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Use of *FOXJ1CreER^{2T}* mice for inducible deletion of embryonic node gene expression

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Abstract

The ciliated cells of the node of the mouse embryo contribute to the establishment of left-right patterning via generation of leftward laminar fluid flow and initiation of a left-sided morphogen gradient. Here, we identify *FOXJ1CreER^{2T}* mice in which expression of Cre recombinase is directed to ciliated node cells. The data demonstrate that *foxj1* is expressed specifically in the node throughout the developmental window critical for left-right patterning. In transgenic embryos, Cre expression is detected by immunohistochemistry in ciliated cells of the node. *Rosa26R* reporter mice, in which expression of *lacZ* is activated only after Cre-mediated recombination, demonstrate strong and uniform labeling at the node when crossed with *FOXJ1CreER^{2T}* mice. Cell labeling occurred as early as 0–2 somite stages specifically within cells of the node and recombination was highly efficient in response to tamoxifen. *FOXJ1CreER^{2T}* transgenic mice represent a new genetic tool for the analysis of node-specific gene expression and will also be valuable in the study of node cell lineage and temporal cell fate mapping.

Generation of mice in which tissue-specific loss of gene function occurs is a powerful tool for dissecting gene function. The use of the site specific recombination using the Cre/*loxP* recombinase system to excise genes flanked by *loxP* sites has allowed cell type- and tissue-specific gene knockout (Lewandoski, 2001; Nagy, 2000). However, this technology is limited by the availability of transgenic mouse lines expressing Cre recombinase in the cells or tissues of interest.

The node of the mouse embryo is a transient embryonic structure that is important for the generation of left-right patterning. The cells of the node contain monocilia that function in the establishment of left-right asymmetry by generating nodal flow (McGrath *et al.*, 2003; Nonaka *et al.*, 2002; reviewed in Hirokawa *et al.*, 2006). There is direct evidence in zebrafish that impairment of cilia function in the organizer, using morpholinos targeted to dorsal forerunner cells/Kupffer's vesicle, disrupts left-right patterning (Essner *et al.*, 2005). In mouse, strains have been created to allow inactivation of gene activity specifically within large, non-ciliated crown cells surrounding the node, thus disrupting important asymmetric regulatory pathways (Brennan *et al.*, 2002; Saijoh *et al.*, 2003). However, delineation of the specific role(s) of the ciliated cells of the node has been difficult to study in mouse due to lack of reagents for cell type specific gene inactivation. Here we examine the ability of *FOXJ1CreER^{2T}* mice to generate node cell-specific recombination.

Foxj1 is a member of the forkhead box family of transcription factors which is expressed in ciliated cells of the respiratory, reproductive and central nervous systems (Hackett *et al.*,

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1995; Lim *et al.*, 1997). This transcription factor has an important role in ciliogenesis and is necessary for basal bodies to anchor to the apical cytoskeleton (Gomperts *et al.*, 2004). *Foxj1* has previously been shown to be expressed in the node of the presomite embryo (Zhang *et al.*, 2004), and targeted deletion of *foxj1* results in abnormal cardiac looping and randomization of *nodal* and *pitx2* gene expression in the lateral plate mesoderm, indicating the requirement of *foxj1* for the development of proper left-right patterning (Brody *et al.*, 2000; Chen *et al.*, 1998).

To further evaluate gene expression during critical stages of left-right patterning, a *foxj1* riboprobe was generated and sense and antisense probes were tested in late headfold (LHF) and E9.5 embryos. The antisense probe showed specific staining in the region of the node (n = 2/2) and in the choroid plexus (n = 6/6) at the respective stages (data not shown), in accordance with published data (Zhang *et al.*, 2004). Subsequently, gene expression was evaluated from late streak through 14 somite stage by whole mount in situ hybridization (WISH)(Fig. 1 and Table 1). The data indicate that *foxj1* is expressed at least as early as late streak stages of gastrulation and continues to be highly expressed in early head fold (EHF), LHF and 0–4 somite stage embryos. Expression subsequently decreases, but continues to be present in a majority of embryos until 8 somite stage. These results indicate that *foxj1* is expressed during the initiation of left-right signaling at the node and persists during critical stages of signal transduction in the lateral plate mesoderm. Expression at these stages was specifically restricted to the node region, suggesting that the *foxj1* promoter might be a useful reagent for directing node-specific gene expression.

Previous studies have demonstrated the ability of a *foxj1* promoter to direct expression to ciliated structures of the lung epithelium (Rawlins *et al.*, 2007; Zhang *et al.*, 2007). To further investigate node-specific expression, we utilized transgenic mice in which a 1 Kb *FOXJ1* human promoter drives expression of a tamoxifen-inducible Cre recombinase. The node of a mouse embryo is morphological distinguishable by its teardrop shape and small cell size. In addition, the node stains strongly with anti-acetylated tubulin, which labels microtubules as well as cilia at the node. Surrounding the node are large crown cells which lack monocilia. In these cells, molecular evidence of asymmetry can be identified at E8. Immunohistochemistry was performed at 0–2 somite stages using an anti-Cre antibody and anti-acetylated tubulin to label the node (Fig. 2). Cre expression was detected in cells of the node at this stage (n = 7), but not in non-transgenic littermate control embryos (n = 5).

To assess whether Cre expression at the node mediates recombination, *FOXJ1CreER*^{2T} mice were mated to *Rosa26R* reporter mice. Embryos were harvested at E7.75 through E8.5 and processed for X-gal staining. The results indicate that recombination occurs in node cells as early as 0 somite stage, with robust recombination occurring by 4 somite stage (Fig. 3). Sections through the node indicate greater than 80% labeling, suggesting high levels of recombination in response to the tamoxifen dosing regimen. Two transgenic lines were evaluated and both showed similar levels of recombination. Line 79 had no extra-nodal lacZ expression in embryos analyzed from 0–18 somites (n = 10), whereas line 24 exhibited extra-nodal staining in approximately 15% of embryos at these stages (n = 20)(Fig. 3c). These transgenic mice should therefore be useful for node-specific gene deletion. It should be noted that transgenic mice which utilize a FOXJ1 promoter to drive Cre without tamoxifen induction have also been shown to direct expression to ciliated epithelium of the lung (Zhang *et al.*, 2007), and it would be of interest to test node-specific expression in these mice as well.

In addition to the functional importance of *FOXJ1CreER*^{2T} mice for gene inactivation, this tool can be exploited for lineage labeling due to the irreversible nature of recombination. As proof of principle, *FOXJ1CreER*^{2T} embryos were harvested at 12–18 somite stages and used for whole mount in situ hybridization (WISH) with a *Cre* riboprobe. As expected from *foxj1*

WISH data, in which expression is lost after the 11 somite stage, no node or notochord *Cre* transcript expression was identified (data not shown). In contrast, X-gal staining is present at these stages in *FOXJ1CreER^{2T}::Rosa26R* mice (Fig. 4), indicating that this strategy will be useful in cell fate mapping experiments. The inducible nature of Cre recombinase in these mice in response to tamoxifen will allow independent investigation of temporal versus lineage specific gene expression. The ability to lineage label using *FOXJ1CreER^{2T}* mice therefore provides a tool to address technically difficult questions related to ciliated node cell progeny.

In summary, the *FOXJ1CreER^{2T}* transgenic mice direct Cre expression to ciliated cells of the ventral node in a specific fashion. Onset of Cre expression and recombination occurs during developmental windows in which left-right patterning signal transduction pathways function, indicating that these mice will be a useful tool to further test gene function in ciliated node cells.

MATERIALS AND METHODS

Transgenic mice and tamoxifen induced Cre recombination

FOXJ1CreER^{2T} mice have been described previously (Rawlins *et al.*, 2007). Two independent lines of *FOXJ1CreER^{2T}* mice were used for all experiments. Both lines showed similar levels of recombination. The *Rosa26R* (*Gt(ROSA)26Sor^{tm1Sor}*) reporter line (Soriano, 1999) was obtained from Jackson Laboratories. Tamoxifen free base (Sigma) was diluted at a concentration of 10mg/ml in corn oil. Pregnant females were injected with two doses of tamoxifen intraperitoneally. The first dose of 75 mg/kg body weight was injected at E5.5 and the second dose of 40 mg/kg body weight was injected at E6.5.

Whole mount in situ hybridization and X-gal staining

A riboprobe was created using the following primers for PCR amplification of a 236 bp fragment of *foxj1* exon 1 (NM_008240) upstream of the conserved forkhead domain: 5′ GTCAGGATCCGCTGCAGGAATTCTCCATTC 3′; 5′ TAATCTCGAGTGGTGGCATAGTCCACGTC 3′. The PCR fragment was cloned into BamHI and Xba1 sites of pBluescript (restriction sites in primers underlined). Sense and antisense riboprobes were prepared using a digoxigenin RNA labeling kit (Roche) as per manufacturer's instructions. WISH was performed as described previously (Purandare *et al.*, 2002). X-gal staining was performed according to standard procedures after a 20 minute fixation in 4% paraformaldehyde (PFA) in PBS. For sections, X-gal stained embryos were embedded in plastic and sectioned at 8um with a glass knife as described previously (Ware *et al.*, 2006).

Whole-mount Immunohistochemistry

WT and *FOXJ1CreER^{2T}* embryos were dissected at E7.75–E8 in cold PBS and fixed with 4% PFA in PBS overnight. After fixation, embryos were dehydrated with a graded methanol series and embryos with 0–2 somites were selected for further analysis. After stepwise rehydration, embryos were permeabilized with 0.2% Triton X-100 in PBS (PBT), blocked in 3% bovine serum albumin in PBT for 1 hour, and subsequently incubated with primary antibodies overnight at 4°C with gentle rocking. After thorough washing with PBT, embryos were stained with secondary antibodies. After washing, embryos were mounted in VECTASHIELD HardSet mounting medium (Vector Laboratories Inc.) and imaged on a confocal laser-scanning microscope (Nikon PCM 2000) at 60X magnification. Primary antibodies include mouse monoclonal anti-acetylated tubulin clone 6-11B-1 (Sigma) used at 1:200 dilution and rabbit polyclonal anti-Cre antibody (Novagen, San Diego, CA) used at 1:1000 for whole mount embryos. Secondary antibodies include goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (Molecular Probes) used at 1:150 dilution.

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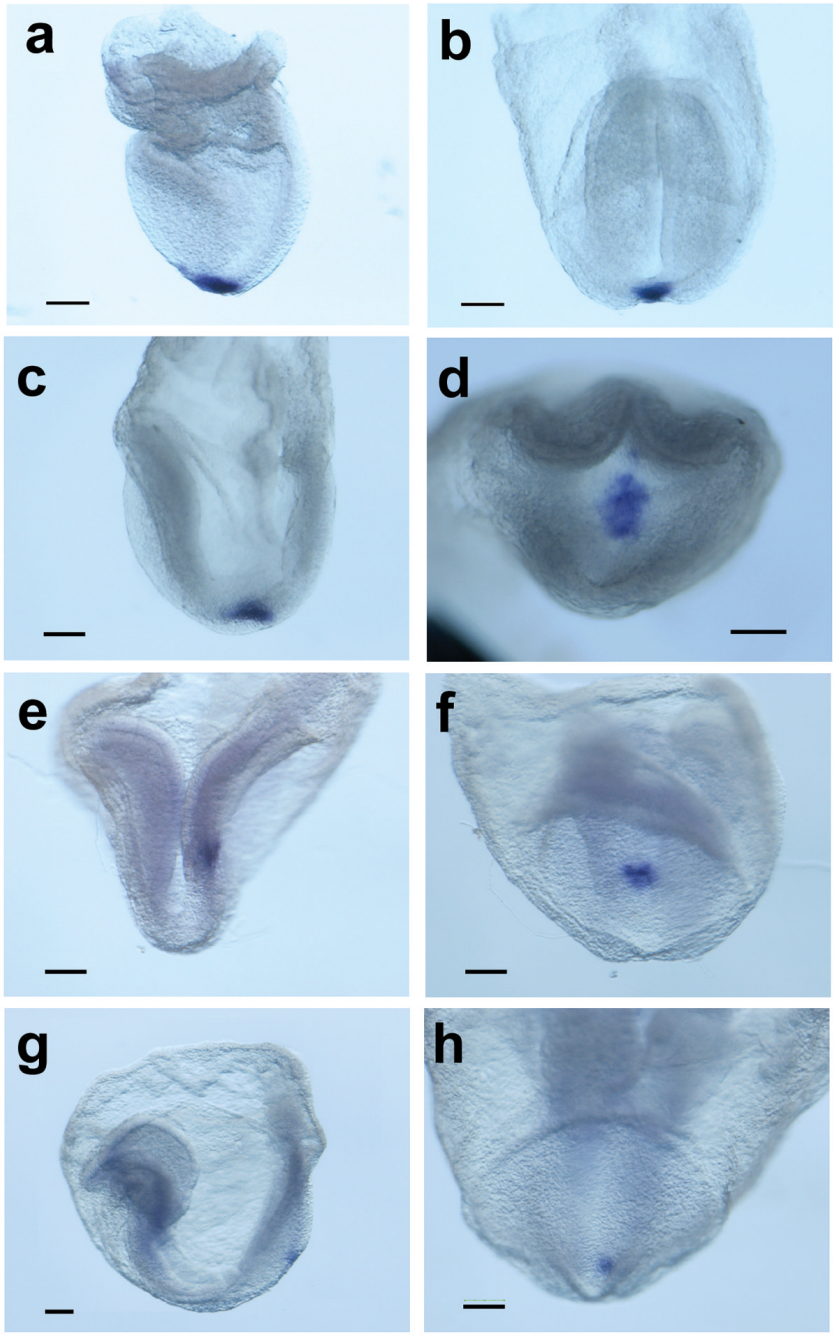

FIG. 1.

Node-specific expression of *foxj1*. Whole mount in situ hybridization using a *foxj1* riboprobe demonstrates expression exclusively in cells of the ventral node in (a) late streak stage (b) late bud stage (c) early headfold (d) late headfold/0 somite (e, f) 0–2 somite (g, h) 4–6 somite and (i, j) 8–10 somite embryos. Panels f, h, and j show distal or posterior views of the node of the embryos pictured in a left lateral view in e, g, and i. Scale bar, 100 μm.

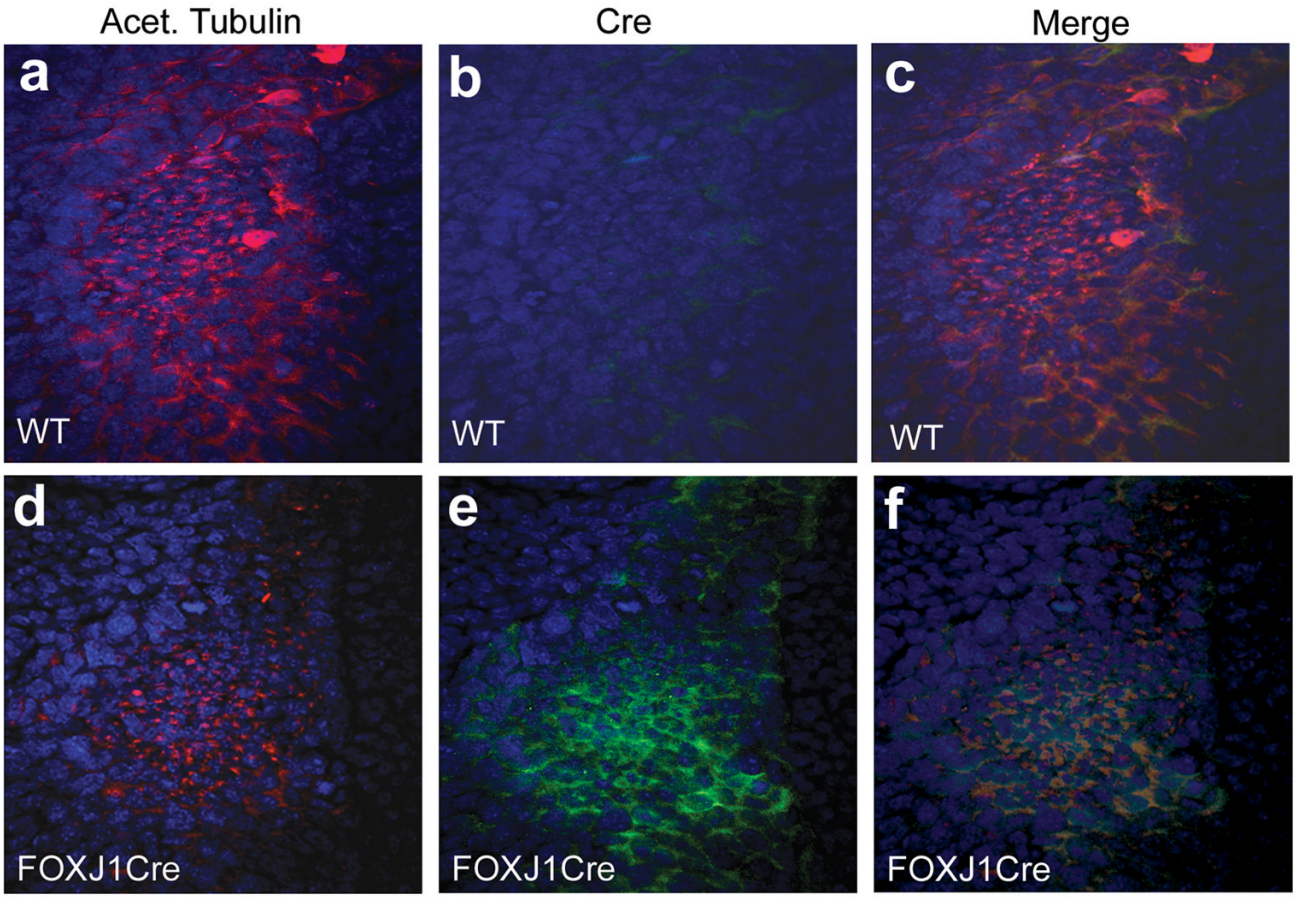

FIG. 2.

Cre immunohistochemistry. The node of E7.75–E8 embryos is highlighted by staining with anti-acetylated tubulin (a, d) in wild type and *FOXJ1CreER*^{2T} embryos. Cre staining is identified within the node of *FOXJ1CreER*^{2T} embryos (e), but is not found in wt embryos (b). WT, wild type.

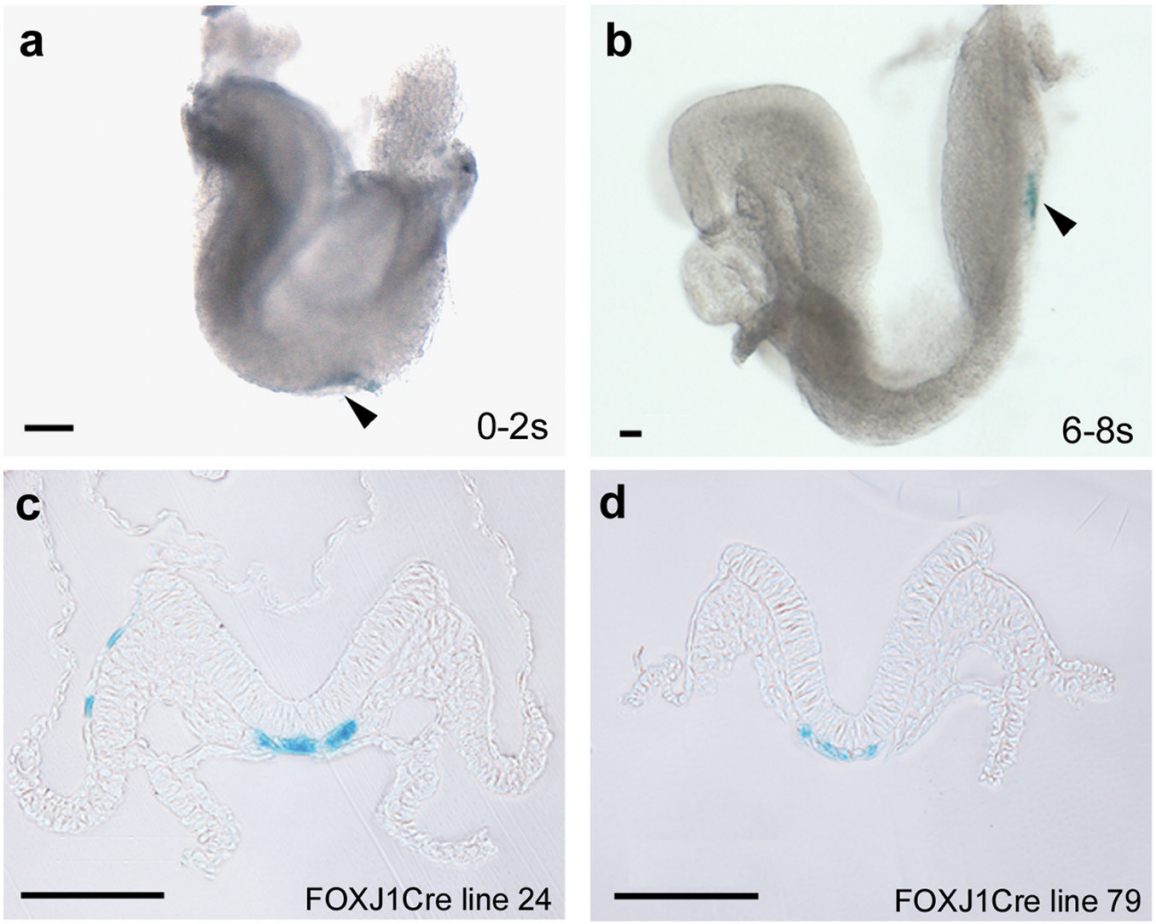

FIG. 3.

X-gal staining is present in the node in *FOXJ1CreER^{2T}::R26R* embryos. X-gal staining is visible in the node of whole embryos (a, b at arrowhead) and in sections (c, d). In a small subset of embryos of line 24, extra-nodal X-gal staining was present in a few cells (c). Scale bar, 100 μm.

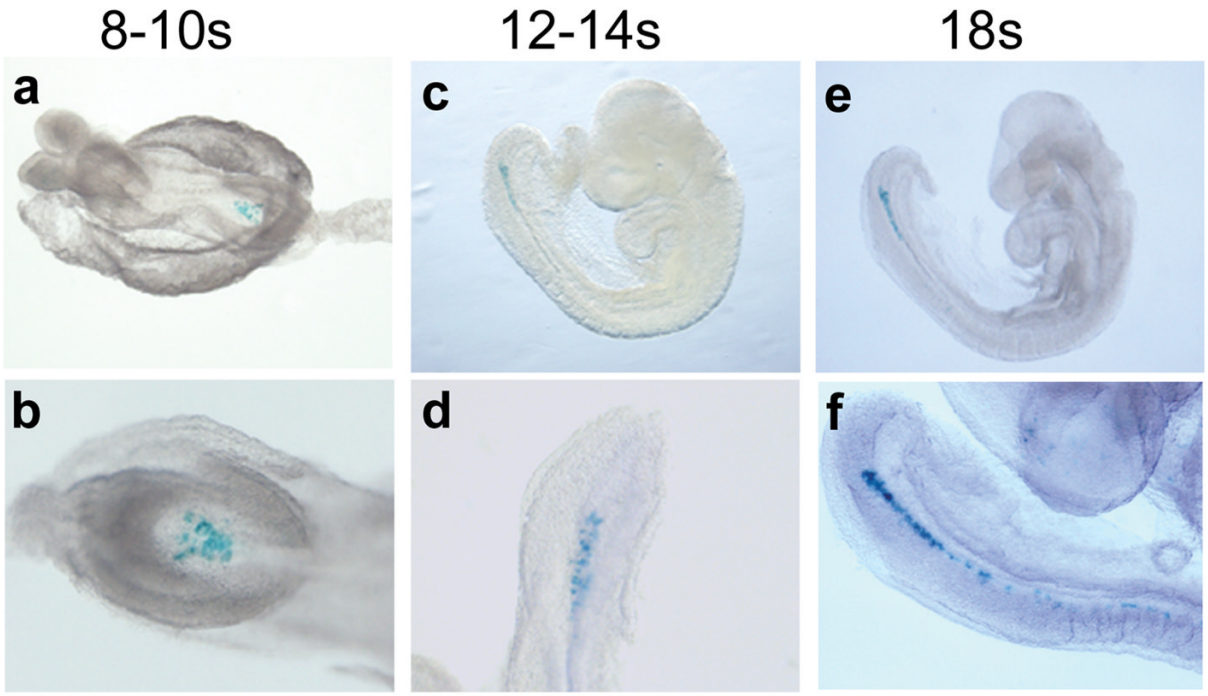

FIG. 4.

Fate mapping of ciliated cells from the node. Females were dosed with tamoxifen at E6.5 and embryos harvested at somite stages as shown. Labeled cells are found in the node at 8–10 somite stage (a, whole embryo; b, high power view of the node) and in the node, notochord, and tailbud at later somite stages (c–f).

Table 1

Stage specific expression of *foxj1* in the node

Stage	# analyzed	# staining	Percent
EHF-LHF	9	9	100
0–4 somite	12	12	100
4–6 somite	6	5	83
6–8 somite	10	8	80
9–10 somite	4	1	25
11–14 somite	3	0	0

EHF, early headfold; LHF, late headfold