Self-organization of a human organizer by combined Wnt and Nodal signalling

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In amniotes, the development of the primitive streak and its accompanying ‘organizer’ define the first stages of gastrulation. Although these structures have been characterized in detail in model organisms, the human primitive streak and organizer remain a mystery. When stimulated with BMP4, micropatterned colonies of human embryonic stem cells self-organize to generate early embryonic germ layers1. Here we show that, in the same type of colonies, Wnt signalling is sufficient to induce a primitive streak, and stimulation with Wnt and Activin is sufficient to induce an organizer, as characterized by embryo-like sharp boundary formation, markers of epithelial-to-mesenchymal transition and expression of the organizer-specific transcription factor GSC.

The pioneering experiments of Spemann and Mangold demonstrated that a small group of cells located on the dorsal side of the early amphibian embryo have the ability to induce and ‘organize’ a complete secondary axis when transplanted to the ventral side of another embryo2. This led to the concept of the ‘organizer’, and later discovery of embryonic tissue with similar organizer activity in fish, birds, and rodents3,4–7 demonstrated that this early embryonic activity is evolutionarily conserved. Cells in the organizers of all species studied to date exhibit the same behaviour during axis induction: they (i) contribute autonomously to axial and paraxial mesoderm, including head process and notochord, and (ii) induce neural fate non-autonomously in their neighbours. While cells exhibiting some molecular characteristics of an organizer have been generated in vitro from human embryonic stem cells8–10 (hESCs), these functional properties have not been demonstrated and the organizer in humans remains undefined.

Owing to the ethical limitations of working with early human embryos, the only way to search for the human organizer is via human embryonic stem cells (hESCs). We have previously shown that grown on geometrically confined discs, hESCs respond to BMP4 by differentiating and self-organizing into concentric rings of embryonic germ layers: with ectoderm in the centre, extra-embryonic tissue at the edge, and mesoderm and endoderm in between1. These embryo-like ‘gastruloids’ are robust and amenable to analysis with subcellular resolution.

During mouse gastrulation, BMP4 signalling activates the Wnt pathway which in turn activates the Activin–Nodal pathway (Fig. 1a), at both the transcriptional and signalling levels8. Since it has been shown in mouse and other vertebrates that these three pathways are the most critical pathways for organizer formation9–12, we first investigated whether this hierarchy was conserved in human gastruloids. Using RNA sequencing analysis (RNA-seq), we found that among the 19 Wnt genes in the human genome, only WNT3 is markedly and immediately induced upon stimulation with BMP4 (Fig. 1b). Reverse transcription with quantitative PCR (qPCR) analysis shows that activation of Wnt signalling directly induces NODAL expression (Fig. 1c). Further qPCR analysis showed that NODAL induction was reduced when the Nodal pathway was inhibited with SB431542 (SB) and IWP2, which antagonizes Nodal and BMP4 signalling respectively. Activin acts on Nodal, and Nodal then acts on Wnt, creating a positive feedback between Wnt and Nodal (Fig. 1d).

Fig. 1 | Primitive streak signalling in hESCs follows the BMP4 to Wnt to Nodal hierarchy. a, Model of the proposed hierarchy of signalling that initiates the primitive streak in hESCs, along with indication of the steps at which the inhibitors SB431542 (SB) and IWP2 may act. As in the mouse, BMP4 acts on Wnt, and Wnt then acts on Nodal. b, RNA-seq analysis of Wnt ligand expression, in pluripotency and after 4 h of stimulation with BMP4, in 500-μm diameter hESC micropatterns. The results show that overall Wnt expression is low in the pluripotent state and that WNT3 is the only strongly induced Wnt in hESCs. c, qPCR analysis showing expression of WNT3 and NODAL in micropatterned hESC colonies after 4 h of stimulation as indicated. Data are mean ± s.d. of n = 3 independently performed replicates. DLDN193189. d, Pie sectors are of representative 1,000-μm-diameter micropatterned hESC colonies stimulated with BMP4, BMP4 + IWP2, or BMP4 + SB. Colonies were fixed 48 h after stimulation and stained for germ layer molecular markers. All micropattern experiments were performed on at least three separate occasions with similar results, and unless mentioned otherwise, all other micropattern experiments are shown 1,000-μm in diameter. Staining is quantified in Extended Data Fig. 1c.

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inhibitor SB431542 (SB) was present. Together with the observation that Activin induces NODAL expression, this suggests the presence of a Nodal feedback loop, as in the mouse\textsuperscript{11}. Additionally, no direct BMP4 induction by either Wnt or Nodal signalling was observed (Extended Data Fig. 1b). This demonstrates that the transcriptional hierarchy of BMP4 to WNT3 to Nodal is evolutionarily conserved in hESCs.

To establish whether the hierarchy of signalling activity was also conserved, we challenged the self-organizing activity of BMP4 with SB and the Wnt inhibitor IWP2. Neither inhibitor had an effect in the absence of BMP4, but either IWP2 or SB prevented BMP4-induced formation of mesoderm (indicated by BRA expression) and endoderm (indicated by SOX17 expression; Fig. 1d and Extended Data Fig. 1c). Thus both Wnt and Activin–Nodal signalling are necessary for mesendodermal induction and patterning downstream of BMP4.

To determine whether either Wnt or Activin–Nodal signalling alone were sufficient as well as necessary for the organizing activity of BMP4, hESC colonies were stimulated with either WNT3A or Activin. After 48 h of treatment with WNT3A, cells at the periphery of colonies differentiated into mesoderm and endoderm (Fig. 2a, Extended Data Fig. 2a), whereas cells in the centre of colonies maintained their pluripotent epiblast fate (indicated by SOX2 and NANOG expression) (Fig. 2a, b) rather than differentiating into ectoderm. After 48 h of treatment with Activin, however, the cells showed no sign of differentiation or self-organization, and all maintained the same morphology and expression of pluripotency markers (Fig. 2a, b).

As it is unlikely that Activin–Nodal has no effect during human gastrulation, we presented WNT3A in two combinations that represent the opposite extremes of an Activin–Nodal gradient: either as WNT3A + Activin or as WNT3A + SB. Consistent with studies in model systems and human and mouse embryonic stem cells\textsuperscript{12,13}, we found that Activin–Nodal signalling acts as a modifier of mesoderm and endoderm patterning—in the presence of Activin, cells on the periphery converted to endoderm (SOX17) with no mesoderm (BRA) expression, whereas in the presence of SB all cells converted to mesoderm (BRA), with no endoderm (SOX17) expression (Fig. 2a; Extended Data Fig. 2a). As with WNT3A treatment alone, both sets of colonies had sharp morphological boundaries that became much more pronounced after 48 h (Fig. 2c). The expression profile of Collagen IV\textsuperscript{14} and the 3D organization of cells around this boundary (Fig. 2d–h) was highly reminiscent of the morphological signature of a primitive streak. Confirming the primitive streak-like nature of these structures, we found evidence of an epithelial–mesenchymal transition (EMT), with expression of SNAIL and a switch from expression of E-cadherin to N-cadherin, in which the mesendodermal fates are later established (Fig. 2i, Extended Data Fig. 2b). Taken together, these results show that Wnt signalling is necessary and sufficient to induce a primitive streak, and that Activin–Nodal signalling acts as a modifier that controls the timing of EMT and patterning of mesoderm versus endoderm.

Having identified primitive streak in our human gastruloids, we next investigated whether an organizer subpopulation was also present. In mouse, the organizer is located in the anterior primitive streak in what is thought to be the region with highest Nodal signalling\textsuperscript{15}. We found that treatment with either WNT3A alone or with combined WNT3A and Activin results in co-expression of OTX2 and FOXA2 in the same micropattern region that expresses SOX17. This combination of markers is characteristic of anterior primitive streak in mouse. However, as in the mouse, only the condition with the highest Nodal signalling, that is, combined treatment with WNT3A and Activin, results in the expression of the organizer-specific marker GSC (Fig. 2, k, Extended Data Fig. 3c). This combined treatment also leads to the highest expression of genes for key secreted inhibitors that are known to be produced by the organizer and its derivatives\textsuperscript{16}, such as CHRD, DKK1, CER1, LEFTY1 and LEFTY2, as well as the highest expression of NODAL, which at later stages in mouse is also specific to the organizer (Extended Data Fig. 4d).

The induction of characteristic organizer markers, the emergence of a sharp Collagen IV-based morphological boundary dividing the...
primitive streak and epiblast regions, and the induction of EMT are all evidence that support induction of a human organizer in an early primitive streak by treatment with WNT3A and Activin. However, as originally defined by the classic amphibian experiments, an organizer is determined functionally as a group of cells that can induce a secondary axis when grafted ectopically into host embryos. In this context, the grafted cells should contribute directly to the ectopic axis (autonomously), and induce neural tissue in the cells of the host (non-autonomously). In order to test for the most stringent and functional definition of an organizer, we used an ex ovo cross-species transplantation strategy based on previous mammalian organizer studies, grafting fluorescent reporter micropatterned hESC colonies treated for 24 or 48 h with WNT3A and Activin into the marginal zone of early chick culture embryos (stage HH2 to HH3+). We used 500-μm diameter micropatterns rather than 1,000-μm diameter micropatterns as these had a higher proportion of GSC+ cells, and we performed grafts at both 24 h and 48 h after treatment, as GSC first becomes apparent and is co-expressed with BRA 24 h after treatment (Extended Data Fig. 4a–c). For the reporter line, we used the CRISPR–Cas9 generated RUES2-GLR (germ layer reporter) cell line (see Extended Data Figs. 5, 6 and Methods).

We found that RUES2-GLR grafts survived, mingled with host cells, and induced and contributed to a secondary axis that became apparent between 24 and 48 h after grafting (Fig. 3b–l). Both the live cell reporter and a human-specific nuclear antigen (HNA) revealed that the human cells directly contributed to the ectopic axis autonomously and continued to differentiate in their new environment, contributing both BRA+ and SOX17+ cells (Fig. 3h, i, m). This mirrors previous observations in mouse-to-mouse organizer grafting experiments. Confocal cross-sections of these secondary axes revealed self-organizing features directly resembling those found in the early chick and mouse embryo, including correct layering of germ layers and central elongated notochord-like structures composed partly or entirely of graft-derived cells (Fig. 3n–r; Supplementary Video 1). Analysis of molecular markers also established that the human cells induced neural tissue in the chick non-autonomously; SOX2 and SOX3 were ectopically induced in chick cells that surrounded the human cells (Fig. 3e–g, i–k, s). RNA in situ hybridization and antibody staining for HOXB1, GBX2 and OTX2 established that the neural tissue was predominantly posterior in nature (Fig. 3t–v). Since in the mouse the early-gastrula organizer and late-streak node also do not induce anterior neural structures when grafted to another mouse embryo, this result suggests that our human organizer is closer to these organizer stages than to the mouse mid-gastrula organizer. As controls, RUES2-GLR grafts derived from hESCs treated with WNT3A, WNT3A plus SB, BMP4, or medium alone exhibited lower survival rates and did not induce chick neural markers (Table 1 and Extended Data Fig. 5a). Taken together with the morphological, cellular and molecular evidence described above, this functional test in an embryonic environment provides the most stringent evidence for the induction of a human organizer. It also highlights that the organizer itself can be obtained in vitro by self-organization when grafted to another mouse embryo, this result suggests that our culture embryos 19 (stage HH2 to HH3+). For the reporter line, we used the CRISPR–Cas9 generated RUES2-GLR (germ layer reporter) cell line (see Extended Data Figs. 5, 6 and Methods).

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Our ability to generate a human organizer closes the loop that was initiated by classical experimental embryologists working on amphibian systems nearly 100 years ago, and demonstrates that the

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**Table 1 | Induction of chick neural tissue by hESC micropatterns**

| Survival | SOX2 | SOX3 | GBX2 | HOXB1 | OTX2 |
|----------|------|------|------|-------|------|
| Control (medium only) | 4/15 | 0/15 | — | — | — |
| WNT3A + Activin, 24 h | 273/300***| 10/19*** | 5/6 | 9/10 | 0/14 |
| WNT3A + Activin, 48 h | 37/40*** | 6/15*** | 6/8 | — | — |
| WNT3A, 48 h | 8/15 | 0/15 | — | — | — |
| WNT3A + SB, 48 h | 7/15 | 0/15 | — | — | — |
| BMP4, 48 h | 14/15*** | 0/15 | — | — | — |

Survival column indicates whether treated and grafted hESC cells from micropatterned RUES2-GLR colonies were detected in the live host chick 24 h after the graft. Many of these grafts were used to optimize the antibodies and probes listed in the remaining columns. Example control grafts are shown in Extended Data Fig. 7. Statistical analysis (Fisher’s exact test, compared to the control condition). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

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**Fig. 3 | Human organizer induces secondary axis in chick embryo.**

a, Schematic showing the strategy for the hESC-chick xenograft.

b–g, Secondary axis induced by a micropatterned RUES2-GLR colony, stimulated for 24 h, and grafted into a HH3-stage chick. SOX17-tomato (red) live marker at 0, 24 and 38 h after grafting (b–d); SOX2 (green) is prominent in the tip of the secondary axis 48 h after grafting (f, g), and does not overlap with the hESCs (red, human nuclear antigen (HNA); e, g). Scale bar, 200 μm. h–k, A different 24-h-stimulated micropatterned hESC colony inducing a secondary axis in a chick host 27 h after grafting. Image of SOX17-tomato hESC cells in the live grafted embryo (red, h). The fixed embryo, stained for HNA (red; i, k) and SOX2 (green; j, k). Scale bars, 500 μm (h), 200 μm (i–k). l–r, Example of secondary axis induction from a 24-h-stimulated micropatterned hESC colony with more complete self-organizing structures, 27 h after grafting. Image of SOX17-tomato hESC cells in the live grafted embryo (red). m, Confocal section of secondary axis stained with DAPI (grey), HNA (red) and BRA (green). n–r, Confocal projection of the region indicated by the line in m, also showing staining for SOX17 (blue). The merged image (r) shows how the secondary axis is layered, with epiblast chick cells on top of a layer of human BRA+ cells, which in turn rest on a layer of human SOX17+ cells, in a similar arrangement to that of epiblast, mesoderm and endoderm layers in a gastrulating mouse or chick embryo. Scale bars, 500 μm (l), 100 μm (m) and 50 μm (n–r). a–v, RNA in situ hybridization in the grafted embryo, s. Chicken Sox3 is expressed throughout the neural tube and head in the host chick, as well as in the induced secondary axis. t. OTX2 is expressed in the host forebrain but is absent in the graft-induced tissue (indicated by the arrowhead). u, HOXB1 is expressed in the host and in the graft-induced secondary axis. v. GBX2 is expressed in the host and in the graft-induced secondary axis. w, x. Magnified view of the region indicated in v; GBX2 mRNA expression (x), and the secondary axis and tdTomato-hESCs (red) in the fixed tissue (w). The arrow shows the location of the hESC graft. Scale bars, 500 μm (s–v), 250 μm (w, x). All experiments were performed at least three times with similar results; exact numbers of replicates and measures of reproducibility are shown in Table 1.
concept of the organizer is evolutionarily conserved from frogs to humans. Our chick experiments also define an in vivo platform to validate results obtained in an in vitro gastruloid platform, and may be generally applicable to test and explore other aspects of early human development.

Online content
Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0150-y.

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Author contributions I.M., A.H.B. and E.D.S. conceptualized the work and wrote the paper. I.M. performed stem cell experiments. I.M., M. and T.Y.K. performed chick experiments. A. R. conceived, generated and validated the RUES2-GLR cell line. All authors reviewed the manuscript.

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METHODS
No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Ethics statement. This work was conducted according to protocols approved by the Tri-Institutional Stem Cell Initiative Embryonic Stem Cell Research Oversight Committee (Tri-SCI ESCRO), an independent committee charged with oversight of research with human pluripotent stem cells, in accordance with university policies, and guidelines from the US National Academy of Sciences (NAS) and the International Society for Stem Cell Research (ISSCR). The Tri-SCI ESCRO is composed of members with scientific and bioethical expertise. The ESCRO review of these protocols was conducted before the May 2016 issuance of the ISSCR guidelines, but the review addressed the issues of growth and culture of human–chick chimaeras and in vitro culture of embryo-like structures and anticipated the ISSCR guidelines (specifically recommendations 2.1.3 and 2.1.5, which are pertinent to this study). As part of these protocols, the human cells transplants were limited to <10% compared to host animal at any given stage, and no chicken–human chimaeras were allowed to hatch. Additionally, the researchers considered that the self-organised structures that arose from the experiments lacked human organonomic potential owing to the in vitro culture lacking the necessary non-embryonic tissues or support that are present in vivo. The ESCRO Committee also reviewed and approved the NIH grants HD080699 and GM101653 that funded part of this study, and approved the initial derivation of the RUES2 cell line which is listed in the NIH Human Embryonic Stem Cell Registry.

Cell culture. hESCs (RUES2 cell line) were grown in HUES medium conditioned by mouse embryonic fibroblasts (MEF-CM) and supplemented with 20 ng/ml bFGF. Mycoplasma testing was carried out before experiments and again at two-month intervals. For maintenance, cells were grown on GelTrex (1:40 dilution, Invitrogen)-coated tissue culture dishes (BD Biosciences). The dishes were coated overnight at 4°C and then incubated at 37°C for at least 20 min before the cells were seeded. Cells were passaged using Gentle Cell Dissociation Reagent (Stem Cell Technologies, 07174).

Micropatterned cell culture. Micropatterned coverslips were made according to a new protocol devised in our laboratory that reduced operating costs. First, 22 × 22 mm no. 1 coverslips were spin-coated with a thin layer of polydimethylsiloxane (PDMS; Momentive, RTV615A) and left to set overnight. They were then coated with 5 μg/ml laminin 521 (Biolamina) diluted in PBS with calcium and magnesium (PBS ± ×) for 2 h at 37°C. After two washes with PBS ± ×, coverslips were placed under a positive feature UV Quartz Mask (Applied Image) in a home-made UV oven. Laminin not protected by the features in the mask was burned off by 10 min of deep UV application (185-nm wavelength). Coverslips were then removed, washed twice more with PBS ± ×, and then left at 4°C overnight in 1% F127–Pluronic (Sigma) solution in PBS ± ×. The now patterned coverslips were dried for 30 min at room temperature. The coverslips were then immersed in 100 μM ROCK inhibitor Y-27632 (Abcam). Coverslips were placed in 35-mm plastic tissue culture dishes, and 1 × 10^5 cells in 2 ml of medium were used for each coverslip. After 1 h the ROCK inhibitor was removed and replaced with standard growth medium supplemented with penicillin–streptomycin (Life Technologies) for 7 min. Cells were washed once with growth medium, washed again with PBS, and then re-suspended in growth medium with 10 μM ROCK inhibitor X-217632 (Abcam). Coverslips were placed in 35-mm plastic tissue culture dishes, and 1 × 10^5 cells in 2 ml of medium were used for each coverslip. After 1 h the ROCK inhibitor was removed and replaced with standard growth medium supplemented with penicillin–streptomycin (Life Technologies). Cells were stimulated or treated with the following ligands or small molecules 12 h after seeding: 20 ng/ml bFGF, 2 μM SB431542, 2 μM ROCK inhibitor, 2 mM sodium 2-phosphonovalerate, and 2 μM THZ214 (Stem Cell Technologies, 07174).

Chick RNA in situ hybridization. Chicken SOX3 probe was kindly provided by F. M. Vieceli, and whole-mount RNA in situ hybridization was performed using previously described procedures21. In brief, the embryos were fixed overnight in 4% paraformaldehyde in PBS 24–48 h after the grafting. The embryos were then washed three times with PBS + 0.1% Tween-20, and then dehydrated through a methanol series (25% methanol/PBS, 50% methanol/PBS, 75% methanol/PBS, 100% methanol) and rehydrated (100% methanol, 75% methanol/PBS, 50% methanol/PBS, 25% methanol/PBS). In 15-min steps at room temperature. Next, the embryos were incubated with 10μg/ml Proteinase K for 5 min, rinsed twice in PBS + 0.1% Tween-20, incubated in 2 mg/ml glycine in PBS + 0.1% Tween-20, washed twice in PBS + 0.1% Tween-20 for 5 min each and post-fixed for 20 min in 4% paraformaldehyde + 0.2% glutaraldehyde in PBS. The embryos were then hybridized at 70°C using antisense RNA probes for chicken SOX3, OXT2, HOXB1 or Gbx2 labeled with digoxigenin-11-UTP. The probe was localized using alkaline phosphatase-conjugated antibodies and the signal was developed with BM-purple.

Microscopy and image analysis. Images were acquired with either a Zeiss Axio Observer and a 20× 0.8-NA lens, or with a Leica SP8 inverted confocal microscope with a 40× 1.1-NA water-immersion objective. Image analysis and stitching was performed with ImageJ and custom Matlab routines. Images used in Supplementary Video 1 and Extended Data Fig. 8 were also deconvolved with Autoreconstruct software and analysed in IARIS. In these images the notochord-like feature was identified by a combination of manual and fast automatic classification based on DAPI morphology, and cells belonging to this structure were segmented and false-coloured with the assistance of custom Python 3D segmentation software written by C. D. Jessberger.

qPCR data. RNA was collected in Trizol at indicated time points from either micropatterned colonies or from small un-patterned colonies. Total RNA was purified using the RNeasy mini kit (Qiagen). qPCR was performed as described previously20. Primer sequences are listed in Supplementary Table 2.

Transplantation of human organizer into chick host. Fertilized white leghorn chicken eggs were incubated at 37–38°C and 50% humidity and staged according to Hamburger and Hamilton25. Chick embryos were removed from the egg and set up in early-chick culture18, with Pannett–Compton saline solution as final wash and residual liquid in the culture. Instead of growing on home-made micropatterned coverslips, hESCs were grown on EMB CYTOO coverslips, as these had 500-μm diameter micropatterns. All other culture details were as described. Once grown to the indicated time with the indicated stimulation conditions, 500-μm diameter colonies were peeled off whole with tungsten needles (Fine Science Tools). These colonies were washed twice with Pannett–Compton solution to remove culture growth factors and ligands. Colonies were then moved to chick embryos and grafted into the marginal zone between the area opaca and area pellucida approximately 90° away from the site of primitive streak initiation, following the example of a typical host–organizer transplant protocol22. After grafting, the newly formed patterned colonies or from small un-patterned colonies. Total RNA was isolated from the grafts and single-stranded cDNA was generated with a random hexamer primer (Supplementary Table 4) was cloned into a Cas9-nickase cassette (loxP-PGK-Neo-pA-loxP); and (iv) a right homology arm containing a 1-kb sequence immediately upstream of the HOXB1 or SOX3 start codon was inserted into the P2A cassette (loxP-PGK-Neo-pA-loxP). Then, two independent hESC lines were reprogrammed to induce cellular reprogramming by removing the ROCK inhibitor and to develop and were imaged live one day later and were ultimately fixed between 24 and 48 h after the graft. Owing to background from the agar mount and chick autofluorescence, only the SOX17-tdTomato marker had sufficient signal to noise ratio to be imaged live. In all steps penicillin–streptomycin was used to minimize the chances of bacterial contamination. I.M. and T.Y.K. were able to replicate the human–chick grafts independently, and the grafts could also be replicated by an experienced embryologist in our lab who was not involved in this study.

Generation and validation of RUES2–GLR line. CRISPR–Cas9 technology was used to generate a single hESC line containing three independent fate reporters (SOX2–mCitrine, BRA–mCerulean and SOX17–tdTomato). The already established and registered RUES2 hESC line (NHHeESC-09-0013) was used as the parental line. In order to achieve three independent targeting events in the same line, we approached each gene sequentially, since the efficiencies of recombination were not high enough for simultaneous targeting. First, for SOX17 targeting, we generated a homology donor plasmid (pSOX17-HomDon) containing: (i) a left homology arm containing a 1-kb sequence immediately upstream of the SOX17 stop codon; (ii) a P2A-H2B-tdTomato cassette; (iii) a floxed Neomycin selection cassette (loxP-PGK-Neo-PA-LoxP); and (iv) a right homology arm containing a 1-kb sequence immediately downstream of the SOX17 stop codon. Note that, since the H2B-tdTomato is separated from the SOX17 gene by a self-cleaving P2A peptide, the expressed fluorescent reporter is not fused to SOX17, and therefore it will only be a reporter of the activation of the SOX17, as the two proteins may have different half-lives. We initially tried to use a direct fusion of SOX17 and tdTomato, but the fusion made the protein not localize correctly to the nucleus, and we therefore decided to use a self-cleaving strategy. All DNA fragments were amplified from pre-existing plasmids or genomic DNA using Q5 polymerase with BsaI and BbsI restriction enzyme sites (Supplementary Table 3). All DNA ligation protocols. A single-guide RNA (sgRNA) recognizing a sequence near the stop codon of SOX17 (Supplementary Table 4) was cloned into a Cas9-nickase expression vector (pX335 from the Zhang laboratory, Addgene plasmid #42335).
This plasmid, together with the pSOX17-HomDon plasmid, was nucleofected into RUES2 cells using a Nucleofector II instrument and Cell Line Nucleofector Kit L (Lonza). Geneticin (a neomycin analogue) was added to the cultures five days after nucleofection, and maintained in the medium for a further seven days to ensure selection of correctly targeted clones. Colonies derived from single geneticin-resistant cells were picked and expanded for screening. PCR amplification and Sanger sequencing were used to identify correctly heterozygously targeted clones, with no unwanted mutations in the SOX17-sgRNA target site, both in the targeted and in the untargeted alleles. Positive clones were also validated for karyotyping (Giemsa banding). The top four potential off-target sites of the sgRNA were PCR amplified and Sanger sequenced to ensure no unwanted mutations were present. The pluripotency status and absence of differentiation of the clones were validated through immunofluorescence staining. Once a validated SOX17-tdTom clone was identified, its youngest frozen stock was thawed to undergo BRA gene targeting. Targeting of BRA followed a similar strategy as SOX17, but using a Puromycin resistance cassette. Colonies derived from single puromycin-resistant cells were screened and validated as with the previous SOX17 targeting. After a fully validated double-targeted clone (SOX17-tdTomato and BRA-mCerulean) was identified, it underwent sequential SOX2 targeting. Unlike in the cases of SOX17 and BRA, for SOX2 targeting, the direct fusion of mCitrine with SOX2 did not affect its localization or function, and therefore the SOX2–mCitrine reporter constitutes a faithful reporter of both the ‘on’ and ‘off’ expression rates. The SOX2 homology donor consists of: (i) a 1-kb left homology arm; (ii) an mCitrine-T2A-blasticidin cassette; and (iii) a 1-kb right homology arm. Colonies derived from single blasticidin-resistant cells were screened and validated as with the previous SOX17 and BRA targetings.

**RUES2-GLR time-lapse imaging.** Cultures of RUES2-GLR cells were dissociated to single cells from growth plates with StemPro Accutase (Life Technologies), washed, and then re-suspended in MEF-CM with 10μM Y-27632 (Abcam). CYTOO micropatterned chips were placed in 35-mm tissue culture plastic dishes, and 8 × 10^5 cells in 2 ml medium were added to each coverslip. After 1 h Y-27632 was removed and replaced with standard MEF-CM, supplemented with penicillin–streptomycin (Life Technologies), and incubated overnight. The following morning the micropatterned coverslip was carefully removed from the dish and placed in a coverslip holder (CYTOOchambers from CYTOO), to which 1 ml MEF-CM, penicillin–streptomycin, 50 ng/ml BMP4 was added to induce differentiation. Immediately after addition of medium, the holder was transferred to a spinning disk confocal microscope (CellVoyager CV1000, Yokogawa), in which fluorescent images were acquired every 30 min for 2 days. Multichannel time-lapse videos were generated from the raw images using ImageJ analysis software.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Code availability statement.** Details or copies of the custom code are available upon request.

**Data availability.** All relevant data are available from the authors and the Source Data represented graphically in the figures are available in the online version of this paper. RNA-seq data are from a previously published dataset, which is available from the GEO database under accession number GSE77057.

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22. Hamburger, V. & Hamilton, H. L. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88, 49–92 (1951).

23. Psychoyos, D. & Finnell, R. Assay for neural induction in the chick embryo. *J. Vis. Exp.* https://doi.org/10.3791/1027 (2009).
Extended Data Fig. 1 | Controls for investigating hESC primitive streak initiation hierarchy. a, Micropatterned hESC colonies were stimulated with IWP2, SB or blank medium. Colonies were fixed after 48 h and stained for germ layer molecular markers. This experiment was repeated at least three times independently with similar results.

b, qPCR for BMP4 of unpatterned small colonies stimulated for 4 h as specified. Consistent with the model hierarchy, there was no marked induction of BMP4 by Activin, WNT3A, or BMP4. Data are mean ± s.d. of three biologically independent replicates. c, Quantification of expression data in Fig. 1d. Here, and in all other analyses unless stated otherwise, nuclei were segmented using DAPI and the intensity of immunofluorescence signal for each marker was normalized to the DAPI intensity. Single-cell expression data was binned radially and averaged. The final radial profile represents the mean ± s.d. of $n = 25$ colonies.
Extended Data Fig. 2 | Primitive streak germ layer quantification and EMT timing. a, Quantification of data presented in Fig. 2a. The radial profile represents the mean ± s.d. of n = 25 colonies. b, Micropatterned hESC colonies were stimulated with BMP4, WNT3A, WNT3A + SB, or WNT3A + Activin, fixed after 12, 24, 36 or 48 h and stained for the primitive streak molecular markers SNAIL, E-cadherin (E-CAD) and N-cadherin (N-CAD). Note that colonies stimulated with WNT3A and WNT3A + Activin turn on expression of EMT markers more rapidly than those stimulated with BMP4 or WNT3A + SB, and have mostly downregulated SNAIL after 48 h. This experiment was repeated at least three times independently with similar results.
Extended Data Fig. 3 | Further micropattern fate characterization. 

**a, b,** Micropatterned hESC colonies were stimulated with BMP4, WNT3A, WNT3A + SB, or WNT3A + Activin, fixed at 24 or 48 h after stimulation, and stained for EOMES (a) or PITX2 (b). EOMES expression was highest in cells stimulated with WNT3A or WNT3A + Activin and was also dynamic, with the highest expression levels occurring 24 h after stimulation, coinciding with the onset of primitive streak marker expression (Extended Data Fig. 2b). PITX2 is not highly expressed in any of the tested conditions. This experiment was repeated at least three times independently with similar results. 

**c,** Quantification of data in Fig. 2j, k and Extended Data Fig. 3a, b. The radial profile represents the mean ± s.d. of *n* = 25 colonies.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Further characterization of the induced organizer. a, Micropatterned hESC colonies (1,000-μm and 500-μm diameter) stimulated with WNT3A + Activin, fixed 24 h after stimulation and stained for GSC and BRA. Note that, as previously observed\(^1\) for BMP4 induction, shrinking the colony size results in removal of the central micropattern fate region, resulting here in a higher proportion of GSC-expressing cells. This experiment was repeated at least three times independently with similar results. b, Quantification of a. The radial profile represents the mean ± s.d. of \(n = 25\) colonies. c, Scatter plot of single-cell expression of GSC versus BRA. Note that at 24 h most cells co-express BRA and GSC, but by 48 h GSC expression is increased and BRA expression is decreased. We therefore grafted micropatterns at 24 h as well as at 48 h post-stimulation, reasoning that earlier coexpression of BRA and GSC would result in increased graft contribution to axial mesoderm structures. d, qPCR of additional organizer markers. RNA samples were collected from 500-μm-diameter micropatterns stimulated with BMP4, WNT3A, WNT3A + SB, or WNT3A + Activin for 24 or 48 h. With the exception of NOG, the characteristic organizer-secreted inhibitors DKK1, CER1, CHRD, LEFTY1 and LEFTY2, are all most highly expressed in the WNT3A + Activin condition. The high induction of NOG by BMP4 in hESCs has been noted before\(^2\), and may represent a species difference between human and mouse. NODAL, which in mouse is restricted to the organizer later in gastrulation, is also most highly expressed in WNT3A + Activin conditions. Data are mean ± s.d. of three biologically independent replicates.
Extended Data Fig. 5 | Generation and validation of the RUES2-GLR cell line. a, Sequencing of the targeted alleles of **SOX17**, **BRA** and **SOX2** genes. No indels were detected. b, The RUES2-GLR cell line maintains pluripotency normally, as assessed by staining of typical pluripotency markers (OCT4, NANOG and SOX2). Scale bar, 100 μm. This experiment was repeated at least two times independently with similar results. c, The RUES2-GLR cell line was karyotypically normal.
Extended Data Fig. 6 | Functional validation of the RUES2-GLR cell line. a, Specificity of germ layer reporters. When induced to differentiate to individual germ fates, only the specific reporter was turned on. SOX2–mCitrine was expressed during pluripotency and three days after neural (ectoderm) differentiation, BRA–mCerulean expression commenced after three days of mesodermal differentiation and SOX17-tdTomato reporter was active after three days of endodermal differentiation. Scale bar, 100 μm. b, Snapshots of a time-lapse imaging of the RUES2-GLR cells in micropatterns after treatment with 50 ng ml$^{-1}$ BMP4, showing how differentiation starts from the edges and extends inwards. Scale bar, 100 μm. c, RUES2-GLR colonies reproducibly generate the typical self-organized concentric rings of germ layers when induced to differentiate with a step presentation of 50 ng ml$^{-1}$ BMP4 in micropatterns. Scale bar, 200 μm. These experiments were repeated at least three times independently with similar results.
Extended Data Fig. 7 | Control chick grafts. a, Representative grafts for control conditions. With the exception of the BMP4 control condition, grafted hESC colonies were static, with the colonies either growing or dying in place. With BMP4 treatment, the colonies were frequently elongated, possibly owing to hESC migration. There was no induction of SOX2 in the host cells in any of the control conditions. Note that in the case of the WNT3A + SB graft shown, two colonies were grafted into two different locations. Scale bar, 500 μm. Experiments were repeated at least three times independently with similar results. b, Confocal sections showing co-expression of SOX17 (tdTomato) and FOXA2 or OTX2 in human cells that contribute to the secondary axes induced by a 24-h WNT3A + Activin-stimulated micropatterned hESC colony. Scale bar, 20 μm. Experiments were repeated at least three times independently with similar results.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Further characterization of the induced secondary axis. a, Examples of classifying the notochord-like feature (NLF) based on morphology. At $z = +19\mu$m, the NLF appears as a tighter and brighter rod of cells running north to south that is also distinct and somewhat separated from the surrounding chick epiblast. At $z = +46\mu$m, paired elongated cells stick out ahead of the other cells in a continuation of the originally identified NLF. Other cells belonging to the NLF between $z = +46\mu$m and $z = +19\mu$m are obscured or out of focus in this section, but can be easily identified in individual sections at the other $z$ positions. Scale bar, 100 $\mu$m. b, Snapshots from Supplementary Video 1. From top to bottom: yellow shows co-localization of BRA (green) and human (red) cells; purple shows co-localization of Sox17:tdTomato (blue) with human (red) cells; cross-section shows that chick and human cells arrange themselves into germ layers properly, and that they flank the central notochord-like feature indicated by the arrow (cyan); a proportion of human mesoderm cells contribute to part of the notochord-like structure, while the cyan-coloured cells without HNA (red) shows that the remainder of the NLF is composed of host cells. c, Two examples of donor hESC grafts contributing to the induced notochord-like feature, imaged in a live chick embryo 27 h (left) and 23 h (right) after grafting. Scale bars, 200 $\mu$m (left), 100 $\mu$m (right). Similar notochord-like features were observed in at least ten independent biological replicates.
Life Sciences Reporting Summary

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1. Sample size
   Describe how sample size was determined.
   No minimum sample size was calculated prior to start of experiments. Final sample size was determined by measuring the change in standard deviation and mean across samples of various sizes and selecting the sample size at which this change became less than the measurement error.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.
   Our chick results could be replicated by 3 people independently, one of which was not involved in this study. It could be replicated across many batches of eggs at different times of the year. Our micropattern data could be reproduced by at least 5 other people in the lab not involved in this study, and was robust across biological replicates performed on different days.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   For grafting experiments where there were multiple experimental groups at the same time embryos were allocated randomly into these groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Experimenters had no further control over the outcome of the graft or development of the micropattern once the initial stimulus so no blinding was necessary.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   | □         |

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided  
  *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- □ Test values indicating whether an effect is present  
  *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
- □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- □ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Policy information about availability of computer code

Describe the software used to analyze the data in this study.

Image analysis was performed with ImageJ and custom written Matlab or Python routines (Matlab v2014b, Python 3.6.0). For Supplemental Video 1 and Supplemental Figure 8 Autoquant X and Imaris 8.4.1 software were used for visualization.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

No unique materials used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Information related the antibodies we used can be found in a list in our Methods section. All antibodies used have been tested and validated by the supplier and also by other researchers in the field. The list of antibodies used in our Methods is copied here:

- BRACHYURY R&D Systems AF-2085 1:300
- CDX2 Abcam Ab-15258 1:50
- E-CADHERIN Cell Signalling 3195 1:200
- GOOSECOID R&D AF-4086 1:100
- NANOG R&D Systems AF-1997 1:200
- N-CADHERIN BioLegend 350802 1:200
- SNAIL R&D Systems AF-3639 1:200
- SOX17 R&D Systems AF-1924 1:200
- SOX2 Cell Signalling 3579 1:200
- COLLAGEN IV Abcam Ab-6586 1:100
- OTX2 SCBT 30659 1:200
- FOXA2 SCBT 6554 1:200
- PITX2 Abcam Ab-55599 1:100
- EOMES Abcam Ab-23345 1:100
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. The RUES2 cell line was created in our lab and is listed in the NIH Human Embryonic Stem Cell Registry. It was derived under approval from the Tri-Institutional Stem Cell Initiative Embryonic Stem Cell Research Oversight (Tri-SCI ESCRO) Committee, an independent committee charged with oversight of research with human pluripotent stem cells and embryos to ensure conformance with University policies, and guidelines from the U.S. National Academy of Sciences (NAS).
   b. Describe the method of cell line authentication used. The cell line was derived in our lab and so was known by us to be authentic. The line was also whole genome sequenced.
   c. Report whether the cell lines were tested for mycoplasma contamination. Mycoplasma testing details are included in our methods section. All cell lines tested negative for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide all relevant details on animals and/or animal-derived materials used in the study. Fertilized white leghorn chicken eggs from Charles River Laboratories were used in this study. They arrived fresh and were incubated and staged till they reached the appropriate developmental stage (HH1-3+).

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants. No human research participants were used.