Ubiquitous expression of Sry induces embryonic lethality related to suppression of Tie2/Tek expression

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ABSTRACT

Sry (sex-determining region on the Y chromosome) is a mammalian sex-determining gene on the Y chromosome. In mice, the transient expression of Sry in supporting cell precursor cells between 10.5 and 12.5 days post-coitus (dpc) triggers the differentiation of Sertoli cells from granulosa cells. The importance of the strict regulation of Sry expression remains unknown. Thus, we attempted to produce a Sry ubiquitous-expressing transgenic (Tg) mouse in which foreign Sry is driven by the CAG (cytomegalovirus immediate-early enhancer, chicken beta-actin promoter, and the fusion intron of chicken beta-actin and rabbit beta-globin)-Sry gene for ubiquitous expressing Sry. A low rate (2/127) of Tg pups was observed, whereas the rate of early-stage transgenic embryos before birth was 19.2% (5/26). The Sry ubiquitous-expressing embryos showed abnormal development. The results suggest that ubiquitous expression of Sry exerts a negative effect on embryonic development. One of the two adult Tg mice showed low levels of Sry expression. The other Tg mouse showed high Sry transgene expression, but was mosaic for the transgene. Developmental analysis of transgenic F1 embryos produced from the mosaic Tg mouse revealed that ubiquitous expression of Sry had a lethal effect on embryonic development around 12.5 dpc. The histological data indicated that ubiquitous expression of Sry induced abnormal cardiovascular development, resulting in embryonic death. Enhanced expression of Sry suppressed endogenous Tie2/Tek (tyrosine kinase with Ig and EGF homology domains 2/tunica interna endothelial cell kinase) expression in Sry-transfected primary cultured cells from wild type embryonic hearts. The results indicate that the tissue-specific and stage-specific expression of Sry is essential for normal embryogenesis.

Keywords: Sry; Tie2/Tek; Transgenic Mice

1. INTRODUCTION

Sry (Sex determining region on the Y chromosome) is a transcription factor with a DNA-binding domain referred to as the high mobility group (HMG), which triggers a gene expression cascade required for initiating male sex differentiation in the bipotential indifferent gonads of mammals [1]. Mouse Sry is expressed for a brief period between 10.5 and 12.5 days post-coitus (dpc) in the supporting cells of undifferentiated gonads that differentiate into Sertoli cells instead of granulosa cells [2-5]. It is well documented that Sry is a trigger and decisive gene for mammalian sex determination. Sry expression induces down- or up-regulation of the expression of various genes linked to the sex-determination cascade and subsequent testicular development. A large number of factors driving gonadal differentiation are encoded by autosomal genes. Testicular development after Sry expression has been shown to be regulated by various genes such as SfI/Ad4bp (steroidogenic factor 1/Adrenal 4 binding protein) [6], Wt1 (Wilms’ tumor suppressor 1) [7,8], Amh/Mis (Anti-Mulerian hormone/Mulerian-inhibiting substance) [9], and Sox9 (Sry-related high-mobility group box 9) [10]. In addition, the regions responsible for stage-specific and tissue-specific regulation of mouse Sry expression have also been investigated [11-14]. However, the gene responsible for gonadal differentiation, i.e., the direct target of Sry, remains to be identified.

Previously, an XX-sex-reversal mouse line carrying the Sry transgene driven by a weak basal Hsp70.3 pro-
moter (Hsp-Sry) was established. Comparison of Hsp-Sry/XY gonads with wild-type/XY and Hsp-Sry/XX gonads has suggested that Sry mRNA expression alone is not likely to provide positional or timing information needed for male-specific Sox9 activation in developing gonads [15]. The ability of Sry to induce testis development is limited to approximately 11.0-11.25 dpc, a time window of only 6 hours after the normal onset of Sry expression in XY gonads [16]. It is generally expected that the tightly regulated spatiotemporal expression profile of Sry during embryogenesis is crucial for assuring the normal development not only of gonads, but also of other fetal organs; however, the precise biological significance of limited Sry expression has yet to be clarified.

It is well established that the phenotype of transgenic (Tg) mice exhibiting ubiquitous expression of a gene of interest can provide evidence for speculations regarding natural biological functions [17-19]. Thus, to evaluate the role(s) played by Sry in development, we utilized transgenic mice. For our specific purposes, we constructed a CAG (cytomegalovirus immediate-early enhancer, chicken beta-actin promoter and fusion intron of chicken beta-actin and rabbit beta-globin)-Sry fusion gene construct that induces strong ubiquitous expression of a gene of interest [20], and we then attempted to generate the corresponding Tg mice (Figure 1). Here, we describe the developmental effects of ubiquitous Sry expression in these Tg mice.

2. MATERIALS AND METHODS

2.1. Animals

The following strains of mice were purchased from a commercial animal breeder (Sankyo Labo-Service Corporation, Inc., Tokyo, Japan): B6C3F1 (C57BL/6NxC3H/HeN), C57BL/6J, and ICR. The mice were kept in an environment with regulated temperature (22-25°C), humidity (40-50%), and illumination cycles (14-h light, 10-h dark), and were provided with food and water ad libitum. The experiments were conducted according to guidelines for the care and use of laboratory animals at the College of Agriculture, the University of Tokyo.

2.2. Tg Mouse Generation

Tg mice were generated by microinjecting DNA into the pronuclei of zygotes collected from the oviducts of superovulated B6C3F1 females that were mated with B6C3F1 males. All methods for generating the Tg mice used here have been described in the protocol reported by Hogan et al. [21]. The construction of pCX-Sry has been described previously [22]. The Sall/BamHI DNA fragment containing the CAG-Sry fusion gene was excised from pCX-Sry and separated by electrophoresis through 1% agarose gel; the fragment was then purified by CsCl ultra-centrifugation. The purified DNA fragment was dissolved in a solution containing 10 mM Tris-HCl (pH 7.4) and 0.25 mM EDTA (pH 7.4) and was used for pronuclear microinjection. To identify Tg founder animals, genomic DNA was isolated from the tip of the tail, and the genomic DNA was screened by polymerase chain reaction (PCR) amplification using the following primers: 5’-CTC-TGC-TAA-CCA-TGT-TCA-TGC-CTT-3’, which span the CAG promoter and enhancer, chicken beta-actin promoter and fusion intron of chicken beta-actin and rabbit beta-globin)-Sry fusion gene construct that induces strong ubiquitous expression of Sry and generate the transgenic (Tg) mice.

2.3. Preparation of Primary Cultured Cells and Transfection of Plasmids

Primary cultured cells were prepared from the hearts of 12.0-dpc embryos according to methods described in our previous paper [12,14]. pCX-Sry and pCAGGS (mock) were transfected using Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer’s
2.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Transcripts of the Sry transgene and endogenous Tie2/Tek (tyrosine kinase with Ig and EGF homology domains 2/ункция внутренняя эндотелиальная клетка эндоцирона) in the embryonic tissues, and Sry-transfected primary cultured cells from the hearts of 12.0-dpc embryos were deter- mined as previously described [12,14,22]. PCR was done using the appropriate primer sets for the target gene: for Sry, forward primer 5’-AAG-CGC-CCC-ATG-AAT-GCA-TTT-ATG-GT-3’ and reverse primer 5’-ACA-CTT-TAG-CCC-TCC-GAT-GAG-GCT-GA-3’. for Tie2/Tek, 5’-TAC-ATA-GGA-GGA-AAC-CTG-TTC-ACC-3’ and 5’-GGA-GGT-AAG-ACT-CGG-TTG-ACA-GTG-3’; for gly- ceraldehyde-3-phosphate dehydrogenase (G3PDH), 5’-TGA-AGG-TCG-GTG-TCA-ACG-GAT-TTG-GC-3’ and 5’-CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC-3’.

2.5. Histology

For the staging of embryos, midday for the vaginal plug was considered to be 0.5 dpc. Part of a limb was re- moved from the embryos for DNA extraction and geno- typing. For the histological analysis, embryos were fixed in 4% paraformaldehyde. After fixation, the embryos were processed for paraffin embedding as previously
described [5]. The embryos prepared in this manner were then sectioned at 6 μm, and the sections were used for hematoxylin and eosin staining.

2.6. Statistical Analysis
The results are expressed as mean ± SEM. The significance of differences between groups was determined by Student’s t-test.

3. RESULTS

3.1. Low Production Rate of Sry Transgenic Mice
The construct containing Sry under the control of the CAG promoter (CAG-Sry) was used for DNA microinjection to produce Tg mice (Figure 2(a)). The CAG promoter was selected because it is a ubiquitously and strongly expressed promoter. In analyses of DNA pups born, the percentage of Tg animals was 1.5% (2/127). Two CAG-Sry Tg lines (female and male) were generated. The female karyotype was XX, and that of the male was XY. Then, in the next experiment, pregnant mice that had undergone a transfer of CAG-Sry microinjected eggs were sacrificed between 11.5 dpc and 17.5 dpc, and the percentage of Tg embryo was investigated. The percentage of Tg embryo taking into consideration both embryonic stages, was approximately 19.2% (5/26). Tg embryo sacrificed at 17.5 dpc showed histological abnormalities (Figure 2(f)), suggesting that the survival rate of Tg embryo was reduced by the ubiquitous expression of Sry.

3.2. Lethal Effect of Sry-Ubiquitous Expression during Embryonic Development
Although the transgene was detected in F1 mice in the case of female CAG-Sry Tg mouse, low transcripts from the transgene were detected by RT-PCR, and female-to-male sex reversal did not occur with the Sry transgene (data not shown). The male Tg mouse was characterized as XY and fertile. No newborn pups resulting from breeding with the male Tg mouse were transgenic. This result suggested that ubiquitous and strong expression of Sry yields embryonic lethality; the mosaic integration of the transgene in the founder Tg mouse could explain why the founder Tg mouse was able to live to adulthood. To further investigate this lack of generation of transgenic offspring, we performed a series analysis of litters at embryonic stages. Tg embryos showing high levels of Sry transgene expression were detected at a rate of 7% (12/175). The rate of occurrence of Tg embryos indicated the mosaic integration of the transgene. Next, we attempted to determine the stage of embryonic development at which the ubiquitous-expression of Sry could exert a negative impact. At 11.5 dpc, control and CAG-Sry Tg embryos displayed no morphological differences (Figures 3(a) and (b)). In contrast, edema and congestion were observed in CAG-Sry Tg embryos at 12.5 dpc (Figure 3(d)). The CAG-Sry Tg embryos were dead by 13.5 dpc (Figure 3(f)). Therefore, the present results indicate that the ubiquitous expression of Sry has a lethal effect on embryonic development at approximately 12.5 dpc. Histological analysis of the genital ridge of CAG-Sry transgenic embryos (XY) at 12.5 dpc showed no testis cord formation (Figure 4(g)), and an enlargement of the diameter of the atrium was also observed (Figure 4(h)). The layer of smooth muscle cells of Tg embryos was thin, compared to that of wild-type embryos, in the dorsal aorta region (Figure 4(d) and (i)). Moreover, the endothelial cells of Tg embryos were abnormally round (Figure 4(j)).

3.3. Suppression of Tie2/Tek Expression by Sry Expression
We also examined the expression levels of Tie2/Tek in Sry-transgenic tissues, because Tie2/Tek has been reported to be involved in cardiovascular development. In wild-type embryos, Tie2/Tek expression levels were highest in the hearts, compared with those of the other two tissue types examined, i.e., brains and gonads, at 12.0 dpc (Figure 5(a)). As regards the rates of expression observed in these three tissues, Tie2/Tek expression levels of Sry-transgenic tissues were lower than that of wild-type tissues (Figure 5(a)). Thus, in these embryonic tissues, Tie2/Tek expression was suppressed by the ubiquitous expression of foreign Sry. This suppression of Tie2/Tek expression by Sry expression was also seen in Sry-transfected primary cultured cells from wild-type embryonic hearts (Figure 5(c)). The present findings suggest that ubiquitous Sry expression exerts a negative effect on cardiovascular development via changes in the expression of other genes, which ultimately results in embryonic lethality. The Tg male became unable to impregnate any females, which rendered it impossible to conduct further analyses of embryos from the Tg male.

4. DISCUSSION
In this study, we used a Tg approach to characterize the biological function of Sry. We generated Tg mice that ubiquitously expressed Sry as a means of elucidating the biological functions of this transcription factor. The efficiency of transgenesis was remarkably low (1.5%) compared with the efficiency of our usual transgenic experiments [23-26]. Furthermore, no newborn pups generated by breeding of the CAG-Sry Tg founder (mosaic for the transgene) carried the CAG-Sry transgene. Time-series analyses of F1 CAG-Sry Tg embryos clearly re-
Figure 3. Comparison of CAG-Sry transgenic embryos to wild-type embryos at different stages. Gross morphology of wild-type (a, c, e) and CAG-Sry Tg (b, d, f) embryos at 11.5 dpc (a, b), 12.5 dpc (c, d) and 13.5 dpc (e, f). Arrow in d shows congestion. Arrowhead in D shows edema. CAG-Sry Tg embryos appear normal up to 11.5 dpc. At 12.5 dpc, the Tg embryos begin to appear deformed and are dead by 13.5 dpc.

The negative effects of the ubiquitous expression of Sry on cardiovascular development; these negative effects were apparent as early as the 12.5 dpc, indicating that neither the spermatogenesis of germ cells, nor embryonic development, may be influenced by the ubiquitous expression of Sry prior to 11.5 dpc. The results also suggest that the precisely regulated expression of Sry in gonads appears to be essential for normal embryogenesis as well as for sex differentiation. Foreign mouse Sry has been shown to induce XX sex reversal [1]. The ability of Sry to induce testis development is limited to approximately 11.0-11.25 dpc [16]. Interestingly, although the gene-regulation system of goat SRY differs from that of the mouse Sry gene [14], transgenic mice with goat SRY showed XX sex reversal [27]. The previous results suggest that tissue-specific and stage-specific Sry expression might not necessarily be required for testis differentiation. Indeed, Sry, when under the control of the Hsp70.3 promoter (which induces weak yet broad expression), induced XX sex reversal [15]. In this study, it remained unclear whether or not the CAG-Sry transgene could induce testis development, because gonadal development had already stopped prior to testis cord formation (Figure 4(g)).

Knockout-mouse disrupted genes related to cardiovascular development (e.g., fetal liver kinase-1, fms-like tyrosine kinase-1, vascular endothelial growth factor, Tie2/Tek, and angiopeptin-1) have been associated with embryonic lethality at 8.5-12.5 dpc [28-32]. Ubiquitous Sry expression might influence the expression of these genes after 11.5 dpc. A Tie2/Tek promoter region analysis suggested that the Octamer-binding protein-1 (Oct-1) co-factor complex mediates the expression of Tie2/Tek [33,34]. There are 10 SOX (Sry-related High Mobility Group box) binding motifs, AACAA(T/A), within 5 kb of the 5’-flanking region of mouse Tie2/Tek. It is probable that the SOX-Oct complex regulates Tie2/Tek expression. Interestingly, Tie2/Tek expression was found to be downregulated in the heart of CAG-Sry Tg embryos at 12 dpc, and was also downregulated by the transfection of pCX-Sry into primary cultured cells prepared from embryonic mouse hearts (Figure 5). The SOX transcription factor family contains 20 (human and mouse) members, which have been classified into 8 groups [35]. SoxF genes (Sox7, Sox17, and Sox18) are expressed in endothelial cells and are required for vascularization [36-42]. As the morphology of endothelial cells was found to be malignant in CAG-Sry Tg embryos (Figure 3(j)), the CAG-Sry transgene might interrupt the function of SoxF genes in endothelial cells by competition with SoxF genes, thereby inducing abnormal development of the cardiovascular system; this was the case with a Sox18 mutant (Ra, Ra1, Ra9, and Ragl), which acted as a dominant negative [43-45]. It has been reported that Tie2/Tek knockout mice exhibit embryonic lethality accompanied...
Figure 4. Histological analysis of CAG-Sry transgenic embryos and wild-type littermates. Hematoxylin and eosin staining of whole embryos (a, f), gonad regions (b, g), heart regions (c, h) and dorsal aortic regions (d, e, i, j) of wild-type XY embryos (a to e) and Tg XY embryos (f to j) from breeding of wild-type female mouse and male CAG-Sry Tg mouse are shown. Tg embryos show the accumulation of blood cells in the cardinal veins (f, arrow). In the sections of genital ridge region, the tubule structure (arrow) is observed in gonad region of wild-type XY embryo (b). There is no tubule structure in gonad region of Tg XY embryo (g). Arrow shows bleeding in abdominal cavity. Sections of heart region of wild-type (c) and Tg (h) embryos show enlarged atrium in Tg embryo (arrow). Thin layer of muscle cells of aortic region (indicated by two arrows) is observed in section of Tg embryo (i) compared with that of wild-type embryo (d). Normal endothelial cells of aorta show flat morphology (arrows in e) and abnormal round shape of endothelial cells are observed in sections of Tg embryo (arrows in j). bar, 50 μm.

by abnormal cardiovascular development [31]. In the present study, it was revealed that Tie2/Tek expression was suppressed by enhanced Sry expression in both the Sry-transgenic heart and Sry-transfected primary cultured heart cells. As hypothesis of mutant Sox18 by Downes and Koopman [45], Sry proteins might act as dominant negative form by disruptive interaction with co-factor(s) of Sox18 (Figure 5).

In conclusion, we demonstrated that the tissue-specific and stage-specific expression of Sry is essential for normal embryogenesis, and in particular for cardiovascular development.
Figure 5. Effects of Sry-transgene on endogenous Tie2/Tek expression. (a) Tie2/Tek expression levels relative to those of G3PDH in the CAG-Sry transgenic tissues at 12.0 dpc. Expression levels of Tie2/Tek in the Sry transgenic tissues were reduced (n = 1). (b) Determination of expression of Sry transgene in the primary cultured cells collected from embryonic hearts at 12.0 dpc. pCX-Sry: Sry-transfected cells, Mock: mock-transfected cells, G3PDH: a house-keeping gene used as a reference, RT-: no reverse transcription. (c) Tie2/Tek expression levels relative to those of G3PDH. Tie2/Tek expression levels were reduced in the Sry-transfected primary cultured cells. *: P < 0.05, Vertical bars indicate the means ± SEM (n = 3). (d-f) The hypothesized effects of ubiquitous expression of Sry on the function of Sox18. Sox18 binds to the upstream regulatory sequence of a Tie2/Tek gene. The functional trans-activation domain is shown as opening that complement in shape its respective interacting co-factor (d). When affected by mutation, the domain is depicted as non-complementary opening (e). In the case of CAG-Sry Tg mice, Sox18 is replaced by Sry proteins which act as if mutant Sox18 (f). Successful interaction of Sox18 domain with its co-factor is indicated by an arrow, while disrupted interaction is indicated by a crossed arrow.
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REFERENCES


