Snail family genes are required for left–right asymmetry determination, but not neural crest formation, in mice

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Snail family genes encode zinc finger transcriptional repressors that are key regulators of epithelial–mesenchymal transitions in vertebrates, including the transitions that generate the mesoderm and neural crest. Here, we show that, contrary to observations in frog and avian embryos, the Snail family genes Snail (Snai1) and Slug (Snai2) are not required for formation and delamination of the neural crest in mice. However, embryos with conditional inactivation of Snai1 function exhibit defects in left–right asymmetry determination. This work demonstrates that although some aspects of Snail family gene function, such as a role in left–right asymmetry determination, appear to be evolutionarily conserved, their role in neural crest cell formation and delamination is not.

Slug | development | evolution

Neural crest cells are a migratory cell population that forms at the border between the neural plate and the embryonic ectoderm. These cells undergo an epithelial–mesenchymal transition (EMT), delaminate from the neural epithelium, and migrate throughout the embryo, differentiating at their destination sites into a wide array of cell types. Subsequent to the specification of neural crest progenitors at the neural plate border, a group of genes that primarily encode transcription factors, including the Snail family genes Snail (Snai1) and Slug (Snai2), are induced in neural crest progenitor cells (1–4). Snail family genes have been implicated in regulating EMT during both embryonic development and metastasis of epithelial tumors (5–8). Recent evidence indicates that they also regulate other aspects of cellular function, including cell migration, cell proliferation, and protection from apoptosis (8). In frog and avian embryos, functional analyses have demonstrated that Snail family genes are required for formation and delamination of neural crest cells. In chick embryos, Slug expression is required for neural crest cell delamination (9), whereas in Xenopus embryos the Snai1 gene functions upstream of the Snai2 gene during neural crest cell formation (10, 11). We demonstrated previously that Snail2-deficient mice do not exhibit neural crest defects (12). It has been widely proposed that the absence of a neural crest phenotype in Snai2−/− mice reflects an absence of Snail2 expression in premigratory neural crest cells in the mouse and possible functional redundancy with the Snail1 gene (1–3, 13, 14). We demonstrate here that neither Snail1 nor Snail2 function is required for the generation, delamination, or migration of neural crest cells during early embryogenesis in mice. However, we show that the Snai1 gene plays a critical role in the establishment of left–right asymmetry.

Results and Discussion

The Meox2-Cre Transgenic Line Efficiently Deletes the Snai1flox Allele by Embryonic Day (E) 8.5. The Snai1 gene is expressed at high levels in the primitive streak and parietal endoderm at E7.5. Subsequently, Snai1 expression becomes prominent in the presomitic mesoderm, allantois, and in the dorsal aspects of the neural folds (see Fig. 5, which is published as supporting information on the PNAS web site). By E8.5, Snai1 expression is detected in the somites and becomes more prominent in the neural folds. The Snai1 gene also is expressed at this stage in the right lateral plate mesoderm (ref. 13 and Fig. 5). By E9.5, Snai1 expression also is observed in the branchial arches, limb buds, and endocardium.

We described recently the generation of Snai1−/− null mutant embryos (15). Snai1−/− embryos exhibit defects in mesoderm formation and die shortly after E7.5, likely because of defects in the extraembryonic membranes. This early embryonic lethality precluded investigation of the role of the Snai1 gene in developmental events occurring later in embryogenesis, such as formation and delamination of neural crest cells. To circumvent the early lethality of Snai1−/− embryos, we used a conditional null Snai1 allele (Snai1flox) (16). Snai1 genomic sequences were deleted by using Meox2-Cre mice, which express Cre recombinase in the embryon proper, but not in most extraembryonic membranes (17). We assessed the efficiency of deletion of the Snai1flox allele by whole-mount in situ hybridization with a Snai1 riboprobe. This analysis demonstrated that by E8.0, the Meox2-Cre, Snai1flox; null (hereafter designated Snai1-cko for Snai1 conditional knockout) mutant embryos lacked any detectable Snai1 expression (Fig. 5). Isolation of litters at different gestational stages revealed that, in contrast to Snai1−/− null embryos, the Snai1-cko embryos could survive as late as E9.5, when they subsequently died because of several vascular defects.

Snail Family Genes Snai1 and Snai2 Are Not Required for Neural Crest Cell Formation or Delamination. We initially examined Snai1-cko embryos for defects in neural crest cell formation and delamination. Development of neural crest cell-derived structures, such as the branchial arches, appeared morphologically normally in Snai1-cko embryos (Fig. 18). Expression of the neural crest marker Crabp1 demonstrated that neural crest cells were able to delaminate and migrate away from the dorsal neural tube of Snai1-cko embryos (Fig. 1 B and E). Similar results were obtained by using the markers Max1, Sox10, and Wnt1 (Fig. 1 H, K, and N). These results clearly demonstrate that the Snai1 gene is not essential for neural crest cell formation or delamination in mice. Although the Snai2 gene is normally expressed only in migratory neural crest cells in mice (12, 13), we analyzed the Snai1 conditional mutation on the Snai2-null mutant background (12) to exclude the possibility that compensation by the Snai2 gene permitted neural crest formation and delamination in Snai1-cko embryos. However, analysis of Snai1-cko Snai2−/− double-mutant embryos revealed patterns of neural crest cell marker gene expression similar to those of Snai1-cko embryos (Fig. 1 C, F, I, L, and O). We also established neural explant...
cultures to assess the ability of mutant neural crest cells to delaminate from the cranial neural folds in vitro. In cultures established from both Snai1-cko and Snai1-cko Snai2−/− embryos, migratory cells expressing the neural crest cell marker P75 were observed emigrating from the explants (Fig. 1 P–R). We conclude that at least through E9.5, neither the Snai1 nor the Snai2 gene, alone or in combination, is required for neural crest formation and delamination in mice.

**Primitve Streak Defects in Snai1-cko Embryos.** In contrast to the apparently normal generation of neural crest cells in Snai1-cko mutants, these embryos exhibited a number of defects in tissues derived from mesoderm. Snai1-cko embryos isolated at E8.5 exhibited a poorly formed allantois that failed to fuse with the chorion and a prominent posterior bulge extruding dorsally in close proximity to the primitive streak (Fig. 2). Analysis of the expression of several mesodermal markers, including T, Wnt3a,
and reversed heart looping in 40% (29/73) of cases, whereas in the remaining 23% (17/73) of cases the mutant embryos had a mostly vertical heart tube that could not be scored unambiguously. Similarly, axial rotation was reversed in 46% (17/37) of mutant embryos, independent of heart looping status. Control embryos exhibited no defects in either heart looping (0/41) or axial rotation (0/22). These data indicated that there is a randomization of left–right specification in Snail-cko embryos. In addition, we observed altered polarity of Hand1 expression in the heart of some Snail-cko embryos (Fig. 3F), further demonstrating the randomization in specification of the left–right axis in Snail-cko embryos.

To better understand the molecular basis for the laterality defects of Snail-cko embryos, we examined expression of the Pitx2, Lefty1, Lefty2, and Nodal genes in E8.0 Snail-cko and control littermate embryos. These genes are expressed on the left side of the embryo and form part of an evolutionarily conserved signaling cascade responsible for left–right morphogenesis (20). Snail-cko embryos consistently displayed bilateral expression of the Nodal, Lefty2, and Pitx2 genes (Fig. 4), which in wild-type embryos are normally expressed in the left lateral plate mesoderm. Lefty2 expression was particularly pronounced in the posterior of the Snail-cko embryos (Fig. 4 D and E), overlapping the normal Snail expression domain. The Nodal gene displayed a dynamic expression pattern in Snail-cko embryos, beginning just before the formation of the first epithelial somite. Very young (0–2 somites) embryos exhibited pronounced bilateral, posterior Nodal expression (Fig. 4 G and H), whereas in slightly older Snail-cko embryos (2–4 somites) expression was restricted to discreet rostral domains (Fig. 4 I and J). By the 4–6 somite stage, Nodal expression in the lateral plate mesoderm was lost completely (data not shown). These observations suggest that Snail-cko mutant embryos have a dynamically altered pattern of Nodal expression, with a rapid progression of bilateral expression moving from posterior to anterior. The dynamic pattern of Nodal expression is restricted to the lateral plate mesoderm, because perinodal expression is stable throughout this period. The bilateral expression of the Nodal, Lefty2, and Pitx2 genes suggests that the Snail gene plays a key role in regulation of asymmetric gene expression in the lateral plate mesoderm.

Snail-cko embryos did not express the Lefty1 gene (Fig. 4 D and E), which in wild-type embryos is expressed along the embryonic midline in the prospective floor plate (Fig. 4C). Proper restriction of left-specific signals to the left side of the embryo requires a structurally intact midline, and the Lefty1 gene has been proposed to function as a molecular barrier at the midline to prevent the diffusion of left-specific signals to the right side of the embryo. The lack of Lefty1 expression at the midline of Snail-cko embryos does not appear to be secondary to a loss of midline integrity, as assessed by expression of the T and Shh genes (see Fig. 7 B and D, which is published as supporting information on the PNAS web site). Another cause of laterality defects in mammals is mutations that affect the development and/or function of the node. However, node monocilia were observed on both control and Snail-cko nodes at E7.5 (see Fig. 8 A and B, which is published as supporting information on the PNAS web site). Moreover, perinodal expression of both Nodal and Cerl2, which in wild-type embryos are asymmetrically enhanced on the left and right side of the node, respectively, was observed in the Snail-cko nodes (Figs. 3 G–J and 8 D and F). We conclude that the Snail gene functions downstream of the initial left–right symmetry-breaking event, and regulates expression of laterality genes in the lateral plate mesoderm.

These results demonstrate that although some aspects of Snail family gene function, such as a role in left–right asymmetry determination, appear to be evolutionarily conserved, other
is lost in the posterior and restricted to the anterior portion of the lateral plate mesoderm. Note that normally in the perinodal region, but bilaterally in the lateral plate mesoderm, focused in the posterior domain. By the 2–4 somite stage (A vs. B), expression of Nodal in the region around the node (arrowhead) and left lateral plate mesoderm. In mutant embryos at 0–2 somites (A vs. B), transient, bilateral expression of Nodal in the lateral plate mesoderm of Snai1-cko embryos. Control embryos (C) express Lefty1 at the midline and Lefty2 in the left lateral plate mesoderm. Snai1-cko embryos (D and E) do not express Lefty1 and exhibit bilateral expression of Lefty2, which is particularly pronounced in the posterior region of the embryo. Note the varying degree of Lefty2 expression extending to the anterior of the embryo (D vs. E). (F–J) Transient, bilateral expression of Nodal in the lateral plate mesoderm of Snai1-cko embryos. Control embryos (F) between the 2 and 8 somite stage express Nodal in the region around the node (arrowheads) and left lateral plate mesoderm. In mutant embryos at 0–2 somites (G and H), Nodal is expressed normally in the perinodal region, but bilaterally in the lateral plate mesoderm, focused in the posterior domain. By the 2–4 somite stage (I and J), expression is lost in the posterior and restricted to the anterior portion of the lateral plate mesoderm. Note that Nodal expression around the node (arrowheads) appears normal at both stages in mutant embryos (G–J).

Aspects are not. Although a role for the Snail gene in regulating left–right asymmetry is conserved between mouse and avian embryos, it is not clear that identical mechanisms are operative in each situation. The bilateral expression of the left-specific gene Pitx2 in Snai1-cko embryos mirrors what is seen in chick embryos exposed to high levels of Snail antisense oligonucleotides (18, 19). In these experiments, Snail antisense treatment had no effect on Nodal expression, which suggested that the Snail gene acts either downstream of or in parallel with Nodal. Our findings, however, show a dynamic bilateral Nodal expression pattern in Snai1-cko mutant mouse embryos that moves rapidly from posterior to anterior before disappearing. Further work will be required to determine whether the Snail gene regulates left–right determination in mammalian and avian embryos by similar or distinct mechanisms.

Our results also demonstrate that neither the Snail1 nor the Snail2 gene is required, alone or in combination, for the formation, delamination, and initial stages of migration of neural crest cells during embryogenesis in mice. This finding is surprising, given the demonstrated role for these genes during neural crest cell formation and migration in Xenopus and avian embryos. Because of lethality caused by vascular defects subsequent to E9.5, we were unable to assess the requirement for these genes in neural crest cells generated in more posterior regions of the embryo or in terminal differentiation of neural crest cells. A number of papers and reviews have speculated that the absence of a neural crest phenotype in Snail2-deficient mice is likely due to functional redundancy with the Snail1 gene, along with the interchange in sites of expression of the Snail1 and Snail2 genes in various vertebrate species (1–3, 13, 14). The work described here demonstrates that there has not been an interchange of function between the Snail1 and Snail2 genes during neural crest cell formation and delamination in mouse and chick embryos. Rather, Snail family genes in mice are not required for these processes, at least through 9.5 days of gestation.

Materials and Methods

Mice. Targeted null alleles of the Snai1 (15) and Snai2 (12) genes have been described. Construction and characterization of the Snai1flox conditional allele also has been described (16), and Snai1flox/flox mice were maintained as homozygotes. Meox2-Cre mice (17) were obtained from The Jackson Laboratory. For the experiments described in this work, typically, male mice heterozygous for both the Meox2-Cre allele and the Snail1 null allele were crossed to Snai1flox/flox females, and embryos were isolated at varying gestational stages. Embryos of the genotype Meox2-Cre/+; Snai1flox/null were designated Snai1-cko embryos in this work. Embryos were genotyped by PCR of DNA isolated from the yolk sac or the entire embryo subsequent to in situ hybridization.

In Situ Hybridization. Whole-mount in situ hybridization was performed as described (21). Radioactive in situ hybridization was performed essentially as described (21), except 33P UTP was used instead of 35S as the labeled nucleotide. Exposure times for each probe were determined empirically. Digoxigenin-labeled riboprobes were generated by using the Roche labeling kit according to the manufacturer’s instructions. Radioactive probes were made by using the Promega Riboprobe kit, using 33P-labeled UTP (PerkinElmer).

Immunofluorescence. Histology and immunofluorescence was performed on standard 7-μm sections of paraformaldehyde-
in situ TUNEL analysis, samples were analyzed by using the fluorescein set mounting medium with DAPI (Vector Laboratories). For experiments. Samples were mounted by using Vectashield hard-ary antibodies (Invitrogen) were used at a 1:400 dilution for both placed on fibronectin-coated coverslips (Fisher) in six-well culture dishes containing culture medium (DMEM 4.5 g/liter glucose, 10 mM L-glutamine, Pen/Strep, and 10% FBS). Experiments were allowed to grow for 48 h before being fixed in 4% paraformaldehyde for 10 min at room temperature. For p75 immunofluorescence, fixed cells were permeabilized and blocked in 0.1% Triton X-100/10% goat serum/PBS for 1–2 h, washed, and incubated with anti-p75 (Chemicon) diluted in the blocking buffer at 1:100. Alexa Fluor 546-labeled secondary antibodies (Invitrogen) were used, and slides were mounted with DAPI.

Neural Epithelium Explant Cultures. Dorsal neural folds were isolated and cultured from E8.5 mutant and control embryos essentially as described (22). Briefly, embryos were isolated and treated in 1 mg/ml dispase (GIBCO) for 20 min at room temperature. The embryos then were washed in culture medium, and cranial neural folds were dissected away from the rest of the embryo, which was used for genotyping. Neural epithelium were teased away from the dorsal aspect of the cranial neural folds and placed on fibronectin-coated coverslips (Fisher) in six-well culture dishes containing culture medium (DMEM 4.5 g/liter glucose, 10 mM L-glutamine, Pen/Strep, and 10% FBS). Experiments were allowed to grow for 48 h before being fixed in 4% paraformaldehyde for 10 min at room temperature. For p75 immunofluorescence, fixed cells were permeabilized and blocked in 0.1% Triton X-100/10% goat serum/PBS for 1–2 h, washed, and incubated with anti-p75 (Chemicon) diluted in the blocking buffer at 1:100. Alexa Fluor 546-labeled secondary antibodies (Invitrogen) were used, and slides were mounted with DAPI.

Electron Microscopy. E7.5 and E9.5 embryos were fixed overnight at 4°C with 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.2). After washes in PB, embryos were postfixed in 1% osmium tetroxide in 0.1 M PB for 2 h at 4°C, dehydrated, and dried under CO2. Embryos then were mounted, sputter-coated with gold to 15 nm, and examined at 20 kV with a 3000N scanning electron microscope (Hitachi, Tokyo).

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