FGF8 functions in the specification of the right body side of the chick

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Left–right asymmetry in vertebrate embryos is first recognisable using molecular markers that encode secreted proteins or transcription factors. The asymmetry becomes morphologically obvious in the turning of the embryo and in the development of the heart, the gut and other visceral organs. In the chick embryo, a signalling pathway for the specification of the left body side was demonstrated. Here, Sonic hedgehog (Shh) protein is the first asymmetric signal identified in the node [1,2]. Further downstream in this pathway are the left-specific genes *nodal*, *lefty-1*, *lefty-2* and *Pitx2* [1,3–5]. On the right body side, a function of the activin pathway is indicated by the right-sided expression of cActRIIa [1,6]. We detected that another key molecule in vertebrate development, fibroblast growth factor 8 (FGF8) [7,8], is expressed asymmetrically on the right side of the posterior node. We demonstrate that transcription of *FGF8* is induced by activin and the FGF8 protein inhibits the expression of *nodal* and *Pitx2* and leads to expression of the chicken snail related gene (*cSnR*) [9]. Left-sided application of FGF8 randomises the direction of heart looping.

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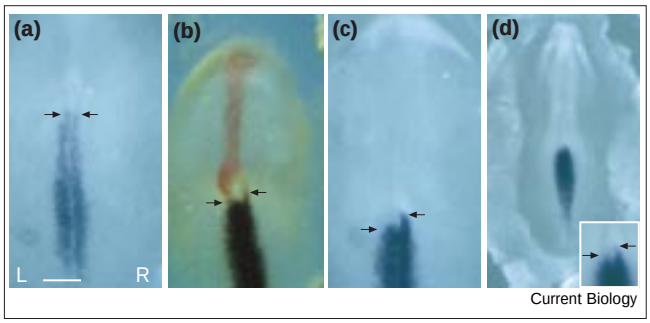
Results and discussion

Figure 1 shows the expression of *FGF8* during the early development of chick embryos. *FGF8* was strongly expressed in the anterior two thirds of the primitive streak at an early stage of streak development (Hamburger–Hamilton stage 3; not shown). Expression was low in, or absent from, Hensen's node at the fully extended streak stage and at the head-process stage (Figure 1a). With the first appearance of the headfold, expression of *FGF8* was detected in the right but not in the left posterior portion of the node (Figure 1b). This asymmetry became more pronounced at the one-somite stage (Figure 1c) and was still detected in four-somite-stage embryos (Figure 1d). A direct comparison of *FGF8* and *SHH* expression by double *in situ* analysis revealed a transcript-free zone in the midline including the primitive pit at the headfold stage (Figure 1b). The asymmetric *FGF8* expression occurred later and more posteriorly than the asymmetric domains of *cActRIIa* and *SHH* [1].

Figure 1

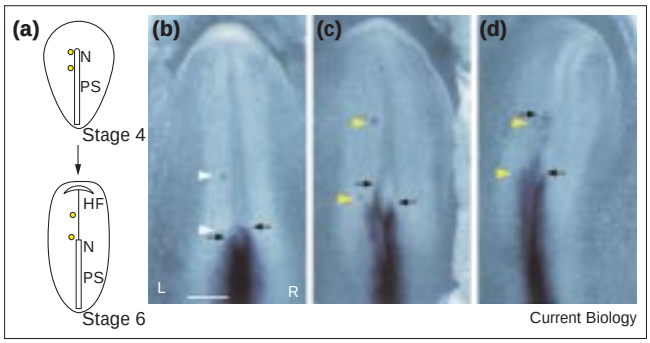


Left–right asymmetry of *FGF8* expression in the chick node. A whole-mount *in situ* analysis of chick embryos with an *FGF8* (blue) or a *SHH* (red) probe is shown in dorsal views. Arrows indicate the anterior levels of *FGF8* expression; the left (L) and right (R) sides are indicated. (a) The head process stage at Hamburger–Hamilton stage 5 [14]. There is no *FGF8* expression in the node. (b) The headfold stage, Hamburger–Hamilton stage 6. Note that there is right-sided *FGF8* expression in the posterior node, and left-sided expression of *SHH*, with a transcript-free zone in-between. (c) The one-somite stage, Hamburger–Hamilton stage 7. Note the pronounced asymmetric *FGF8* expression. (d) The four-somite stage, Hamburger–Hamilton stage 8. Note the persistent asymmetric *FGF8* expression (a higher magnification is shown in the inset). The scale bar indicates 290 μm in (a–c) and 580 μm in (d).

We analysed whether or not the activin pathway controls asymmetric expression of *FGF8* in the node. Beads soaked in activin A were implanted close to the left side of the node or the anterior primitive streak of cultured embryos at Hamburger–Hamilton stages 4 or 5 (Figure 2a). This ectopic application of activin extended the expression of *FGF8* from the left primitive ridge anteriorly into the left side of the node, and further into the epiblast (13 out of 16 with activin beads, 0 out of 6 with control beads; Figure 2b,c). Thus, the temporally successive expression of *cActRIIa* and *FGF8* on the right side of the node appeared not to be an independent event, but rather to be the result of an inductive interaction.

To study possible interactions between *SHH* and *FGF8*, sources of *FGF8* (n = 19) or Shh (n = 45) were implanted close to the left or the right side, respectively, of the

Figure 2

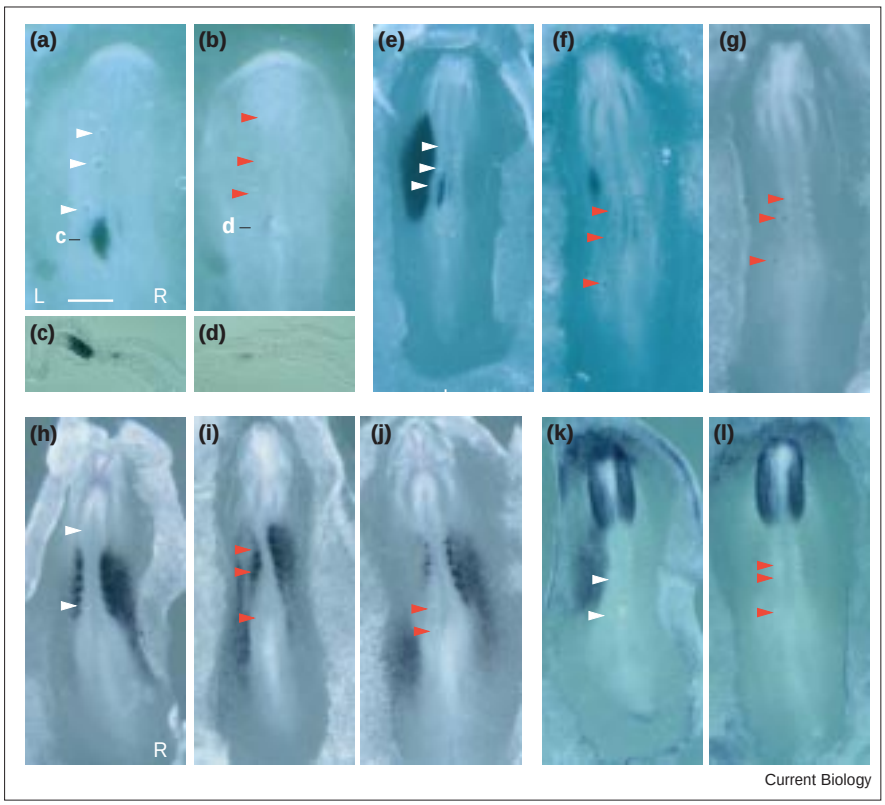


Effect of activin A protein on the expression of *FGF8*. Black arrows indicate the anterior levels of *FGF8* expression. **(a)** Schematics of chick embryos depict the sites of bead implantation at Hamburger–Hamilton stage 4 and their location after development of the embryo to stage 6. N, node; PS, primitive streak; HF, headfold. **(b)** Control beads (white arrowheads) have no effect on the asymmetric *FGF8* expression. **(c,d)** Activin A coated beads (yellow arrowheads) induce ectopic, left-sided expression of *FGF8*. The scale bar indicates 290 μm (b–d). L indicates the left side, R the right side.

stage 4 node (Figure 2a). We never detected any effect on the asymmetrically expressed gene, a finding which may be linked to the observation of a gap between the expression domains of *SHH* and *FGF8* in the node at stage 6 (Figure 1b). These data indicate that the distribution of Shh to the left and FGF8 to the right portion of the node does not involve regulatory interactions between these two factors.

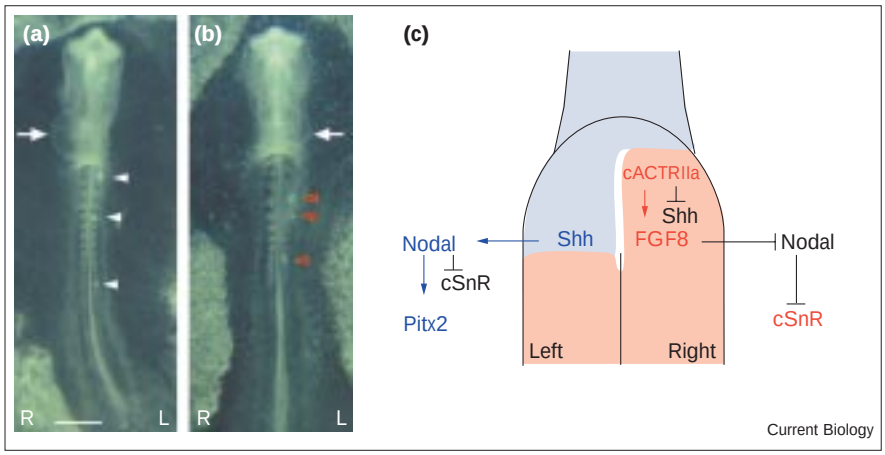
Next, we investigated whether the absence of *nodal* expression from the right side of the embryo could be due to a repressing effect of FGF8. Beads loaded with FGF8 protein were transplanted into stage 4 or stage 5 embryos close to the left side of the node or the anterior primitive streak ($n = 22$; see Figure 2a). Node cells that are fated to become the left paraxial mesoderm should thus come under the influence of FGF8, as is normally the case only on the right side. Embryos with beads adjacent to the node at the time of fixation always showed a strong reduction or absence of the left-sided *nodal* domain (18 out of 18 treated embryos, 0 out of 12 control embryos; Figure 3a–g). In embryos at stage 6, the early, paraxial expression domain of *nodal* close to the regressing node was suppressed (Figure 3a–d). At later stages of development (stage 8), the *nodal* expression in the lateral plate mesoderm was also inhibited, in spite of the relatively large distance between the source of FGF8 and the lateral *nodal* domain (Figure 3e–g). Thus, the observation that the lateral *nodal* domain is induced by the midline signal Shh via a paraxial expression domain on the left [1], also applies to its repression by the midline signal FGF8 on the right. These results

Figure 3



Effect of FGF8 protein on the expression of asymmetrically expressed genes. BSA-loaded control beads (white arrowheads) or FGF8-loaded beads (red arrowheads) were implanted at Hamburger–Hamilton stage 4–5 adjacent to the node and the anterior primitive streak (see Figure 2a). Embryos are shown in dorsal views. **(a–g)** Effect of FGF8 on *nodal* expression. (a,b) Note the repression of the paraxial *nodal* domain by FGF8-loaded beads. The corresponding sections shown in (c) and (d) reveal strongly reduced *nodal* expression in the paraxial mesoderm. (e–g) Note the repression of *nodal* in the lateral mesoderm resulting from FGF8 beads close to the midline. **(h–j)** Note the ectopic expression of *cSnR* at the five-somite stage in the lateral mesoderm of the left side as a consequence of FGF8 bead implantation. **(k,l)** Note the absence of *Pitx2* RNA from the lateral plate mesoderm resulting from implanted FGF8 beads. The scale bar indicates 454 μm in (a,b), 458 μm in (e–g) and 539 μm in (h–l). L indicates the left side, R the right side.

Figure 4



(a,b) Ectopic FGF8 causes randomisation of heart situs. The embryos are shown in a ventral view. BSA-loaded control beads (white arrowheads) or FGF8-loaded beads (red arrowheads) were implanted at Hamburger–Hamilton stage 4 (see Figure 2). Another bead was inserted adjacent to the node at stage 7. Note left-sided heart looping resulting from FGF8 beads implanted on the left side close to the midline in (b), in comparison to right-sided heart looping in control embryos ((a), white arrows). The scale bar indicates 973 μm in (a,b). L indicates the left side, R the right side. (c) Schematic representation of the molecular pathways in left–right patterning with blue denoting the expression domain of SHH and red the expression domain of *FGF8*. In the chick, Shh signalling is essential for left-sided development by positively regulating *Pitx2* via *nodal*. FGF8 functions in the development of the right side downstream of activin and leads to right-sided *cSnR* expression, possibly via repression of *nodal*.

suggest that FGF8 is a molecular cause of the absence of nodal protein on the right side of the embryo. Its effect can be mimicked by FGF4 (7 out of 8 embryos tested) and FGF1 (5 out of 8), but not by FGF7 (0 out of 7).

In the chick at stage 8, RNA of the chicken snail related gene (*cSnR*) is found in both the left and right somites as well as in a large domain that is restricted to the right lateral mesoderm, where no *nodal* expression occurs [9]. On the left side, *nodal* induces the transcription of *Pitx2* in the lateral mesoderm [3,4]. We tested the transcriptional response of *cSnR* and *Pitx2* to FGF8 on the left side of the embryo by the implantation of beads close to the streak and the node (stages 4–5). When the embryos were allowed to develop up to the five-somite stage (stage 8), ectopic *cSnR* expression was observed in the left lateral mesoderm (12 out of 18 treated embryos, 0 out of 6 control embryos). In some specimens the size of the ectopic *cSnR* domain resembled that of the right domain, but in many cases it was smaller than the right domain and was induced only in the posterior, lateral mesoderm (Figure 3h–j). The impact of FGF8 beads on *Pitx2* expression was analysed in similar experiments. In contrast to control embryos, the embryos with implanted FGF8 beads did not express *Pitx2* in the left lateral mesoderm (7 out of 10 FGF8-treated; 0 out of 4 controls; Figure 3k,l).

We examined whether or not left-sided FGF8 application was able to affect the looping of the heart. FGF8 beads were implanted close to the left side of the node at stage 4 or 5, and in a second operation again at stage 6 or 7. Cultured embryos were grown to the beating-heart stage (stage 11). Thus, an FGF8 source was constantly present next to the left side of the node. In all 25 controls we observed only correct, right-sided heart-looping (Figure 4a), but we obtained 6 out of 25 specimens in the FGF8-treated group with left-sided looping (Figure 4b), and 3 out of 25 specimens with a symmetric heart. These findings indicate a randomisation of heart looping, and demonstrate a function for *FGF8* in establishing the left–right asymmetry of the heart.

Several recent findings indicate connections between activin, FGFs, mesoderm induction and patterning. Thus, laterality defects in murine *ActRIIB* mutants (which lack the activin RIIB receptor) indicate that the role of the activin signalling in the development of the right body side may not be restricted to the chick [10]. FGF signalling is not only required for mesoderm induction by activin [11,12], but also plays a major role in mesoderm patterning in the trunk [13]. Heart defects are reported for murine *FGF8* compound heterozygous mutants, which carry a hypomorphic and a null allele of *FGF8*, but homozygous null mutant embryos did not develop far enough for the detection of left–right patterning defects [8]. Our study has demonstrated a role for FGF8 in the promotion of right-sided and the suppression of left-sided development in the avian embryo (Figure 4c). The *FGF8* gene appeared to function downstream of activin, as indicated by its slightly later transcription on the right side of the node, and by the fact that it can be ectopically induced by activin. The direct or indirect repression of the *nodal* and the *Pitx2* genes, and the upregulation of *cSnR* gene, identified FGF8 as a node-derived signal responsible for the absence or presence, respectively, of these transcription factors in the right lateral mesoderm. Given that we found no interaction between the SHH and the *FGF8* genes, the 'decision' between the left and the right pathways appears to occur at a higher level, possibly between *activin* and SHH [1]. Common to both the left and the right pathways is the

central role of the node and its molecular architecture as well as the transfer of left–right information via paraxial mesoderm to the lateral plate mesoderm.

Materials and methods

White Leghorn chick embryos were staged according to Hamburger and Hamilton [14]. For the ectopic application of proteins, beads or cell aggregates were implanted between the ectoderm and the endoderm in cultured embryos as described [15]. Heparin acrylic beads (Sigma) were washed in phosphate-buffered saline (PBS) and soaked in 1 μg/μl FGF1, FGF4, FGF7 or FGF8b (R&D Systems) in 0.1% bovine serum albumin in PBS, or in 13 U/μl recombinant bovine activin A (Innogenetics) in PBS. Control beads were incubated in 0.1% BSA in PBS or PBS alone, respectively. Implantation of Shh-producing cells has been described earlier [15]. Whole-mount *in situ* hybridisation was performed as in [16] with the following probes: *FGF8* [7], *nodal* [1], *Pitx2* [4], *Shh* [17] and *cSnR* [9].

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