A strategy to discover new organizers identifies a putative heart organizer

Claire Anderson, Mohsin A.F. Khan, Frances Wong, Tatiana Solovieva, Nidia M.M. Oliveira, Richard A. Baldock, Cheryll Tickle, Dave W. Burt & Claudio D. Stern

Organizers are regions of the embryo that can both induce new fates and impart pattern on other regions. So far, surprisingly few organizers have been discovered, considering the number of patterned tissue types generated during development. This may be because their discovery has relied on transplantation and ablation experiments. Here we describe a new approach, using chick embryos, to discover organizers based on a common gene expression signature, and use it to uncover the anterior intestinal portal (AIP) endoderm as a putative heart organizer. We show that the AIP can induce cardiac identity from non-cardiac mesoderm and that it can pattern this by specifying ventricular and suppressing atrial regional identity. We also uncover some of the signals responsible. The method holds promise as a tool to discover other novel organizers acting during development.
Organizers are formally defined as signalling regions, unique in being able both to induce and to pattern adjacent tissue. The dorsal lip of the blastopore, which can induce a complete secondary axis in amphibian embryos, was the first organizer to be discovered, in 1924 (ref. 2). Given the complexity of the vertebrate embryo, one might expect that many organizers should exist, but only a few others have been described: Hensen’s node (the amniote equivalent of the dorsal lip of the blastopore, which can induce and pattern the central nervous system), the notochord/floor-plate (which can induce and organize different sets of neurons in the neural tube), the zone of polarizing activity (ZPA, which can induce a patterned set of limb elements) and the midbrain–hindbrain boundary (isthmus, which can specify and pattern the adjacent regions of the midbrain/tectum and hindbrain/cerebellum). One reason why so few organizers have been found could be that their discovery requires grafting appropriate tissues at the right time and place, and there are many possible combinations.

It has been shown that in some cases organizers can substitute for each other. Most strikingly, a graft of Hensen’s node from an early (primitive streak stage) embryo into the anterior limb bud of a much later embryo can mimic the action of the limb organizer, the ZPA, by inducing and patterning a full set of skeletal elements including the digits. This raises the intriguing possibility that organizers may share a genetic signature (‘synexpression’) that confers them with inducing and patterning properties. If this is the case, we should be able to use this genetic signature to point to potential new organizing regions. In this study, we tested this idea by comparing the transcriptomes of three known amniote organizers (Hensen’s node, the notochord/floor-plate and the ZPA). This defines a synexpression set of 48 transcripts that are either enriched or depleted in organizers, which we then used to explore the embryo for other regions of synexpression. This suggested that the endoderm of the anterior intestinal portal (AIP) might be a new organizer. The AIP is an endodermal invagination appearing with the head-fold, which moves caudally down the embryo to form the foregut. As it does so, it is closely associated with the developing heart tube. We therefore performed a series of experiments to test whether the AIP can act as an organizer of the heart. Ablation and transplantation experiments in vivo, along with co-culture in vitro, revealed that the AIP endoderm can induce heart fates from non-cardiac mesoderm as well as pattern the heart field by specifying ventricular and suppressing atrial regional identity.

Results
Defining a gene signature for organizers. To investigate whether organizers have a common genetic signature of enriched and depleted transcripts, we designed a differential microarray screen in chick embryos, comparing three known organizers to their most similar non-organizer tissue. First, we chose stage HH10 3+ 4 Hensen’s node, which induces and patterns the neural plate, compared with the posterior primitive streak, which cannot (Fig. 1a). We also sampled the later node (HH6), which has lost its ability to induce a full neural plate. This three-way comparison was designed to reveal transcripts associated with full organizer function. Second, we selected the notochord and floor-plate at HH10-11, responsible for organizing the dorso-ventral axis of the neural tube, and compared this to the dorsal neural tube (Fig. 1b). Finally, we compared the posterior wing bud, containing the ZPA, to the anterior wing bud at HH20-21 and HH24 (Fig. 1c). Each comparison generated a list of transcripts significantly enriched or depleted in that organizer (≥ 1.2-fold the log2 of the expression level, and a false discovery rate ≤ 0.05. The dataset was submitted to ArrayExpress with the title ‘Microarray analysis of chick embryonic tissues: gastrulation, neural tube/notochord and limb development’ and given accession number E-MTAB-4048. These lists were then combined using a Boolean algorithm to find enriched or depleted transcripts common to all three organizers. This approach uncovers relative changes in expression between...
organizer cells and neighbouring regions, even if the absolute level of expression is widely different at different stages: a putative organizer gene set of 31 enriched and 17 depleted transcripts is revealed (Supplementary Fig. 1a–d and Supplementary Data 1). Interestingly, over 60% of the enriched genes encode membrane-associated or secreted molecules and 7% represent transcription factors, whereas transcription factors are much more frequent (38%) among the depleted genes (Supplementary Fig. 1c). One possible interpretation of this is that the synexpression set is enriched for signalling molecules emitted by the organizer and depleted of transcriptional repressors that suppress the organizer state.

The AIP endoderm as a candidate organizer. Next, we used this set to verify the expression of the selected genes (Fig. 1d–i, Supplementary Figs 2 and 3), as well as to explore whether the synexpression signature occurs in other places and stages during development. A large-scale in situ hybridization analysis of the 48 genes was undertaken from pre-primitive streak stage up to HH27; these expression patterns can be browsed on eChick atlas12 (www.echickatlas.org). One embryonic region appropriately expresses 35 genes from the organizer gene set: the endoderm of the AIP. At some stage between HH7-14, 20/31 enriched transcripts are detected (Supplementary Fig. 4a–t), 15/17 organizer-depleted genes are appropriately absent from the early AIP (Supplementary Fig. 4u–k), and SOCS2 and BTG2 are absent before HH10 and HH14 respectively (Supplementary Fig. 4l,m). Thus, the AIP shares a similar transcriptional profile to other known organizers. There is substantial evidence that the early endoderm adjacent to the bilateral cardiac mesoderm is required for normal heart formation in chick, amphibian and fish embryos13,14, but less is known about the AIP endoderm of later stages. Genetic or manual ablation of the AIP endoderm in mouse and chick embryos results in cardiac biftida15–19 (failure of the bilateral heart progenitors to fuse in the midline), rotation defects16 and downregulation of early cardiac genes15, indicating that a functional AIP endoderm is required for normal heart formation, but its inducing and patterning abilities have not been tested. Could the AIP be an organizer of the heart?

The AIP can induce cardiac identity from non-cardiac mesoderm. We first confirmed that ablation of the AIP at HH8 does indeed cause cardiac biftida and abnormal heart rotation (Supplementary Fig. 5). Next, to test if the AIP can induce cardiac identity, a necessary prerequisite is to identify a suitable responding tissue. Fate and specification maps indicate that the paraxial mesoderm adjacent to Hensen’s node at HH5 (prospective head mesoderm) is neither fated nor specified as heart20–24, but is competent to respond to cardiac inducing signals22. We compared this mesoderm (named #3) with four other regions of HH5 mesoderm (Supplementary Figs 6a and 7a): #3 does not contribute to the heart when grafted homotopically (Supplementary Fig. 7b–h), and is the only region of mesoderm that does not express cardiac markers when explanted either in vitro (Fig. 2 f,i,o,r,u,x,a’,b’ and Supplementary Table 1) or in a host embryo (Supplementary Fig. 7j–v and Supplementary Table 2). Mesoderm #3 is therefore suitable for testing whether the early AIP can induce heart fate. The AIP induces early cardiac markers, MYOCD, NKX2.5 (Fig. 2j,m and Supplementary Fig. 8b,c), GATA4, TBX5, ISL1 and MEF2C in explants of #3-mesoderm (Supplementary Fig. 8d–g), while co-culture with control (Cont.), non-AIP endoderm from the lateral embryo, largely does not (Fig. 2k,n, Supplementary Fig. 8b–h, Supplementary Tables 2 and 3). Strikingly, spontaneous beating is observed after 48 h of in vitro co-culture of #3-mesoderm with AIP (Supplementary Movie 1), but not with control, non-AIP endoderm. Together these results show that the AIP endoderm can induce cardiac identity in mesoderm that is not otherwise fated to become part of the heart.

Patterning by inducing ventricular identity and suppressing atrial character. Many of the known early heart markers are normally expressed in the bilateral cardiac mesoderm before the AIP forms (around stage HH5; Supplementary Fig. 6a–f), whereas regional markers of anterior–posterior heart tube patterning are observed later (after HH9; Supplementary Fig. 6v–n). Ventricular markers (VMHC1/MYH15, IRX4 and NPPB) are initially expressed in the medial splanchnic mesoderm25–27 immediately adjacent to the AIP (Supplementary Fig. 6d,f,g), and the atrial marker AMHC1 is in the lateral splanchnic mesoderm28 (Supplementary Fig. 6c). In #3-mesoderm explants cultured either in a host embryo or in vitro, AIP induces VMHC1/MYH15, IRX4 and patchy NPPB expression (Fig. 2b–d,p,s,x, Supplementary Tables 2 and 3). AIP also induces GLA5 (Fig. 2e,y), which marks the anterior ventricular myocardium, endocardium and outflow tract (Supplementary Fig. 6l). AIP does not induce AMHC1 (Fig. 2f,b’ or SHOX2 (Fig. 2g,e’), a marker of the sinoatrial node (Supplementary Fig. 6f,p’). These results show that the AIP can induce ventricular cardiac identity in mesoderm not destined to this fate.

Previous tissue recombination experiments using HH5-6 chick explants have demonstrated that anterior-lateral endoderm adjacent to the early cardiogenic mesoderm can induce heart gene expression in posterior primitive streak29. Are the induction properties of the HH8 AIP endoderm distinct to the early anterior-lateral endoderm? To test this, we co-cultured HH5 anterior-lateral endoderm with non-cardiac #3-mesoderm (Supplementary Fig. 9a and Supplementary Table 4). Unlike AIP endoderm, anterior-lateral endoderm does not induce ventricular markers (Supplementary Fig. 9b–e) nor does it induce AMHC1 (Supplementary Fig. 9f). These data show that the anterior-lateral endoderm is different to the AIP endoderm and that the ability to induce ventricular character is a property specific to the AIP endoderm.

To test if the AIP patterns the heart tube by specifying ventricular identity, AIP from transgenic GFP donors was grafted over the cardiogenic mesoderm of a host embryo (Fig. 3a). Strikingly, this expands the expression of VMHC1/MYH15 and IRX4 posteriorly into the prospective atrial region (Fig. 3c–l), while expression of the atrial marker AMHC1 is abolished (Fig. 3m–q). Moreover, the AIP represses AMHC1 in #1-mesoderm which would normally express it (Fig. 3r–t and Supplementary Table 3); since IRX4 is known to repress AMHC1 (refs 26,30), this repression by the AIP may be either direct or indirect, due to prior AIP-mediated induction of IRX4. Thus, the AIP not only induces ventricular identity, but also actively blocks atrial character. The ability to induce a new fate in responding tissues and to impart a spatial pattern are properties shared by other known organizers1. Taken together, these results show that the AIP endoderm is distinct from other endodermal regions, in that it can both induce cardiac identity in mesoderm not destined for this fate and also pattern heart mesoderm by promoting ventricular and suppressing atrial regional character.

Common signals between organizers. Gifts of either Hensen’s node3, notochord or floor-plate31 into the anterior limb bud can induce extra digits, suggesting that at least some signals (an obvious one being SHH) may be common to different organizers. We therefore tested whether the heart-inducing or ventricle-specifying functions of the AIP can be mimicked by...
other organizers. We find that Hensen’s node induces patchy expression of VMHC1/MYH15 but not MYOCD in #3-mesoderm cultured in a host (Supplementary Fig. 10a,b and Supplementary Table 5), as well as in explants (Supplementary Fig. 10c,d and Supplementary Table 5). Hensen’s node has also been shown to induce ectopic VMHC1, but not AMHCl in the germinal crescent and the lateral plate mesoderm32, comparable to the ability of the AIP to induce ventricular markers. Conversely, to test whether the AIP can mimic the neural inducing activity of Hensen’s node, we grafted AIP endoderm into the area opaca33 at HH3 + : this induces the early neural marker SOX3, but not the later neural plate marker SOX2 (Supplementary Fig. 10e,f). Finally, we tested whether the AIP can mimic a ZPA graft into the anterior wing bud: strikingly, the AIP causes duplication of digit-2 (Supplementary Fig. 10h). Therefore, the AIP is partly interchangeable with other organizers at earlier and later stages of development, some of which could be related to its expression of SHH (Fig. 11) or FGF8 (ref. 34). However it seems that the ability of organizers to substitute for each other is limited.

**Signals from the AIP endoderm.** The inducing and patterning activities of other known organizers rely on multiple signals,
acting together and/or sequentially\textsuperscript{35–37}. FGF8 (refs 38,39) and SHH (refs 40,41) play important roles in other organizers; both have been implicated in cardiogenesis\textsuperscript{34,42} and both are expressed in the AIP. However, our syngeneic set contains many other secreted or membrane-associated factors that have not hitherto been implicated either in the activity of other organizers or in cardiac gene induction or heart patterning. Four of these are expressed in the early AIP: NRPI, FBLN7, KIRREL3 and VTN (Fig. 1j,k,m,n). To investigate their activity, we co-cultured #3-mesoderm with pellets of cells transfected with expression plasmids encoding these four factors. This combination partly mimics the AIP, sometimes inducing patchy expression of the ventricular markers VMHCl/MYH15 (Fig. 4a) and NPPB (Fig. 4b), but not IRX4 (Supplementary Fig. 11f) or other early cardiac markers (Fig. 4c, Supplementary Fig. 11a–e and Supplementary Table 6).

These results suggest that other signals are required, possibly SHH and FGF8, but also considerable complexity, not unlike other organizers, which are believed to emit different signals at different times to account for their various inducing and patterning functions\textsuperscript{35–37}. Accordingly, some gene expression profiles change over time as the organizer changes its properties. Although HH8 AIP endoderm was used in co-cultures, the AIP continues its developmental programme, expressing transcripts that are detected in the later AIP (Supplementary Fig. 12). HH8 AIP cultured either alone or with #3-mesoderm expresses genes, including those that encode secreted or membrane-associated molecules normally observed in the HH10 AIP (Supplementary Fig. 4j,k), after 24h (Supplementary Fig. 12a–d) and those expressed in the HH12–13 AIP (Supplementary Fig. 4q,s) after 48h (Supplementary Fig. 12e–h). These results suggest that the signalling properties of the AIP endoderm mature over the culture period as they do in the embryo.

Taken together, our results implicate the AIP as an organizer of the heart, and suggest that a complex combination of signals account for its heart inducing and patterning functions at early stages of heart tube formation.

Discussion

Our results suggest that organizers do share a common genetic signature, and that this property can be used to identify putative new organizers acting during development. The choice of the three organizers to compare was relatively arbitrary and perhaps we should have used a different well-characterized organizer, the mid-hindbrain boundary (MHB) or isthmic organizer\textsuperscript{3,7} instead of the comparison between notochord/floor-plate with dorsal neural tube (particularly as the latter is also a signalling region).

The main reason why we did not use the MHB is that it is difficult

Figure 3 | AIP specifies ventricular regional identity and represses atrial character. (a) AIP (green, c,d,h,i,m,n brown, e,g,j,1-o,q) from a HH8 GFP chick was grafted heterotopically onto cardiogenic mesoderm of a HH5 wild-type chick host (c,h,m, T0) and grown for 12h (d,i,m, T12). (c–q) AIP expands VMHCl/MYH15 (e–g) and IRX4 (j–l) expression (red arrowheads) and abolishes AMHC1 (o–q, black arrows). (b) In vitro co-culture of #1-mesoderm with a HH5 chick with HH8 GFP-AIP or control (Cont.) endoderm (brown) for 48h. AMHC1 expression in #1-mesoderm (r, red arrowhead). AIP represses AMHC1 in #1-mesoderm (s, black arrow), Cont. does not (t, red arrowhead). *P ≤ 0.05 using two-tailed Fisher’s exact test. Scale bars, 0.5 mm in whole-mounts and 0.1 mm in explants and sections.

Figure 4 | Secreted molecules induce ventricular markers. (a–c) HH5 #3-mesoderm co-cultured in a chick host with cell pellets transfected with NRPI + FBLN7 + KIRREL3 + VTN (red) either overnight (a,b) or for 6–9h (c). VMHCl/MYH15 and NPPB are induced in #3-mesoderm (a,b, red arrowheads) but MYOCd is not (c, black arrows). Control pellets (red; pCAB) do not induce VMHCl/MYH15, NPPB or MYOCd (a–c, black arrows). Scale bars, 0.5 mm in whole-mounts and 0.1 mm in insets and sections.
to identify a non-organizer tissue that is similar enough to this boundary in every other respect. The organizer gene set could be refined further using this tissue in future. Nevertheless, this study is an important proof of principle and, based on the synexpression of 48 genes, we uncover the AIP endoderm as a putative organizer because it can both induce cardiac identity and pattern the heart field by specifying regional characteristics (inducing ventricular and suppressing atrial character).

Previous studies have concluded that the endoderm is required for heart development (reviewed in ref. 13). Many of these studies investigated the early anterior-lateral endoderm adjacent to the cardiac mesoderm at HH4-6 (refs 34,43–48). Tissue recombination experiments have shown that this endoderm can reprogram non-cardiac tissues to heart identity29 and BMPs, FGFs and inhibition of WNT signalling have been implicated in this process in this study. However, it has since been shown that these tissues express MYOCD (ref. 43), GATA (ref. 54) and TBX20 (ref. 14) transcription factors, and can express WNT and VMHC1/MYH15 following culture in vitro (Supplementary Table 1) leaving open the possibility that these experiments do not demonstrate a true change of fate in this tissue. Widespread expression of some early cardiac markers makes it difficult to find a responding tissue suitable for an induction assay (neither being fated to give rise to heart nor express any of the markers being assessed as a result of the induction). Here we use anterior-medial mesoderm (Fig. 5a), which is neither fated nor specified as heart and can be used to assess both induction and patterning rigorously.

The expression patterns of early cardiac markers, like NKX2.5 (ref. 29), and the signalling molecules previously implicated in their induction (BMP2/4/7 (ref. 22), FGF8 (ref. 34) and CRESCENT (ref. 49)) indicate that initial signals act at an early stage of cardiogenesis (HH4-6, Fig. 5a) before the AIP forms. Our results show that the AIP from a later stage embryo can also induce heart identity (reprogramming non-heart #3-mesoderm from an earlier stage donor) as well as pattern it by specifying ventricular and suppressing atrial character. During normal development, the AIP is likely to be responsible for the later patterning functions (Fig. 5b), but our results show that it can also mimic the activity of earlier endoderm. This could be due to the presence of AIP precursors within this early endoderm and the continued expression of BMP2 (ref. 22) and FGF8 (ref. 34). This is reminiscent of other organizers, for example, Hensen’s node can initiate the entire process of neural induction, even though the first neural inducing signals begin before gastrulation, long before Hensen’s node can be defined. This could be a general feature of organizers, perhaps because they produce many signals. However, the signals emitted by organizers also change over time as their patterning properties evolve, which is seen in organizers like Hensen’s node, as well as in the AIP. Heart progenitor cells become determined to either the ventricular or atrial compartment at HH8 (refs 28,57,58), consistent with the timing of signals from the AIP endoderm that induce ventricular and repress atrial character (Fig. 5b). Retinoic acid signalling from the posterior lateral plate mesoderm is known to control the allocation of cardiac progenitor cells to the atrial lineage28,57,59. We have shown that signals from the AIP endoderm, NRP1, FBLN7, KIRREL3 and VTN when combined can sometimes induce VMHC1/MYH15 and NPPB (Fig. 5c), but whether these signals also repress atrial identity remains to be tested. BMP2/4, FGF4 and Wnt inhibitors have also been shown to induce VMHC1 (refs 22,32,49), although the results of these studies do not rule out induction of heart identity rather than regionalization. Additional regional markers need to be tested to resolve this. Interestingly, both VTN and NRP1 have other, much later roles in heart patterning.

There is conflicting evidence about the extent to which cells that make up the AIP roll around it (involution) and turn over rapidly, or whether they remain in place as it advances caudally.

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**Figure 5 | Successive roles of the endoderm in heart induction and patterning.** Each diagram represents a stage in development; in each, tissue interactions are depicted on the left, signals on the right. (a) Initially (HH 4–6), the anterior-lateral endoderm (including some prospective AIP, green) is required for the adjacent cardiac mesoderm (light blue) to express NKX2.5, MEF2C and MYOC29 and MYOC23. BMP2 and FGF8 signalling from the anterior-lateral endoderm induce NKX2.5, GATA, MEF2 and HAND transcription factors22,34,51,52. The anterior endoderm expresses WNT inhibitors including Crescent, which can induce NKX2.5 (ref. 49). (b) At HH8, progenitor cells are becoming determined as either atrial or ventricular28,57,58. The AIP endoderm induces ventricular character and represses atrial identity. Signals from the AIP endoderm, including NRP1, FBLN7, KIRREL3 and VTN described here (red text and arrows) and previously reported BMP2, FGF4 and Wnt inhibitors22,32,49 (black text and arrows), induce some ventricular markers, which are integrated with atrial-inducing retinoic acid signalling from the posterior lateral plate mesoderm28,57,59. The signals from the AIP endoderm that repress atrial identity are unknown. (c) At HH9, when regional markers of anterior-posterior heart tube patterning begin to be expressed, AIP endoderm induces ventricular markers VMHC1/MYH15, IRX4, NPPB and GJA5 (the latter expressed from HH12) and represses the atrial marker AMHC1. Four secreted molecules from the AIP endoderm, NRP1, FBLN7, KIRREL3 and VTN can induce VMHC1/MYH15 and NPPB.
down the embryo. Neither possibility is incompatible with the AIP being an organizer. The cellular composition of Hensen’s node constantly changes as cells move in and out of it, but it also contains resident cells and similar findings have been made for the ZPA of the limb. In all these cases the organizer property, as well as molecular markers for it, remain in the region rather than the limb at HH20/21 (HH20 AL) and HH24 (HH24 AL). The limb mesenchyme for the limb segments was enriched in the posterior limb bud (containing the ZPA) at either HH20/21 or HH24.

The dataset was submitted to ArrayExpress with the title ‘Microarray analysis of chick embryonic tissues: gastrulation, neural tube/notochord and limb development’ and given accession number E-MTAB-4048.

Embryo manipulation. Fertilized Brown Bovin Gold hens’ eggs (Henry Stewart, UK) were incubated at 38°C and staged according to Hamburger and Hamilton (HH). The following tissues were collected (three separate samples of each) and RNA extracted by standard methods: Hensen’s Node at HH 3/4 (HH4 HN and HH5 HN) and HH6 HN, posterior primitive streak at HH 3/4 (HH4 PS), HH10/11 notochord and adjacent ventral neural tube/forebrain (VNT) and the corresponding dorsal neural tube (DNT), the posterior third of the limb at HH20/21 (HH20 PL) and HH24 (HH24 PL) and the anterior third of the limb at HH20/21 (HH20 AL) and HH24 (HH24 AL).

Hybridization to Affymetrix microarrays was conducted by ARK-Genomics at the Roslin Institute, University of Edinburgh, as described in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Inc.). Briefly, 15 µg of total RNA from each sample was reverse transcribed using a T7-oligo (dT) primer in the first-strand complementary DNA (cDNA) synthesis. After RNA-Ase-H treatment, second-strand cDNA synthesis was carried out. The cDNA was then purified and used as a template in the subsequent in vitro transcription reaction. After transcription, the cDNA was blunt-ended and incorporated into biotinylated cTP and UTP using T7 polymerase. The biotinylated cDNA targets were fragmented and hybridized to the Affymetrix GeneChip Chicken Genome Array, which provides good coverage of the chicken genome (32,773 targets were fragmented and hybridized to the Affymetrix GeneChip Chicken Genome Array, which provides good coverage of the chicken genome (32,773 genes)). Twenty-seven arrays were used in separate samples of each) and RNA extracted by standard methods: Hensen’s Node (HN) and anterior-medial endoderm (AM) were harvested from HH5/C0 and kept on ice until needed. Mesoderm (#1–5, from both the left and the right sides and used randomly) was harvested from HH5/C0 to HH9/C0 embryos. Host embryos ranged from HH4 to HH6. Grafts were placed on the right or the left side of host embryos; no differences were noted in the results. All grafting, the embryos were placed on ice for 1-6 h prior to grafting (12–18 h). Limb grafts were performed in ovo at HH20–22 and incubated for 5 days.

Delivery of factors using transfected cells. Chicken NRPI (NM_204782.1; F primer CCGCCTCTGGAAAG; R primer CATTACGGTTAATCTCTG), FBLN7 (XP_003649034.1, formerly FIBULIN 7), and GJA5 (NM_205061.1; F primer CTCTGCTCAGTCACAGTAG with 5’ end labeling) probes and antibodies

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METHODS

Comparison of samples was performed as follows:

HH4 HN versus HH4 PS;
HH6 HN versus HH4 PS;
HH4 HN versus HH6 HN;
VNT versus DNT;
HH20 PL versus HH20 AL;
HH24 PL versus HH24 AL.

Probes with a fold change (FC) ≥1.2 (log2) and a false discovery rate ≤0.05 were deemed significant. The results of these individual comparisons (using significant enrichment or depletion as the indicator, rather than absolute amounts) were then combined using the Boolean algorithm: (HH4 HN versus HH4 PS AND HH4 HN versus HH6 HN OR HH6 HN versus HH4 PS) AND VNT versus DNT AND (HH20 PL versus HH20 AL OR HH24 PL versus HH24 AL). Probes that are differentially enriched or depleted significantly in this algorithm comprise the organizer gene set. The first set of comparisons selects genes enriched or depleted in the early (HH3/4) Hensen’s node compared with the posterior streak, some of which remain expressed the same way at the later (HH5/6) stage. The second set of comparisons targeted the enrichment or depletion in the posterior limb bud (containing the ZPA) at either HH20/21 or HH24.

In vitro culture of explants. Tissues were excised from chicken and quail embryos as described above, and cultured in vitro for 24 or 48 h at 37°C with 5% CO2 on a 0.4 µm Nucleopore filter (Whatman, WHA101007) floating on DMEM (Gibco) (ref. 17). Endoderm was excised from donor embryos using 0.12% w/v trypsin (porcine trypsin 1:250, Sigma) in Pannett-Compton saline mixed with 30 G syringe needles, washed with saline and kept on ice until needed. Mesoderm (#1–5, from both the left and the right sides and used randomly) was harvested from HH5/C0 to HH9/C0 embryos. Host embryos were placed on the right or the left side of host embryos; no differences were noted in the results. All experiments were performed on embryos obtained by co-culturing the explants with the mesoderm.

Probe and antibodies. Whole-mount in situ hybridization and immunohistochemistry were performed as described. 19,23,25,26,27. Probes were used as follows: MYOD (ref. 43), B5X5 (ref. 75), IRX4 (ref. 26), DSL1 (ref. 76), PITX2 (ref. 77). The cDNA for NOTO (NM_001287381.1; R primer 5’ end labeling) was kindly provided by T. Brand and S. Knochel (ref. 86), kindly provided by the originating laboratories (ref. 87). Following whole-mount in situ hybridization, embryos were incubated in QCPN (Developmental Studies Hybridoma Bank, diluted 1:5) and/or anti-GFP (1:1000) and/or anti-VMHC1 (MYH15) (ref. 73) antibodies (73%) homology to VMHC1 (MYH15) were kindly provided by T. Brand and S. Knochel.

Probes and antibodies.
Scoring of co-cultures. Explant cultures (Supplementary Tables 1–6) were considered ‘positive’ if high-level expression was observed throughout the majority of the explant. The n-numbers in the figures represent those that were scored as positive. The n-numbers that include positive and patchy expression are indicated. Explants with no detectable expression or very weak expression (perhaps due to non-specific staining, constitutive expression and/or marginal induction) were scored as negative. Explants with high-level expression in a small patch (some of which could be due to contamination) were excluded from P-value calculations, unless indicated. To calculate P values, positive (plus patchy where specified) and negative counts were compared across two conditions (#3 alone versus #3 + AIP or #3 + Cont.) using two-tailed Fisher’s exact test with a 2 × 2 contingency table. P values ≤ 0.05 were deemed significant.

Data availability. The microarray data have been deposited in the ArrayExpress database under accession code E-MTAB-4048. All other data supporting the findings of this study are available within the article and its Supplementary information files or from the corresponding author upon reasonable request.

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