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Secondary neurulation-fated cells in the tail bud undergo self-renewal and
tubulogenesis regulated by a Sox2 gradient

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1 **Abstract**

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3   **During amniote development, anterior and posterior components of the**

4 **neural tube form by primary neurulation (PN) and secondary neurulation (SN),**

5 **respectively. Unlike PN, SN proceeds by the mesenchymal-to-epithelial transition**

6 **of SN precursors in the tail bud, a critical structure for the axial elongation. Our**

7 **direct cell labeling delineates non-overlapping territories of SN- and mesodermal**

8 **precursors in the chicken tail bud. SN-fated precursors are further divided into**

9 **self-renewing and differentiating cells, a decision regulated by graded expression**

10 **levels of Sox2. Whereas Sox2 is confined to SN precursors, Brachyury (T) is widely**

11 **and uniformly distributed in the tail bud, indicating that Sox2^{+}/Brachyury^{+} cells**

12 **are neural-fated and not mesodermal. These results uncover multiple steps during**

13 **the neural posterior elongation, including precocious segregation of SN precursors,**

14 **their self-renewal, and regulation by graded Sox 2.**

15

Introduction

In amniotes (mammals, birds and reptiles), the neural tube is composed of a neuroepithelium that forms through two processes along the anterior posterior (AP) axis. These processes are primary neurulation (PN) and secondary neurulation (SN). The PN occurs in the anterior region of the body comprising the future brain and spinal cord in thoracic regions, where an epithelial cell sheet (neural plate) invaginates to make a tubular structure (Colas & Schoenwolf, 2001, Copp *et al.*, 2003, Saitsu *et al.*, 2004). In contrast, the SN-mediated neural tube, located posterior to the lumbar/hind limbs (in chickens and humans), is achieved by the mesenchymal-to-epithelial transition (MET) of SN precursors in the tail bud (Hughes & Freeman, 1974, Pasteels, 1937, Schoenwolf, 1979, Holmdahl, 1938). The tail bud is a transient structure located at the posterior end of the embryo during axial elongation, and it continuously supplies cells to the region of the neural tube undergoing SN (designated as the secondary neural tube). The secondary neural tube eventually provides innervations that govern physiological functions for a wide variety of organs including the colon, bladder, genitalia, and tail movement (Copp *et al.*, 2015, Le Douarin & Kalcheim, 1999, Snell, 1995, Stiefel *et al.*, 2007). In addition, a failure of SN is implicated to be a cause of neural tube defects (NTDs) including *spina bifida*, which are among the most common congenital malformations in humans (Copp *et al.*, 2015, Copp *et al.*, 2013, Dady *et al.*, 2014, Detrait *et al.*, 2005, Greenberg *et al.*, 2011, Marks & Khoshnood, 1998). It is therefore important to elucidate the mechanisms by which the secondary neural tube formation is regulated. However, the ways that the SN precursors accomplish the tasks of neural differentiation and tubular morphogenesis remain poorly understood.

The tail bud also provides cells that form the paraxial mesoderm of the posterior embryo, as well as the midline components of the notochord and floor plate during the axial elongation (Cambray & Wilson, 2002, Catala *et al.*, 1996, Catala *et al.*, 1995). However, how these different lineages become specifically located in the tail bud remains undetermined, although several different hypotheses have been proposed. A main reason for our ignorance is that cells constituting the tail bud are mesenchymal in shape, hampering morphological delineation between the lineages (Colas & Schoenwolf, 2001, McGrew *et al.*, 2008). To solve this long-standing question, a cell-labeling technique that directly visualizes SN precursors must be useful.

We previously used direct cell labeling in the embryonic chicken tail bud

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1   (Shimokita & Takahashi, 2011) to show that SN-precursors are derived from a specific

2   region of the epiblast, called the presumptive SN (preSN). This preSN is located along

3   the midline posterior to the Hensen's node at Hamburger and Hamilton stage 8 (HH 8;

4   Fig. 1A-D). The preSN at this stage is unique for two features. First, this specific region

5   of epiblast is not underlain by the primitive streak. Rather, the anterior end of the

6   primitive streak is apart posteriorly from the preSN by 200-250 µm (Fig. 1A). Second,

7   the preSN lacks the underlying basement membrane, thereby allowing preSN-epithelial

8   cells to ingress by the epithelial-to-mesenchymal transition (EMT) in a manner similar

9   to mesodermal ingression from the primitive streak. Labeling either by DiI or by

10   EGFP-electroporation revealed that the preSN-derived ingressed cells constitute the tail

11   bud where they remain mesenchymal until they are incorporated into the secondary

12   neural tube (Shimokita & Takahashi, 2011). Little or no mesodermal cells are derived

13   from the preSN region.

14   By extending these findings, we noticed that despite the massive elongation of

15   the secondary neural tube that continuously receives SN precursors, the amount of

16   labeled SN precursors in the tail bud remains constant as seen at HH14 (23ss) and

17   HH17 (30ss) (Fig. 1B-D). This raised the possibility that SN precursors would possess a

18   self-renewing ability in the tail bud. In this study, we have tested this possibility by

19   following a precise delineation of the SN-fated cell population in the tail bud. We have

20   found that the SN-precursors in the tail bud are composed of two groups of cells,

21   self-renewing cells and neural-specified transitional cells. The SN precursor territory

22   including these two subpopulations expresses both Sox 2 and Brachyury, with the Sox 2

23   level decreasing posteriorly. This Sox 2 gradient plays roles in the regulation of SN

24   precursors in the tail bud.

25

26   **Material and Methods**

27   **Chicken embryos**

28   Fertilized chicken eggs were obtained from Shiroyama poultry farm

29   (Sagamihara, Japan). Embryos were staged according to Hamburger and Hamilton

30   (Hamburger & Hamilton, 1951) or the somite number. All animal experiments were

31   conducted with the ethical approval by Kyoto University (No. H2620).

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Vector constructions

pCAGGS- EGFP was as previously described (Momose *et al.*, 1999). cDNAs encoding Sox2 and Sox3HMG-EnR were provided by Drs. K. Fukuda (Tokyo Metropolitan University) and Y. Sasai (CDB Riken), respectively. The cDNAs were individually subcloned into the Mul I-Nhe I site of pBI-EGFP or pBI-DsRed vector (Watanabe *et al.*, 2007).

In ovo **electroporation**

An anode and cathode were prepared with a platinum wire (diameter of 0.3– 0.5 mm) and a sharpened tungsten needle (40 μm diameter at the tip), respectively. The *in ovo* electroporation was carried out at HH8 according to the method previously reported (Momose *et al.*, 1999, Nakaya *et al.*, 2004) with slight modifications: the anode was inserted in between the embryo and yolk, and a DNA solution containing 2% fast green FCF (Nakarai) was laid on the epiblast, followed by electric charges five times of 5 V, 25 ms with 100 ms intervals (Electro Square Porator ECM830; BTX) using the cathode. Maximal attention was paid so that the DNA solution did not spread into the primitive streak, from which mesoderm arises.

PKH-labeling

The presumptive SN region in the epiblast or primitive streak of HH 8 (6 somites stage) embryos were labeled with PKH26 Red Fluorescent Cell Linker (Sigma) or PKH67 Green Fluorescent Cell Linker (Sigma) using a micropipette pulled from a 1 mm glass capillary in a vertical micropipette puller (model PC-10; Narishige).

In situ **hybridization**

Whole-mount in situ hybridization was performed as previously described (Henrique *et al.*, 1995, Atsuta & Takahashi, 2015) with some modifications. Embryos were fixed overnight in phosphate-buffered saline, PBS (0.1M Tris-HCl [pH 7.5], 0.15M NaCl) containing 4% paraformaldehyde (PFA), 100 mmol∕L 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.4), 2 mmol∕L ethylene glycol tetraacetic acid (EGTA), and 1 mmol∕L $MgSO_4 \cdot 7H_2O$. After washing twice in 0.1 % Tween 20 in PBS (PBST), specimens were dehydrated by a successively graduated series of methanol in PBST (40%–100%). They were subsequently treated with proteinase K (20 μg∕mL) for

1   15 min, followed by refixation in 4% PFA in PBS for 20 min at room temperature (RT).

2   After two PBST washes, the embryos were transferred to hybridization buffer

3   (ULTRAhyb, Ambion) and prehybridized for 1 hr at 68 °C. Hybridization was carried

4   out overnight at 68 °C in the hybridization buffer containing a digoxigenin

5   (DIG)-labeled RNA probe. The embryos were washed three times for 30 min each in

6   50% formamide, 5 x standard saline citrate (SSC), 1% ethylenediaminetetraacetic acid

7   (EDTA), 0.2% Tween 20, 0.5% 3-[(3- Cholamidopropyl) dimethylammonio]

8   propanesulfonate (CHAPS), at 68 °C. They were further washed in 0.1 mol∕L Maleic

9   Acid (pH 7.4), 0.15 mol∕L NaCl, 1% Tween 20 (MABT) at least three times, prior to

10   preblocking in 2% BBR in MABT. Hybridization was carried out by incubating

11   embryos in blocking solution, which contained alkaline phosphatase-conjugated

12   anti-DIG antibody (Roche), overnight. After the embryos were extensively washed in

13   MABT for at least 5 h with several changes of solutions, they were processed to NTMT

14   (100 mmol∕L Tris-HCl [pH 9.5], 100 mmol /L NaCl, 50 mmol∕L MgCl$_2$, 0.1% Tween

15   20). The alkaline phosphatase activity was visualized by incubating embryos in NTMT

16   containing 0.45 mg∕mL nitroblue-tetrazolium chloride (Roche) and 0.175 mg∕mL

17   5-bromo-4-chloro-3-indolyl phosphatase (Roche). After stopping the color reaction,

18   embryos were post-fixed in 0.1% glutaraldehyde /4% PFA in PBS for 30 min at 4 °C.

19

20   **Immunohistochemistry**

21   Embryos were fixed for 2 hours in PBS containing 3 % PFA at 4°C, followed

22   by preparation of cryostat sections 10 μm thick. The detection of the Sox2 or Brachyury

23   proteins (T) was performed as follows: after pre-blocking with 2 % blocking reagent

24   (Roche)/PBS for 1 hr at RT, the sections were incubated at 4 °C overnight with either of

25   the following antibodies in 1% blocking reagent (BBR, Roche)∕PBS: anti-Sox2 mouse

26   polyclonal antibody (ab5603; Millipore) (Oginuma *et al.*, 2017) diluted 1:100, and

27   anti-Brachyury goat polyclonal antibody (AF2018; R&D Systems) (Nakanoh *et al.*,

28   2017) diluted 1:100, anti-Fibronectin antibody (F3648; Sigma) (Yoshino *et al.*, 2014),

29   anti-GFP antibody (11814460001, Roche) diluted 1:300. After washing three times in

30   PBST, the specimens were reacted with either of the following second antibodies:

31   anti-rabbit IgG-Alexa 568 conjugated donkey antibody (A10042, Invitrogen), anti-goat

32   IgG-Alexa 488 conjugated donkey antibody (A11055, Invitrogen), anti-rabbit

33   IgG-Alexa 647 conjugated donkey antibody (A31573, Invitrogen), anti-mouse

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1 IgG-Alexa 488 conjugated donkey antibody (R37114, Invitrogen) diluted 1:400 with

2 1% blocking reagent /PBS for 1 hr at RT. The reaction was terminated by washing three

3 times in PBS, and the sections were sealed by VectaShield (Funakoshi).

4

5 **Results**

6 **SN precursors are segregated from mesodermal cells in the tail bud at HH14**

7 To precisely locate the SN precursors and other lineages in the tail bud, we

8 labeled the preSN region by PKH 67 (green) and the anterior primitive streak by

9 PKH26 (red) in a single HH8 embryo having 6-somite (6ss). Dorsal views of these

10 embryos at HH14 (24ss) showed a domain of preSN-derived cells that was distinct from

11 that of anterior primitive streak-derived paraxial mesodermal precursors in the tail bud

12 (Fig. 1A-D, n=123; Fig. 1E, F, n=26). The preSN-derived cells remained restricted to

13 the secondary neural tube-forming territory with little, if any, contribution to the

14 mesoderm. In sagittal histological sections, the reciprocal segregation is also obvious

15 between the SN- and mesodermal populations in the tail bud: SN precursors (green)

16 occupy the anterior half of the tail bud with the posterior half being populated by

17 mesodermal cells (red) (Fig. 1G-J, n=12). As expected, neither SN- nor paraxial

18 mesodermal precursors were found in the chord neural hinge (CNH), known to give rise

19 specifically to the notochord and floor plate (Catala *et al*., 1995). These observations

20 demonstrate that a vast majority of cells of the tail bud are differentially fated at least at

21 HH14. The segregation between SN- and mesoderm territories in the tail bud was

22 retained at HH17 (30ss), where mesodermal population was located more ventrally to

23 the SN precursors, and was not overtly visible in the dorsal view (Fig. 1D; n=4).

24

25 **SN precursors in the tail bud contain self-renewing cells**

26 Since SN precursors are constantly present in the tail bud during the

27 secondary neural tube elongation, we reasoned that these precursors contain

28 self-renewing cells. To test this, we prepared HH17 (30ss) embryos in which SN

29 precursors had been EGFP-labeled (Fig. 2A). From these EGFP-embryos, a piece was

30 dissected from the SN precursor domain of the tail bud, and transplanted into the

31 corresponding region of the tail bud at the presumptive 30-32 somite level of a

32 non-electroporated HH14 embryo (24ss) (Fig. 2A-D; n=8). Even though the

33 experimental approaches in this study allowed us to specifically trace the EGFP-labeled

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1   SN-precursors, we paid maximal attention not to take contamination of unlabeled

2   ventral cells in the tail bud that contain CNH and some paraxial mesoderm-fated cells.

3   When these manipulated embryos were allowed to develop until HH17 (30ss),

4   EGFP-positive cells were, as expected, found in the epithelialized neural tube posterior

5   to somite level 31-33. Importantly, EGFP signals were also detected in the

6   mesenchymal population in the tail bud (Fig. 2C, E; 6 out of 8). We further performed a

7   serial transplantation to a third host embryo (Fig. 3F, G; n=6), and observed again

8   EGFP-positive cells both in the secondary neural tube posterior to the 31-33 somite

9   level and in the tail bud (4 out of 6). These results indicate the existence of

10   self-renewing SN precursors in the tail bud.

11   To more precisely locate these self-renewing cells in the SN precursor

12   territory of the tail bud, we performed similar experiments, but this time we separated

13   the anterior and posterior halves of the SN precursor domain, and transplanted each of

14   them into its equivalent domain of stage-matched embryos (HH14). When the anterior

15   half was transplanted, EGFP-positive cells were found solely in the differentiated neural

16   tube posterior to the presumptive somite level 31-33 in HH17 embryos (Fig. 2J-L; 6 out

17   of 7). In clear contrast, EGFP-cells from the posterior half of the SN precursor domain

18   were found in both the epithelialized neural tube and undifferentiated mesenchymal

19   population in the tail bud (Fig. 2M-O; 5 out of 7). Thus, SN precursors in the tail bud

20   are subdivided at least into two different subpopulations: one is anteriorly located and

21   specified to neural epithelialization/differentiation; and the other is the compartment of

22   posterior cells maintained as undifferentiated. When the posterior half was transplanted,

23   EGFP-positive cells in the secondary neural tube were located posteriorly to somite pair

24   35 and not to somite pairs 31-33, thereby suggesting that it takes 3-6 hrs for the

25   self-renewing cells to be incorporated to the forming neural tube (one cycle of somite

26   segmentation is 90 min in chickens) (Palmeirim *et al*., 1997).

27

28   **Graded expression of Sox 2 in SN precursors**

29   To explore the molecular mechanisms by which the two different

30   subpopulations of SN precursors are regulated and distinguished, we paid attention to

31   the expression of the *Sox2* gene, known to be active at the earliest stages of PN and SN

32   neural tube formation (Uchikawa *et al*., 2011). Indeed, the presence of *Sox2* mRNA is

33   concomitant with neural epithelialization during the SN process (Shimokita &

8

1   Takahashi, 2011). In the current study, we carefully examined expression levels of Sox

2   2 in the tail bud of HH14 (23ss) chicken embryos. In situ hybridization for *Sox2* mRNA

3   revealed expression in the *anterior* domain of SN precursors, whereas signals were

4   barely detectable in the *posterior* domain (Fig. 3A, B; n=6). We therefore switched to

5   immunohistochemistry using anti-Sox2 antibody, allowing sensitive detection of the

6   Sox2 protein. Confocal microscopic analyses virtualized nuclear Sox2 protein both in

7   the anterior and posterior domains of SN precursors with graded signals deceasing

8   posteriorly. This notion was supported by the quantitative evaluation (Fig. 3C, F; n=15).

9   We also compared the staining pattern of Sox2 with that of Brachyury (T)

10   protein in the tail bud of HH14 embryos. It was previously shown that in very early

11   mouse embryos, a specific epiblastic region that gives rise to both neural and

12   mesodermal progenitors (NMPs) expresses both Sox2 and Brachyury (Bra), and since

13   then, the $Sox2^+/Bra^+$ double-positive signal has been proposed as a marker for NMPs

14   (Olivera-Martinez *et al*., 2012, Wymeersch *et al*., 2016, Gouti *et al*., 2014). Contrasting

15   with these proposals, we found that the Brachyury protein was widely distributed in the

16   tail bud as shown in the sagittal section in Fig. 3D (n=10). Most importantly, the Bra^+

17   region included the $Sox2^+$ precursor territory, that is, almost the entire anterior half of

18   the Bra^+ domain was $Sox2^+/Bra^+$ double positive. Furthermore, unlike Sox2, the signal

19   intensity of Bra was relatively uniform throughout in the tail bud, although it dropped

20   abruptly following neural epithelialization, as expected (Fig. 3G; n=6). Thus, the

21   mesenchymal cells of the HH14 tail bud were positive for Bra regardless of their

22   developmental fate.

23   To further corroborate our observation that Sox2 expression was restricted to

24   the neural fate in the tail bud, we double stained a sagittal section of HH14 tail bud for

25   the Sox2 protein (red) and the fate of the anterior primitive streak-derived mesoderm

26   (PKH-labeling). As expected, the mesoderm territory was negative for Sox2 (Fig. 3H;

27   n=14).

28   These results revealed an intimate correlation between the differential levels

29   Sox2 expression and different states of SN precursors in the tail bud: low and high

30   levels of Sox 2 for self-renewing- and SN-specified populations, respectively.

31

32   **Augmented level of Sox 2 expression directed SN precursors to precocious**

33   **differentiation/epithelialization**

1   To know whether differential levels Sox2 expression are functionally related
2   to the two groups SN precursors, we experimentally elevated the level of Sox2
3   expression in these cells. Similar to the experiments shown in Fig 1, the *in ovo*
4   electroporation was carried out at HH8 (6ss) to target the preSN epiblastic region.
5   However, if the overexpressed Sox 2 inhibited EMT/ingression of the epiblast, the
6   electroporated cells would end up in neural epithelia at later stages, which might
7   mislead interpretation. To avoid this possible confusion, we switched on the *Sox2*
8   expression after the electroporated preSN-derived cells ingressed as mesenchyme, using
9   the Tet-on expression system (Watanabe, et al., 2007).

10   As shown in Fig. 4A, three plasmids were co-electroporated:
11   pCAGGS-mCherry (constitutively active), pBI-Sox2/EGFP (BI, previously called TRE,
12   is a bidirectional promoter induced by doxycycline, an analog of tetracycline; Dox), and
13   pCAGGS-Tet-On 3G (transcriptional activator which binds the tetracycline responsive
14   element (TRE) only in the presence of Dox). When electroporated embryos reached
15   HH14 (22ss), a Dox solution was administered. At the time of administration (0 hr), a
16   decent amount of ingressed cells were already found in the tail bud, which expressed
17   mCherry but not EGFP (Fig. 4A, B, E; n=7).

18   By 3 hrs post Dox at HH14 (24ss), EGFP/Sox2 started to be expressed in SN
19   precursors in the tail bud, which is consistent with the previous report that a TRE-driven
20   gene starts to be expressed after 3 hr of Dox injection (Watanabe, et al., 2007). It is
21   known that the *in ovo* electroporation in chickens yields a mosaic pattern of transfected
22   cells, and also that if two different plasmids, mCherry and EGFP, are co-electroporated,
23   three types of transfected cells emerge: one double positive (mCherry$^+$/EGFP$^+$), and two
24   single positives (mCherry$^+$/EGFP$^-$ or mCherry$^-$/EGFP$^+$). Indeed, at 3 hrs post Dox, these
25   three types of cells were observed in *EGFP*/*Sox2*-electroporated SN precursors in the
26   tail bud, which was comparable to EGFP-electroporated control cells. (Fig. 4A, C, F;
27   n=7).

28   In contrast to 3 hrs, at 9 hrs post Dox, that is 6 hrs after the onset of *Sox 2*
29   expression, distribution patterns of *EGFP*/*Sox2*-electroporated SN precursors were
30   significantly different from those in control *EGFP*-electroporated embryos. In the
31   control tail bud, all the three types, mCherry$^+$/EGFP$^+$, mCherry$^+$/EGFP$^-$ and
32   mCherry$^-$/EGFP$^+$ cells, were observed (Fig. 4E, F; n=7). However, in
33   *EGFP*/*Sox2*-electroporated embryos, only mCherry$^+$ but no EGFP$^+$ cells were detected

1   in the tail bud (Fig. 4J, K; n=11). In addition, although the anteriorly formed neural tube

2   contained double-positive mCherry$^+$/EGFP$^+$ cells, and single positive mCherry$^-$/EGFP$^+$

3   or mCherry$^+$/EGFP$^-$ cells, the nasant area of epithelialized cells at the boundary between

4   the tail bud was preferentially occupied only by mCherry$^+$/EGFP$^-$ cells (Fig 4. J, K, near

5   white arrow). Since we observed no sign of apoptosis in the tail bud in these embryos

6   (not shown), it is most likely that the Sox2-elevated SN precursors underwent

7   precocious differentiation/epithelialization.

8

9   **Inhibition of Sox2 prevented both differentiation/epithelialization and self-renewal**

10 **ability of SN precursors**

11   We further asked whether Sox2-deprived SN precursors would remain in a

12 self-renewing state in the tail bud. To this end, we used Sox3HMG-EnR, known to

13 repress endogenous Sox B type genes (Sox 1 to Sox 3) (Bylund *et al*., 2003, Sasai,

14 2001). Since SN precursors in the tail bud express Sox2, but not Sox1 or Sox3

15 (Uchikawa *et al*., 2011), electroporated Sox3HMG-EnR was considered to repress the

16 activity of Sox 2. Using the Tet-on expression system shown in Fig. 4, *Sox3HMG-EnR*

17 was Dox-induced to be expressed in a temporally controlled manner. This time, we

18 electroporated pBI-Sox3HMG-EnR/ DsRed (which are driven bidirectionally by TRE),

19 pCAGGS-EGFP, and pCAGGS-Tet-On3G. In addition, to precisely track the

20 *Sox3HMG-EnR*-electroporated cells during secondary neural tube formation, we

21 dissected gene-electroporated SN precursors from a tail bud of HH14 (22ss) embryo,

22 and transplanted them homotopically into stage-matched non-electroporated embryos,

23 into which Dox solution had been administered (Fig. 5A, B; n=5).

24   By 12 hrs post Dox, when embryos reached HH16, DsRed (Sox3HMG-EnR)

25 signals were detected (the onset of DsRed detection after Dox was delayed compared to

26 mCherry due to the protein configuration) (Fig. 5A, C; n=5). Notably, at this stage, only

27 a few DsRed$^+$ cells were recognized in the tail bud probably due to the cell death of

28 *Sox3HMG-EnR*-expressing cells, and even this remnant of remaining cells failed to

29 participate in the secondary neural tube (Fig. 5C, D; n=5). It clearly contrasts with the

30 observations shown in Fig 2, where transplanted EGFP$^+$ (normal) cells were actively

31 incorporated into the secondary neural tube. It is unlikely that Sox2-inhibited cells were

32 converted to mesoderm. In transverse sections, Sox 2-inhibited cells (red) in the anterior

33 domain of SN precursors, which would normally express a high level of Sox 2, were

11

1   located near the neural tube within the territory of SN precursors (Fig. 5E, F; n=4).

2   These sections also confirmed that the posterior domain of SN precursors, which would

3   normally be of low Sox 2, did not contain Sox 2-inhibited cells (Fig. 5D', G, H; n=4).

4   The distribution of single positive EGFP$^+$ cells, which did not receive the

5   *Sox3HMG-EnR* gene, was comparable to normal cells (Fig. 5D compare to Fig. 2; n=4).

6   Collectively, these results suggest that a high level of Sox2 is required for the anterior

7   SN precursors to correctly be incorporated into the secondary neural tube. And even the

8   low level of Sox 2 activity in the posterior SN precursors is indispensable, particularly

9   for the self-renewing cells to survive.

10   The loss of *Sox3HMG-EnR*-electroporated cells was specific to SN precursors

11   since when the expression of this gene was Dox-induced in already epithelialized cells

12   in the secondary neural tube, no detectable effects were seen until HH16 (Fig. 5I; n=7).

13

14   **Discussion**

15   Using the direct labeling of SN precursors, SN-specific transplantations, and

16   SN-specific gene manipulations, we have uncovered cellular and molecular mechanisms

17   underlying the SN-mediated neural elongation in the posterior region of chicken

18   embryos. SN precursors occupy the anterior half of the tail bud at HH14, whereas the

19   posterior half is populated by paraxial mesodermal precursors. The SN precursor

20   territory was further divided into two distinct regions; one is its posterior half with

21   self-renewing cells, and the other is the anterior half more specified to the neural

22   tubulogenesis (Fig. 6). Sox2 plays an important role in the regulation of these two

23   distinct states of cells: high and low levels of Sox2 activities endow the neural-specified

24   and self-renewing states, respectively.

25

26   **A discrete territory of SN precursors in the tail bud**

27   The tail bud has long been appreciated to be critical for the posterior body

28   elongation, and the existence of precursors has been shown that give rise to the

29   secondary neural tube, and/or paraxial mesoderm, and the chord neural hinge (CNH),

30   although their precise positions in the tail bud have been under debates (Cambray &

31   Wilson, 2002, Catala *et al.*, 1996, Catala *et al.*, 1995, McGrew *et al.*, 2008, Selleck &

32   Stern, 1991, Olivera-Martinez *et al.*, 2012, Tucker & Slack, 1995). Recent advances in

33   genetic labeling of earlier epiblast cells in mice proposed that neuromesodermal

1 common progenitors (NMPs) exist not only in the early epiblast but also in the tail bud

2 (Tzouanacou *et al.*, 2009, Wymeersch *et al.*, 2016). However, no direct fate mapping of

3 the tail bud-constituting cells has been conducted in amniotes.

4 In this study, we have extended our previous findings that the SN precursors

5 can directly be traced in the tail bud (Shimokita and Takahashi, 2011). These SN

6 precursors derive from the preSN region in the epiblast at HH8, which is located in

7 between Hensen's node and the anterior end of the primitive streak (Fig. 1). By

8 focusing on the tail bud at HH14 (24ss), which displays a representative feature of this

9 structure, we have delineated SN- and mesoderm-harboring regions. The SN precursors

10 occupy the anterior half, whereas the posterior half is populated by mesodermal cells

11 derived from the anterior primitive streak, leaving the CNH unlabeled (Fig. 1J). This

12 delineation is striking to us because several previous studies of axial elongation

13 postulated prominent NMPs in the tail bud, although these studies traced more naïve

14 cells residing in the epiblast at earlier stages than those used in the current study

15 (Selleck & Stern, 1991, Wymeersch *et al.*, 2016, Taniguchi *et al.*, 2017). Thus, our

16 study clarified, for the first time, different fates of mesenchymal cell populations in the

17 tail bud, at least at the HH14 (24ss) stage. Our results do not exclude the existence of

18 NMPs in the tail bud, since preSN-labeling rarely yields cells located in the medial edge

19 of presomitic mesoderm (see Fig. 4E). Nevertheless, a vast majority of the tail bud cells

20 are differentially fated to either SN, mesoderm, or CNH.

21

22 **Posteriorly positioned SN precursors possess self-renewing ability**

23 The SN precursors are constantly present in the tail bud despite their

24 continuous participation in the secondary neural tube formation, and we had reasoned

25 that SN precursors in the tail bud might contain stem (-like) cells. Specific tracing of the

26 SN precursors in the tail bud has facilitated to test this, and we have indeed shown that

27 such stem cells exist in the tail bud by a serial transplantation of EGFP-labeled SN

28 precursors (Fig. 2A). Furthermore, these self-renewing cells are preferentially located in

29 the posterior half of the SN precursor territory (Fig. 2, Fig. 6). These posterior cells

30 undergo both self-renewal and its subsequent neural tubulogenesis (epithelialization),

31 whereas the anterior half cells only participate in the tubulogenesis without staying in

32 the tail bud. Thus, the SN precursors contain at least two subpopulations, one is

33 posteriorly positioned self-renewing cells, and the other is anteriorly located cells in a

13

1   transitional state (Fig. 6). This is the first identification of SN stem cell population in

2   the tail bud. Whether a single stem cell generates both self- and differentiating cells

3   awaits further analyses.

4

5   **AP gradient of Sox2 expression regulates the binary decision between**

6   **self-renewing and transitional states of SN precursors**

7   Sox2 displays a posteriorly decreasing gradient of expression within the SN

8   precursor territory in the tail bud, which contrasts with a relatively steady level of

9   Brachyury (T) expression both in the SN- and mesodermal precursors (Fig. 3). We have

10   provided evidence that the AP gradient of Sox2 is important for the binary decision

11   between the self-renewing and transitional states of SN precursors. With

12   gain-of-function experiments, we have found that a high level of Sox2 directs cells to

13   the differentiation/epithelialization of the secondary neural tube. Loss-of-function

14   experiments have unexpectedly shown that even a low level of Sox2 is important for the

15   posterior SN precursors. Sox2 has been implicated in normal neural development,

16   pluripotency of stem cells, and survival of neural progenitors (Feng *et al.*, 2013, Hagey

17   & Muhr, 2014, Hutton & Pevny, 2011, Kondoh & Lovell-Badge, 2016). In particular, a

18   low level of Sox2 is important for the proliferation of neural stem cells in adult brain

19   (Hagey & Muhr, 2014). It is possible that the self-renewing SN precursors identified in

20   the current study might serve as a novel source for therapeutic treatments of neural

21   diseases.

22   It is yet to be determined what generates the AP gradient of Sox 2 expression

23   in the SN precursor territory. It is known that the preSN region in the epiblast of HH8

24   embryo, from which SN precursors arise, is devoid of Sox2, although its adjacent neural

25   plate/epiblast (i.e. primary neurulation tissues) expresses this gene, and also that this

26   preSN-specific inactivation of Sox2 requires BMP4 (Takemoto *et al.*, 2006). It is

27   conceivable that the AP gradient of Sox 2 expression in the tail bud is also regulated by

28   BMPs expressed in the tail bud and its surrounding tissues (Takemoto *et al.*, 2006).

29   Previous studies of axial elongation have regarded $Sox2^+/Bra^+$ double positive

30   signal as a marker for an NMPs cell population in the early epiblast (Olivera-Martinez

31   *et al.*, 2012, Wymeersch *et al.*, 2016, Delfino-Machin *et al.*, 2005, Tsakiridis *et al.*,

32   2014, Yoshida *et al.*, 2014). However, in the current study, we have explicitly

33   demonstrated that a majority of the SN precursors in the tail bud are $Sox2^+/Bra^+$

14

1   double-positive, even though they are *not* NMPs. Thus, the fate of Sox2$^+$/Bra$^+$ double

2   positive cells must vary according to the developmental context. It is also possible that

3   Sox2$^+$/Bra$^+$ cells might have high potential to easily change their fate upon encountering

4   external signals. We are currently investigating differentiation potential of SN- and

5   paraxial mesodermal precursors in the tail bud based on the developmental lineages

6   delineated in the current study.

7   　　　　Lastly, it is of interest speculating that the self-renewing ability of the SN

8   precursors might reflect the stemness of adjacent mesoderm (Iimura & Pourquie, 2006).

9   When mesodermal cells that are derived from the anterior primitive streak of HH4-6

10   embryos give rise to the medial half of somites, they do so via the tail bud where some

11   of these cells stay for a long period of time and serve as self-renewing cells (Iimura &

12   Pourquie, 2006). According to our findings, such mesodermal stem cells should be

13   those located in the posterior half of the tail bud at HH14, and more ventrally at HH17

14   (Fig. 1). In this way, both the mesodermal stem cells contributing to the medial half of

15   somites and the SN stem cells found in this study are possibly adjacent to each other in

16   the tail bud (Fig. 6). It is plausible that these differentially fated but similarly

17   self-renewing cells would share signals for the endowment of the stemness. Whether

18   these signals include Fgfs and Wnts, which are predominantly expressed in the posterior

19   region of the elongating body awaits further analyses.

20

21   **Acknowledgements**

28

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1   **Figure legends**

2   **Figure 1**

3   Segregation of SN- and mesoderm-fated cells in the tail bud at HH14. (A) A
4   fate map of the HH8 epiblast caudal to the Hensen's node (HN) modified from
5   Shimokita and Takahashi (2011). A specific region called presumptive secondary
6   neurulation (preSN, purple) gives rise solely to secondary neurulation (SN), which
7   undergoes posteriorly to 27th somite level. The EGFP-positive neural tube anterior to
8   27th is of primary neurulation. (B) *In ovo* electroporation conducted at HH8 (see also
9   Materials and Methods). (C, D) EGFP-electroporated preSN gave rise to EGFP-positive
10   cells in the SN-forming cells but not in the mesoderm as seen at HH14 and HH17. (E)
11   Simultaneous labeling by different colors of PKHs of preSN and anterior primitive
12   streak (mesoderm) in a single embryo. (F) Dorsal view, and (G-I) sagittal sections at
13   HH14. Little overlapping between SN (green)- and mesoderm (red)-fated cells in the
14   tail bud. CNH, notochord and floor plate were unlabeled. (J) Schematic trace of SN- and
15   mesodermal precursors in the tail bud. NT, neural tube; TB, tail bud; CNH, chord neural
16   hinge; NC, notochord, *, background. Scale bar; 100 μm.

17

18   **Figure 2**

19   Transplantation with SN-fated cells revealed self-renewing cells in the
20   posterior half of the SN-territory. (A-E) EGFP-expressing SN-fated cells were dissected
21   from the tail bud of HH17 embryo, and transplanted into a corresponding region of the
22   tail bud of HH14 embryos. (F-H) When the host embryo developed to HH17, similar
23   transplantation was repeated. (I) The EGFP-positive SN precursor territory in the tail
24   bud was subdivided into the anterior and posterior halves, and each of them was
25   isotopically transplanted into a host embryo (HH14).   (J-L) The anterior SN precursors
26   underwent neural tube differentiation without remaining in the tail bud (bracket). (M-O)
27   The posterior SN precursors underwent not only neural tube differentiation, but also
28   remained as mesenchymal cells in the tail bud (white arrowheads). A white small dot
29   indicates the position of 31-33 somite level. White arrow is the boundary between the
30   posterior end of epithelialized neural tube and mesenchymal SN precursors of the tail
31   bud.

32

33

20

1 **Figure 3**

2   Graded expression levels of Sox2 in the SN precursor territory in the HH14

3 tail bud. (A, B) *In situ* hybridization for *Sox2* mRNA. Dorsal view (A) and sagittal

4 section (B). (C-D) Immunohistochemistry for Sox2 and Brachyury (T) proteins in a

5 para-sagittal section. (F, G) Graded expression of Sox2 was quantitatively shown,

6 whereas the expression level of Brachyury was largely steady in the tail bud. (I-K) The

7 Sox2-positive area overlaps with the SN-fated territory, but not with mesodermal cells

8 derived from the anterior primitive streak (PKH67-labeled). Scale bar; 100 μm.

9

10 **Figure 4**

11   An experimentally augmented level of Sox2 caused precocious differentiation

12 of SN precursors. (A) *Sox2* cDNA was overexpressed in a temporally controlled manner

13 using the Tet-on system. The preSN region of HH8 embryos was electroporated with

14 the plasmids indicated. When the embryo reached HH14, at which gene-electroporated

15 cells emerged as mesenchyme in the forming tail bud, a Dox solution was administered

16 (0hr). Note that at 3 hrs post-Dox in both control and Sox2-overexpressing embryos,

17 three types of cells, green (EGFP), red (mCherry) and yellow (EGFP and mCherry)

18 were observed in the tail bud (see text for details). However, at 9 hrs,

19 Sox2-overexpressing cells were found solely in the formed neural tube, and not in the

20 tail bud.

21

22 **Figure 5**

23   Sox2-deprived SN precursors failed to participate in the secondary

24 neurulation. (A) *In ovo* electroporation and tet-on induced expression of *Sox3HMG-EnR*

25 were carried out in a way similar to that shown in Fig. 4. In addition, Sox2-deprived SN

26 precursors were precisely tracked following isotopic transplantation. (B) At the time of

27 transplantation (0 hr), no signal for the expression of Sox3HMG-EnR was seen yet. (C)

28 By 12 hrs post Dox, *Sox3HMG-EnR*-expressing SN precursors cells (red), whose

29 number was smaller than that of control (EGFP) cells, failed to be incorporated into the

30 secondary neural tube formation (E, F). (G, H) In the posterior region of the SN

31 precursor territory, no Sox3HMG-EnR-expressing cells were observed. Scale bar; 50

32 μm.

33

21

1 **Figure 6**

2   A summary diagram of the SN precursors in the tail bud of chicken embryos.

3 SN-fated cells populate the anterior half of the mesenchymal mass of the tail bud,

4 whereas the rest of the tail bud is populated by mesodermal precursors (paraxial

5 mesoderm) and chord neural hinge (CNH). The SN precursor territory (light blue) is

6 further divided into two subpopulations: self-renewing cells posteriorly and more

7 neural-specified transitional cells anteriorly. Sox 2 displays posteriorly decreasing

8 levels of expression in the SN precursor territory. High and low levels of Sox 2 are

9 important for neural differentiation/epithelialization of transitional zone, and survival of

10 self-renewing cells, respectively.

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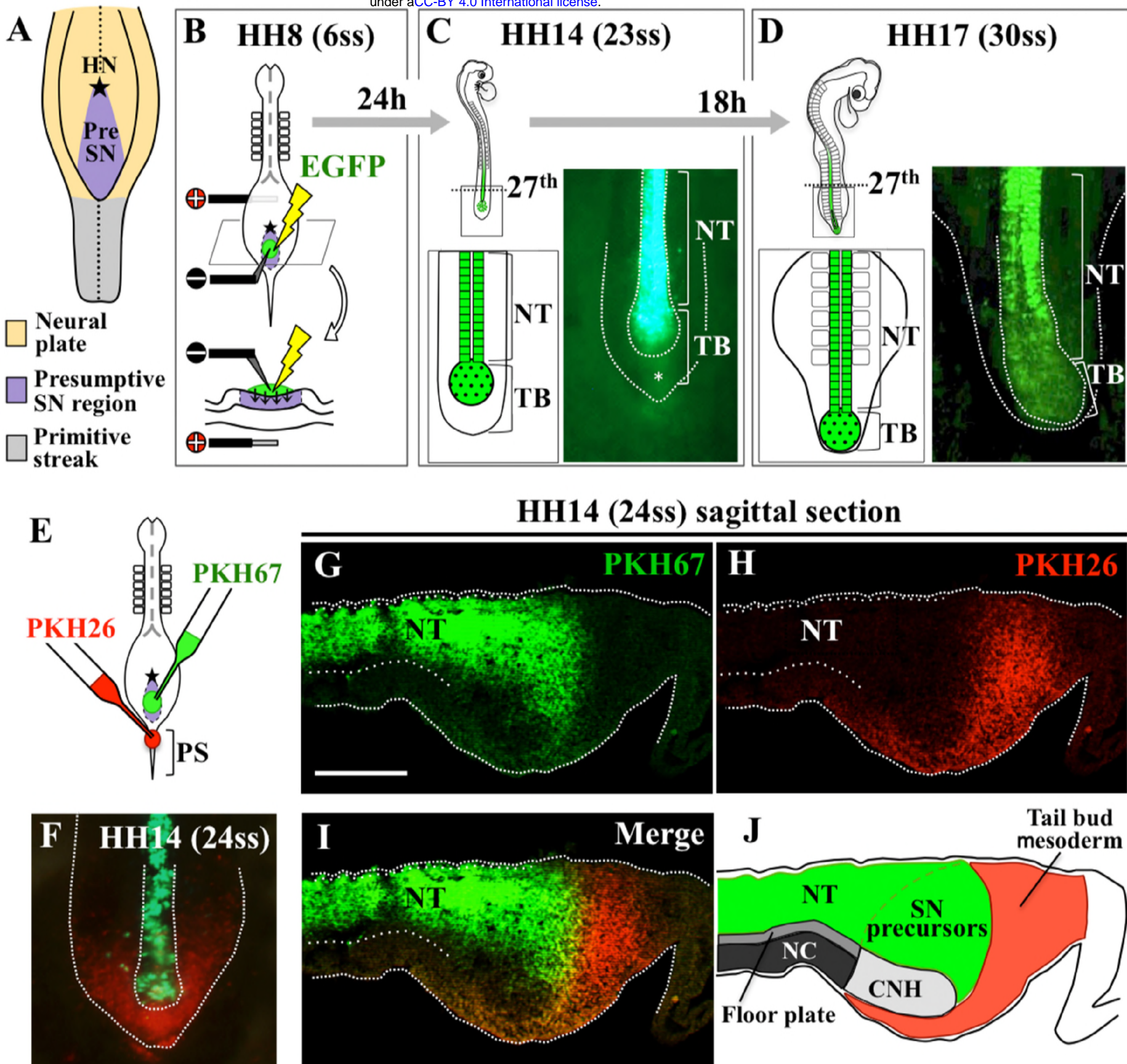
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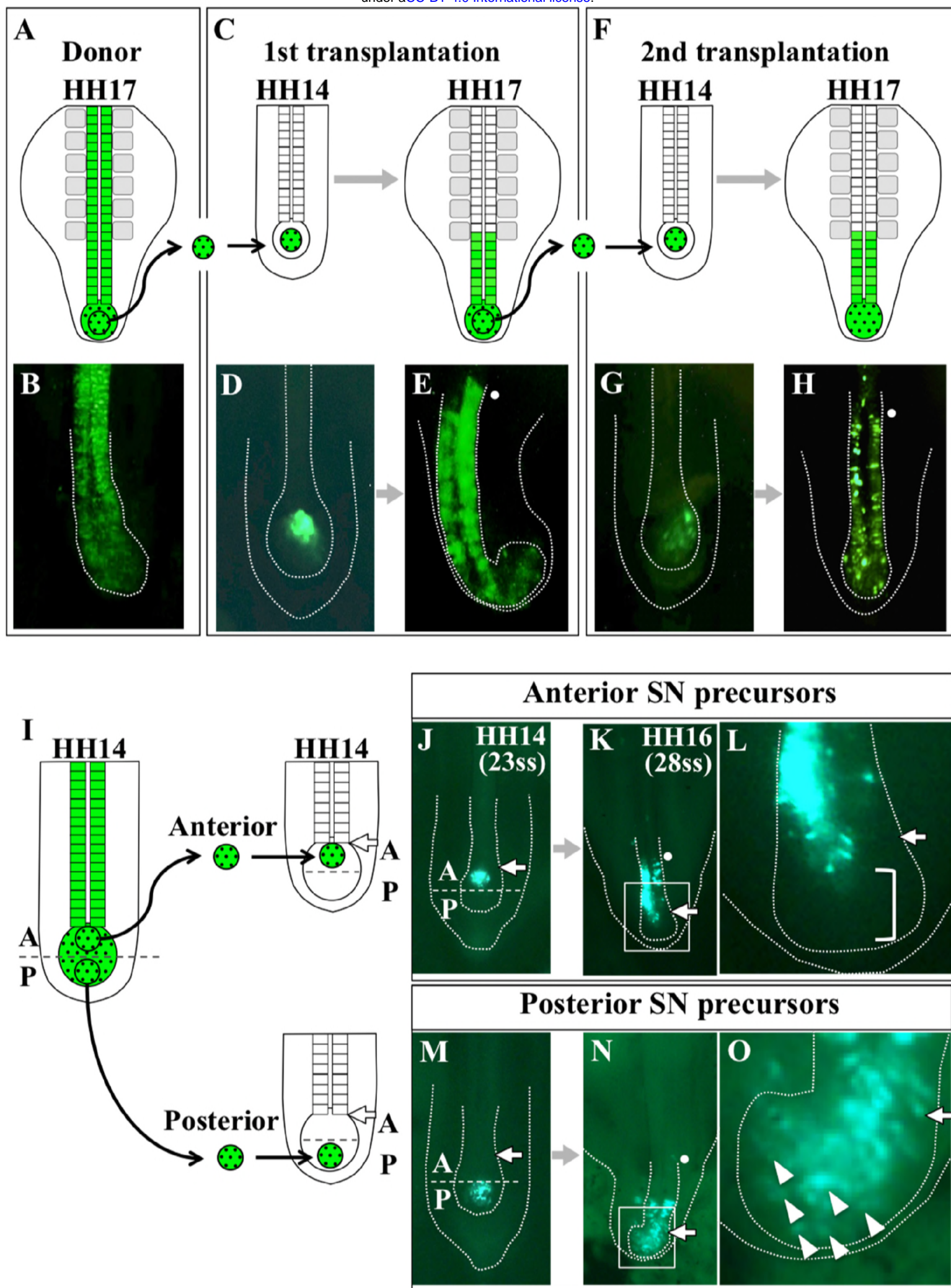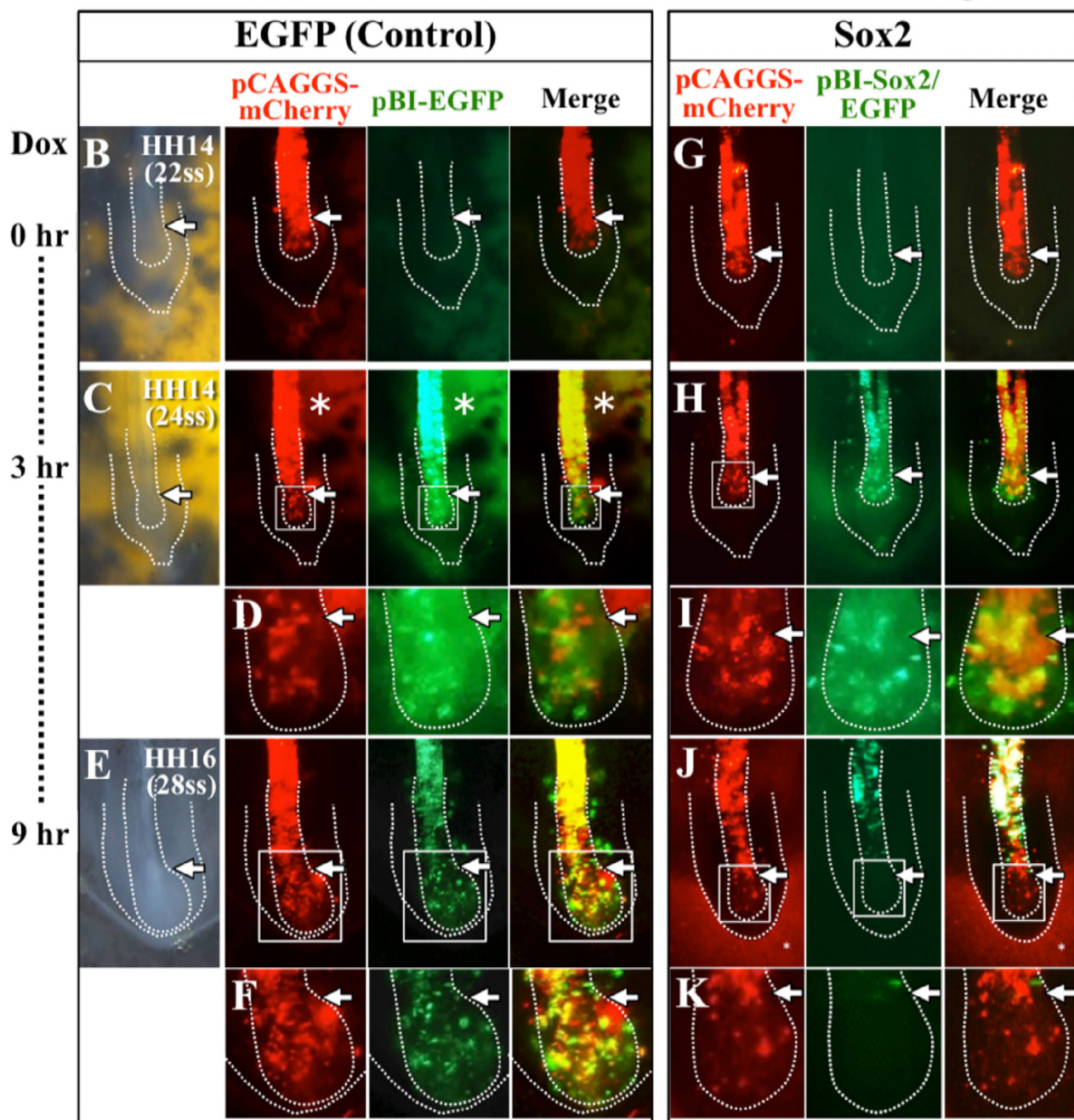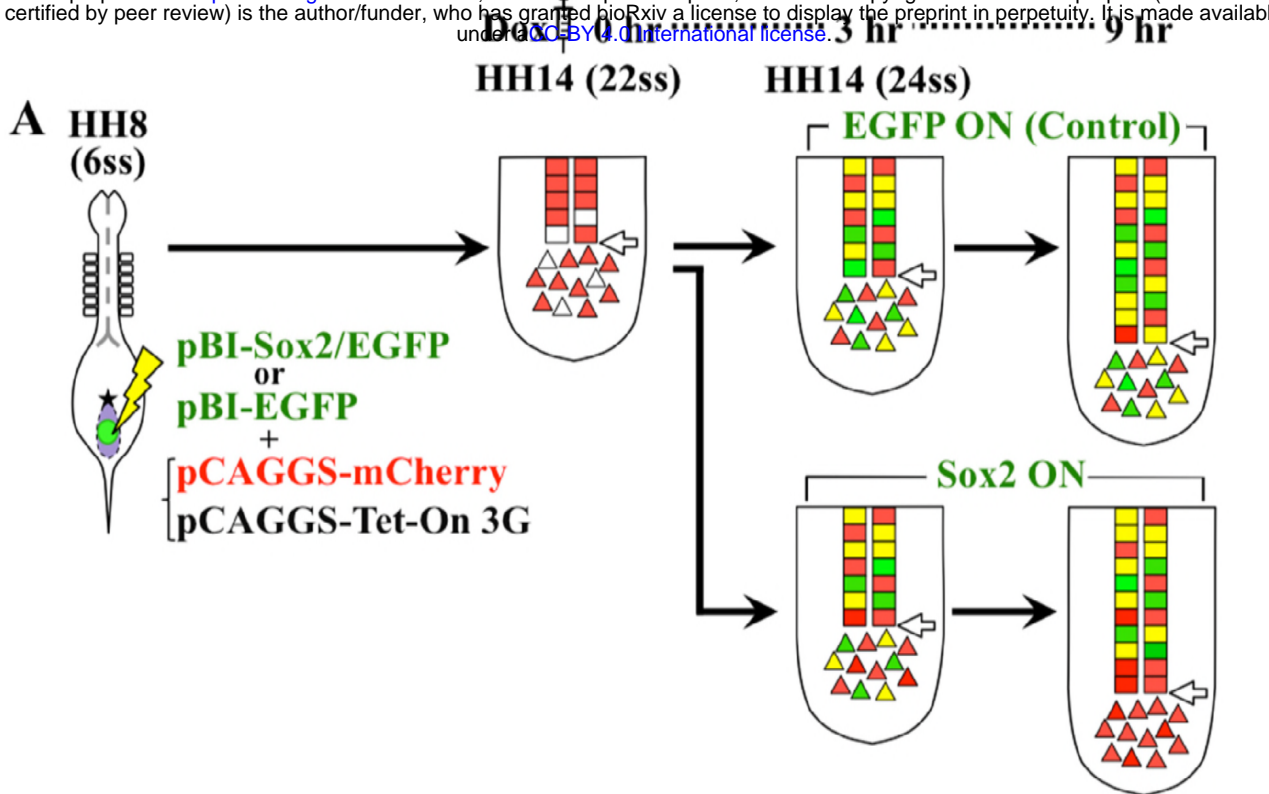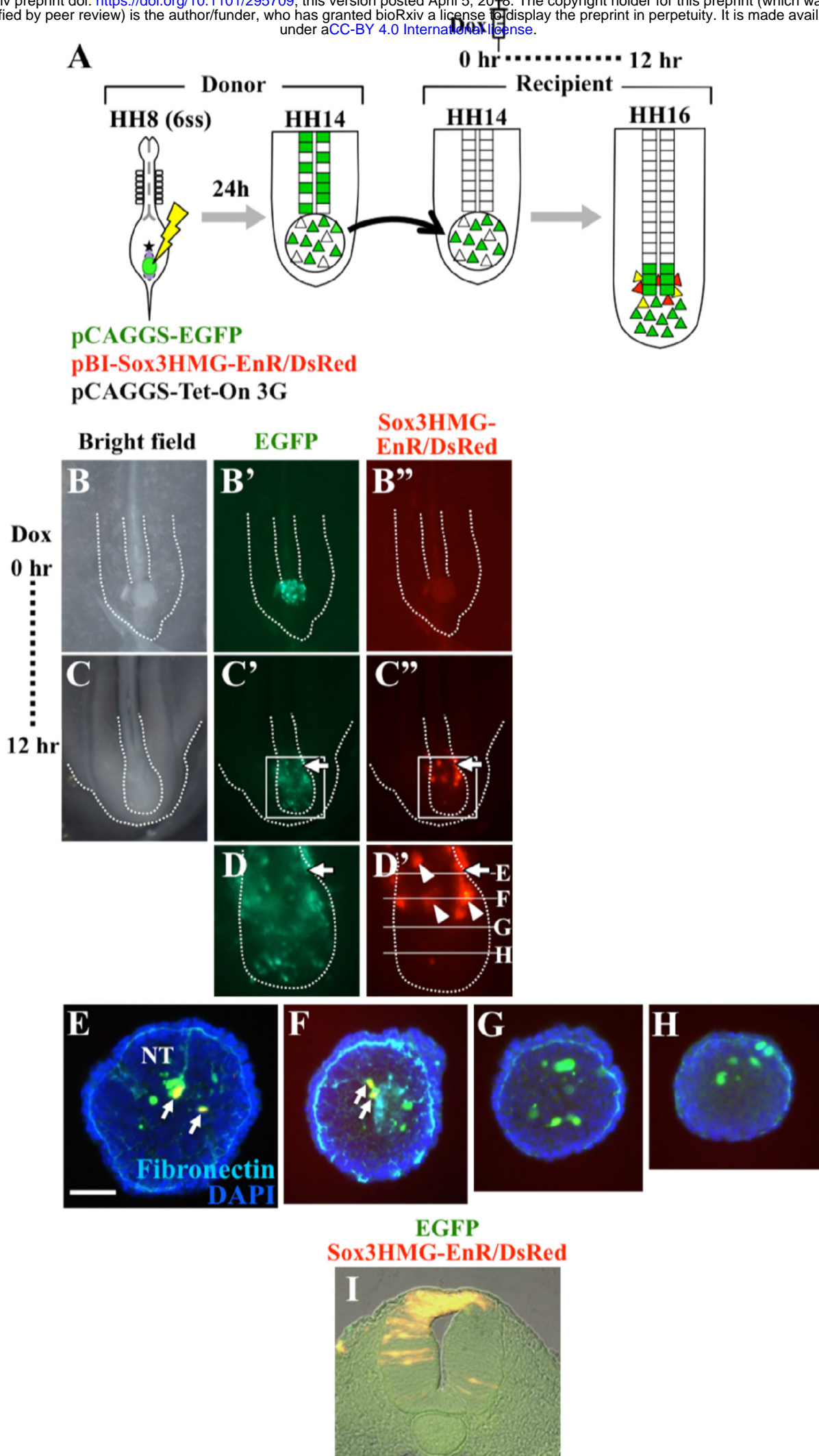
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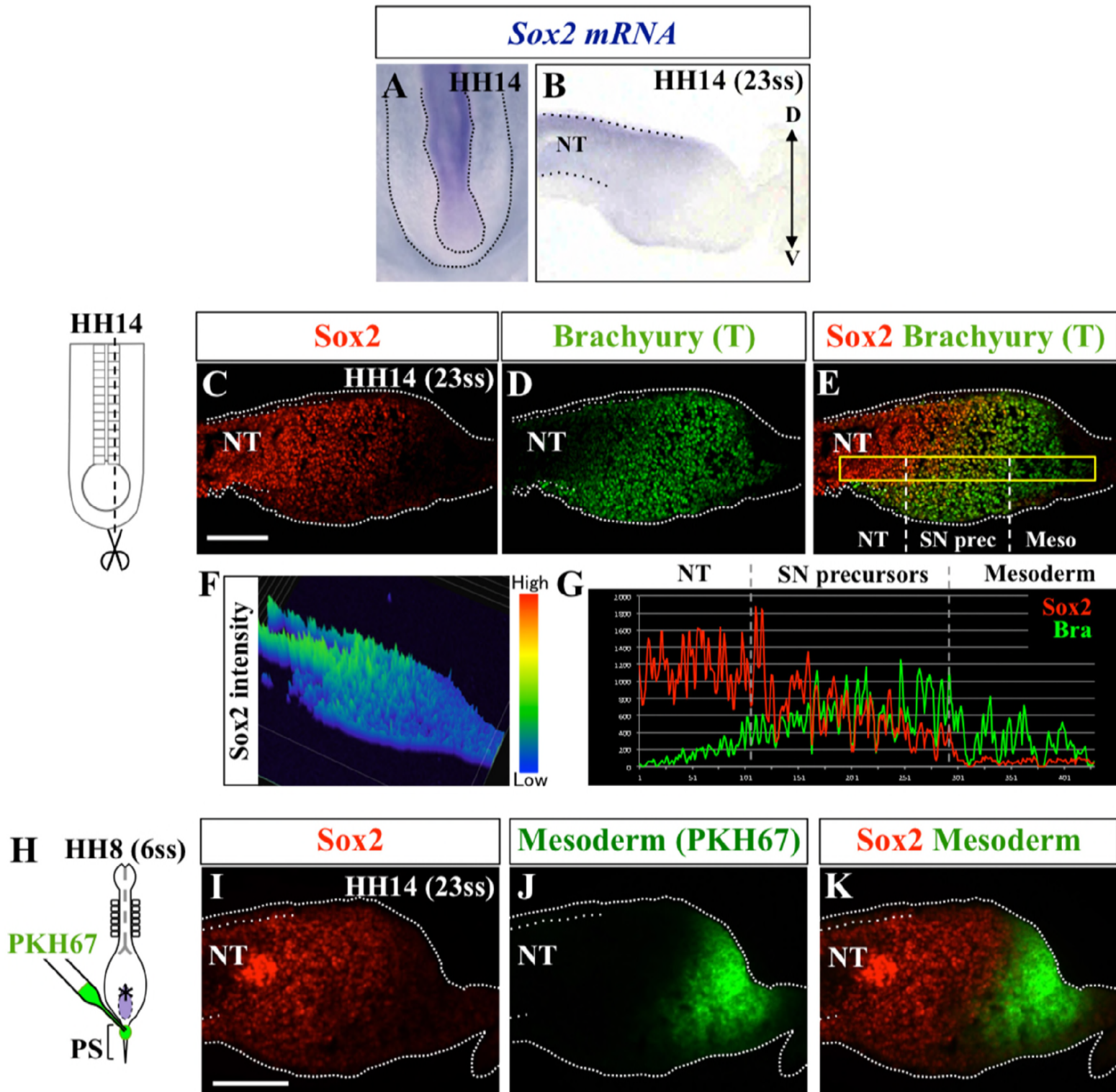
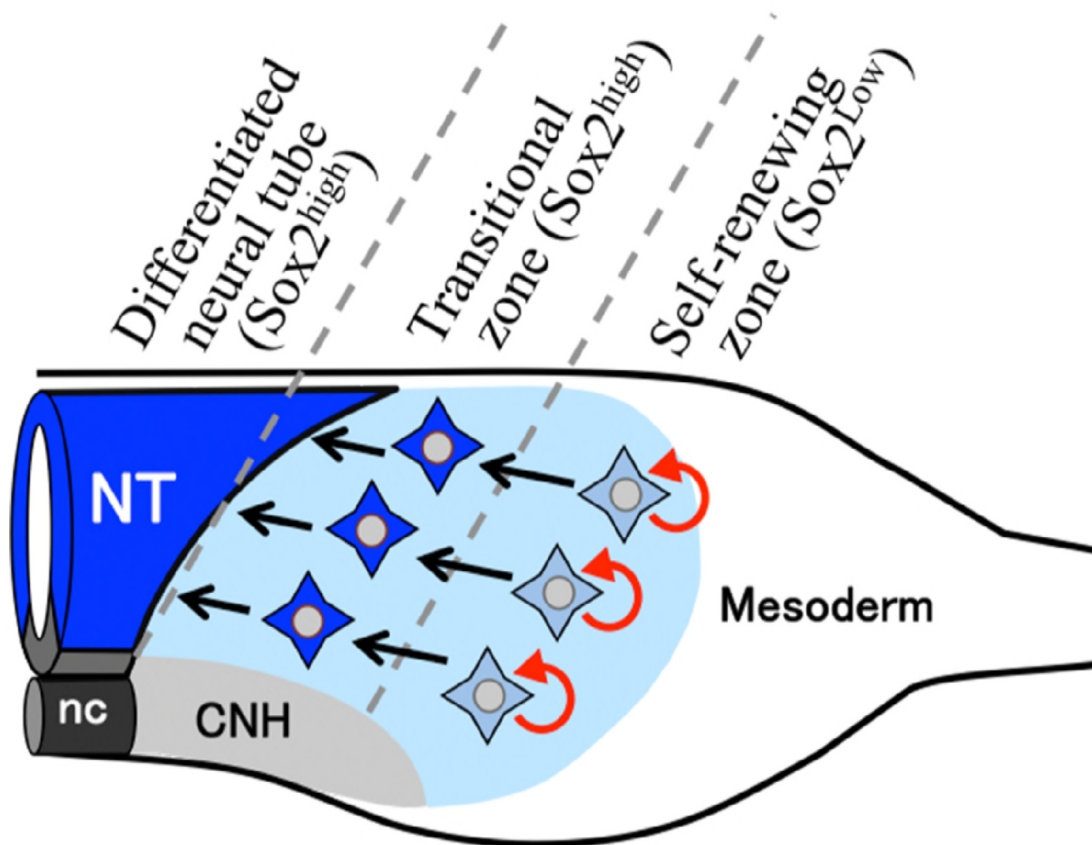Fig. 3 Kawachi et al.

Fig. 4 Kawachi et al.

Fig. 5 Kawachi et al.

Fig. 6 Kawachi et. al.