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Shared regulatory programs suggest retention of blastula-stage potential in neural crest cells

Elsy Buitrago-Delgado,[1]* Kara Nordin,[1]* Anjali Rao,[1] Lauren Geary,[1] Carole LaBonne[1,2]†

[1]Department of Molecular Biosciences, Northwestern University, Evanston, Il 60208, USA. [2]Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Evanston, Il 60208, USA.

*These authors contributed equally to this work.

†Corresponding author: clabonne@northwestern.edu

Neural crest cells, unique to vertebrates, arise in the ectoderm but can generate cell types typically categorized as mesodermal. This broad developmental potential persists past the time when most ectoderm-derived cells become lineage restricted. The ability of neural crest to contribute mesodermal derivatives to the bauplan has raised questions about how this apparent gain in potential is achieved. Here we describe shared molecular underpinnings of potency in neural crest and blastula cells. We show that in *Xenopus*, key neural crest regulatory factors are also expressed in blastula animal pole cells and promote pluripotency in both cell types. We suggest that neural crest cells may have evolved as a consequence of a subset of blastula cells retaining activity of the regulatory network underlying pluripotency.

Embryogenesis initiates with a totipotent fertilized egg cell, and subsequent development is characterized by progressive restrictions in cellular potential. At blastula stages, when the three primary germ layers are forming, chordate embryos still possess populations of pluripotent cells that are capable of differentiating into all somatic cell types. In mammals these are inner cell mass cells, whereas in *Xenopus* these cells are the deep/inner cells of the blastula roof, also termed animal pole cells (*1*). The pluripotency of blastula cells is transient; as embryogenesis proceeds into gastrulation, their potential becomes rapidly restricted into one of three cell types: ectoderm, mesoderm and endoderm. In all vertebrate species, a population of stem cell-like progenitors, called neural crest cells, represents an exception to this loss of potential. These cells arise from ectoderm positioned at the neural plate border, but in addition to ectodermal cell types can also differentiate into cartilage, bone, connective tissue, smooth muscle, pericytes and adipocytes, all of which are also formed by the mesoderm. Neural crest cells represent a major vertebrate innovation, collectively contributing to many of the features that distinguish vertebrates from non-vertebrate chordates, including much of the craniofacial skeleton, the chromaffin cells of the adrenal medulla and spinal nerve (dorsal root) ganglia. Because neural crest, despite its ectodermal origins, forms numerous cell types considered mesodermal, it has been likened to a fourth germ layer that renders vertebrates quadroblastic, and endows them with the potential to form a diversity of new cell types (*2*).

Much effort has been directed toward determining the developmental mechanisms by which a classic embryonic induction leads to the formation of the neural crest; cells that seemingly possess greater developmental potential than those from which they were derived embryologically or evolutionarily. Under current models these cells appear to defy the paradigm of progressive restriction in potential, and thus far no mechanism has been found to explain their apparent gain in potential. An alternative, more parsimonious, model for the origins of neural crest cells might be that they selectively retain the regulatory circuitry responsible for the pluripotency of their blastula precursors; a selective retention of earlier features. This model is supported by a shared requirement for Myc protein, and its transcriptional target Id3, in both neural crest cell genesis and ES cell pluripotency (*3–6*). We recently found that another neural crest regulatory transcription factor, *Sox5,* is initially expressed in blastula cells where it functions as a BMP R-Smad co-factor (*7*), providing an additional link between neural crest cells and pluripotent blastula cells. Based on these observations, we decided to systematically test the alternative model in *Xenopus.*

Neural crest shares regulatory circuitry with pluripotent blastula cells

In mammals, Pou5F1 (Oct4), Sox2 and Nanog, constitute a core pluripotency network essential for maintaining the uncommitted state of blastula cells (*8–13*). In *Xenopus*, the Pou5F1 factors expressed in ectoderm are Pou5F3.1 (Oct91), Pou5F3.2 (Oct25) and Pou5F3.3 (Oct60) (*14*, *15*). The functional role of Nanog in *Xenopus* is assumed by Ventx factors (Vent1/2) (*16*). These factors, along with *Sox2*, and the closely related *Sox3*, are expressed in blastula cells (*17*) (Fig. 1A). We wondered if other neural crest regulatory factors besides *Myc*, *Id3* and *Sox5* were co-expressed with the core pluripotency network in *Xenopus* blastula cells. We found that *Id3*, *TF-AP2, Ets1, FoxD3 and Snail1* were co-expressed with the core pluripotency factors (Fig. 1B). *FoxD3* and *Snail1* are also expressed in murine ES cells (*18*, *19*), providing further molecular links between neural crest factors and pluripo-

tency. While these neural crest and pluripotency factors exhibited broad expression during blastula stages, their expression became progressively restricted as lineage determination progressed, with several genes, including *Oct60, Sox3, Vent2, Ets1, Zic1*, *Pax3*, and *Snail1*, showing enhanced expression at the neural plate border by late gastrula stages (fig. S1, A and B). We found *Vent2* was co-expressed with *Snail2* at late gastrula/neurula stages when neural crest cells retain their full developmental potential, but was down-regulated as these cells begin to migrate and lose multipotency (fig. S1C).

Explanted blastula animal pole cells retain full developmental potential until the onset of gastrulation, when they lose competence to form mesoderm and endoderm (*20*, *21*). We thus examined whether expression of regulatory factors present in pluripotent blastula cells is lost when explants age and their developmental potential becomes restricted. *Oct60, Sox3*, *FoxD3*, and *Myc* expression was high in blastula-stage explants but reduced by late gastrula stages, correlating with loss of potential (Fig. 1C). Not all potency factors were down-regulated as these cells lost plasticity; expression of *Vent2* and *Id3* was unchanged as explants aged from blastula to gastrula stages (Fig. 1C). This suggests a concentration-dependent signature of regulatory factors may be essential to retaining broad developmental potential and preventing lineage restriction, consistent with findings in mouse that specific threshold concentrations of Oct4 (50-150% of endogenous levels) support pluripotency, while levels outside this range lead to differentiation (*9*, *22*).

Neural crest factors are required for pluripotency in blastula cells

Given that neural crest potency factors are co-expressed with core pluripotency factors in blastula cells, we asked if they were required to maintain expression of these pluripotency factors. Blocking Snail1 function in the animal pole of blastula embryos cells led to loss of expression of factors linked to the neural crest state, such as *TF-AP2* and *Id3* (Fig. 2A and fig. S3A). Expression of *Oct/Sox/Vent* network components was also lost (Fig. 2A and fig. S3A). We obtained similar results when Sox5 function was blocked in animal pole cells (Fig. 2B and fig. S3B). Thus, neural crest regulatory factors are not merely expressed in pluripotent blastula cells, but also function there to maintain expression of core pluripotency factors.

The developmental plasticity of amphibian animal pole cells was first demonstrated by Peter Nieuwkoop, whose recombinant assay drove current understanding of mesendoderm formation (*23*) (fig. S1D). Since we found that neural crest factors such as Snail1 are required for maintaining expression of factors linked to pluripotency, we hypothesized that cells lacking Snail1 function would lack competence to respond to endogenous inducing signals. To test this, animal pole explants from control blastulae, or blastulae in which Snail1 function had been blocked, were recombined with vegetal tissue from sibling embryos. Control recombinants robustly expressed mesodermal markers *Brachyury* and *MyoD*, whereas animal pole cells blocked for Snail1 function showed dramatically diminished responsiveness (Fig. 3, A and B, and fig. S3, C and D). Similar results were observed with animal pole cells depleted of Sox5 (Fig. 3, C and D, and fig. S3, C and D). As with conjugation to vegetal tissue, treatment of pluripotent blastula cells with low/moderate doses of activin instructs them to form mesoderm, and this responsiveness is also lost in cells depleted of Snail or Sox5 function (Fig. 3, E and G, and figs. S1E; S2, A and C; and S3, E and F).

Since Snail factors have endogenous roles in mesoderm formation, a more demanding test of their contributions to pluripotency was to ask if blastula cells lacking Snail1 function consequently lose their capacity to form endoderm. Blastula explants adopt endodermal fates in response to high activin concentrations, expressing endoderm-specific genes such as *Endodermin* and *Sox17*. However, blastula explants depleted of Snail function could no longer form endoderm (Fig. 3F and figs. S2B and S3, G and H). Snail proteins are neither expressed in, nor function in, endoderm endogenously, thus loss of activin-mediated endoderm induction likely reflects a general lack of competence of Snail depleted animal pole cells to respond to endoderm-inducing signals. Similar results were found when Sox5 was depleted from blastula cells (Fig. 3H and figs. S2D and S3, G and H).

Reprogrammed neural crest can form endoderm

Given that neural crest potency factors are expressed in pluripotent blastula cells and required for expression of core pluripotency factors, we further explored the link between the neural crest state and the pluripotent blastula state. Specifically, we asked if establishing a neural crest state is sufficient to confer pluripotency on, or prevent loss of pluripotency in, descendants of blastula animal pole cells. Animal pole cells explanted at blastula stages are initially competent to give rise to all somatic cell types, but lose pluripotency by gastrula stages. Established protocols exist for converting blastula animal pole explants to a neural plate border or neural crest state. Combined expression of Pax3 and Zic1 efficiently converts explants to neural plate border (*24–26*) whereas Snail2 together with Wnt signaling is sufficient to establish a neural crest state (*27*, *28*). We therefore asked if converting these explants to a neural plate border or neural crest state would be sufficient to prevent loss of competence and extend the pluripotency of these cells (Fig. S1F).

As predicted, explants treated at blastula stages with mesoderm-inducing concentrations of activin robustly expressed the mesoderm-specific *MyoD* gene, but if explants were aged to gastrula stages before treatment they were unable to form mesoderm (Fig. 4A). By contrast, explants converted to a neural plate border state retained their potency and formed mesoderm in response to either early or late activin treatment (Fig. 4A and fig. S4A). We also tested whether this change in plasticity extended to endoderm

formation. When blastula-derived cells were treated with endoderm-inducing doses of activin, identical results were achieved (Fig. 4B). Explants treated with high activin at blastula states expressed the endodermal markers *Endodermin* and *Sox17* but were unable to do so when treated at gastrula stages (Fig. 4B and fig. S2E). By contrast, Pax3/Zic1 programmed explants retain the ability to form endoderm even when treated at gastrula stages (Fig. 4B and figs. S2E and S4, C and E). Similarly, blastula-derived cells programmed to a neural crest state with Snail2/Wnt8 retain competence to form mesoderm and endoderm through gastrula stages (Fig. 4, C and D, and figs. S2F and S4, B, D and F). The ability of neural plate border/neural crest factors to prevent loss of pluripotency in animal pole derived cells, combined with the requirement of these factors for the normal plasticity of these cells at blastula stages, suggests a close link between the molecular networks controlling the potency of neural crest and blastula cells.

Endogenous neural crest can form endoderm

If such a link exists, one prediction is that cells isolated from the neural plate border region of an intact neurula-stage embryo should also exhibit broad developmental potential, including the capacity to form both mesoderm and endoderm. This capacity should exist even though there is currently no evidence that neural plate border-derived cells form endoderm during normal development. To test this prediction we isolated neural plate border cells from neurula stage embryos, and cultured them in vitro (fig. S1G). As expected, when these cells were cultured without inducers they did not express the mesodermal markers *MyoD* or *Brachyury,* or the endodermal markers *Endodermin* or *Sox17* (Fig. 5, A to D). However, treatment of neural plate border explants with concentrations of activin sufficient to induce mesoderm or endoderm in pluripotent blastula cells elicited strong expression of all of these genes (Fig. 5, A to D, and figs. S2G and S4, G to J). These findings demonstrate that endogenous neural crest cells possess a much greater degree of potency/plasticity than has previously been appreciated, including an unexpected capacity for endoderm formation.

Discussion

Longstanding models for neural crest formation posit that inductive interactions endow these cells with greater developmental potential than the cells they were derived from, developmentally or evolutionarily (*25–28*) (fig S5A). This classic view implies a unique reversal of trajectory in Waddington's landscape of progressive restriction of developmental potential (*29*). Based upon our findings reported here, we suggest instead a revised model in which neural crest cells are an example of cellular neoteny (*30*). Select cells with the pluripotent potential characteristic of the blastula state persist to neurula stages where they can be induced to form the highly diverse lineages that derive from the neural crest (fig. S5B). This retention of pluripotency long after other cells have become fate restricted has endowed the neural crest with the capacity to contribute the novel attributes characteristic of vertebrates to the simple chordate bauplan. Mechanistically, we propose that neural crest cells arose as a consequence of their retention of all or part of a regulatory network that controls pluripotency in the blastula cells from which they were derived.

Our model is consistent with, and helps explain, an earlier study of avian embryos that detected expression of genes associated with the neural crest state, such as *Pax7*, in the medial epiblast at early gastrula stages (*31*). Those findings were interpreted at the time as evidence that neural crest induction occurs earlier than previously believed, but we suggest it reflects the retention of pluripotency in a subset of avian epiblast cells. Our work further suggests that transcription factors, such as Pax7 or Snail1, previously considered/defined as neural plate border or neural crest factors should instead be viewed as pluripotency maintenance factors. Future studies should address how such factors function to retain potential to contribute to all three germ layers in cells that will become the neural crest. The previously unrecognized capacity of neural crest cells to express endodermal markers in culture raises the question of whether they also contribute endodermal cell types endogenously. Perhaps neural crest contributions to otherwise endodermal organs, such as the parafollicular cells of the thryroid (*32*), should be considered a contribution of endoderm.

The model for formation of neural crest cells proposed here provides a framework for future studies in basal chordates to probe the earliest evolutionary origins of these cells. Ascidians, for example, possess a cell lineage that arises from the neural plate border and expresses genes such as *Snail*, *Id*, *FoxD* and *Ap2*, all of which we find shared between pluripotent blastula cells and neural crest. This a9.49 lineage in ascidians may be homologous to the neural crest lineage in vertebrates (*33*). Investigating shared and divergent aspects of pluripotency network components in these and other protochordate and basal vertebrate models should therefore shed light on when and how pluripotency was retained in cells that become neural crest, and thus provide insight into the evolutionary origins of vertebrates.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S5

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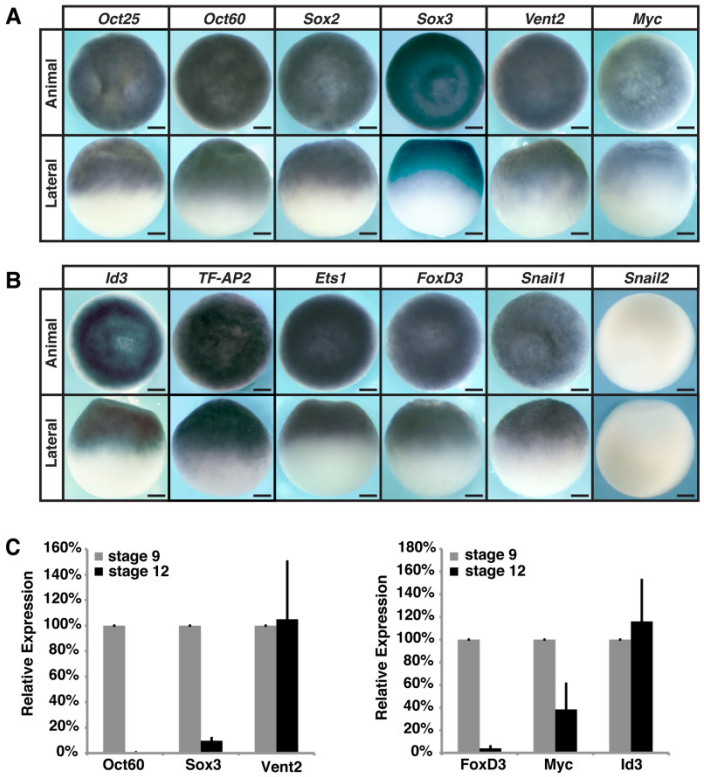

Fig. 1. Neural crest cells and pluripotent blastula cells share a common regulatory circuitry. (**A** and **B**) In situ hybridization of wildtype blastula (stage 9) *Xenopus* embryos examining expression of genes associated with pluripotency (A) or neural crest formation (B). Scale bars, 250 μM. (**C**) qRT-PCR of wildtype ectodermal explants examining relative expression of pluripotency genes and neural crest genes over developmental time.

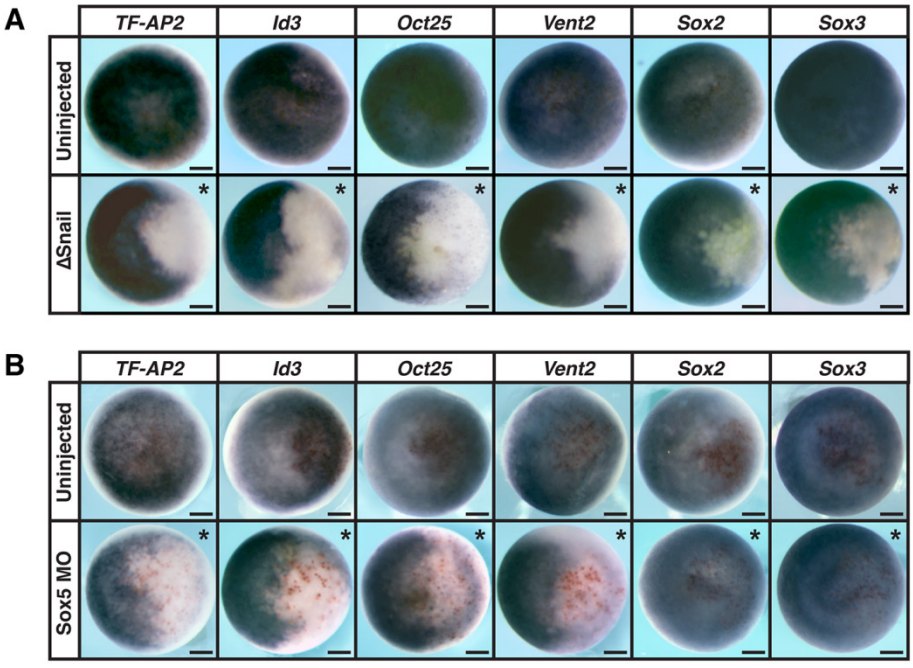

Fig. 2. Neural crest regulatory factors are required for the expression of blastula pluripotency factors. (**A** and **B**) In situ hybridization of embryos injected with ΔSnail mRNA (A) or Sox5 MO (B). Embryos were collected at blastula stages (stage 9) and examined for expression of genes associated with pluripotency/neural crest formation. Asterisk denotes injected side with β-gal staining (red) serving as a lineage tracer. Scale bars, 250 μM.

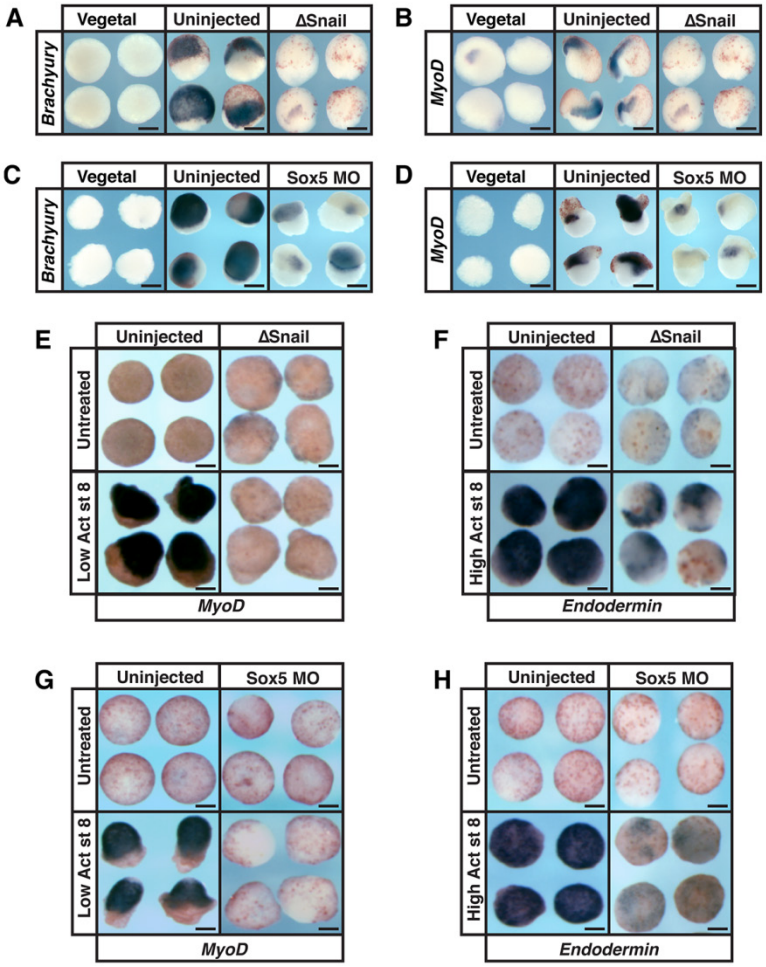

Fig. 3. Neural crest regulatory factors are required for pluripotency of blastula cells. (**A** to **D**) Nieuwkoop recombinant assay examining expression of *Brachyury* [(A) and (C)] and *MyoD* [(B) and (D)] after depleting Snail1 [(A) and (B)] or Sox5 function [(C) and (D)]. Recombinants were harvested at gastrulation stages for *Brachyury* expression (stage 11.5) or early neurula stages (stage 13/14) for *MyoD* expression. (**E** to **H**) Ectodermal explant assay examining expression of *MyoD* [(E) and (G)] and *Endodermin* [(F) and (H)]. Explants were injected with ΔSnail mRNA [(E) and (F)] or Sox5 MO [(G) and (H)] and cultured with or without activin until early neurula stages for *MyoD* expression (stage 13/14) and midgastrula stages (stage 11.5) for *Endodermin* expression. Scale bars, 250 μM.

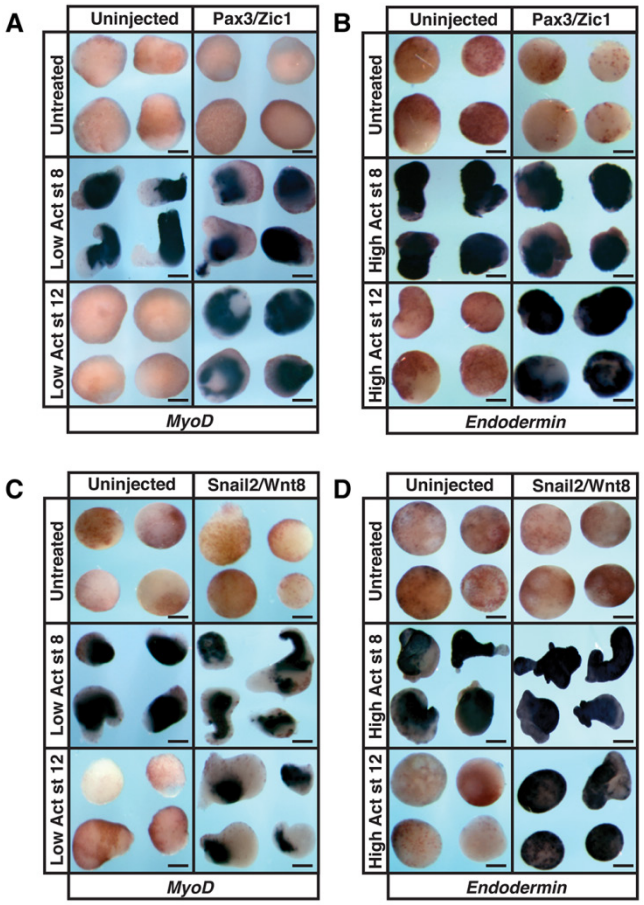

Fig. 4. Establishing a neural crest state prevents loss of potency in blastula-derived cells. (A to D) Ectodermal explant assay examining expression of *MyoD* [(A) and (C)] or *Endodermin* [(B) and (D)] in embryos that were injected with Pax3-GR/Zic1-GR mRNA [(A) and (B)] or Snail2/Wnt8 mRNA [(C) and (D)]. Explants were treated with activin at either stage 8 or 12 and cultured until late neurula stages (stage 18). Scale bars, 250 μM.

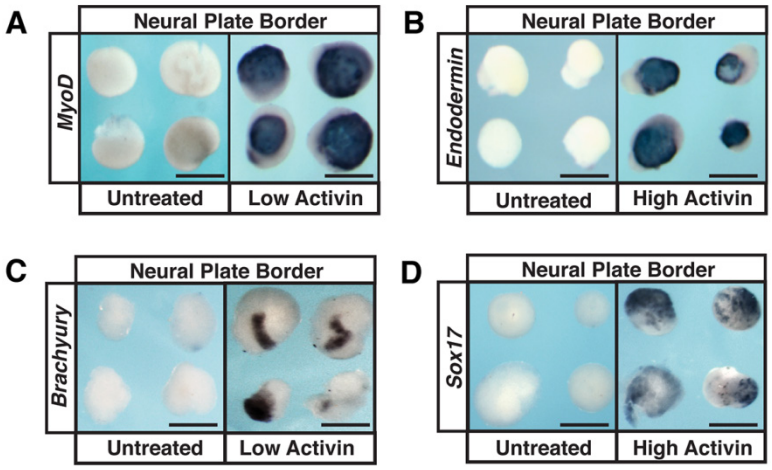

Fig. 5. Neural crest cells possess the capacity for endoderm formation. (A to D) In situ hybridization examining at late neurula stages (stage 18) expression of *MyoD* (A), *Endodermin* (B), *Brachyury* (C), and *Sox17* (D) in neural plate border/neural crest tissue treated with or without activin. Scale bars, 250 μM.