Supplementary Materials for

Construction of a Vertebrate Embryo from Two Opposing Morphogen Gradients

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Materials and Methods

Engineering of opposing BMP and Nodal morphogen gradients

Gradients of BMP and Nodal were engineered at the animal pole of blastula embryos by injecting, as previously described (3, 4), their corresponding mRNAs into two different animal pole blastomeres at the 128-cell stage. By diffusing from their secreting source and by stimulating their cognate receptor, BMP and Nodal form gradients of morphogen activity, maximal in proximity to the secreting clones and progressively decreasing further away from their source. The Nodal signaling gradient pathway has been generated by injecting 10 pg of Nodal-related 2 (Ndr2, cyclops) mRNA. Ndr2 has been described to act at short range but, when stimulating animal pole cells, it activates transcription, after the midblastula transition, of nodal-related 1 (Ndr1, squint), known to act at long range (12). Therefore the final Nodal activity gradient results from the activity of both Ndr2, synthesized from the injected mRNA, and Ndr1, induced as a direct response of cells stimulated by Ndr2. We therefore use “Nodal” as a generic term to describe the sum of Ndr1 and Ndr2 activities that stimulate animal pole cells.

The establishment of a gradient of Nodal activity at the animal pole of the blastula/gastrula by the clone of cells derived from the blastomere that had been injected with Nodal mRNA is demonstrated by the concentric expression pattern of Nodal downstream target genes, with those known to require the highest level of stimulation (endoderm and prechordal plate markers) expressed close to the secreting clone while those induced by a lower amount (dorsal and dorso-lateral mesoderm) observed to be expressed in more distant cells.

Stimulation of the BMP pathway was performed by injecting 100 pg of bmp2b mRNA or 20 pg of a 1:1 mix of bmp2b and bmp7 mRNAs. The functionally active BMP morphogen has been shown to be a bmp2b/bmp7 heterodimer (28, 29). Therefore, after injection of the bmp2b mRNA, the morphogen forms by heterodimerization between the BMP2b synthesized from the injected mRNA and the BMP7 translated from endogenous mRNAs transcribed after the MBT in the cells derived from the injected blastomere.

The optimal distance between the injected blastomeres has been experimentally determined and found to correspond to injection into two blastomeres of the animal pole blastula at the 128-cell stage separated by one non-injected blastomere. This condition allows induction of complete secondary embryonic axes in more that 50% of injected embryos. Injection in adjacent cells results in Nodal and BMP clones overlapping extensively, resulting into the formation of partial secondary axes lacking dorsal mesendodermal structures. Injection into blastomeres separated by two or more uninjected blastomeres strongly reduces the frequency of complete secondary embryonic axes that have been observed.

All experiments involving generation of Nodal and/or BMP gradients by injection of animal pole blastomeres have been performed at least in triplicate on 150 embryos each time. After optimal conditions have been defined, every in situ marker used has been tested on 50 embryos for each condition and stage (reproduced 3 times).

Labeling of injected blastomeres

To track cells derived from injected blastomeres, 50 pg of mRNA coding for Green
Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP) have been coinjected together with Nodal and BMP mRNA. To visualize BMP secreting cells in experiments where injected embryos were analyzed by in situ hybridization, cells were labeled by coinjection of 50 pg of LacZ mRNA or 50 pg of GFP mRNA. Localization of β-galactosidase activity was performed as previously described (30). GFP was localized by HRP immunohistochemistry using an anti-GFP polyclonal primary antibody made in rabbits (ab290 from abcam) used at a dilution of 1/100,000 and an HRP Goat Anti-Rabbit secondary antibody used at a dilution of 1/2000 followed by detection of HRP using the DAB peroxidase substrate kit (Vector Laboratories, Inc).

**In situ hybridization**

Single and double color in situ hybridization have been performed as previously described (31). The liv1 (slc39a6) probe contains the sequence of the second exon of the slc39a6 gene cloned into the pCR-Topo vector. All other probes have been generated from cDNA clones inserted into the pBS vector; antisense mRNA was synthesized using the T7 RNA polymerase. The expression pattern during embryonic development for all molecular markers used is available at [http://zfin.org](http://zfin.org).

**Labeling of blastula/early gastrula embryonic margin**

To differentially label the animal pole territory and the embryonic margin, 50 pg of mRNA coding for the photoactivable fluorescent Kaede protein was injected at the one-cell stage. This was followed, at the 128-cell stage, by injection of BMP and Nodal mRNAs into two different animal pole blastomeres. At the late blastula stage, the fluorescence emission spectrum of the Kaede protein was irreversibly converted, as previously described (32), from green to red by irradiation of the embryonic margin with UV light. Embryos were examined immediately after photoconversion to verify that the fluorescence of the Kaede protein was photoconverted from green to red at the embryonic margin. Embryos were further analyzed at 24hpf for localization of the green and red fluorescent signals looking for the presence or absence of red labeled cells in the secondary embryonic axis.

**Explantation of animal pole and in vitro culture of explants**

Using a syringe needle (27G1/2 Becton Dickinson) as a tool, the animal pole region of early embryos injected with Nodal and/or BMP at the 128-cell stage or of uninjected controls was cut from the blastula at the 512-cell stage in a Petri dish coated with 1.5% agarose filled with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media with 25 mM Hepes, 2.5 mM L-Glutamine and supplemented with 7 mM CaCl2, 1 mM sodium pyruvate, 50 µg/ml gentamycin, 1x antibiotic-antimycotic (Gibco) and 3% heat inactivated fetal bovine serum (FBS). Animal pole explants corresponding roughly to 1/3 of the blastula were allowed to form a sphere in this dish for a few minutes and then placed in another Petri dish containing fresh culture medium and incubated at 28.5°C. Fusion of two animal pole explants is obtained by apposing the two freshly cut surfaces (as shown in fig. S10A). After a few minutes, the two pieces of tissue form a single mass of cells which is placed in another Petri dish with fresh culture medium and incubated at 28.5°C.
Time-lapse imaging
For time-lapse imaging, live embryos or embryoids were placed in a glass bottom culture dish (MatsTek corp.) properly oriented in one drop of 0.1% agarose /Danieau buffer 0.3x, then covered with a solution of Agarose 0.1% (supplemented with DMEM/F12 – 3% FBS and 7 mM CaCl₂ for embryoids). A coverslip was used to seal the preparation. Using a Leica TCS LSI confocal macroscope and the LAS AF (Leica Application Suite Advanced Fluorescence) software, time-lapse movies were made of z-stack captured every 5 minutes in bright field, green channel (for GFP) and red channel (for RFP). Z-slices were acquired with a z-interval of 10 µm.

Supplementary references

Fig. S1
Organizing a vertebrate embryo with gradients of BMP and Nodal activity (A) Spemann-Mangold grafting experiment (1), illustrated in the middle panel for a zebrafish embryo, or gain of function of ventral morphogen antagonists at the ventral margin results in the formation of a mirror image duplication (right panel) of the gradient of ventralizing factors present in wild-type embryo (left panel). (B) The BMP/Nodal ratio of activity varies continuously from ventral to dorsal along the embryonic margin in the wild-type embryo (left panel). As previously reported (8), stimulation of animal pole cells with a given ratio of BMP and Nodal or a graft onto the animal pole of a corresponding domain of the embryonic margin induces different parts of the embryonic axis. A high ratio or a graft of ventral margin organizes a tail; a low ratio or graft of the dorso-lateral margin organizes a posterior head while a moderate ratio or a graft of lateral margin organizes trunk structures. Stimulation of the animal pole by Nodal or a graft of the dorsal margin induces formation of axial mesendodermal tissues. (C) Engineering two opposing gradients of BMP and Nodal at the animal pole generated by clones secreting these factors recapitulates, at the animal pole, the continuous variation of BMP/Nodal activity ratios present at the wild-type embryonic margin and generates conditions sufficient for the organization of a complete secondary embryonic axis.
Fig. S2
The induced secondary embryonic axis is built from animal pole cells.
(A) Embryo injected at the one cell-stage with Kaede mRNA and at the 128-cell stage with BMP and Nodal mRNAs into two different blastomeres and subsequently observed at late blastula. ap: animal pole, m: embryonic margin. (B, C) At late blastula, Kaede fluorescence is converted from green to red by UV irradiation of the embryonic margin. (B) Red channel, (C) Merge of red and green channels. (D to G) Same embryo at 24 hpf showing (D) primary (i) and secondary (ii) embryonic axes observed for fluorescence of (E) unmodified Kaede protein (green channel) or (F) photoconverted Kaede protein (red channel). (G) Merge of green and red channels. The secondary embryonic axis is devoid of red-labeled cells and is built only with green-labeled cells originating from the animal pole.
Fig. S3
BMP, by itself, does not induce formation of ectopic embryonic structures at the animal pole.

(A, B) Clones derived from one animal pole blastomere injected with both BMP2b and GFP mRNAs (A) spread in the ectoderm at gastrulation, (B) populate the epidermis at 24hpf without affecting embryo morphology or (C) stay at the animal pole at gastrulation, (D) lead to dorsal expansion of the epidermis and decrease of anterior neural tissues at 24hpf. (E, F) wild-type embryos at (E) gastrulation and (F) 24 hpf. Embryos are shown in lateral view.
Fig. S4
Expression of Nodal stimulates generation of an attracting center at the animal pole of the blastula/gastrula.
Left panels: wild-type embryos at blastula (40% epiboly) or gastrula (shield, 60% epiboly) stages displaying two fluorescent clones of cells derived from two blastomeres injected at the 128-cell stage with RFP or GFP mRNA that spreads within the ectoderm during the gastrula stage. Right panels: embryos at the same developmental stages injected at the 128-cell stage with GFP mRNA in two blastomeres (green cells) on both sides of a central blastomere injected with a combination of Nodal and RFP mRNAs (red cells). Stimulation by Nodal induces an attracting center, toward which surrounding cells converge.
**Fig. S5**
Stimulation of the animal pole by Nodal induces expression of dorsal and dorso-lateral marginal genes.

Stimulation of the animal pole by Nodal (+ Nodal) induces expression of dorsal specific genes such as (A) goosecoid (gsc) and (B) chordin (chd) at the dome stage. At the beginning of gastrulation, the expression patterns of genes induced by Nodal are organized in concentric circles. Expression of the panmesodermal marker notail (ntl) forms a ring (C). Double color *in situ* hybridizations reveals that genes requiring the highest level of Nodal activity such as sox32 (endodermal marker, D) and frzb (prechordal plate marker, E), are expressed in the center of the ntl expressing ring (labelled in red). Expression of the notochordal marker sonic hedgehog a (shha) is induced in the inner part of the ntl expressing ring (F) while wnt8a, which is excluded from dorsal but is present in dorso-lateral marginal cells is induced in the outer part of the ntl expression domain (G). Nodal does not induce expression of lateral and ventral genes such as eve1 (H). At late gastrulation, the ectoderm present around the growing notochord expressing ntl (red) is of dorsal identity and expresses the hindbrain marker gbx1 (I). The embryos in the upper panels have been injected with Nodal RNA in one animal pole blastomere and embryos in the lower panels correspond to the expression of the genes indicated in the low left corner in wild-type (WT) embryos. Developmental stages are indicated at the bottom. The red dashed line indicates the domain of the dorso/dorso-lateral embryonic margin, which is induced when Nodal stimulates the animal pole. Embryos are shown in animal pole view, dorsal to the right. Scale bar in (A) 100 µm except for upper panels (C to G) 35 µm.
**Fig. S6**

**Stimulation of the animal pole by Nodal and BMP induces lateral and ventral cell identity.**

(A, B) Expression of the paraxial mesoderm marker *myf5* demonstrates the presence of dorso-lateral mesoderm in the structure induced by stimulation of animal pole cells by Nodal and BMP. (A) Animal pole view. BMP secreting cells are visualized using LacZ staining (light blue). (B) Optical cross-section of the structure growing at the animal pole of the embryo shown in (A). (C) Expression of *myf5* in wild-type (WT) embryo. Embryo is in vegetal pole view, dorsal to the right. pm: paraxial mesoderm. (D) Expression of the epidermal (ventral ectoderm) marker *foxi1* at late gastrula stage in an embryo injected with Nodal and BMP and for which, at the onset of gastrulation, the BMP secreting clone was located on the dorsal part of the animal pole. ep(i): epidermis of the primary embryo, ep(ii): epidermis of the secondary embryo. (E, F) Double color *in situ* hybridization at late gastrula stage (80% epiboly) with *ntl* (red, notochord) and *gbx1* (black, hindbrain) in an embryo injected with Nodal and BMP, with the BMP secreting clone in the ventral part of the animal pole at the onset of gastrulation. The interruption of the expression of *gbx1* (between arrowheads in F) indicates that ventral ectodermal identity has been induced by BMP. (F) is a high magnification of the animal pole of the embryo shown in (E). Scale bar in (D): 200 µm (C to E), 60µm (A, B, F).
Antero-Posterior polarity and elongation of the secondary embryonic axes induced by Nodal or by Nodal and BMP

(A, B) Double color in situ hybridization revealing the anterior (a) - posterior (p) polarity (gbx1, hindbrain marker being anterior and ntl, mesodermal marker being posterior) of the primary (green arrows) and secondary (red arrows) axes induced by Nodal (A) or by Nodal and BMP (B). (C, D) Expression of wnt8a and (E, F) of fgf8a at gastrula stage visualized by in situ hybridization in embryos in which the animal pole has been stimulated by Nodal. Triangles illustrate the action on the antero (a)-posterior (p) polarity of Wnt8a and FGF8a, which act as morphogens, from the embryonic margin (green) and from the blastopore lip induced by Nodal (red). (G) Stimulated solely by Nodal, the induced radially symmetrical structure extends out of the animal pole (arrow) parallel to the animal-vegetal axis of the egg (doted line). (H) In the presence of Nodal and BMP signaling centers, at late gastula stage, the prechordal plate (pp) expressing frzb and the notochord (n) expressing ntl migrate away from the BMP secreting cells. ppi: prechordal plate of the primary axis. This results in a rotation of the a-p axis of the extending structure of about 45° relative to the animal-vegetal axis (blue arrow). Consequently, when the induced embryonic axis elongates posteriorly (arrow), it extends in the direction of the BMP secreting center, which becomes located in its ventral-posterior position. Scale bar in (G): 250 µm (G, H), 200 µm (A to F).
Fig. S8
Correlation between the relative position of the BMP and Nodal secreting clones and the orientation of the secondary embryonic axis induced at the animal pole.

(A, B) When (A) the orientation of the vector between the Nodal and the BMP secreting clones (yellow arrow) is parallel to the D-V axis (white arrow) of the embryonic margin, where Nodal is strong dorsally and BMP strong ventrally, (B) the primary (i, blue arrow) and the secondary (ii, red arrow) axes are parallel. (C, D) When (C) the Nodal-BMP vector is perpendicular to the D-V axis, the secondary embryonic axis grows (D) perpendicular to the primary axis. (E, F) When (E) the Nodal-BMP vector is antiparallel to the D-V axis, primary and secondary axes grow (F) in opposite directions.

sh: embryonic shield, (A, C, E) animal pole views at the shield stage, dorsal to the right, (B, D, F) lateral views at 30hpf.
Characterization of animal pole explants of early zebrafish blastulae. Gene expression analysis by in situ hybridization at gastrula stage in uninjected animal pole explants for the Nodal (ndr2, lft1); FGF (fgf8, spry4), Wnt (wnt8a, sp5l), retinoic acid, (aldh1a2), non canonical Wnt (wnt11) and BMP (bmp2b, bmp4, bmp7a, ved, szl, bambi) signalling pathways, as well as the dorsal BMP superfamily member admp, an epidermal marker (foxi1), neural plate markers (zic1, pax2a), an endoderm marker (sox32), a notochord marker (flh), a muscle marker (msgn1), a ventral marginal marker (eve1), a dorsal marginal marker (gsc) and a prechordal plate marker (hgg1). Altogether, explants do not express ligands or downstream targets of the Nodal, Wnt or FGF pathways, which are activated at the early embryonic margin. Similarly, expression of aldh1a2, an enzyme involved in retinoic acid biosynthesis, as well as expression of molecular markers of endoderm, mesoderm and dorsal ectoderm are absent. Expression of genes coding for BMP as well as expression of BMP target genes is observed in all cells of the explants, which have a ventral ectodermal identity and express epidermal markers such as foxi1. Explants have been fixed for analysis by in situ hybridization when their siblings have reached the midgastrula stage. Scale bars: 200 µm.
Fig. S10
Formation of embryoids from two fused animal pole explants. (A) Fusion of two animal pole explants derived from an embryo injected at the 1-cell stage with fluorescein dextran (green) and an embryo injected at the 1-cell stage with rhodamine dextran (red) and with BMP and Nodal mRNAs into two separate blastomeres of the animal pole (ap) at the 128-cell stage prior to isolation and fusion of the two animal explants. This results in the formation of a single mass of cells containing two animal pole regions for which the animal (a) – vegetal (v) axes (arrowheads) are oriented in opposite directions. At the gastrula stage a blastopore forms in that part of the fused explant derived from the explant injected with BMP and Nodal mRNAs (red) while a cavity (blastocoele) is apparent in the part (green) derived from the fluorescein dextran injected embryo. At 24 hpf, the mesendoderm of the resulting embryo is labelled in red and derives from the explant obtained from the embryo injected with BMP and Nodal while the anterior central nervous system and the head epidermis is mainly formed from cells derived from the explant obtained from the fluorescein dextran injected embryo (green). (B) Volume of single and double explants compared to the volume of a whole embryo at the sphere stage that was removed from the yolk. About 6,000 cells are present in a complete embryo at the sphere stage. Single explants contain roughly 2,000 cells and double explants contain roughly 4,000 cells. (C) Size comparison of embryoids derived from single explants or from double explants with the size of a wild-type embryo at 24 hpf. ap: animal pole, bl: blastopore, br: brain, cns: central nervous system e: eye, ep: epidermis, n: notochord. Scale bars: 200 µm.
**Movie S1**
Secondary embryonic axis induced by the combination of Nodal and BMP activity gradients. Observed at 30 hpf, the secondary embryonic axis (bottom) includes a complete head, a beating heart and shows spontaneous muscle contractions indicative of a functional nervous system independent of muscle contractions of the primary axis (on top). Remarkably, the antero-posterior orientation of this induced secondary axis is the reverse of the antero-posterior orientation of the primary axis.

**Movie S2**
Time-lapse imaging of ectopic gastrulation induced by Nodal at the animal pole. Embryos injected with 10 pg of Nodal mRNA and 50 pg of red fluorescent protein (RFP) mRNA in one animal pole blastomere at the 128-cell stage and observed under Leica TCS/LSI confocal macroscope during 4 hrs from germ ring to late gastrulation. Nodal secreting cells (red) allow visualization of the internalization of animal pole cells and formation of the blastopore and blastopore lip. Cells that do not internalize and remain superficial at the animal pole are cells derived from the injected blastomere that participate in the formation of the enveloping layer (EVL) at the early blastula stage.

**Movie S3**
Time-lapse imaging of ectopic gastrulation induced by Nodal and BMP at the animal pole. Embryos injected with 10 pg of Nodal mRNA and 50 pg of RFP mRNA in one blastomere and with 100 pg of BMP2b mRNA and 50 pg of GFP mRNA in another blastomere of the animal pole at the 128-cell stage were observed under a Leica TCS/LSI confocal macroscope for 3 hrs from germ ring to mid-gastrula stage. Red labeling: Nodal secreting cells; green labeling: BMP secreting cells. Green cells close to the Nodal signaling center are attracted by and converge toward the Nodal secreting cells, while more distant cells are displaced vegetally and disperse in the ectoderm.

**Movie S4**
Time-lapse imaging of the gastrulation induced by Nodal in animal pole explants cultured *in vitro*. Animal pole injected with 10 pg of Nodal mRNA and 50 pg of GFP mRNA in one blastomere at the 128-cell stage, was explanted, placed in culture medium and observed under a Leica TCS/LSI confocal macroscope for 4 hrs, from germ ring to late gastrula stage.
References and Notes


