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Characterization of Sry-related HMG box group F genes in zebrafish hematopoiesis

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Objective. **The roles of Sry-related HMG box (Sox) genes in zebrafish hematopoiesis are not clearly defined. In this study, we have characterized the sequence homology, gene expression, hematopoietic functions, and regulation of sox genes in F group (SoxF) in zebrafish embryos.**

Materials and Methods. **Expression of zebrafish SoxF genes were analyzed by whole-mount in situ hybridization, reverse transcription polymerase chain reaction, and real-time reverse transcription polymerase chain reaction of erythroid cells obtained from *Tg(gata1:GFP)* embryos by fluorescence-activated cell sorting. Roles of SoxF genes were analyzed in zebrafish embryos using morpholino knockdown and analyzed by whole-mount in situ hybridization and real-time reverse transcription polymerase chain reaction. Embryo patterning and vascular development were analyzed.**

Results. **All members, except *sox17*, contained a putative β-catenin binding site. *sox7* and *18* expressed primarily in the vasculature. *sox17* expressed in the intermediate cell mass and its knockdown significantly reduced primitive erythropoiesis at 18 hours post-fertilization (hpf). Definitive hematopoiesis was unaffected. Concomitant *sox7* and *sox18* knockdown disrupted vasculogenesis and angiogenesis, but not hematopoiesis. *sox32* knockdown delayed medial migration of hematopoietic and endothelial progenitors at 18 hpf and abolished *cmyb* expression at the caudal hematopoietic tissue at 48 hpf. These defects could be prevented by delaying its knockdown using a caged *sox32* morpholino uncaged at 10 hpf. Knockdown of SoxF genes significantly upregulated their own expression and that of *sox32* also upregulated *sox18* expression.**

Conclusions. ***sox17*** **helped to maintain primitive hematopoiesis, whereas *sox7* and *sox18* regulated angiogenesis and vasculogenesis. *sox32* affected both vascular and hematopoietic development through its effects on medial migration of the hematopoietic and endothelial progenitors.**

The Sry-related high-mobility-group box (Sox) gene family is a group of transcription factors sharing a high-mobility-group (HMG) box domain with >50% homology to that of sex-determining-region on the Y chromosome (Sry). The HMG box domain binds to the minor groove of DNA with preference for the sequence (A/T)AACAAT [1] and induces a large bend in the DNA strand [2]. This bending enhances the recruitment and binding of other transcription factors adjacent to the HMG box binding sites. In vertebrates, >20 sox genes have been identified. They are arranged in eight groups (A–H) based on sequence homology with those in *Drosophila melanogaster* and *Caenorhabditis elegans*. They also contain functional domains outside the HMG box at the carboxyl and amino terminal regions, which are conserved among members of the same group [3].

Information about sox gene functions was derived mostly from studies in gene targeting in mice and position cloning for human syndromes [4,5]. They are involved in

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diverse embryonic processes, including male sex determination, neural, skeletal and hair development, definitive endoderm development, cardiogenesis and angiogenesis, and, more recently, hematopoiesis [6–8]. *sox2* is also involved in maintaining pluripotency of stem cells, as exemplified by early studies in embryonic stem cells [9] and more recently in induced pluripotent stem cells [10].

In most vertebrates, the group F sox (SoxF) genes comprise *sox7*, *sox17* and *sox18*. Both *sox7* and *sox17* are expressed in the extra-embryonic endoderm in mouse embryos and *sox17* are also expressed in the definitive endoderm. Gene-targeted deletion of *sox17* in mice resulted in defective definitive endoderm [11]. Constitutive expression of *sox7* and *sox17* in human embryonic stem cells induces commitment of these cells to extra-embryonic endoderm and definitive endoderm lineage, respectively, highlighting the importance of these genes in early embryonic development [12]. *sox7* and *sox18* are involved in cardiogenesis and angiogenesis in xenopus [13] and zebrafish [14–16]. *sox18* could also play a role in lymphatic development in mice [17]. A divergent SoxF member known as *sox32* was found exclusively in teleosts and a loss-of-function *sox32* mutant *casanova* was deficient in gut endoderm development [18]. Despite the diverse function of sox genes during embryonic development, their role in hematopoiesis was unclear. Recently, conditional deletion of *sox17* in mice resulted in a lack of fetal definitive hematopoietic stem cells [8]. *sox7* expression was also detected in mouse hemangioblast population and was involved in the regulation of hematopoietic specification from mesodermal precursors [7].

Zebrafish has emerged as a model organism for the study of hematopoiesis during embryonic development. In this organism, primitive hematopoiesis occurs in the intermediate cell mass (ICM) in the first few days of life, followed by definitive hematopoiesis, which arises in the ventral wall of dorsal aorta (DA) at 24 hours post-fertilization (hpf). The genetic program of hematopoiesis is highly conserved and the embryos are amendable to genetic manipulation. In this study, we made use of the zebrafish model to investigate the gene expression, hematopoietic functions, and regulation of SoxF genes during embryonic development.

Materials and methods

Maintenance of zebrafish and collection of embryos

Wild-type zebrafish were obtained from local fish farms and maintained at 28°C as described [19]. Transgenic fish lines *Tg(TOP:dGFP)*^{w25} and *Tg(fli1a:EGFP)* were obtained from ZFIN (Eugene, OR, USA). Embryos obtained by natural spawning were maintained in embryo medium at 28.5°C and staged according to Kimmel et al. [20]. *Tg(gata1:GFP)* was a generous gift from Dr. Anming Meng (Tsinghua University, Beijing, China). The protocols for whole-mount in situ hybridization, morpholino, and messenger RNA microinjection, as well as real-time quantitative reverse transcription polymerase chain reaction (RT-PCR), have been described previously [21]. Embryos at 12, 14, and 18 hpf were staged according to the number of somites. This study has been approved by the Committee of the Use of Laboratory and Research Animals in the University of Hong Kong.

Sequence analysis of SoxF genes

Amino acid sequences for human SOX7 (NP_113627.1), SOX18 (NP_060889.1), and SOX17 (NP_071899.1) and zebrafish sox7 (NP_001074291.1), sox18 (XP_001337702.1), sox17 (NP_571362.2), and sox32 (NP_571926.1) were obtained from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). These sequences were aligned with ClustalW (http://align.genome.jp/) using default parameters and the final output was generated with BioEdit (version 7.0.5.3).

Synthesis of zebrafish SoxF riboprobes for whole-mount in situ hybridization

mRNA from 24hpf zebrafish embryos was reverse transcribed using the SuperScript II RT kit (Invitrogen, Carlsbad, CA, USA) and segments of complementary DNA of *sox7*, *sox17*, *sox18* and *sox32* were amplified (Supplementary Table E1; online only, available at www.exphem.org). The amplified products were subcloned into pGEM-T vector (pGEM-T Vector Systems, Promega, Madison, WI, USA). Anti-sense and sense riboprobes were synthesized from these clones by in vitro transcription using SP6 and T7 RNA polymerase (Roche Applied Science, Indianapolis, IN, USA).

Morpholino knockdown of zebrafish SoxF genes

Morpholinos that specifically bind to the start codon and the donor splice site of *sox17* were designed by Genetools LLC (Philomath, OR, USA). Morpholinos for *sox7*, *sox18,* and *sox32* have been described previously [14,22]. The standard morpholino, which did not match any known zebrafish gene, served as the control (Supplementary Table E1; online only, available at www.exphem.org). To generate mRNA for rescue experiments, the full coding region of *sox7*, *sox17,* and *sox18* were amplified from complementary DNA derived from 18hpf zebrafish embryos (Supplementary Table E1; online only, available at www.exphem.org) and the amplified products were subcloned into pGEM-T vector and in vitro transcribed into sense mRNA with a poly(A) tail (mMessage mMachine and poly(A) tailing kits; Ambion, Austin, TX, USA). Silent mutations around the translational start site were generated for *sox17* transcript using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) so that the translation of the injected mRNA was not inhibited by the morpholino. To test the molecular targeting by *sox17* morpholino, a fusion transcript containing the morpholino binding site and enhanced green fluorescent protein was generated by subcloning a partial region of *sox17* into the pcDNA3.1/CT-GFP-TOPO vector using the CT-GFP Fusion TOPO Expression Kit (Invitrogen). Plasmid containing the fusion gene was injected directly into the embryos at 1-cell stage.

Caged morpholino knockdown

Conditional *sox32* knockdown at defined developmental stage was accomplished using caged morpholino (Photomorph; SuperNova Life Science, Auburn, WA, USA) as described previously [23]. A molar ratio of caging strand to morpholino of 5:1 were mixed together, incubated at 70°C for 30 minutes, then at 4°C overnight.