Chapter 5
The Evolutionary Aspects of the Mammalian Sex-Determining Gene *SRY*

Shingo Miyawaki and Makoto Tachibana

Abstract In eutherian mammals, gonadal sex is determined by the presence or absence of the *sex-determining region Y* (*SRY*) gene, which locates in the male-specific region of the Y chromosome. Although the process of sex determination triggered by *SRY* is conserved among animal species, its genome sequence is highly diverse. Thus, *SRY* represents a unique, dynamic, and rapid evolutionary process. Since its discovery 30 years ago, *SRY*, including mouse *Sry*, has been believed to be a single-exon gene. Recently, we identified a previously undiscovered second exon of mouse *Sry* and a corresponding novel transcript, the two-exon *Sry* (*Sry-T*). The discovery of *Sry-T* provided new insights into the genetic evolution of *Sry*. In this review, we discuss the genetic evolution of *Sry* sequences based on the discovery of *Sry-T*.

Keywords Sex development · *Sry* · Gene evolution

5.1 The Mammalian Sex Determination Gene *SRY*

The development of male and female sexes is essential for the survival of almost all animal species. Sex in mammals is determined by a combination of sex chromosomes. Given that an XX chromosome results in female and an XY chromosome results in male, it has been thought that there is a male factor (sex-determining factor) in the Y chromosome (McLaren 1988; Welshons and Russell 1959).

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Sex-determining region Y (*SRY*) is a sex-determining gene in mammals (Gubbay et al. 1990; Sinclair et al. 1990; Koopman et al. 1991). Koopman et al. (1991) experimentally demonstrated that *Sry* alone is sufficient to trigger testis development in mice. In that experiment, insertion of a DNA fragment of the Y chromosome containing *Sry* and forcible expression of *Sry* resulted in testes formation in chromosomally female (XX-type) mice. This experiment also revealed that all of the sequences necessary for *Sry* expression are present within this 14.5-kb region. Human patients exhibiting XY gonadal dysgenesis and loss-of-function studies in mice, pig, and rabbit have demonstrated that *SRY*/*Sry* disruption causes sex reversal, such that chromosomal males (XY-type) are anatomically female (Berta et al. 1990; Jäger et al. 1990; Sinclair et al. 1990; Lovell-Badge and Robertson 1990; Wang et al. 2013; Kato et al. 2013; Song et al. 2017; Kurtz et al. 2021). These experiments suggested that SRY functions as the master regulator of male sex determination in all eutherian mammals (Waters et al. 2007).

5.2 Function of *Sry* in Sex Determination

Identification of the sex determination gene *SRY*/*Sry* led to an understanding of the mechanism of sex determination in mammals. Differentiation of Sertoli cells is the first step in male sex development in mammals (Albrecht and Eicher 2001; Koopman et al. 1990), with this process driven by *Sry* expression in the supporting cell lineage in the sexually undifferentiated gonad. Sertoli cells are essential for the coordination of testicular development and form the testis cords that encapsulate and direct germ cell differentiation as well as orchestrate the differentiation of other somatic cells, including Leydig cells, which represent a steroidogenic cell lineage (Habert et al. 2001). Sequential morphological and functional differentiation is subsequently induced under the influence of sex hormones (Fig. 5.1).

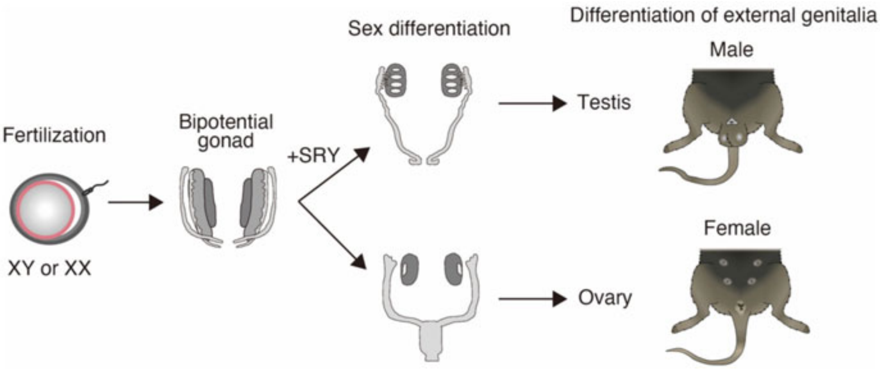
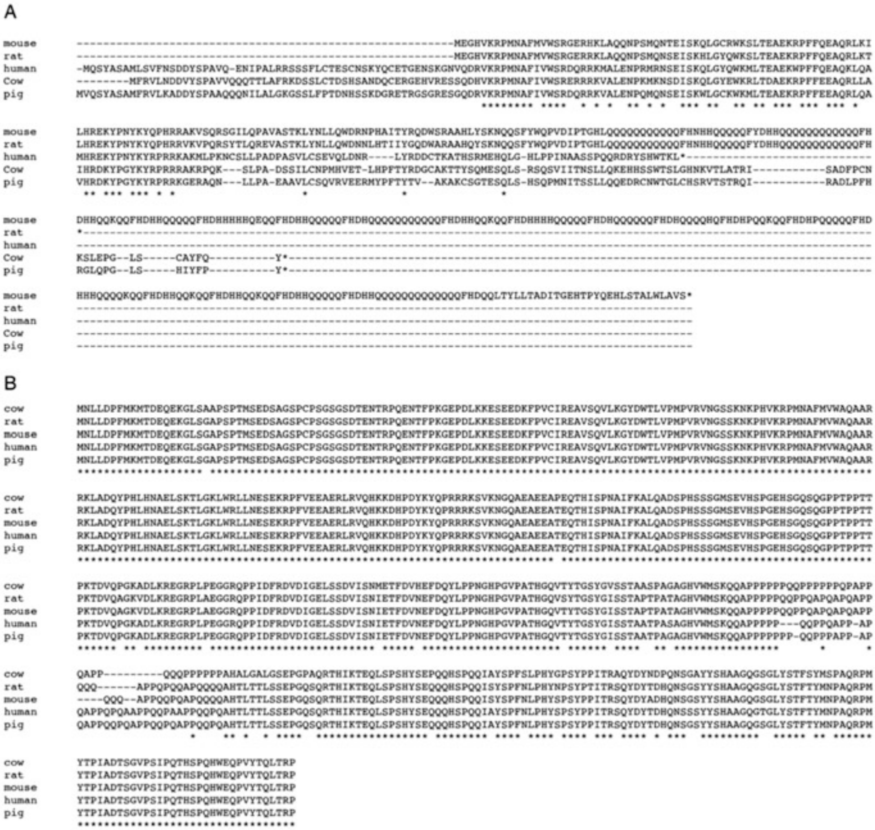


Fig. 5.1 Mammalian sex determination

In mice, *Sry* expression is strictly controlled in a cell-type- and temporal-specific manner. *Sry* expression starts at embryonic day 10.5 (E10.5) in pre-Sertoli cells of XY genital ridges, reaches a peak at E11.5, and declines by E12.5 (Hacker et al. 1995; Jeske et al. 1995; Koopman et al. 1990). If *Sry* expression is delayed by as little as a few hours, testis differentiation is disrupted or blocked completely in favor of ovarian development (Bullejos and Koopman 2001, 2005; Hiramatsu et al. 2009). *SRY*/*Sry* is more broadly expressed in animal species (human, rats, sheep, and rabbits) other than mice; however, recent studies using RNA sequencing (RNA-seq) analysis revealed that *SRY*/*Sry* also has a temporary expression pattern in humans and rats (Guo et al. 2021; Lecluze et al. 2020; Prokop et al. 2020). These observations indicate that sex determination in mammals is highly dependent on accurate transcription of *Sry*. Epigenetic regulation (i.e., histone modification and/or DNA methylation) is a mechanism that ensures accurate transcription and plays an important role in regulating *Sry* expression (Okashita and Tachibana 2021). In support of this view, complete or partial sex reversal has been observed in mice harboring knockouts of several epigenetic factors (Carré et al. 2018; Kuroki et al. 2013, 2017; Miyawaki and Tachibana 2019; Okashita et al. 2019).

A transgenic study demonstrated that ectopic expression of *SRY-box transcription factor 9* (*Sox9*), which belongs to the *Sry*-type high-mobility group box (Sox) family, can initiate the testis-determining pathway in XX mice (Vidal et al. 2001). In the developing testis, the critical function of *Sry* is to upregulate *Sox9* transcription (Sekido and Lovell-Badge 2008). SRY protein contains a high-mobility group (HMG) box DNA-binding domain that binds to regulatory elements upstream of *Sox9*. The testis-specific enhancer of *Sox9* (TES), a 3.2-kb element mapping to a 13-kb section at the 5′ end of the transcription start site of *Sox9*, and its 1.4-kb core region TESCO, activates *Sox9* expression *in cis* in Sertoli cells (Sekido and Lovell-Badge 2008). SRY and nuclear receptor subfamily 5 group A member 1 bind TESCO directly and activate *Sox9* expression. Recently, enhancer 13 (Enh13), a 557-bp element located at the 5′ end of the *Sox9* gene, was identified as a key enhancer of *Sox9* (Gonen et al. 2018). Mouse Enh13 contains a single consensus SRY-binding site, and targeted deletion of Enh13 reduced *Sox9* expression to a level equivalent to that in XX gonads and resulted in male-to-female sex reversal in mice (Gonen et al. 2018). These findings provided the first evidence that deletion of a noncoding genomic region causes sex reversal. Because most human XY females harbor mutations in the HMG box of SRY, its DNA-binding activity is essential for sex determination (Harley et al. 1994; Harley and Goodfellow 1994). Thus, future studies may reveal the existence of noncoding genomic regions that are necessary for sex determination in humans.

SOX9 activates a network of gene activity required for testis development while simultaneously impeding a network required for ovarian development (Warr and Greenfield 2012). Additionally, SRY represses the R-spondin1–Wnt/β-catenin signaling pathway that drives ovarian development (Bernard et al. 2008; Capel 2006; Lau and Li 2009; McElreavey et al. 1993). These two alternative gene regulatory networks confer the bipotential gonad its unique ability to differentiate into two morphologically and functionally distinct organs (Larney et al. 2014). In summary,



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A

mouse      ---------------------------------------------------------MEGHVKRPMNAFMVWSRGERHKLAQQNPSMQNTEISKQLGCRWKSLTEAEKRPFFQEAQRLKI
rat        ---------------------------------------------------------MEGHVKRPMNAFMVWSRGERRKLAQQNPSMQNSEISKHLGYQWKSLTEAEKRPFFQEAQRLKT
human      -MQSYASAMLSVFNSDDYSPAVQ-ENIPALRRSSSFLCTESCNSKYQCETGENSKGNVQDRVKRPMNAFIVWSRDQRRKMALENPRMRNSEISKQLGYQWKMLTEAEKWPFFQEAQKLQA
Cow        --------MFRVLNDDVYSPAVVQQQTTLAFRKDSSLCTDSHSANDQCERGEHVRESSQDHVKRPMNAFIVWSRERRRKVALENPKMKNSDISKQLGYEWKRLTDAEKRPFFEEAQRLLA
pig        MVQSYASAMFRVLKADDYSPAAQQQNILALGKGSSLFPTDNHSSKDGRETRGSGRESGQDRVKRPMNAFIVWSRDQRRKVALENPQMQNSEISKWLGCKWKMLTEAEKRPFFEEAQRLQA
                                                                        ******** ****  * * *  ** * *  *** **  ** ** *** *** *** *

mouse      LHREKYPNYKYQPHRRAKVSQRSGILQPAVASTKLYNLLQWDRNPHAITYRQDWSRAAHLYSKNQQSFYWQPVDIPTGHLQQQQQQQQQQQFHNHHQQQQQFYDHHQQQQQQQQQQQQFH
rat        LHREKYPNYKYQPHRRVKVPQRSYTLQREVASTKLYNLLQWDNNLHTIIYGQDWARAAHQYSKNQQSFYWQPVDIPTGHLQQQQQQQQQQQFHNHHQQQQQFYDHHQQQQQQQQQQQQFH
human      MHREKYPNYKYRPRRKAKMLPKNCSLLPADPASVLCSEVQLDNR----LYRDDCTKATHSRMEHQLG-HLPPINAASSPQQRDRYSHWTKL*----------------------------
Cow        IHRDKYPGYKYRPRRRAKRPQK---SLPA-DSSILCNPMHVET-LHPFTYRDGCAKTTYSQMESQLS-RSQSVIITNSLLQKEHHSSWTSLGHNKVTLATRI-----------SADFPCN
pig        VHRDKYPGYKYRPRRKGERAQN---LLPA-EAAVLCSQVRVEERMYPFTYTV--AKAKCSGTESQLS-HSQPMNITSSLLQQEDRCNWTGLCHSRVTSTRQI-----------RADLPFH
            ** *** *** * *                   *              *              *

mouse      DHHQQKQQFHDHHQQQQQFHDHHHHHQEQQFHDHHQQQQQFHDHQQQQQQQQQQQFHDHHQQKQQFHDHHHHQQQQQFHDHQQQQQQFHDHQQQQHQFHDHPQQKQQFHDHPQQQQQFHD
rat        *-----------------------------------------------------------------------------------------------------------------------
human      ------------------------------------------------------------------------------------------------------------------------
Cow        KSLEPG--LS-----CAYFQ----------Y*----------------------------------------------------------------------------------------
pig        RGLQPG--LS-----HIYFP----------Y*----------------------------------------------------------------------------------------

mouse      HHHQQQQKQQFHDHHQQKQQFHDHHQQKQQFHDHHQQQQQFHDHHQQQQQQQQQQQQQFHDQQLTYLLTADITGEHTPYQEHLSTALWLAVS*
rat        ---------------------------------------------------------------------------------------------
human      ---------------------------------------------------------------------------------------------
Cow        ---------------------------------------------------------------------------------------------
pig        ---------------------------------------------------------------------------------------------

B

cow        MNLLDPFMKMTDEQEKGLSAAPSPTMSEDSAGSPCPSGSGSDTENTRPQENTFPKGEPDLKKESEEDKFPVCIREAVSQVLKGYDWTLVPMPVRVNGSSKNKPHVKRPMNAFMVWAQAAR
rat        MNLLDPFMKMTDEQEKGLSGAPSPTMSEDSAGSPCPSGSGSDTENTRPQENTFPKGEPDLKKESEEDKFPVCIREAVSQVLKGYDWTLVPMPVRVNGSSKNKPHVKRPMNAFMVWAQAAR
mouse      MNLLDPFMKMTDEQEKGLSGAPSPTMSEDSAGSPCPSGSGSDTENTRPQENTFPKGEPDLKKESEEDKFPVCIREAVSQVLKGYDWTLVPMPVRVNGSSKNKPHVKRPMNAFMVWAQAAR
human      MNLLDPFMKMTDEQEKGLSGAPSPTMSEDSAGSPCPSGSGSDTENTRPQENTFPKGEPDLKKESEEDKFPVCIREAVSQVLKGYDWTLVPMPVRVNGSSKNKPHVKRPMNAFMVWAQAAR
pig        MNLLDPFMKMTDEQEKGLSGAPSPTMSEDSAGSPCPSGSGSDTENTRPQENTFPKGEPDLKKESEEDKFPVCIREAVSQVLKGYDWTLVPMPVRVNGSSKNKPHVKRPMNAFMVWAQAAR
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cow        RKLADQYPHLHNAELSKTLGKLWRLLNESEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKSVKNGQAEAEEAPEQTHISPNAIFKALQADSPHSSSGMSEVHSPGEHSGQSQGPPTPPTT
rat        RKLADQYPHLHNAELSKTLGKLWRLLNESEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKSVKNGQAEAEEATEQTHISPNAIFKALQADSPHSSSGMSEVHSPGEHSGQSQGPPTPPTT
mouse      RKLADQYPHLHNAELSKTLGKLWRLLNESEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKSVKNGQAEAEEATEQTHISPNAIFKALQADSPHSSSGMSEVHSPGEHSGQSQGPPTPPTT
human      RKLADQYPHLHNAELSKTLGKLWRLLNESEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKSVKNGQAEAEEATEQTHISPNAIFKALQADSPHSSSGMSEVHSPGEHSGQSQGPPTPPTT
pig        RKLADQYPHLHNAELSKTLGKLWRLLNESEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKSVKNGQAEAEEATEQTHISPNAIFKALQADSPHSSSGMSEVHSPGEHSGQSQGPPTPPTT
           ************************************************************************ ***********************************************

cow        PKTDVQPGKADLKREGRPLPEGGRQPPIDFRDVDIGELSSDVISNMETFDVHEFDQYLPPNGHPGVPATHGQVTYTGSYGVSSTAASPAGAGHVWMSKQQAPPPPPPQQPPPPPPQPAPP
rat        PKTDVQAGKVDLKREGRPLAEGGRQPPIDFRDVDIGELSSDVISNIETFDVNEFDQYLPPNGHPGVPATHGQVSYTGSYGISSTAPTPATAGHVWMSKQQAPPPPPQQPPQAPQAPQAPP
mouse      PKTDVQAGKVDLKREGRPLAEGGRQPPIDFRDVDIGELSSDVISNIETFDVNEFDQYLPPNGHPGVPATHGQVTYTGSYGISSTAPTPATAGHVWMSKQQAPPPPPQQPPQAPQAPQAPP
human      PKTDVQPGKADLKREGRPLPEGGRQPPIDFRDVDIGELSSDVISNIETFDVNEFDQYLPPNGHPGVPATHGQVTYTGSYGISSTAATPASAGHVWMSKQQAPPPPP---QQPPQAPP-AP
pig        PKTDVQPGKADLKREGRPLPEGGRQPPIDFRDVDIGELSSDVISNIETFDVNEFDQYLPPNGHPGVPATHGQVTYTGSYGISSTAATPAGAGHVWMSKQQAPPPPPPP-QQPPPAPP-AP
           ****** ** ********* ************************* ***** ********************* ****** ****  ** ****************      *      *

cow        QAPP---------QQQPPPPPPAHALGALGSEPGPAQRTHIKTEQLSPSHYSEPQQHSPQQIAYSPFSLPHYGPSYPPITRAQYDYNDPQNSGAYYSHAAGQGSGLYSTFSYMSPAQRPM
rat        QQQ------APPQPQQAPQQQQAHTLTTLSSEPGQSQRTHIKTEQLSPSHYSEQQQHSPQQISYSPFNLPHYNPSYPTITRSQYDYTDHQNSGSYYSHAAGQGSGLYSTFTYMNPAQRPM
mouse      ----QQQ--APPQQPQAPQQQQAHTLTTLSSEPGQSQRTHIKTEQLSPSHYSEQQQHSPQQISYSPFNLPHYSPSYPPITRSQYDYADHQNSGSYYSHAAGQGSGLYSTFTYMNPAQRPM
human      QAPPQPQAAPPQQPAAPPQQPQAHTLTTLSSEPGQSQRTHIKTEQLSPSHYSEQQQHSPQQIAYSPFNLPHYSPSYPPITRSQYDYTDHQNSSSYYSHAAGQGTGLYSTFTYMNPAQRPM
pig        QAPPQQPQAPPQQPQAPPQQPQAHTLTTLSSEPGQSQRTHIKTEQLSPSHYSEQQQHSPQQIAYSPFNLPHYSPSYPPITRSQYDYTDHQNSGSYYSHAAGQGSGLYSTFTYMNPAQRPM
                            *    ** *  * ****  ***************** ******** **** **** **** *** **** * ***  *********  ***** ** ******

cow        YTPIADTSGVPSIPQTHSPQHWEQPVYTQLTRP
rat        YTPIADTSGVPSIPQTHSPQHWEQPVYTQLTRP
mouse      YTPIADTSGVPSIPQTHSPQHWEQPVYTQLTRP
human      YTPIADTSGVPSIPQTHSPQHWEQPVYTQLTRP
pig        YTPIADTSGVPSIPQTHSPQHWEQPVYTQLTRP
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Fig. 5.2 Sequence comparison of SRY and SOX9. Amino acid sequences of SRY (**a**) and SOX9 (**b**) were aligned across the indicated animal species. Although the amino acid sequence of SRY is less conserved, that of SOX9 is highly conserved

the gene regulatory network for testis development is activated and that for ovarian development is repressed by the SRY–SOX9 axis. The amino acid sequence of SOX9 is highly conserved across animal species (Fig. 5.2), and these sex determination processes are consistent among different species (Nagahama et al. 2021). However, the sequence of *SRY* is diverse across species (Fig. 5.2).

5.3 Genetic Structure of *Sry*

Since the identification of the *SRY*/*Sry* gene in humans and mice, *SRY* has been identified in a variety of animal species. There is a high diversity of *SRY* sequences among animal species. SRY contains a highly conserved HMG DNA-binding domain and less-conserved N- and C-terminal domains (Tucker and Lundrigan

1993; Zhao and Koopman 2012). Mouse *Sry* has a unique CAG repeat sequence at the C-terminus (Bowles et al. 1999), and sequence comparison with rat and spiny rat *Sry* suggests that the CAG repeat sequence was not added to the C-terminus but inserted or extended just before the C-terminal sequence conserved in rodents (Ogata et al. 2019; Roy 2021). CAG repeats encode the polyglutamine (polyQ) amino acid sequence, which is essential for *Sry* transcription (Bowles et al. 1999). These findings suggest that the *Sry* sequence acquired CAGs to maintain transcriptional activity in rodents.

SRY/*Sry* is a single-copy gene in mice, humans, and horses (Behlke et al. 1993; Hacker et al. 1995; Janečka et al. 2018) but amplified in several species. For example, there are at least 11 copies of *Sry* in rats and two copies of *SRY* in dogs, rabbits, and pigs (Turner et al. 2007; Prokop et al. 2013; Geraldes et al. 2010; Skinner et al. 2016). Recent studies in rats showed that distinct copies of *Sry* are expressed (Prokop et al. 2020); however, it is unclear whether all of the *Sry* copies are required for sex determination or whether any of them act as true sex-determining genes. In a knockout study in pigs, a frameshift mutation in one copy of the *SRY* gene did not induce male-to-female sex reversal. These results suggest that expression from one *SRY* copy is sufficient for the development of male genitalia, or that only one of the two *SRY* copies is expressed, and the frameshift mutation was introduced into the unexpressed *SRY* copy (Kurtz et al. 2021).

Mouse *Sry* is embedded in a large palindromic sequence, which is also called an inverted repeat sequence. There is an almost indistinguishable 50-kb palindromic sequence flanking mouse *Sry* (Gubbay et al. 1992), with the palindromic nature of the *Sry* locus capable of potentially leading to the formation of a stem-loop structure. In this situation, juxtaposing the DNA breakpoint within the palindromic sequence might induce deletion of *Sry*, resulting in the generation of XY female mice (Gubbay et al. 1992). Until the recent establishment of genome-editing technology, the palindromic nature of the *Sry* locus made it difficult to manipulate endogenous *Sry* by conventional homologous recombination techniques.

Hacker et al. (1995) analyzed a mouse line termed 32.10 with 12 copies of L741 and concluded that *Sry* is a single-exon gene that encodes only one protein (SRY). Similarly, analysis of gonads during mouse development concluded that *Sry* is a single exon (Jeske et al. 1995). In humans and pigs, it was confirmed that the SRY protein is encoded by a single exon (Behlke et al. 1993; Daneau et al. 1996). Therefore, the conclusion that *SRY*/*Sry* is a single-exon gene was recognized as a confirmed fact that was not doubted for three decades. However, employment of comprehensive transcriptomics approaches identified a cryptic second exon within the flanking palindrome that is essential for male development (Miyawaki et al. 2020).

5.4 Discovery of Two-Exon *Sry* (*Sry-T*)

The genomic sequence of *SRY*, especially at the C-terminal coding sequence, is highly diverse. Various studies have discussed the sequence diversity of *SRY* (Larney et al. 2015; Roy 2021; Tucker and Lundrigan 1993; Zhao et al. 2014). Recently, we highlighted the previously unaddressed nature of the C-terminal sequences of mouse SRY (Miyawaki et al. 2020). In this section, we focus on the latest findings related to the discovery of a cryptic second exon of *Sry* and discuss the genetic evolution of *Sry* sequences.

5.4.1 Identification of Mouse Sry *Exon2*

First, we describe our discovery of two-exon SRY (SRY-T), which is the bona fide testis-determining factor in mice. During our previous study on regulation of *Sry* expression, we established a method to selectively collect gonadal somatic cells expressing *Sry* (Kuroki et al. 2013). Using this method, we conducted gene expression analysis (RNA-seq) of *Sry*-expressing cells, which led to the identification of an uncharacterized sequence transcribed from the 3′ palindromic sequence. We initially named this unknown sequence “*Srx*.”

To characterize *Srx*, we performed comprehensive transcriptome analyses of embryonic gonadal somatic cells during the sex-determining period. Initially, we assumed that *Srx* would be a long noncoding RNA (lncRNA); however, analysis of the transcription start site using cap analysis of gene expression sequencing showed no transcription start signal at the 5′ end of *Srx*, suggesting that it might not be a lncRNA. We then performed long-read RNA-seq and found that this unknown transcript was spliced to the previously known sequence of *Sry* at the typical 5′ GT/3′ AG splice-site sequences with an internal polypyrimidine tract. These results clearly showed that *Srx* was not an independent transcript but was the previously unknown second exon of *Sry*. Sequencing data indicated that two-types of mRNA were transcribed from the *Sry* locus: the known single-exon type (*Sry-S*) and a novel two-exon type (*Sry-T*) (Fig. 5.3).

The SRY-T protein shares an HMG DNA-binding domain and polyQ sequences with the amino acid sequence of SRY-S. The 15 amino acids in the SRY-T C-terminus are encoded by the second exon, whereas the absence of splicing results in 18 different amino acids at the SRY-S C-terminus.

5.4.2 Sry-T *Is Essential for Testis Development*

To investigate the role of *Sry-T* in sex determination, we generated *Sry-T*-deficient mice in which *Sry* exon2 was deleted by genome editing using the CRISPR/Cas9

Fig. 5.3 Exon–intron structure of the mouse *Sry* locus. Transcripts specific for *Sry-S* (blue) and *Sry-T* (red) are shown along with the 5′ GT/3′ AG splicing motifs. TE: retrotransposon-derived sequence

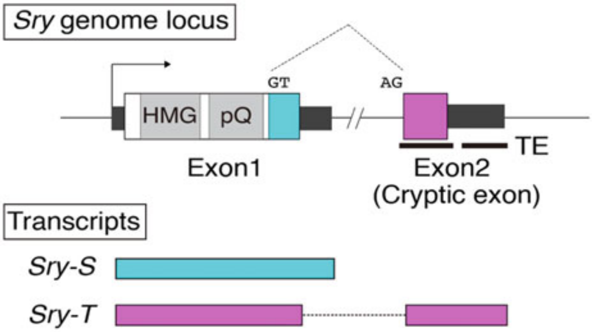

Fig. 5.4 SRY-T is a necessary and sufficient sex determinant. *Sry-T*-deficient XY mice exhibit male-to-female sex reversal (left). *Sry-T*-expressing XX mice develop as males (right)

system. As a result, *Sry-T*-deficient mice showed a sex-reversal phenotype despite expressing *Sry-S*, indicating that *Sry-T* is essential for male sex determination. Furthermore, when *Sry-T* or *Sry-S* was expressed in XX mice under the same conditions, only mice expressing *Sry-T* underwent female-to-male sex reversal. These experiments demonstrated SRY-T as a necessary and sufficient sex determinant in vivo (Fig. 5.4).

5.4.3 Existence of a "Degron" in the SRY-S C-Terminus

As shown in previous studies, *Sry* transgenes encoding only SRY-S can induce testis development in XX mice under experimental conditions (Koopman et al. 1991; Bowles et al. 1999; Washburn et al. 2001; Zhao et al. 2014). However, our studies revealed that *Sry-S* alone could not induce male development under physiological conditions. To investigate the cause of the insufficiency of *Sry-S* for sex determination, we focused on the difference in the C-termini of SRY-S and SRY-T. We generated lentiviral constructs encoding blue fluorescent protein (BFP) and enhanced green fluorescent protein (EGFP) fused to either the C-terminus of SRY-S (termed S18) or SRY-T (termed T15), both of which were translated from the same transcript, and then transduced them into human embryonic kidney cell line 293T (HEK293T). The effect of S18 or T15 on protein stability was evaluated by examining the EGFP/BFP ratio. Although EGFP-T15 expression levels were similar to those of non-fused EGFP, EGFP-S18 expression levels were $<10\%$ those of non-fused EGFP. Further detailed investigation revealed that a protein-degrading sequence called a "degron" was present at the SRY-S C-terminus (Miyawaki et al. 2020). Because the amino acid at the second-to-last residue position is valine, the SRY-S degron was assumed to be a (V-2)-type degron (Koren et al. 2018). In (V-2)-type degrons, this valine residue is essential for degron activity. Replacement of the valine in the -2 site of endogenously expressed SRY-S with proline suppressed SRY-S degradation. Furthermore, the corresponding mice (*Sry-S*:V394P; *Sry*-exon2Δ mice) developed as males, even though they lacked SRY-T. These results showed that SRY-S is insufficient for male development under physiological conditions, because the C-terminal degron sequence makes the protein unstable.

5.4.4 Reconsidering Previous Research in Light of Sry-T Discovery

In the first experiment identifying *Sry* as a sex-determining gene in 1991, insertion of multiple copies of a DNA fragment (L741) encoding only *Sry-S* was successful in producing XX males. Of the eight XX individuals with transgenes, two mice changed their sex to male, whereas the others did not (Koopman et al. 1991). Other studies also reported variations in the ability to cause XX sex reversal on the part of the 14-kb *Sry* transgene lacking the second exon (Bowles et al. 1999; Zhao et al. 2014), which might reflect an inability to control the copy number and integration site of the transgene. Only mice expressing SRY-S in an amount sufficient to overcome its destabilization were able to become male. Furthermore, another study showed that a single copy of the L741 transgene at an autosomal locus (*Col1a1*) did not induce XX sex reversal (Quinn et al. 2014). In that article, the existence of an unknown DNA region necessary for males had been predicted.

Why was the second exon missed in previous studies? One reason might be attributable to the existence of the palindromic sequence. Specifically, the second exon was hidden by the palindrome sequences. As noted, there exists a 50-kb palindromic sequence flanking mouse *Sry* (Gubbay et al. 1992). When duplicate mapped reads are excluded from the mapping of sequence reads to the genome, the transcript corresponding to the second exon is not mapped. When duplicate mapping is allowed, this transcript appears on both sides of the palindrome sequences. Various other palindromic sequences are known to exist in the Y chromosome and reportedly contribute to its structural maintenance (Soh et al. 2014). To date, no functional sequences have been identified in the palindromic sequence. Given that the second exon of *Sry* is located within the palindromic sequence, this exon was identified as the first functional sequence existing within the palindromic sequence.

5.5 Evolution of the Y Chromosome and *Sry*

The discovery of *Sry* exon2 also provides new insights into the evolution of sex-determining genes. The Y chromosome, which contains *Sry*, lost its genes during the evolutionary process (Graves 2006; Lahn and Page 1999). It is believed that the X chromosome and Y chromosome share a common ancestor (Ohno 1967). After the ancestral Y chromosome acquired a male-determining gene, other male-advantage alleles accumulated, and recombination was suppressed. Mutations and deletions in non-recombinant regions resulted in rapid degradation of the proto-Y chromosome. As a result, unlike autosomes and the X chromosome, the Y chromosome is normally unable to recombine with homologous chromosomes; therefore, mutations created during the evolutionally process accumulate in the Y chromosome. Due to this accumulation of mutations, the Y chromosome is thought to be facing a crisis related to loss of function in various genes. Notably, in mammals, the Y chromosome appears susceptible to far more mutation, deletion, and insertion events than the rest of the genome (Lindblad-Toh et al. 2005; Makova and Li 2002).

The X chromosome gene *SRY-box transcription factor 3* (*SOX3*), which is believed to share a common ancestor with *SRY*, is a single-exon gene (Stevanovlć et al. 1993; Sutton et al. 2011). In humans and pigs, it has been confirmed that the *SRY* is single-exon genes (Behlke et al. 1993; Daneau et al. 1996). In the ancestor of rodents, *Sry* is also considered to be a single-exon gene; however, during its evolution, *Sry* might have mutated into a degron-coding gene, presumably leading to the loss of its male-determining function (Fig. 5.5). To avoid subsequent crises of species survival, distinct mechanisms could have been invoked between species.

We explain our hypothesis of how rats and mice avoided *Sry* dysfunction as follows. Rat *Sry* harbors of conserved DNA sequence similar to the mouse *Sry-S* sequence encoding the degron. However, because the degron sequence is not translated due to a preceding four-nucleotide insertion and stop codons, rat SRY is not presumed to be destabilized. Additionally, rat *Sry* has been duplicated through gene conversion at transposable elements on the Y chromosome (Prokop et al.

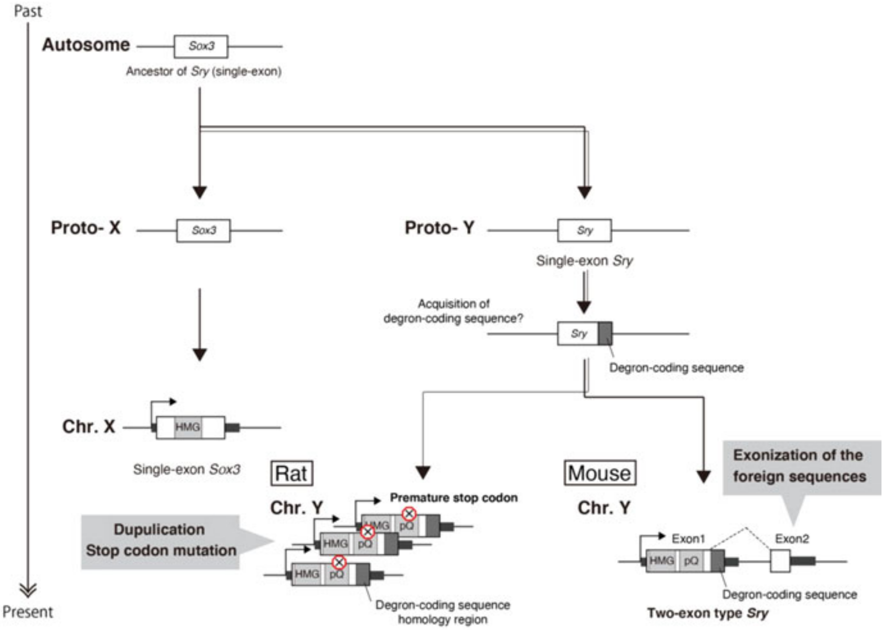

Fig. 5.5 Model for the evolution of the mouse and rat *Sry* locus

2013). At least three *Sry* genes (*Sry*1, *Sry*4A, and *Sry*3C) are expressed at the highest levels at E13, the time of testis differentiation, suggesting that multiple *Sry* genes potentially contribute to testis differentiation in rats (Prokop et al. 2020). These findings suggest that at least in rats, *Sry* might have evolved to avoid degron-mediated SRY dysfunction through gene duplication and early translation termination (Fig. 5.5).

In mice, we hypothesize that a different mechanism has evolved to avoid degron translation by creating a new exon (called exonization) in distal sequences and splicing to excise the degron. Interestingly, the second exon of *Sry* comprises a retrotransposon-derived sequence thought to originate from a retrovirus, suggesting that *Sry-T* was created by the exonization of a retrotransposon-derived sequence. In this case, exonization may have avoided translation of the degron-coding sequence and saved the ancestral *Sry*, which was in danger of losing its function due to the degron. This hypothesis suggests that virus-derived sequences might have evolved the host genes and saved the species from extinction. The discovery of *Sry-T* offer insight into a new aspect of the relationship between viruses and their host organisms. This protective mechanism represents an unusual and intriguing evolutionary mechanism that helps stop vulnerable Y chromosome genes from falling apart.

A previous study showed that although the sequence of the HMG box of SRY is conserved, the SRY C-terminus is rapidly evolving in mice and rats (Tucker and Lundrigan 1993). The hypothesis that the DNA sequence encoding the SRY C-terminus evolved to avoid degrons supports the existence of species-specific adaptive divergence through a process of positive Darwinian selection. From these perspectives, even if the Y chromosome undergoes degradation, *Sry* may persist through continuous evolution.

5.6 Consideration of the Evolution of *Sry* Exons from the Viewpoint of "Sex Spectrum"

The discovery of the second exon of *Sry* and the SRY-S degron offered insight into a part of the evolutionary process of sex-determining genes. The concept of "sex spectrum" explains that all cells/organs possess their own sex that can be located at any intermediate position between a typical male and a typical female. By acquisition of the SRY-S degron, the gonadal sex might have moved to the female side on the spectrum in the ancestor of rodents. However, in mice, the emergence of the second exon, which encodes degron-free SRY-T, moved the gonadal sex back to the male side. To support this concept, our genetic study using single-copy knock-in mouse lines that conditionally express each isoform of *Sry* showed that a copy of *Sry-T* was sufficient to activate the male pathway, whereas that of *Sry-S* was not. Alternatively, in rats, introducing the premature termination codon into *Sry* and increasing the *Sry* copy number might have evolved to move the gonadal sex back to the male side on the spectrum. These data suggest that not only the gene structure of *Sry* but also its sex-determining ability might have dynamically evolved in rodents. It may be worth examining whether the "cryptic" second exon or degron-coding sequence of *Sry* also exists in other mammals.

5.7 Conclusion and Future Directions

In the 30 years since *Sry* was discovered, no one questioned the fact that *Sry* is a single-exon gene. In contrast to this common knowledge, we showed that mouse *Sry* has a cryptic second exon that encodes previously unknown but functionally essential sex determinant, SRY-T. Our results provide insight into a critical neo-functionalization process that appears to be happening in real time; the gain-of-function replacement of SRY-S by SRY-T is currently ongoing in a mouse strain. In many strains of mice, the *Sry* sequence remains incompletely understood; therefore, further studies are warranted to comprehensively understand the evolutionary process of *Sry*.

Despite the high degree of conservation of the sex determination system triggered by SRY, its amino acid sequence shows dynamic evolution, with various species exhibiting novel protein domains, transcripts, and gene amplification. *Sry* is one of the few genes on the Y chromosome that is essential for survival and may be subject to different selection pressures for genetic evolution than autosomal and X chromosomal genes. Therefore, the function of male development may take the first priority, and preservation of the *Sry* DNA sequence may not be of critical importance. In general, the sequences of genes essential for survival tend to be highly conserved; however, despite *Sry* being essential for species survival, it shows sequence diversity among animal species. The diversity of *Sry* sequences in animal species may be the key to understanding the genetic evolution of *Sry*. As a result of these genetic evolutions, the spectrum-like diversity of SRY sequences may have been produced.

Approximately 10 years ago, it was proposed that the genes on the Y chromosome (including *Sry*) were in a state of degeneration and that males would eventually disappear (Bachtrog 2013; Graves 2006). Our findings have a profound influence on this notion.

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