CONSERVED EXPRESSION OF SOX13 ORTHOLOGS IN EARLY VERTEBRATE DEVELOPMENT

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Running head: Sox13 expression in vertebrate development

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Abstract

The skin and nervous tissue is derived from the ectoderm[1, 2]. In *Xenopus*, ectodermal explants (animal caps) from blastula embryos show high tissue plasticity and can differentiate into a variety of tissues *in vitro*. Exploiting this property, we performed a functional screening for factors that can neuralize ectodermal explants, and isolated *Xenopus* Sox13 (XSox13), a member of the Sox (Sry-related high-mobility-group box) transcription factor family. During *Xenopus* embryogenesis, *XSox13* mRNA is expressed in the entire ectoderm at blastula stages and in the organizer region at gastrula stages. Its expression becomes localized to the neural tube during neurulation and then to somites at tailbud stages. Mouse *Sox13*mRNA shows similar expression patterns to the *Xenopus* homolog during embryogenesis: *Sox13* is expressed in the node, an equivalent to the *Xenopus* organizer, at the neural fold stage, exclusively in the nervous tissue at early-mid somite stages, and then showed a segmental expression in the somites at the late somite stage. We next generated *Sox13-LacZ*-knock-in mice, and examined the expression of mouse Sox13in adult tissues by X-gal staining. In contrast to the expression during embryogenesis, *Sox13* is scarcely expressed in the central nervous system in adult. Moreover, *Sox13*-deficient mice showed no apparent abnormalities in neural development. These results suggest that Sox13 expression in early development is conserved in *Xenopus* and mouse and Sox13 plays a redundant role during mouse neural development.

Key words: Sox transcription factor family, Sox13, neural development

Introduction

The skin and nervous tissue is derived from the ectoderm. Neural induction represents the earliest step in the determination of ectodermal cell fates. In vertebrates, bone morphogenetic proteins (BMPs) act as signals of epidermal induction. The inhibition of the BMP signaling pathway in the ectoderm is the hallmark of neural-fate acquisition[3].

To examine the molecular mechanism underlying determination of ectoderm, *Xenopus laevis*(African clawed frog) is a useful model organism, because it is easy to manipulate the gastrula and neurula embryos and to examine gene expression patterns at various developmental stages from blastula to tailbud by whole mount in situ hybridization. In *Xenopus*, neural induction appears to occur at an early stage, from the blastula to gastrula stages, by BMP inhibitors that are secreted from the gastrula organizer[1, 2]. Early patterning along the anteroposterior (AP) axis in the neuroectoderm is thought to be defined by posteriorizing factors, such as retinoic acid (RA), fibroblast growth factors (FGFs) and Wnts[4]. The neuroectoderm subsequently forms the neural plate and then the neural tube.

Ectodermal explants (animal caps) from *Xenopus* blastula embryos show high tissue plasticity and can differentiate into a variety of tissues *in vitro*. Exploiting this unique property of *Xenopus* embryo, we performed a functional screening to identify genes with neutralizing activity of the ectoderm. Among the genes we identified, in this study we focus *Xenopus* Sox13 and its mouse homolog. The Sox (Sry-related high-mobility-group box) gene encodes a transcription factor family, which plays crucial roles in the determination of the cell fate and organogenesis[5]. The Sox family members have a DNA binding high-mobility group (HMG) domain that binds to a core sequence, ATTGTT, and are structurally classified into 8 groups,

A-H[6] . The group D is composed of Sox5, Sox6 and Sox13, and characterized by a group-specific coiled-coil domain in the N-terminal half, which mediates homo- and heterodimerization of the SoxD proteins with each other[5,7]. Although SoxD proteins participate in transcriptional activation and repression in a context-dependent manner, the exact transactivation or transrepression domain has not yet been identified.

Sox13 gain-of-function and loss-of-function mutations have revealed that Sox13 plays a critical role in the γδT cell development by inhibiting canonical Wnt signaling[8]. However, the knowledge on the biological roles of Sox13 is largely limited. Here we compared the expression patterns of Xsox13 and mouse Sox13 during early embryogenesis. Then we examined the expression of Sox13 in adult tissues using *Sox13-LacZ*-knock-in mice. The possible roles of Sox13 in early and late vertebrate development are discussed.

Materials and Methods

Xenopus embryo manipulation

Artificial fertilization and culture of embryonic tissues were performed as described previously[9]. Embryos were staged according to Nieuwkoop and Faber[10]. Animal cap assay was performed as previously described[11, 12].

Construction and functional screening of an Anterior Neuroectoderm library

About 500 pieces of anterior neural plates were dissected from stage 12-12.5 embryos. The neuroectoderm layer was separated from the underlying mesendoderm layer in 1x modified Barth's solution containing 1-2 mg/ml collagenase. After poly (A)+ RNA selection by an

oligo(dT) cellulose column, a cDNA library was made with the Super Script plasmid system for cDNA synthesis and plasmid cloning (Invitrogen) and the pCS105 vector. Two hundred pools, each containing about 200 independent cDNA clones, were prepared, and capped RNA was synthesized from each pool as previously described[11]. Capped RNA (10 ng) was injected into the animal pole region of one-cell stage embryos. Animal caps were dissected at stages 8-9 (blastula stages) and cultured until siblings reached stage 25.

Whole-mount in situ hybridization

Whole-mount in situ hybridization of *Xenopus* embryos was performed according to Harland[13] using an automated system (AIH-101, Aloka). An antisense *XSox13* RNA probe was synthesized by transcribing a NotI-linearized clone pCS105-XSox13 with T7 RNA polymerase. Some stained embryos were embedded in paraffin wax and sectioned at 10-15 mm. Whole-mount in situ hybridization of mouse embryos was performed as described previously[14] with the exception that hybridization was performed at 70°C. Digoxygenin (DIG)-labeled antisense RNA probes were synthesized with the DIG RNA Labeling mix (Roche).

Real-time Reverse transcriptase (RT)-PCR

Animal caps and marginal explants were dissected when siblings reached the tailbud stage, and real-time RT-PCR was carried out as previously described[15] using ABI PRISM 7000 (Applied Biosystems). Each real-time RT-PCR was performed in triplicate. EF-1α was used as an internal control and each bar was normalized to the level of EF-1α expression.

Generation of Sox13-LacZ-Knockin mice

The details of generation *Sox13-LacZ*-Knockin mice was described elsewhere (manuscript in preparation). Briefly, the second exon of the mouse Sox13 gene containing the initiation codon was replaced with the *LacZ* gene.

X-gal staining

Adult mouse tissues were fixed in 4% paraformaldehyde at room temperature for 60 minutes. The fixed tissues were washed in PBS and incubated at 37℃in freshly prepared X gal(5-bromo-4-chloro-3-indolyl-β-D-galactosidase, TAKARA) staining solution (1mg/ml X gal, 34mM potassium ferrocyanide, and 35mM potassium ferricianide) for overnight. After incubation, the tissues were quickly washed in PBS and post-fixed in 4% paraformaldehyde at 4℃ overnight.

RESULTS

Functional screening for neuralizing factors

We screened 200 pools (approximately 40,000 independent clones) for activity to induce a pan-neural marker, *nrp1*, in animal caps by RT-PCR or whole-mount in situ hybridization. Twenty-one of the pools showed such neuralizing activity. These were further sib-selected, and the single clones that were responsible for neuralizing the ectoderm were isolated (Fig.1). We found that these clones included BF2[16], Xsox3[17], Geminin[18],SoxD[19], Xiro3[20] and Zic3[21], which are all expressed in the anterior neuroectoderm and possess neuralizing activity, indicating that our strategy worked well. Among these, we focused on clone A133.

In *Xenopus* embryos, a blastula-stage ectodermal cell can adopt various fates, depending on the signaling inputs it receives. Activation of the BMP pathway in the ectoderm leads to the acquisition of epidermal fates, whereas inhibition of BMP signaling induces neural fates. As shown in Fig.2A, uninjected animal caps developed into atypical epidermis without expression of *nrp1*. When mRNA for noggin, a BMP antagonist, was injected, *nrp-1* was induced in animal caps. Injection of A133 mRNA also induced *nrp1*expression. Consistently, A133 induced *nrp-1* expression in animal caps in a dose-dependent manner r (Fig.2B), but not *β-actin*, a dorsal mesoderm marker (Fig. 2C).

A BLAST search revealed that A133 encoded the C-terminal region of *Xenopus* Sox13 (XSox13). We isolated a full-length clone for the XSox13 by screening a stage 30 head cDNA library using A133 as a probe. Amino acid sequence revealed XSox13 has a leucine-rich domain as well as a HMG domain, both of which are conserved among the E group of the Sox family.

Expression profile of XSox13

Developmental expression patterns of XSox13 were analyzed by RT-PCR using RNA isolated from different stages of *Xenopus* embryos and by whole-mount in situ hybridization. Fig. 3A shows that *XSox13* transcripts are present as maternally expressed genes, and the expression levels are relatively constant throughout early embryogenesis. At the late blastula stage, *XSox13* expression was detected in the entire animal hemisphere (Fig. 3B, C). During gastrulation, *XSox13* was expressed in the dorsal mesoderm above the dorsal lip (Fig.3D). In sagittal sections of embryos at this stage, *XSox13* was strongly expressed in the organizer region (Fig.3E). At the

mid-neurula stage, *XSox13* expression was detected around the blastopore and the anterior ectoderm (Fig.3F). At the late neurula stage, its expression became localized in the neural plate (Fig.3G, I). Examination of a section of a stage 16 embryo confirmed that *XSox13*was expressed in the neural plate, but not in the notochord (Fig.3H). At tailbud stages, *XSox13* was expressed in various regions including the central nervous system (CNS), somites, eyes, otic vesicles, and bronchial arches (Fig. 3J, K, K'), although the expression in the CNS became obscure.

Mouse Sox13 is expressed in neural tissues during embryogenesis.

We next examined mouse *Sox13* expression during early embryogenesis by whole mount in situ hybridization. At E7.5 *Sox13* was expressed in the headfold and allantois, whereas no signals were detected with a sense probe (Fig.4A, B). A cryosection of the embryo revealed that *Sox13* was expressed in the node, an equivalent of the amphibian Spemann organizer (Fig.3C, D). At E8.5 *Sox13* was strongly expressed in the neural tube (Fig.4E). A coronal section of the embryo showed *Sox13* was expressed in the neural tube and foregut (Fig.4F). The *Sox13* expression in the neural tissues continued during somite stages (Fig.4G, H). At E11.5, in addition to the CNS and eyes, *Sox13* showed a segmental expression along the AP axis (Fig.4I). Sagittal sections clarified that *Sox13* was expressed in the somites (Fig.4J, K).

Mouse Sox13 expression in adult tissues

To analyze the role of Sox13 in mouse development, we generated *Sox13-LacZ*-knock-in mice and examined the expression of Sox13 in a variety of adult tissues including the cerebrum, cerebellum, eye, heart, lung, liver, kidney, spleen, stomach, small intestine, colon, celiac lymph

nodes, uterus, and fallopian tube by X-gal staining. In contrast to the expression profile during embryogenesis, *LacZ* expression was scarcely expressed in the CNS in adult. Moreover, *Sox13*-deficient mice showed no apparent abnormalities in neural development. In the lung, *LacZ* was expressed in the epithelium of the terminal bronchiole, but not in alveoli (Fig.5A). The mouse stomach is separated into two well-defined areas, a forestomach with a stratified squamous epithelium and a glandular stomach with a columnar epithelium. As shown in Fig.5B, *LacZ* expression was restricted to the base of the columnar epithelium of the glandular stomach (gastric unit). *LacZ* was also detected in the uterus, where its expression was restricted to the glandular epithelium (Fig.5C). *LacZ* expression was not detected in other organs we examined.

Discussion

Here we isolated XSox13 as a neuralizing factor using the functional screening (Fig.1). When XSox13 was overexpressed in ectodermal explants, *nrp-1*, a panneural marker, was induced without inducing mesoderm in a dose-dependent manner (Fig.2). This is the first report that XSox13 has a neutralizing activity *in vitro*, although it remains to be elucidated whether XSox13 is involved in *Xenopus* neural development *in vivo*. In *Xenopus*, BMP-2, -4, and -7 are expressed in the entire ectoderm at blastula stages and act as signals of epidermal induction[3)]. The inhibition of the BMP signaling pathway in the ectoderm is the hallmark of neural-fate acquisition. We showed that *XSox13* transcripts are also present in the entire ectoderm at blastula stages, suggesting that there are some interactions between the BMP signaling pathway and XSox13. It is an important question whether XSox13 induces neural tissues acting as an antagonist of the BMP signaling pathway. Preliminary data showed that in BMP4-expressed ectodermal explants, the expression of *XSox13* was up-regulated (data not shown). Given that XSox13 antagonizes the BMP pathway, this result suggests that BMP4 induces its own antagonist during neural induction in the ectoderm.

XSox13 is expressed in the Spemann organizer region during gastrulation and its mouse orthologis expressed in the node, an equivalent tissue of the organizer (Fig. 3, 4). The organizer and node play a central role in establishing the embryonic body axes[22)]. In this process, anteroposterior patterning and morphogenic movement of the neural and mesodermal tissues occur cooperatively. In addition to the “organizer” region, XSox13 and mouse Sox13 are also expressed in neural tissues during neurulation stages and expressed in the somite at later stages. These results suggest that the roles of Xsox13 and mouse Sox13 are conserved during

evolution.

Conserved expression in early development between XSox13 and mouse Sox13 imply that their expressions are regulated by a similar mechanism. To examine this possibility, we compared the promoter regions of both organisms. As a result, we found several conserved regions with sequence identity more than 60%. One of the regions of about 600 bp contains several putative binding sites for transcription factors, including Smads, mediators of the TGFβ and BMP signaling pathway (data not shown), Sox. Upregulaton of Sox13 mRNA by BMP in ectodermal explants described above may be mediated through the Smad binding site. The presence of the putative binding site for Sox suggests that the expression of Sox13 is regulated by other Sox family members.

The formation of neural tissues was not affected in *Sox13*-deficient mice, suggesting that Sox13 plays a redundant role for the neural tissue formation. There are more than 30 members of the Sox family in vertebrate and Sox B1 and SoxB2 subgroups are expressed in the CNS[23]. SoxB1 subgroup includes Sox1, Sox2, and Sox3, all of which are co-expressed in proliferating neural progenitors of the embryonic and adult CNS. SoxB2 subgroup includes Sox14 and Sox21; Sox21 is widely expressed in the developing CNS, whereas Sox14 expression is limited to small subset of interneurons in the developing CNS. Sox family members regulate transcription as homodimers or heterodimers with other Sox members or other transcription factors[5]. Thus, we speculated that the lack of Sox13 in developing CNS is compensated by other members of the Sox family.

Sox13-LacZ-knock-in mice have revealed novel expression domains of Sox13 in adult tissues: Sox13 is expressed in the epithelia of the lung, stomach and uterus. The terminal

bronchiole is covered with brush cells and Clara cells which are involved in secretion of surfactant proteins. The glandular stomach is consisted of parietal cells, mucous neck cells and chief cells, which secret pepsinogen, mucus and gastric acid, respectively. The uterine gland is implicated in secretion of mucus. Although Sox13 was not detected in all the secretory epithelia these results suggest that Sox13 is involved in regulation of secretion at least in bronchial, gastric, and uterine epithelia. We are now seeking to identify the Sox13-expressiong cells in these epithelia.

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Figure Legend

Fig.1 Strategy for functional screening for genes with neutralizing activity. The unamplified anterior neuroectoderm cDNA library was divided into small pools which included about 100 independent clones. RNA pools were made from the individual pools and injected into the animal poles of the *Xenopus* embryos. Ectodermal explants, or animal caps, were dissected from the embryos and assayed for the expression of *nrp-1*, a neural marker. The positive pools were further divided into subpools and repeated sib-selection to isolate single clones.

Fig.2 XSox13 showed neutralizing activity in ectodermal explants. (A) Whole-mount in situ analysis for nrp1 in *Xenopus* extodermal explants injected with no mRNA, mRNA for noggin, and mRNA for XSox13. (B) Real-time RT-PCR analysis of animal caps injected with XSox13 mRNA. WE, stage25 whole embryo; NT, no template.

Fig.3 Temporospatial expression of XSox13. (A) Analysis of temporal expression pattern of XSox13 by RT-PCR with RNA from various developmental stages. FGFR, a loading control. Whole mount in situ hybridization analysis of XSox13 during early development (B-K'). (B) Late blastula stage (animal view). (C) Late blastula stage (vegetal view). (D) Mid gastrula stage (vegetal view). (E) Sagittal hemisection of mid gastrula. (F) Neural plate stage (dorsal view; anterior to the top). (G) Neural fold stage (dorsal view; anterior to the top). (H) Coronal section of late neurula. (I) Neural tube stage (dorsal view; anterior to the top). (J) Coronal section of a

neural tube stage embryo (dorsal to the top). (K) Tail bud stage (dorsal view; anterior to the left). (K') The embryo in K with longer staining. DL, dorsal lip; BP, blastopore; NP, neural plate; NC, notochord; CN, cephalic neural crest; NT, neural tube; So, somite, Ey, eye; OV, otic vesicle; BA, branchial arches.

Fig.4 Expression profile of mouse Sox13 during mouse early development. Whole mount in situ hybridization with the sense (A) and antisense (B, E, G, H and I) probes. (A, B) E7.5 embryo (neural-fold stage; A, dorsal view; B, anterior to the left). (C) A cryosection of the E7.5 embryo shown in B. (D) A closer view of the boxed area in C. Note that *Sox13* mRNA is expressed in the node. (E) E8.5 (somite stage, dorsal view). (F) Coronal section of the embryo in E through the plane indicated in E. (G) E9.5 embryo. (H) A dorsal view of the embryo in G (anterior to the top). (I) E11 embryo (40-somite-stage). (J) A sagittal section of the embryo shown in I. Note that (K) A closer view of J. hf, headfold; al, allantois, n, node; nf, neural fold, nt, neural tube; fg, foregut; fb, forebrain; mb, mid-barin; hb, hindbrian; so, somites, ba, branchial arch; fl, forelimb.

Fig.5 X-gal staining of adult mouse tissue. *LacZ* expression in the lung (A), the grandular stomach (B) and the uterus (C). TB, terminal bronchiole; A, alveoli; GU, gastric unit; UG, uterus gland; L, lumen of uterus.

Fig.1

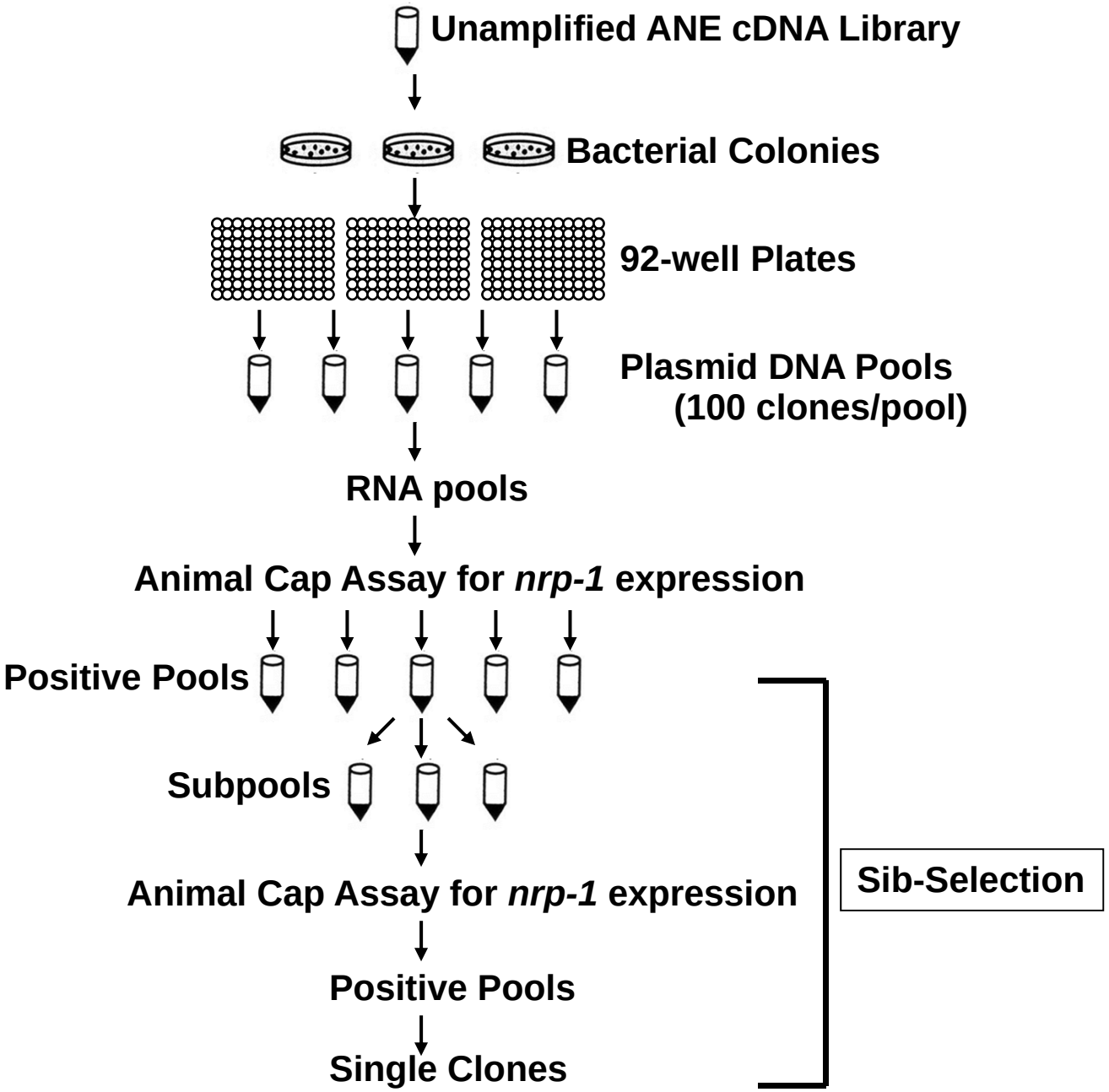
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Fig.2

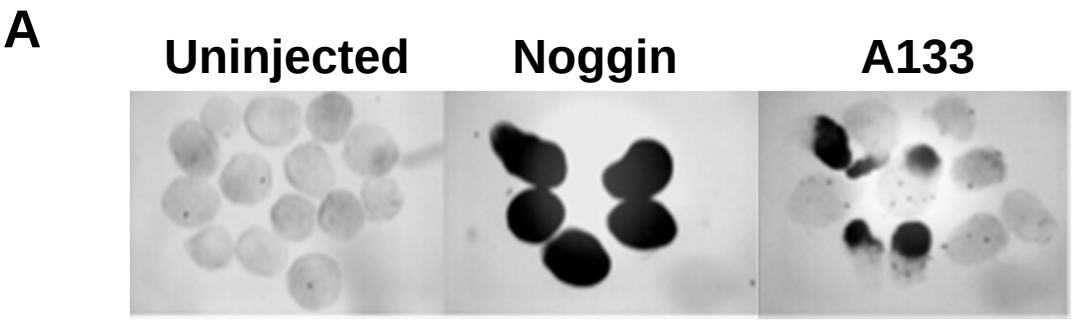
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Fig.3

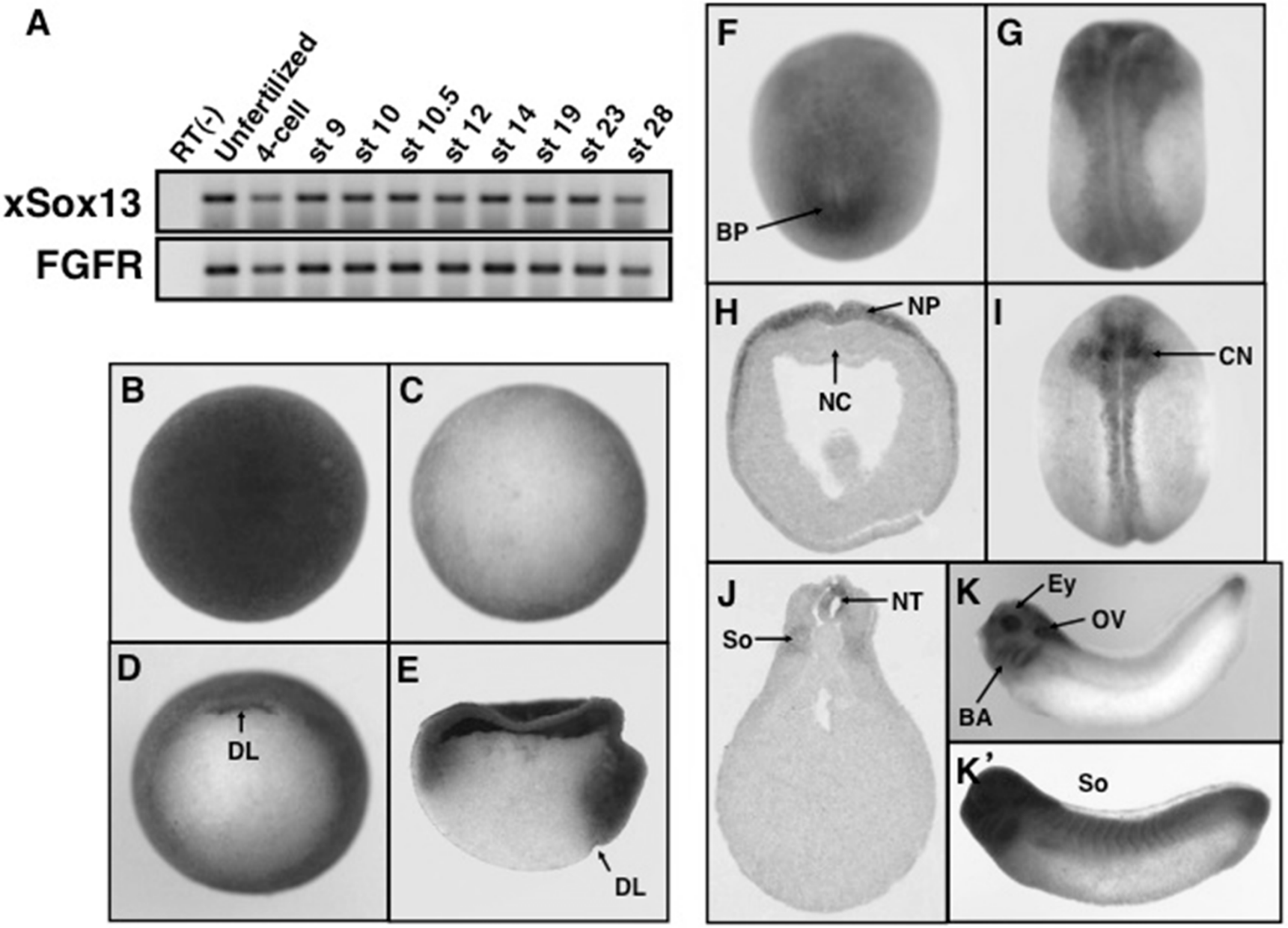
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Fig.4

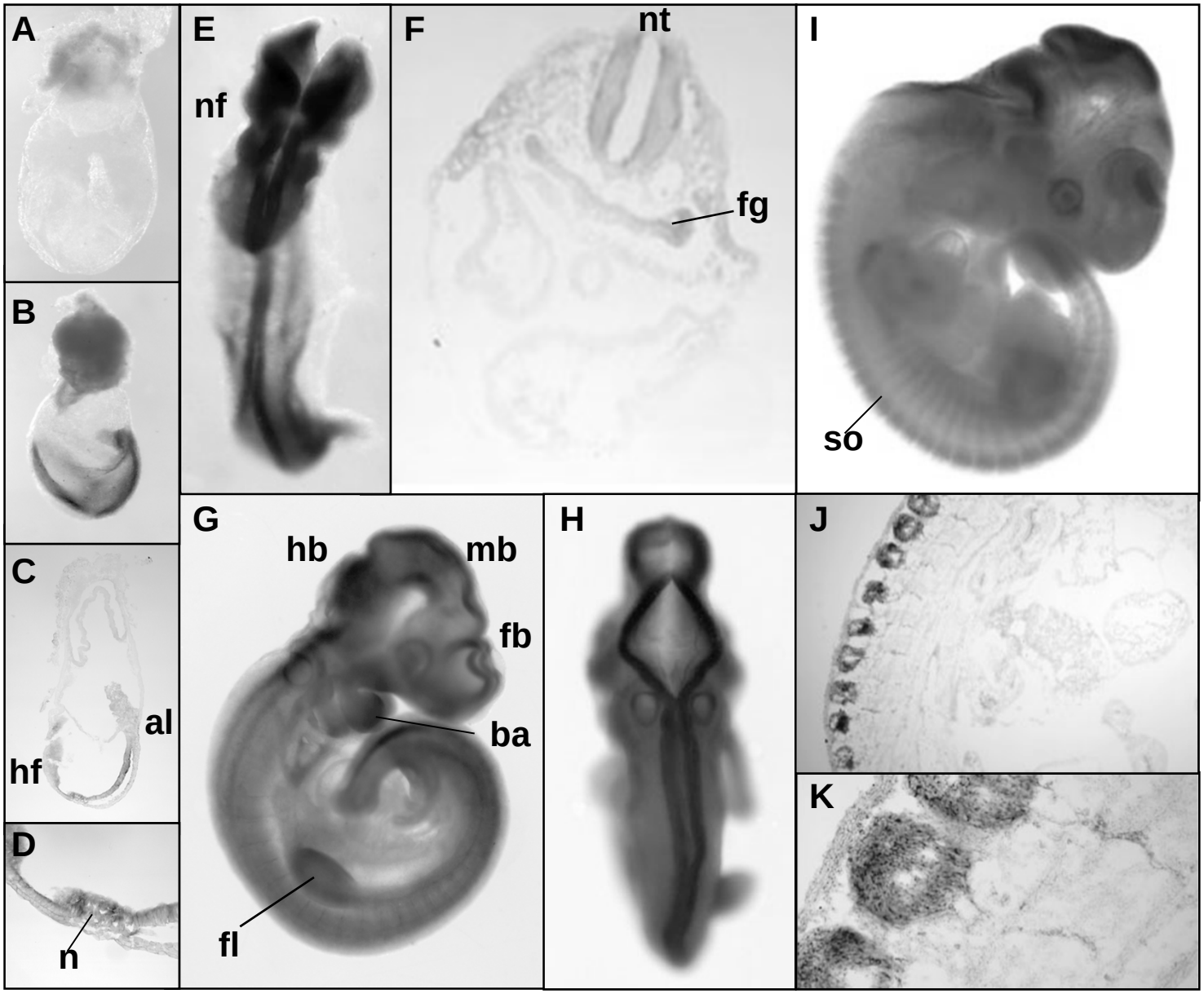
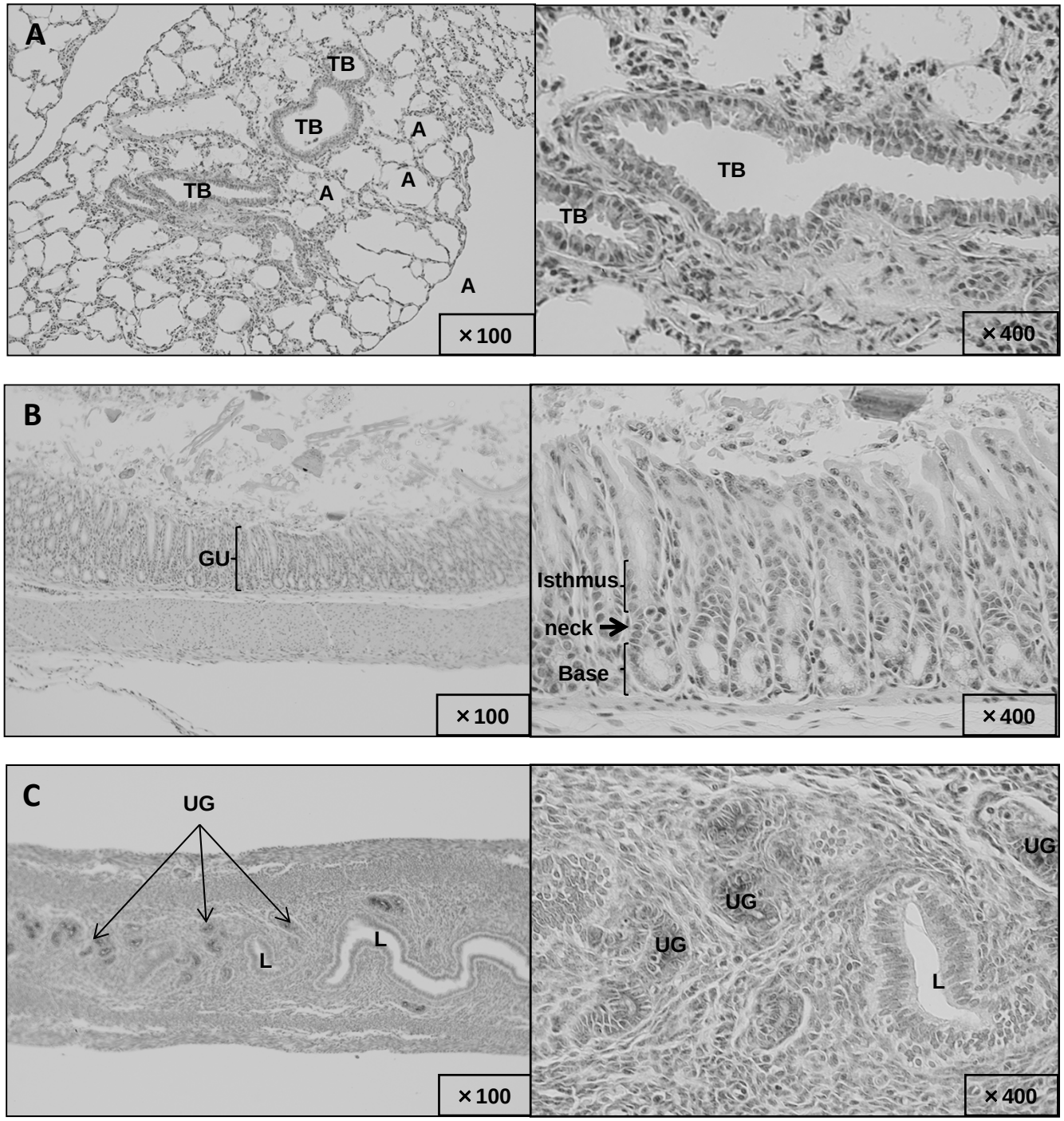


Fig.5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