Live Image Profiling of Neural Crest Lineages in Zebrafish Transgenic Lines

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Zebrafish transgenic lines are important experimental tools for lineage tracing and imaging studies. It is crucial to precisely characterize the cell lineages labeled in transgenic lines to understand their limitations and thus properly interpret the data obtained from their use; only then can we confidently select a line appropriate for our particular research objectives. Here we profiled the cell lineages labeled in the closely related neural crest transgenic lines *Tg(foxd3:GFP)*, *Tg(sox10:eGFP)* and *Tg(sox10:mRFP)*. These fish were crossed to generate embryos, in which *foxd3* and *sox10* transgenic neural crest labeling could be directly compared at the cellular level using live confocal imaging. We have identified key differences in the cell lineages labeled in each line during early neural crest development and demonstrated that the most anterior cranial neural crest cells initially migrating out of neural tube at the level of forebrain and anterior midbrain express *sox10:eGFP* and *sox10:mRFP*, but not *foxd3:GFP*. This differential profile was robustly maintained in the differentiating progeny of the neural crest lineages until 3.5dpf. Our data will enable researchers to make an informed choice in selecting transgenic lines for future neural crest research.

INTRODUCTION

During vertebrate development, multipotent neural crest progenitor cells are generated from the neural plate border between neural and non-neural ectoderm. Following neurulation, neural crest cells are located at the dorsal neural tube, from which they migrate extensively in a rostrocaudal wave, forming distinct streams and differentiating into several distinct tissue types, including neurons, glia, cartilage, bone and melanocytes. (Bronner-Fraser and Fraser, 1988; Knecht and Bronner-Fraser, 2002; Nicole Le Douarin, 1999; Selleck et al., 1993).

The transcription factors *Foxd3* and *Sox10* are both important regulators of early neural crest development. *Foxd3* was shown to be important for cell proliferation, glial differentiation and neural patterning, depending on its embryonic context (Dottori et al., 2001; Dutton et al., 2008; Lister et al., 2006; Mundell et

al., 2012). *Sox10* is believed to act in a dosage-dependent manner in the peripheral nervous system to maintain neural crest stem cell identity by indirectly regulating the expression of basic helix-loop-helix factors (Kim et al., 2003). Its expression is downregulated in differentiating neurons but maintained in glia (Deal et al., 2006; Kim et al., 2003).

Zebrafish transgenic lines expressing fluorescent proteins under the control of *foxd3* or *sox10* promoters are valuable tools in the field of neural crest research (Carney et al., 2006; Dutton et al., 2008; Gilmour et al., 2002; Kirby et al., 2006). Cells can be directly observed as they are born, migrate and differentiate within intact embryos using confocal microscopy: the rapid development of the transparent zebrafish embryo is particularly well suited to such applications. It is important however to carefully profile the lineages labeled in transgenic lines as the promoters and enhancers used may not precisely mimic the endogenous expression profile of the targeted gene. In particular, transgenic lines often maintain marker gene expression longer than the endogenous transcript, thus they may not necessarily reflect the endogenous spatial and temporal expression patterns at later stages of development. This can be a disadvantage in that the dynamic regulation of endogenous gene function cannot be accurately determined. However, these transgenic lines allow cell lineage tracing to be performed easily for specific populations of cells, and also permit dynamic live imaging of labeled tissues and organs during development.

Here we used confocal microscopy to characterize in detail the early neural crest lineages labeled in *Tg(foxd3:GFP)*, *Tg(sox10:eGFP)* and *Tg(sox10:mRFP)* transgenic lines (Carney et al., 2006; Gilmour et al., 2002; Kirby et al., 2006). Our spatial and temporal analysis revealed differences in tissue labelling among these lines, and identified a subpopulation of the most anterior neural crest cells differentially labeled in *foxd3:GFP* embryos. These findings should be taken into consideration when selecting transgenic lines for neural crest imaging studies.

MATERIALS AND METHODS

Zebrafish husbandry

Zebrafish (*Danio rerio*) adults and juveniles of the transgenic

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Fig. 1. Differential labeling of the most anterior cranial neural crest cell lineages in the neural crest transgenic lines. (A-D) Dorsal views of live *Tg(sox10:eGFP/sox10:mRFP)* embryo with anterior to the top left at the 16 somite-stage. Merged brightfield and fluorescent images of embryo at 10 $\times$ (A) and individual fluorescent channels at 20 $\times$ magnification (B-D) show both fluorescent proteins evenly label the cranial neural crest cells from the most anterior to posterior regions. The anterior migrating neural crest streams (1-3) contribute to the first (mandibular), second (hyoid), and third pharyngeal arches respectively. (E-L) Dorsal views of *Tg(foxd3:GFP/sox10:mRFP)* embryo with anterior to the top left. Merged brightfield and fluorescent images of a 13 somite-stage embryo at 10 $\times$ (E) and individual fluorescent channels at 20 $\times$ magnification (F-H) reveal that the most anterior cranial neural crest cells at the midbrain level express only *sox10:mRFP* but not *foxd3:GFP* (arrowhead). The anterior neural crest cells migrate more anteriorly toward the forebrain and ventrally around the eyes and maintain transgene expression, eventually contributing to the trigeminal ganglia and anterior pharyngeal arches [(I-L) 17 somite stage, 10 $\times$ magnification]. (M, N) Lateral views of *Tg(foxd3:GFP/sox10:mRFP)* embryo with anterior to the left at 24 hpf at 10 $\times$ (M) and 20 $\times$ (N) magnification. The olfactory epithelium (split arrowhead) was labeled by *sox10:eGFP*, the otic vesicle by *sox10:mRFP* and maxillary (arrowhead), and mandibular (1, arrow) and caudal pharyngeal arches (2, 3) by both fluorescent proteins. (O, P) Lateral views of *Tg(foxd3:GFP/sox10:mRFP)* embryo with anterior to the left at 24 hpf at 10 $\times$ (O) and 20 $\times$ (P) magnification. *foxd3:GFP* exclusively labeled the pineal complex and its fine process (asterisk) and anterior pigment cells (split arrowhead). The most anterior neural crest derivatives express *sox10:mRFP* but not *foxd3:GFP* (arrowhead). Scale bars, 200 μ m in (A), (E), (I), (M), and (O); 100 μ m in (B-D), (F-H), (J-L), (N), and (P). e, eye; o, otic placode; ov, otic vesicle; 1-7, pharyngeal arches.

reporter strains *Tg(foxd3:gfp)zf15* (Gilmour et al., 2002), *Tg(p7.2sox10:mrp)* (Kirby et al., 2006), and *Tg(-4.9sox10:egfp)ba2* (Carney et al., 2006) were maintained in balanced salt water at 27.5 $^{\circ}$ C in a 14/10 h light/dark cycle. Embryos were

raised in the incubator at 28.5 $^{\circ}$ C and staged by hours post-fertilization (hpf) or days post-fertilization (dpf), according to Kimmel et al. (1995).

eGFP (split arrowhead). (I) Magnified (40 $\times$) view of boxed region from (H), showing trigeminal ganglia, otic vesicle, pectoral fin, and pharyngeal arches (rectangle). (J) Many oligodendrocytes and their processes are labeled by both fluorescent proteins (arrows). However, a dorsal subpopulation of oligodendrocytes are exclusively labeled by *sox10:eGFP* (arrowhead). Scale bars, 200 μ m in (A), (C), and (H); 100 μ m in (B), (I), and (J); 50 μ m in (D-F); 20 μ m in (G). ao, anterior otolith; e, eye; ep, epiphysis; lg, lateral line ganglia; ov, otic vesicle; p, pectoral fin; pa, pharyngeal arch; po, posterior otolith; tg, trigeminal ganglia.

Mounting zebrafish embryos for imaging

Zebrafish embryos were selected for imaging, dechorinated, and anesthetized by adding Tricaine (Sigma 886-86-2) to E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) until animals were unresponsive to touch. A well was prepared by hole-punching 5 layers of electrical tape on a glass slide, and this was filled with 1% agarose (Biosesang 9012-36-6). Embryos were positioned in holes made in the agarose using a heated needle, covered by a drop of 1.5% low melting agarose (Invitrogen 15517-022) in E3 medium and mounted with a glass coverslip. Older embryos were treated with 5% phenylthiourea (PTU, Sigma P7629) to inhibit melanin synthesis.

Confocal microscopy

During live imaging, fluorescence images of the transgenic embryos were acquired at 10 $\times$, 20 $\times$, and 40 $\times$ magnification using a LSM780 NLO confocal microscope with 10 $\times$ dry objective and 20 $\times$ or 40 $\times$ water immersion objective lens (Carl Zeiss, Germany). *eGFP* was detected within a range of 500-550 nm following fluorophore excitation by the 488 nm Ar-laser. For *mRFP*, detection was in the 570-630 range following excitation by the 561 nm HeNe laser. Z-stacks were created in depths of 50-100 μ m with intervals of 2-10 μ m. All acquired images were

processed by the projection of a Z-stack, including adjustment of brightness and contrast, using ZEN2011 software (Carl Zeiss). Panoramic whole embryo images were assembled from multiple 10 $\times$ magnification confocal images.

RESULTS AND DISCUSSION

Sox10:eGFP and *Sox10:mRFP* differentially label developing tissues

We firstly compared the expression of *sox10:eGFP* with that of *sox10:mRFP* by crossing the lines to generate double labeled *Tg(sox10:eGFP/sox10:mRFP)* embryos. Confocal image projections at the 16 somite stage showed that both *sox10:eGFP* and *sox10:mRFP* uniformly labeled the early migrating cranial neural crest cells from the most anterior to most posterior levels (Figs. 1A-1D). Analysis of individual fluorescent channels confirmed that expression of *sox10:mRFP* (Fig. 1C) and *sox10:eGFP* (Fig. 1D) was overlapping in migrating neural crest cells at all axial levels.

At later stages of development however distinct differences between the expression domains of *sox10:eGFP* and *sox10:mRFP* were observed (Figs. 1M, 1N, and 2A-2J). At 20 hpf, the otic placode was specifically labeled by *sox10:mRFP* (Figs. 2A and 2B; arrow) and transgene expression was maintained

Fig. 3. Comparison of labeled cell lineages in *Tg(foxd3:GFP/sox10:mRFP)* embryo at 2.5 dpf. (A) Brightfield lateral view of a live 2.5 dpf embryo at 10× magnification. (B) Fluorescent image projection of whole embryo *foxd3:GFP* and *sox10:mRFP* labelling. Boxed regions are shown in detail in lower panels. (C) Projection image of the caudal hindbrain showing segmentally clustered *foxd3:GFP* positive neurons within the neural tube. Cell bodies are positioned dorsally and processes project toward the ventral lateral neural tube (arrowhead). (D) Boxed region from (C) showing oligodendrocytes labeled with *sox10:mRFP* within the neural tube behind the otic vesicle (arrow). (E) Boxed region from (C) showing neurons in the ventral neural tube caudal to the segmental region of *foxd3:GFP* neurons that exclusively express *sox10:mRFP*. Dividing neurons are indicated by arrowheads. (F-H) A small number of neurons within the spinal cord were selectively labeled by *Foxd3:GFP* [(F, H), white arrow]. *sox10:mRFP* strongly labeled the ventral neural tube and oligodendrocytes within the spinal cord [(F), white arrowhead]. Schwann cell precursors extended their membrane structures labeled by *sox10:mRFP* from the ventral neural tube [(F, G), purple arrows] and the distant processes of the Schwann cells were evenly labeled by both fluorescent proteins [(F-H), purple arrowhead], forming segmentally iterated tubes. (I) Glial precursors associated with the posterior lateral line nerve were labeled by both fluorescent proteins (arrow). Arrowhead indicates pigment cell. (J) Lateral line ganglia precursors are labeled by both fluorescent proteins (arrow). (K) The pectoral fin was exclusively labeled by *sox10:mRFP*. Scale bars, 200 μm in (A, B); 50 μm in (C), (F-J); 20 μm in (D), (E), (K). e, eye; hb, hindbrain; mb, midbrain; nc, notochord; nt, neural tube; ov, otic vesicle; p, pectoral fin; px, pineal complex; y, yolk; ye, yolk extension.

at 45 hpf, after otocyst formation (Fig. 2C). During otic morphogenesis, epithelial protrusions extend from opposing walls of the otocyst to form the semicircular canals and *sox10:mRFP* expression was detected throughout these tissues (Figs. 2D-2F). In contrast, *sox10:eGFP* was expressed in only a small subpopulation of otic cells (Figs. 2E and 2F). Olfactory ensheathing cells derived from neural crest were labeled by *sox10:eGFP* (Figs. 1M, 2A, and 2C; split arrowhead). In addition, glial cells caudal to the olfactory epithelial cells and subpopulations of oligodendrocytes in the brain and spinal cord were also exclusively labeled by *sox10:eGFP* (Figs. 2C and 2H; arrow). The trigeminal ganglia, pharyngeal arches and pectoral fin were all labeled by both fluorescent proteins (Figs. 2C and 2G-2I). The glial cells associated with lateral line ganglia (Ig) and dorsal root ganglia (arrowhead) were also double-labeled (Fig. 2J), while a subpopulation of oligodendrocytes positioned close to the dor-

sal neural tube was exclusively labeled by *sox10:eGFP* (Fig. 2J; arrowhead).

Thus although both *Sox10* transgenic lines appear to similarly label early neural crest populations, clear differences exist in later lineages and in other non-neural crest derived tissues.

***foxd3:GFP* is absent from anterior neural crest lineages**

We next generated *Tg(foxd3:GFP/sox10:mRFP)* embryos by crossing *Tg(foxd3:GFP)* with *Tg(sox10:mRFP)*. At 4 to 6 somite stages, *foxd3:GFP* labeled the mesoderm, including notochord and somites, while *sox10:mRFP* labeled both neural and non-neural ectoderm as well as the neural tube prior to neural crest emigration (data not shown). We observed that the most anterior cranial neural crest cells at the level of the posterior dienkephalon to anterior midbrain expressed *sox10:mRFP* but not *foxd3:GFP* at the 13 somite stage (Figs. 1E-1H; arrowheads).

Fig. 4. Expression profiling of live *Tg(foxd3:egfp/sox10:mRFP)* embryo at 3.5 dpf. (A) Dorsal view of a live whole embryo with anterior to the top. (B) Dorsal view of the cranial region. The pineal complex (arrow) is specifically labeled by *foxd3:GFP*. Boxed regions are shown in detail in other panels. (C) Magnified dorsal view of pineal neurons in the pineal complex (arrow). Also visible is the parapineal organ, a small asymmetric organ attached to the left side of the pineal complex (arrowhead). Oligodendrocytes are shown (asterisk). (D) Lateral view of the pineal complex (arrow) revealed its ventral processes (arrowhead) at 40× magnification. (E) Dorsal view of the midbrain/hindbrain boundary at 40× magnification showing *foxd3:GFP* positive neurons (arrow) at the anterior dorsal region and *sox10:mRFP* positive glial cells (arrowhead) more posteriorly. Split arrowhead indicates neurons labeled with *foxd3:GFP* and oligodendrocytes labeled with *sox10:mRFP*. (F) Dorsal neurons of the caudal hindbrain with ventrally extending processes were strongly labeled by *foxd3:GFP* (arrow) at 40× magnification. (G) Image projection of eye showing *foxd3:GFP* positive rod cells in the outer nuclear layer at 40× magnification. (H) Lateral view of cranial region profiling *foxd3:GFP* and *sox10:mRFP* descendant tissues. *sox10:mRFP* strongly labels the cartilages of maxillary, mandibular and caudal pharyngeal arches (arrow), trigeminal ganglia (asterisk), otic vesicle and pectoral fin. (I) Zebrafish inner ear at 3.5 dpf highlighting the position of the anterior and posterior otoliths. (J) In the spinal cord, *foxd3:GFP* strongly labels a small number of neurons (arrow) while *sox10:mRFP* strongly labels oligodendrocytes (arrowhead). Scale bars, 200 μm in (A, B, and H); 100 μm in (J); 50 μm in (C, F-G, and I); 20 μm in (D). ac, anterior canal; ao, anterior otolith; asc, anterior semicircular canal; e, eye; ep, epiphysis; lsc, lumen of semicircular canal; md, mandibular arch; mh, midbrain-hindbrain boundary; mx, maxillary arch; ov, otic vesicle; p, pectoral fin; pc, posterior canal; po, posterior otolith; psc, posterior semicircular canal; px, pineal complex.

By the 17 somite stage the anterior neural crest cell population exclusively labeled with *sox10:mRFP* had migrated further toward the anterior forebrain and ventrally around the eyes (Figs. 11-1L; arrowheads), and eventually contributed to anterior structures such as the trigeminal ganglia and maxillary and mandibular arches by 24 hpf (Figs. 10O and 1P; arrow). Both *sox10:eGFP* and *sox10:mRFP* strongly labeled anterior neural crest derivatives (Figs. 1M and 1N).

In contrast, *foxd3:GFP*-labeled cells did not contribute to the maxillary arch and the anterior region of the mandibular arch at later stage (Figs. 10O and 1P; arrowhead), but were found in a

subpopulation of the posterior mandibular arch and the more caudal arches (Figs. 10O and 1P; arrow). In 24 hpf embryos, *foxd3:GFP* exclusively labeled the photoreceptive pineal complex and its fine processes (Figs. 10O and 1P; asterisk) as well as a subpopulation of pigment cells, especially around the eye and head, at this stage (Figs. 10O and 1P; split arrowhead) as previously shown (Gilmour et al., 2002; Snelson et al., 2008).

Similar differential transgene expression was evident at 53 hpf (Fig. 3); *foxd3:GFP* exclusively labeled the pineal complex (Fig. 3B), a dorsolateral population of neurons clustered in a segmented pattern in the caudal hindbrain (Figs. 3B and 3C),

and a subpopulation of oligodendrocytes within the neural tube (Figs. 3F and 3H; white arrow). In contrast, *sox10:mRFP* specifically labeled the otic vesicle (Figs. 3A-3C), neurons, and oligodendrocytes of the medial and ventral cranial neural tube (Figs. 3B-3E) and spinal cord (Figs. 3F-3I), and the pectoral fin (Figs. 3B and 3K) as previously shown (Cox et al., 2011; Kirby et al., 2006). A population of Schwann cell precursors migrating from the ventral neural tube along spinal axons towards the lateral line were also labeled by *sox10:mRFP* only (Figs. 3F-3H). These cells appeared to emerge from the spinal cord and join loosely with Schwann cells labeled by both *foxd3:GFP* and *sox10:mRFP* (Figs. 3F-3H; purple arrowhead). The caudally migrating glial precursors of the posterior lateral line nerve were also double labeled in *foxd3:GFP/sox10:mRFP* embryos at 53 hpf (Fig. 3I) as previously shown (Gilmour et al., 2002; Snelson et al., 2008). In the posterior trunk, dorsal root ganglia precursors were labeled by both *foxd3:GFP* and *sox10:mRFP* (Fig. 3J; arrowhead).

Live imaging of *foxd3:GFP/sox10:mRFP* embryos at 3.5 dpf revealed that most tissues labeled with fluorescent proteins at earlier stages strongly maintained their expression (Fig. 4). The pineal complex continued to express *foxd3:GFP* (Figs. 4A and 4B), and expression was also observed in the parapineal organ (Fig. 4C), a small asymmetrically positioned structure attached to the left side of the pineal complex (Snelson et al., 2008). Subpopulations of cells in the midbrain-hindbrain boundary region were differentially labeled in *foxd3* and *sox10* transgenic lines at 3.5 dpf. An anterior population of dorsal neurons was positive for *foxd3:GFP*, while *sox10:mRFP* labeled dorsal glial cells more posteriorly (Fig. 4E). Dorsally located oligodendrocytes with ventrally projecting processes were strongly labeled by *foxd3:GFP* at the level of the caudal otic vesicle at this stage (Fig. 4F; arrow). *foxd3:GFP* also exclusively labeled the rod cells of the outer nuclear layer in the eye (Figs. 4G and 4H). *sox10:mRFP* expression was maintained in the cartilages of maxillary, mandibular and pharyngeal arches, trigeminal ganglia (asterisk), otic vesicle and pectoral fin at 3.5 dpf (Figs. 4H and 4I). A small number of oligodendrocytes inside the neural tube and Schwann cells ventrally extending their processes from the neural tube were labeled by *foxd3:GFP* (Fig. 4J; arrow), while oligodendrocytes and their processes in the ventral neural tube of the spinal cord were highly labeled by *sox10:mRFP* (Fig. 4J; arrowhead).

Foxd3 and Sox10 transcription factors are both essential for neural crest development during early embryogenesis and zebrafish transgenic lines expressing fluorescent proteins under the control of *foxd3* or *sox10* promoters are therefore valuable tools for neural crest research. In this study, we directly compared populations of early neural crest cell lineages and other embryonic tissues labeled with fluorescent proteins in *foxd3:GFP*, *sox10:eGFP*, and *sox10:mRFP* zebrafish lines by live confocal imaging. We identified key differences in spatial and temporal expression of the fluorescent proteins among the three transgenic lineages and discovered that a subpopulation of the most anterior cranial neural crest and their progeny do not express *foxd3:GFP*. The two *sox10* transgenic lines used in this study differ in GFP expression in several different tissues. This may be caused by differences in promoter size: *sox10:mRFP* is expressed under the control of a 7.9 kb promoter sequence located immediately upstream of the Sox10 coding region, while *sox10:eGFP* expression is driven by a smaller 4.9 kb fragment from the 3' end of the same sequence. It is however also possible that the genomic integration sites of the expression constructs may affect promoter activity. Detailed ex-

pression profiling studies such as ours will help researchers select appropriate transgenic lines for future research.

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