos containing a transgene with GFP under the control of pig SRY 5′ flanking sequence were generated. It was found that in the XX transgenic male mice, GFP was detectable indicating that SRY promoter activity does not require factors on the Y chromosome or SRY protein itself. Rather, SRY promoter activity requires factors found within a testicular environment, regardless of the genotype (Daneau et al., 2002).

Recently a novel human gene, KIAA0800, preferentially expressed in the testis, was found to be transactivated by SOX9 (Zhao et al., 2002). The gene encoding KIAA0800 is highly conserved between human and mouse and as yet, a function for KIAA0800 is yet to be elucidated. Analysis of the KIAA0800 promoter identified Sox binding sites to which Sox9 was capable of activating. Further, the promoter contained several elements which repress KIAA0800 transactivation and which contribute to transactivation by Sox9, including Alu element repeats and a poly-T track. Although not tested, it is possible that other Sox proteins also expressed in the testis, including Sox8 (Schepers et al., 2003) may play a role in the transactivation of KIAA0800.

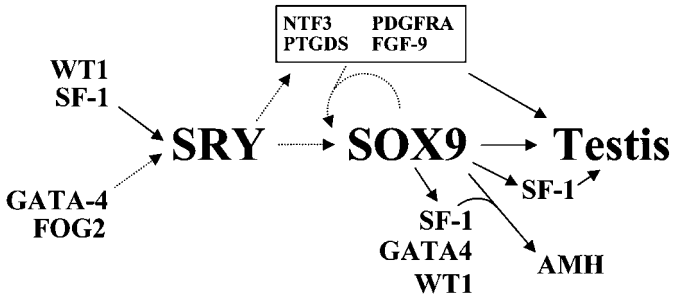

Fig. 2. Genetic pathway of mammalian sex determination in the XY gonad. Solid arrows represent known interactions whereas dashed lines represent hypothesized interactions.

Conclusions

While this review was primarily focused on the roles of two SOX transcription factors, there are a number of other key sex-determining genes, most encoding transcription factors that play roles in mammalian sex determination, many of which are elsewhere discussed within this volume. Synthesizing SRY and SOX9 research led us to formulate a simplistic genetic model of gene regulation that may be taking place in the XY gonad at the time that sex is determined (Fig. 2).

The initiation of SRY expression is critical for the male developmental fate. The early actions of *GATA4* and *Fog2* are important prior to the onset of *Sry* expression. However, the role of these two genes may be indirect when compared with SF-1 or WT1 which directly regulate SRY expression. The up-regulation of SOX9 is the next likely process. Initially, low levels of *Sox9* are detected in both XY and XX gonads possibly maintained by the expression of SF-1. The up-regulation of SOX9 follows the onset of SRY expression, and therefore SOX9 can be placed downstream of SRY in the pathway. Whether this is an indirect or direct action of SRY is not known. SRY can govern at least three cellular events in the XY gonad, and since a number of new genes involved in these processes have been identified (boxed genes in Fig. 2), it may be possible that SRY has a number of targets other than SOX9. As the case for SRY, when *Ntf3* is disrupted there is a decrease in expression levels of *Sox9.* Therefore, additional pathways initiated by SRY may also act to up-regulate SOX9.

Unlike the subsequent drop in expression levels of *Sry* following sex determination observed in the mouse gonad, levels of *Sox9* are sustained, a process possibly governed through an auto-regulatory loop. *Sox9* is capable of up-regulating the expression of *Sf-1* a critical sex-determining gene, and a likely direct *Sox9* target. The sex-determining target of SF-1 downstream of SOX9 is unknown, however as more genes are implicated in sex determination through the ongoing analysis of sex-reversed individuals, mouse transgenic studies and high throughput genomic studies, the number of possible targets of SRY and SOX9 regulation will surely rise.

Acknowledgements

We thank Michael Clarkson, Helena Sim and Louisa Ludbrook for reading of the manuscript.

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