Advances in Bioscience and Biotechnology, 2010, 1, 444-452 **ABB**
doi:10.4236/abb.2010.15058 Published Online December 2010 (http://www.SciRP.org/journal/abb/).

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

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Received 21 October 2010; revised 29 October 2010; accepted 29 October 2010.

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 F_1 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 *Sfl*/*Ad4bp* (steroidogenic factor 1/Adrenal 4 binding protein) [6], *Wt*1 (Wilms' tumor suppressor 1) [7,8], *Amh*/*Mis* (Anti-Mulerian hormone/Mulerian-inhibiting substance) [9], and *Sox*9 (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 *Sox*9 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/6Nx C3H/HeN), C57BL/6J, and ICR. The mice were kept in an environment with regulated temperature (22-25℃), 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 *Sal*I/*Bam*HI DNA fragment containing the CAG-*Sry* fusion gene was excised from pCX-*Sry* and separated by electrophoresis

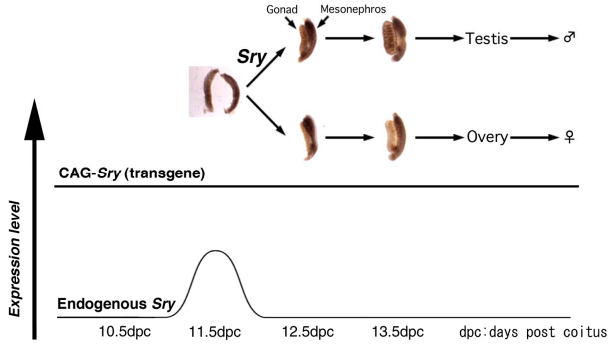

Figure 1. Schematic representation of gonadal sex determination and expression levels of endogenous *Sry* and CAG-*Sry* transgene. In mice, gonads are formed around 10.5 days post-coitus (dpc). *Sry* (Sex-determining region on the Y chromosome) triggers the testis developmental pathway in the bipotential indifferent gonads. *Sry* is expressed in the mouse gonads during a narrow window of development, between 10.5 and 12.5 dpc. In this study 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 *Sry* and generate the transgenic (Tg) mice.

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' and 5'-CCA-CTG-CAG-AAG-GTT-GTA-CAG-TTT-3', which span the CAG promoter and *Sry* coding region (**Figures 2(a)** and **(b)**). The PCR amplifycations were carried out using the following parameters: 35 cycles of 30 s at 94℃, 30 s at 58℃, and 1 min at 72℃. Sex chromosome karyotypes (XX or XY) were determined by PCR using the primers 5'-GCT-CGT-TAA-TTT-CTC-ACG-TTA-GTC-C-3' and 5'-ACA-CTT-TAG-CCC-TCC-GAT-GAG-GCT-GA-3', which span the *Sry* promoter and *Sry* coding region (**Figures 2(c)** and **(d)**). The PCR amplifications were carried out using the following parameters: 35 cycles of 30 s at 94℃, 30 s at 58℃, and 1 min at 72℃.

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

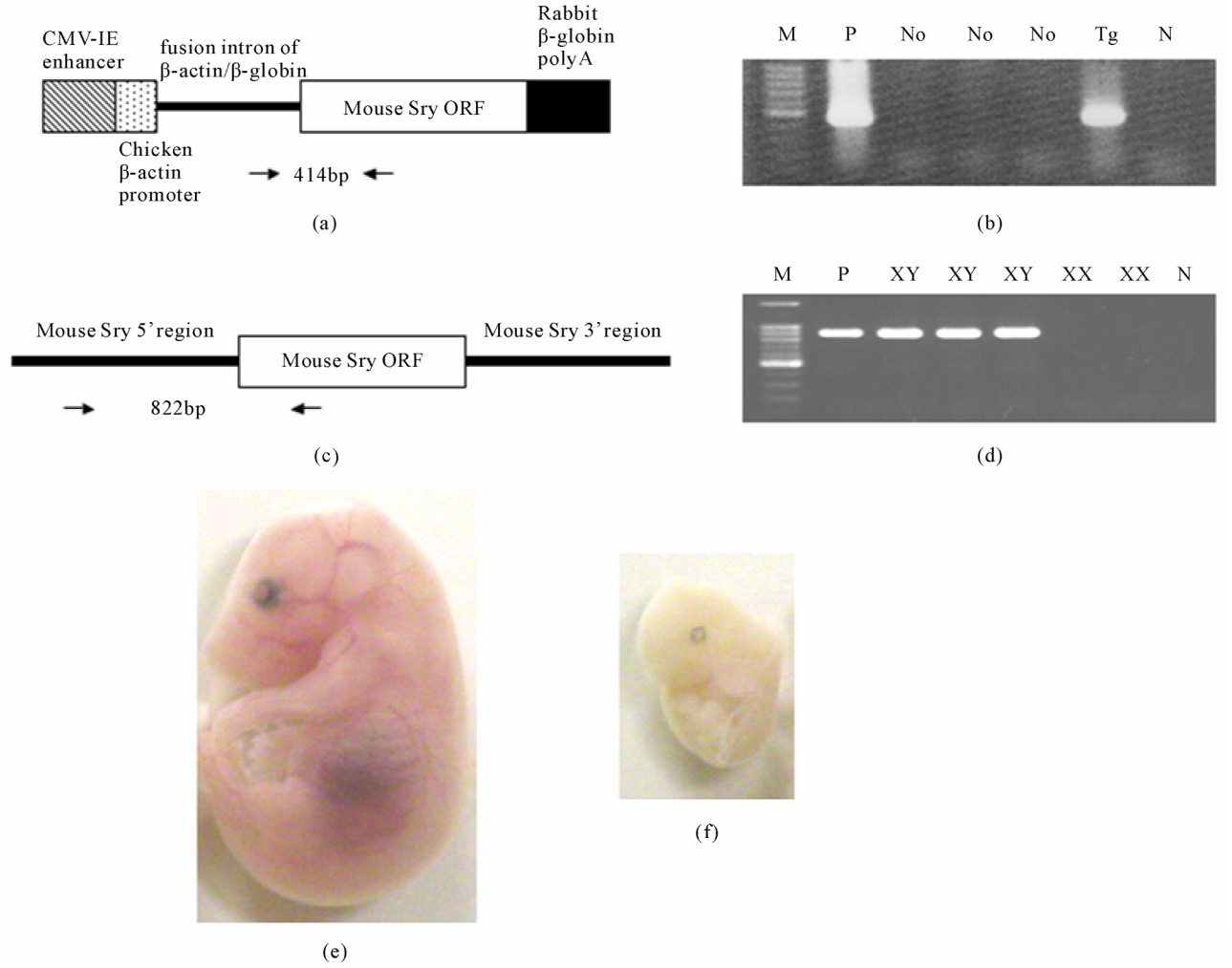

Figure 2. CAG-*Sry* construct and genotyping. (a) A schematic representation of the CAG-*Sry* transgene construct used in the micro-injection. The arrows indicate the location of primers used to identify the transgenic pups or embryos. (b) PCR analysis with genomic DNA to identify Tg mice. (c) A schematic representation of the endogeneous *Sry* gene. The arrows show the primer positions used to identify presence of Y chromosome. (d) PCR analysis with genomic DNA to identify presence of Y chromosome. Embryos were collected from pregnant foster mothers and their developmental level was analyzed. Non-transgenic embryo at 17.5 dpc (e) and Tg embryo at 17.5 dpc (f) were shown. Embryonic death is observed in CAG-*Sry* Tg embryo. Tg, transgenic; No, Non-transgenic; P, positive control; N, Negative control; XX, XX karyotype; XY, XY karyotype; M, size marker.

instructions, and the transfected cells were cultured at 37℃ under 5% CO_2 for 3 days.

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/tunica interna endothelial cell kinase) 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 glyceraldehyde-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 removed from the embryos for DNA extraction and genotyping. 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 embryos sacrificed at 17.5 dpc showed histological abnormalities (**Figure 2(f)**), suggesting that the survival rate of Tg embryos 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 F_1 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 F_1 CAG-*Sry* Tg embryos clearly re-

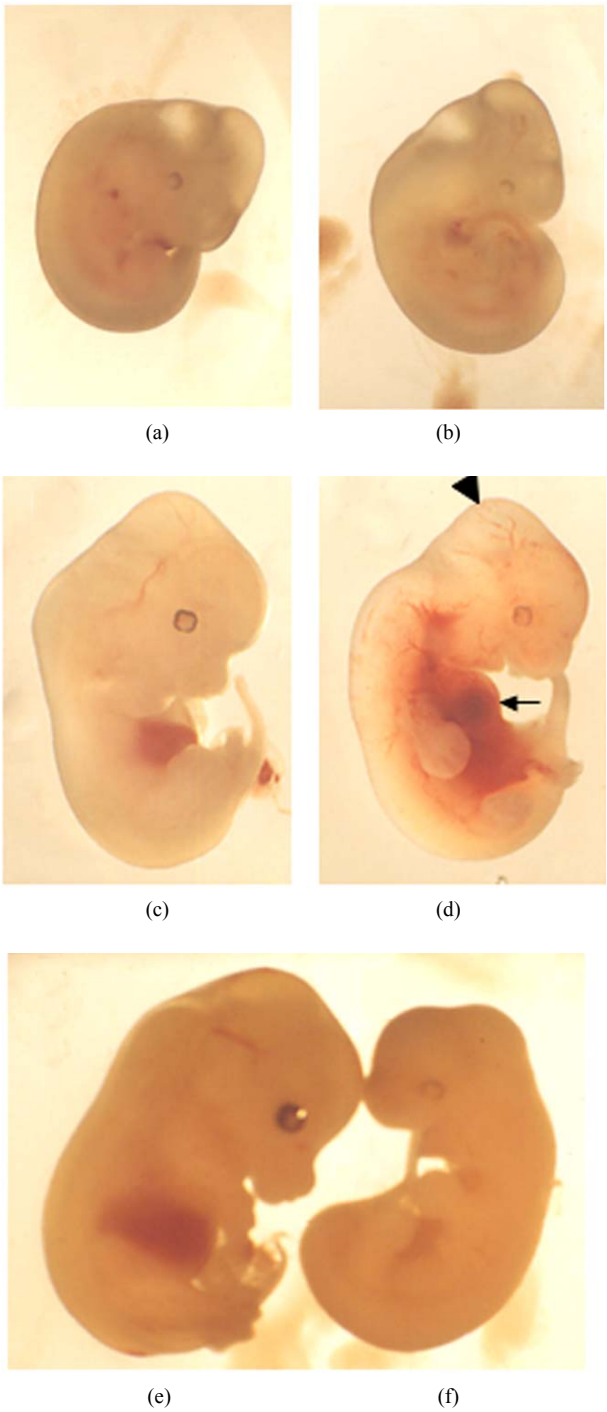

Figure 3. Comparison of CAG-*Sry* transgenic embryos to wild-type embryos at different stages. Gross morphology of wild-type (a, c, e) and CAG-*Sry* Tg (b, d. f) embryos at 11.5 dpc (a, b), 12.5 dpc (c, d) and 13.5 dpc (e, f). Arrow in d shows congestion. Arrowhead in D shows edema. CAG-*Sry* Tg embryos 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.

vealed 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 angiopoietin-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 (*Sox*7, *Sox*17, and *Sox*18) 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 *Sox*18 mutant (Ra, Ra^{J}, Ra^{op} and Ragl), which acted as a dominant negative [43-45]. It has been reported that *Tie2/Tek* knockout mice exhibit embryonic lethality accompanied

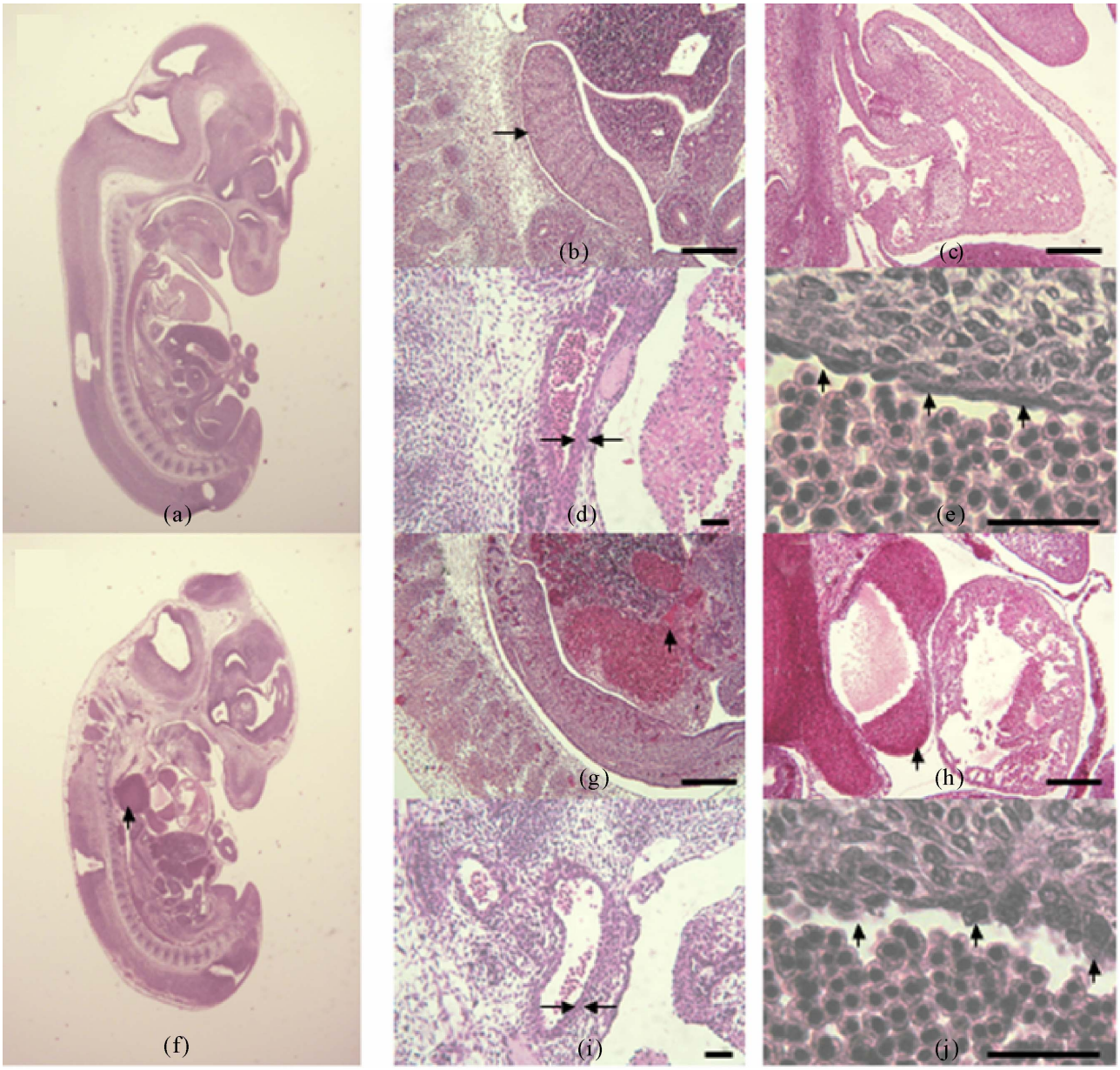

Figure 4. Histological analysis of CAG-*Sry* transgenic embryos and wild-type littermates. Hematoxylin and eosin staining of whole embryos (a, f), gonad regions (b, g), heart regions (c, h) and dorsal aortic regions (d, e, i, j) of wild-type XY embryos (a to e) and Tg XY embryos (f to j) from breeding 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 *Sox*18 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.

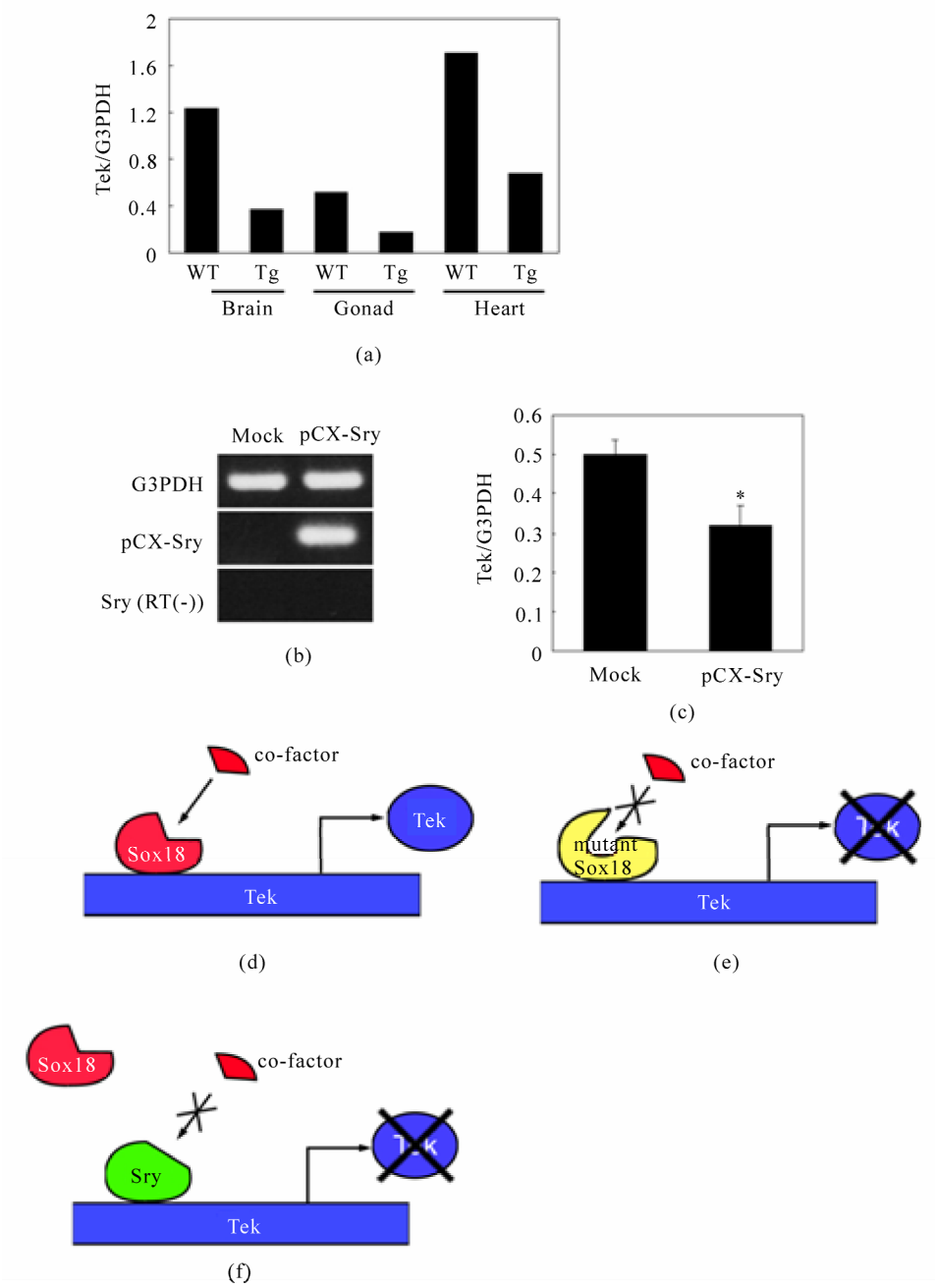

Figure 5. Effects of *Sry*-transgene on endogenous *Tie2/Tek* expression. (a) *Tie2/Tek* expression levels relative to those of *G3PDH* in the CAG-*Sry* transgenic tissues at 12.0 dpc. Expression levels of *Tie2/Tek* in the *Sry* transgenic tissues were reduced (n = 1). (b) Determination of expression 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.

5. ACKNOWLEDGEMENTS

We would like to thank Dr. Toshiyasu Matsui (The University of Tokyo) for expert advice on histology. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No.16380197).

REFERENCES

[1] Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. and Lovell-Badge, R. (1991) Male development of chromosomally female mice transgenic for Sry. *Nature*, **351**, 117-121.

[2] Hacker, A., Capel, B., Goodfellow, P. and Lovell-Badge, R. (1995) Expression of Sry, the mouse sex determining gene. *Development*, **121**, 1603-1614.

[3] Bullejos, M. and Koopman, P. (2001) Spatially dynamic expression of Sry in mouse genital ridges. *Developmental Dynamics*, **221**, 201-205.

[4] Albrecht, K.H. and Eicher, E.M. (2001) Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. *Developmental Biology*, **240**, 92-107.

[5] Ito, M., Yokouchi, K., Yoshida, K., Kano, K., Naito, K., Miyazaki, J. and Tojo, H. (2006) Investigation of the fate of Sry-expressing cells using an *in vivo* Cre/loxP system. *Development Growth and Differentiation*, **48**, 41-47.

[6] Shen, W.H., Moore, C.C., Ikeda, Y., Parker, K.L. and Ingraham, H.A. (1994) Nuclear receptor steroidogenic factor 1 regulates the mullerian inhibiting substance gene: A link to the sex determination cascade. *Cell*, **77**, 651-661.

[7] Toyooka, Y., Tanaka, S.S., Hirota, O., Tanaka, S., Takagi, N., Yamanouchi, K., Tojo, H. and Tachi, C. (1998) Wilms' tumor suppressor gene (WT1) as a target gene of SRY function in a mouse ES cell line transfected with SRY. *International Journal of Developmental Biology*, **42**, 1143-1151.

[8] Hsu, S.Y., Kubo, M., Chun, S.Y., Haluska, F.G., Housman, D.E. and Hsueh, A.J. (1995) Wilms' tumor protein WT1 as an ovarian transcription factor: Decreases in expression during follicle development and repression of inhibin-alpha gene promoter. *Molecular Endocrinology,* **9**, 1356-1366.

[9] Musterberg, A. and Lovell-Badge, R. (1991) Expression of the mouse anti-mulerian hormone gene suggests a role in both male and female sexual differentiation. *Development*, **113**, 613-624.

[10] De Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F. and Berta, P. (1998) Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-mulerian hormone gene. *Molecular and Cellular Biology*, **18**, 6653-6665.

[11] Dolci, S., Grimaldi, P., Geremia, R., Pesce, M. and Rossi, P. (1997) Identification of a promoter region generating Sry circular transcripts both in germ cells from male adult mice and in male mouse embryonal gonads. *Biology of Reproduction*, **57**, 1128-1135.

[12] Ito, M., Yokouchi, K., Naito, K., Endo, H., Hakamata, Y., Miyazaki, J. and Tojo, H. (2002) *In vitro* Cre/loxP system in cells from developing gonads: Investigation of the Sry promoter. *Development Growth and Differentiation*, **44**, 549-557.

[13] Yokouchi, K., Ito, M., Nishino, K., Yamanouchi, K., Naito, K., Suzawa, M., Kato, S., Hakamata, Y., Endo, H. and Tojo, H. (2003) Stage-specific regulatory element of mouse Sry gene. *Molecular Reproduction and Development*, **64**, 389-396.

[14] Ito, M., Yokouchi, K., Naito, K., Endo, H., Hakamata, Y., Miyazaki, J. and Tojo, H. (2005) Detection of elements responsible for stage- and tissue-specific expression of mouse Sry using an in vitro Cre/loxP system. *Biochemical and Biophysical Research Communications*, **337**, 264-270.

[15] Kidokoro, T., Matoba, S., Hiramatsu, R., Fujisawa, M., Kanai-Azuma, M., Taya, C., Kurohmaru, M., Kawakami, H., Hayashi, Y., Kanai, Y. and Yonekawa, H. (2005) Influence on spatiotemporal patterns of a male-specific Sox9 activation by ectopic Sry expression during early phases of testis differentiation in mice. *Developmental Biology*, **278**, 511-525.

[16] Hiramatsu, R., Matoba, S., Kanai-Azuma, M., Tsunekawa, N., Katoh-Fukui, Y., Kurohmaru, M., Morohashi, K., Wilhelm, D., Koopman, P. and Kanai, Y. (2009) A critical time window of Sry action in gonadal sex determination in mice. *Development*, **136**, 129-138.

[17] Ikeda, K., Nukumi, N., Iwamori, T., Osawa, M., Naito, K. and Tojo, H. (2004) Inhibitory function of whey acidic protein in the cell-cycle progression of mouse mammary epithelial cells (EpH4/K6 cells). *Journal of Reproduction and Development,* **50**, 87-96.

[18] Nukumi, N., Ikeda, K., Osawa, M., Iwamori, T., Naito, K. and Tojo, H. (2004) Regulatory function of whey acidic protein in the proliferation of mouse mammary epithelial cells *in vivo* and *in vitro*. *Developmental Biology*, **274**, 31-44.

[19] Iwamori, T., Oosawa, M., Nukumi, N., Kano, K., Sudo, K., Naito, K. and Tojo, H. (2005) Aberrant development of mammary glands, but precocious expression of beta-casein in transgenic females ubiquitously expressing whey acidic protein transgene. *Journal of Reproduction and Development,* **51**, 579-592.

[20] Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**, 193-199.

[21] Hogan, B., Constantini, F. and Lacy, E. (1994) Manipulating the mouse embryo. 2nd Edition, Cold Spring Harbor Laboratory Press, New York.

[22] Ito, M., Miyagishi, M., Murata, C., Kawasaki, H., Baba, T., Tachi, C. and Taira, K. (2006) Down-regulation of endogenous Wt1 expression by Sry transgene in the murine embryonic mesonephros-derived M15 cell line. *Journal of Reproduction and Development*, **52**, 415-427.

[23] Kato, M., Yamanouchi, K., Ikawa, M., Okabe, M., Naito, K. and Tojo, H. (1999) Efficient selection of transgenic mouse embryos using EGFP as a marker gene. *Molecular Reproduction and Development*, **54**, 43-48.

[24] Seo, B.B., Kim, C.H., Yamanouchi, K., Takahashi, M., Sawasaki, T., Tachi, C. and Tojo, H. (2000) Co-injection of restriction enzyme with foreign DNA into the pro-

nucleus for elevating production efficiencies of transgenic animals. *Animal Reproduction Science*, **63**, 113-122.

[25] Kubo, J., Yamanouchi, K., Naito, K. and Tojo, H. (2002) Expression of the gene of interest fused to the EGFP-expressing gene in transgenic mice derived from selected transgenic embryos. *Journal of Experimental Zoology,* **293**, 712-718.

[26] Ito, M., Yamanouchi, K., Naito, K. Calos, M.P. and Tojo, H. Site-specific integration of transgene targeting an endogenous lox-like site in early mouse embryos. *Journal of Applied Genetics*, in press. doi:10.1007/s13353-010-0011-3

[27] Pannetier, M., Tilly, G., Kocer, A., Hudrisier, M., Renault, L., Chesnais, N., Costa, J., Le Provost, F., Vaiman, D., Vilotte, J.L. and Pailhoux, E. (2006) Goat SRY induces testis development in XX transgenic mice. *FEBS Letters,* **580**, 3715-3720.

[28] Fong, G.H., Rossant, J., Gertsenstein, M. and Breitman, M.L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*, **376**, 66-70.

[29] Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L. and Schuh, A.C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature,* **376**, 62-66.

[30] Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J. and Moore, M.W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, **380**, 439-442.

[31] Dumont, D.J., Gradwohl, G., Fong, G.H., Puri, M.C., Gertsenstein, M., Auerbach, A. and Breitman, M.L. (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes and Development*, **8**, 1897-1909.

[32] Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., Yancopoulos, G.D. (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*, **87**, 1171-1180.

[33] Fadel, B.M., Boutet, S.C. and Quertermous, T. (1998) Functional analysis of the endothelial cell-specific Tie2/Tek promoter identifies unique protein-binding elements. *Biochemical Journal*, **330**, 335-343.

[34] Fadel, B.M., Boutet, S.C. and Quertermous, T. (1999) Octamer-dependent *in vivo* expression of the endothelial cell-specific TIE2 gene. *Journal of Biological Chemistry,* **274**, 20376-20383.

[35] Bowles, J., Schepers, G. and Koopman, P. (2000) Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Developmental Biology*, **227**, 239-255.

[36] Zhang, C., Basta, T. and Klymkowsky, M.W. (2005) SOX7 and SOX18 are essential for cardiogenesis in Xenopus. *Developmental. Dynamics*, **234**, 878-891.

[37] Young, N., Hahn, C.N., Poh, A., Dong, C., Wilhelm, D., Olsson, J., Muscat, G.E., Parsons, P., Gamble, J.R. and Koopman, P. (2006) Effect of disrupted SOX18 transcription factor function on tumor growth, vascularization, and endothelial development. *Journal of National Cancer Institute*, **98**, 1060-1067.

[38] Matsui, T., Kanai-Azuma, M., Hara, K., Matoba, S., Hiramatsu, R., Kawakami, H., Kurohmaru, M., Koopman, P. and Kanai, Y. (2006) Redundant roles of Sox17 and Sox18 in postnatal angiogenesis in mice. *Journal of Cellular Science*, **119**, 3513-3526.

[39] Sakamoto, Y., Hara, K., Kanai-Azuma, M., Matsui, T., Miura, Y., Tsunekawa, N., Kurohmaru, M., Saijoh, Y., Koopman, P. and Kanai, Y. (2007) Redundant roles of Sox17 and Sox18 in early cardiovascular development of mouse embryos. *Biochemical Biophysical Research Communications,* **360**, 539-544.

[40] Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R.J. and Schneider, M.D. (2007) Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 3859-3864.

[41] Francois, M., Caprini, A., Hosking, B., Orsenigo, F., Wilhelm, D., Browne, C., Paavonen, K., Karnezis, T., Shayan, R., Downes, M., Davidson, T., Tutt, D., Cheah, K.S., Stacker, S.A., Muscat, G.E., Achen, M.G., Dejana, E. and Koopman, P. (2008) Sox18 induces development of the lymphatic vasculature in mice. *Nature*, **456**, 643-647.

[42] Hosking, B., Francois, M., Wilhelm, D., Orsenigo, F., Caprini, A., Svingen, T., Tutt, D., Davidson, T., Browne, C., Dejana, E. and Koopman, P. (2009) Sox7 and Sox17 are strain-specific modifiers of the lymphangiogenic defects caused by Sox18 dysfunction in mice. *Development*, **136**, 2385-2391.

[43] Pennisi, D., Gardner, J., Chambers, D., Hosking, B., Peters, J., Muscat, G., Abbott, C. and Koopman, P. (2000) Mutations in Sox18 underlie cardiovascular and hair follicle defects in ragged mice. *Nature Genetics*, **24**, 434-437.

[44] James, K., Hosking, B., Gardner, J., Muscat, G.E. and Koopman, P. (2003) Sox18 mutations in the ragged mouse alleles ragged-like and opossum. *Genesis*, **36**, 1-6.

[45] Downes, M. and Koopman, P. (2001) SOX18 and the transcriptional regulation of blood vessel development. *Trends in Cardiovascular Medicine*, **11**, 318-324.

