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FGF8 Acts as a Right Determinant during Establishment of the Left-Right Axis in the Rabbit

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Summary

Background: FGF8 has been implicated in the transfer of left-right (L-R) asymmetry from the embryonic midline (node) to the lateral plate mesoderm (LPM). Surprisingly, opposite roles have been described in chick and mouse. In mouse, FGF8 is required for the left-asymmetric expression of *nodal*, *lefty2*, and *Pitx2*. In chick, FGF8 represses *nodal* and *Pitx2* on the right side. This discrepancy could reflect evolutionary differences between birds and mammals. Alternatively, the right-asymmetric expression of *fgf8*, which is not found in mouse, at the chick node may be a prerequisite of right-sided function. Finally, chick (blastodisc) and mouse (egg cylinder) differ with respect to the topology of the early gastrula/neurula embryo.
Results: The rabbit blastodisc was investigated as an additional mammalian L-R model system. While *nodal*, *lefty*, and *Pitx2* showed asymmetric expression in the left LPM, *fgf8* and all other midline marker genes were symmetrically expressed at the node like in mouse. Left-sided application of FGF8 repressed the endogenous transcription of *nodal* as well as ectopic expression induced by the parallel administration of BMP4. Right-sided inhibition of FGF8 signaling induced bilateral marker gene expression, demonstrating that, in rabbit, FGF8 acts as a right determinant like in chick.
Conclusions: These findings suggest that the anatomy of the early embryo (blastodisc versus egg cylinder) rather than taxonomical differences or asymmetry in expression constitutes an important determinant of FGF8 function in L-R axis formation. The rabbit may provide a useful model for early human embryogenesis, as human embryos develop via a blastodisc as well.

Introduction

Central to the establishment of the L-R body axis is the Nodal cassette, a molecular module conserved from fish to mouse [1–4]. It consists of three asymmetrically expressed genes in the left LPM, the TGFβ-type growth factor *nodal*; the related secreted molecule *lefty2*, a direct target and feedback inhibitor of Nodal signaling; and the *bicoid*-type homeobox transcription factor *Pitx2*, which relays the transient Nodal signal to the organ primordia [1–4].

Events upstream of the Nodal cassette are less well defined. In mouse, the structural and functional integrity of monocilia on ventral node cells is an essential factor upstream of the Nodal cassette [2, 5–7]. It has been hypothesized that the vortical movement of cilia functions in symmetry breakage by asymmetric distribution of a secreted molecule [2, 5, 8]. Although the identity of this molecule has not been revealed, the recent demonstration of inversion of L-R patterning by artificial fluid flow in mouse embryo cultures [9] and the identification of cilia in *Xenopus*, chick, and zebrafish [10] may indicate an evolutionary conserved function of monocilia on vertebrate organizer cells.

The growth factor FGF8 has been implicated in the transfer of the initial asymmetric cue(s) from the node to the periphery, i.e., the left LPM [11, 12]. Most surprisingly, however, embryologic and genetic experiments revealed opposing roles in chick and mouse [11, 12]. In both organisms, *fgf8* is expressed along the forming primitive streak [11–13]. In chick, *fgf8* mRNA in addition asymmetrically localizes to the right but not left side of the node [12]. Embryos cultured with an FGF8 bead introduced on the left side of the node failed to induce the Nodal cassette, while a right-sided bead had no effect, suggesting that, in chick, FGF8 functions to repress the Nodal cassette on the right side [12]. No asymmetries of *fgf8* expression were described in mouse [13]. Knockout embryos die during gastrulation [14], preventing the analysis of laterality defects in *fgf8* null embryos. A hypomorphic allele, however, revealed a role of *fgf8* in L-R axis formation in mouse as well [11]. Embryos were characterized by randomization of heart looping and a right lung isomerism, i.e., a mirror-image duplication of the right lung on the left side, in about 50% of cases [11]. *nodal*, *lefty2*, and *Pitx2* were not expressed in the left LPM at E8.5 in about one half of mutant embryos; the absence of expression implied that, in mouse, FGF8 was required for the induction of the Nodal cassette on the left side [11]. Placement of an FGF8 bead on the right side of early somite-stage embryos resulted in bilateral induction of *nodal* during in vitro culture, whereas a bead on the left side had no effect, establishing FGF8 as a left instructive determinant in mouse [11].

Different effects of FGF8 on laterality decisions in chick and mouse embryos could be attributed to divergent evolution from birds to mammals. Alternatively, the asymmetry in *fgf8* mRNA localization, which is exclusively found in chick, might be required for right-sided function. The anatomy of the gastrula/neurula embryo constitutes an additional difference between the two species. Chick embryos develop via a flat blastodisc, whereas mouse embryos assume a cup-shaped appearance called the egg cylinder [15, 16]. Our analysis in rabbit blastodiscs suggests that the topology of the

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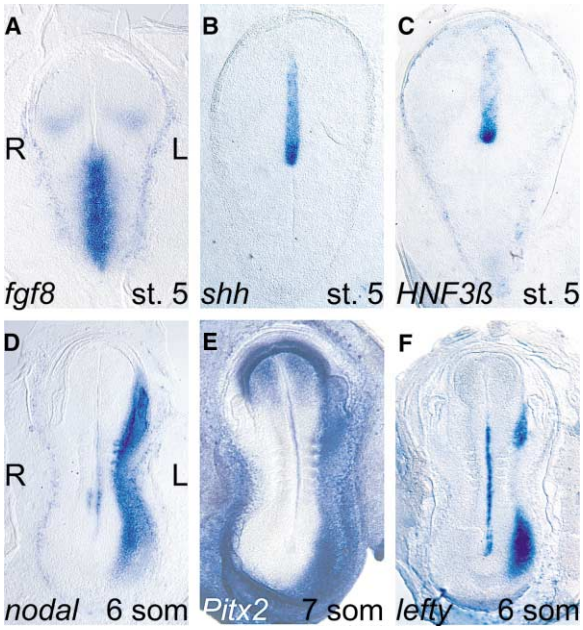

Figure 1. Symmetric Expression of *Fgf8*, *shh*, and *HNF3*β at the Node, and Left-Asymmetric Transcription of *nodal*, *Pitx2*, and *lefty* in the Lateral Plate Mesoderm

(A–C) Symmetric expression of the midline markers genes (A) *fgf8*, (B) *shh*, and (C) *HNF3*β at the node of stage-5 rabbit embryos.
(D–F) Asymmetric mRNA localization of (D) *nodal*, (E) *Pitx2*, and (F) *lefty* in the left LPM of 6-somite rabbit embryos. Please note that the *lefty* probe detected both *lefty1* (midline) and *lefty2* (left LPM).
Nonradioactive whole-mount in situ hybridization of wild-type rabbit embryos. All embryos are shown in ventral view. L, left; R, right; s, somite.

early embryo constitutes an important parameter of FGF8 function.

Results

Symmetric Expression of Midline Marker Genes at the Node and Asymmetric Transcription of the Nodal Cascade Genes in Early Rabbit Embryos

Investigation of FGF8 function in the rabbit required the availabilty of suitable L-R marker genes. Fragments of a number of genes were cloned by PCR, and mRNA expression was analyzed by whole-mount in situ hybridization. Patterns relevant to the study of FGF8 function are depicted in Figure 1. Details of expression profiles will be published elsewhere (A.F. and M.B., unpublished data). Staging of presomite rabbit embryos followed the Hamburger/Hamilton staging system in chick; somite numbers designate later stages. Figure 1A shows *fgf8* expression at stage 5 in the primitive streak and node. In this and all other stages analyzed, no signs of asymmetry were detected (Figure 1A and data not shown). In chick, additional marker genes, such as *shh* and *HNF3*β, for example, display asymmetric expression patterns in or at the node [17]. No such asymmetries were detected for *shh* or *HNF3*β in rabbit embryos (Figures 1B and 1C). Thus, expression patterns of these midline L-R marker genes were symmetrical in rabbit like in mouse and unlike in chick. In contrast, asymmetric mRNA transcription of the Nodal cascade genes *nodal*, *lefty*, and *Pitx2* was conserved in the left LPM (Figures 1D–1F). These genes, therefore, served as useful L-R readout in the experiments described below.

Lability of Laterality in Cultured Prestreak Rabbit Embryos

A second prerequisite to the study of FGF8 function in rabbit was the establishment of conditions that allowed culture of manipulated embryos from stage 5, when *fgf8* mRNA was detected at the node, up to the 3-somite stage, when asymmetric *nodal* transcription in the left LPM first appeared (not shown). A detailed description of culture conditions, which are schematically depicted in Figure 2A, is given in the Experimental Procedures. In a first series of experiments, embyos were taken into culture at stage 5, incubated to the 3- to 5-somite stage, and analyzed for *nodal* transcripts by whole-mount in situ hybridization. Representative examples of cultured embryos are shown in Figure 2B, and results are summarized in Table 1. Cultured embryos developed normally, as assessed by morphological criteria. Notochord, somites, and heart primordia formed as in uncultured embryos of the respective age (Figure 2B and data not shown). Surprisingly, however, cultured embryos in most cases did not reveal the normal expression pattern of *nodal* in the left LPM (Table 1). *nodal* was bilaterally expressed in the LPM or was absent altogether (Figure 2B; Table 1). Placement of PBS-soaked control beads on the right or left side of the node in about one third of the cases prevented expression of *nodal* on the side of bead application (Table 1; Figure 3E).

In mouse, it was reported that laterality cues in embryo cultures become stable only after the onset of neurulation [18]. We therefore cultured rabbit embryos at later stages. Figure 2C and Table 1 show that, in embryos taken into culture at the 1- to 3-somite stage, the normal left-asymmetric expression of *nodal* transcription in the LPM was retained in most cases (Figure 2C; Table 1). When a PBS control bead was placed on the right or left side of the node, wild-type *nodal* expression was observed like in unmanipulated control cultures in most cases (Figure 2C; Table 1). A minor proportion of 15%, however, still showed bilateral *nodal* transcription following culture to the 3- to 6-somite stage (Table 1).

The Nodal Cascade Is Conserved in Cultured Rabbit Embryos

To test if the rabbit embryo was competent to react to L-R axis manipulation at the 1- to 3-somite stage, we introduced Activin-soaked beads into the right LPM, in order to mimick Nodal signaling. Embryos were cultured until 3–6 somites had formed and were analyzed for *nodal* and *Pitx2* expression. In about 50% of the cases, bilateral signals were observed (Figure 2D; Table 1). In summary, these experiments showed that laterality cues in rabbit embryos were not fixed before the 1-somite stage, that the Nodal cascade was conserved in the rabbit, and that the right LPM was competent to react to an ectopic Activin signal up to the 3-somite stage.

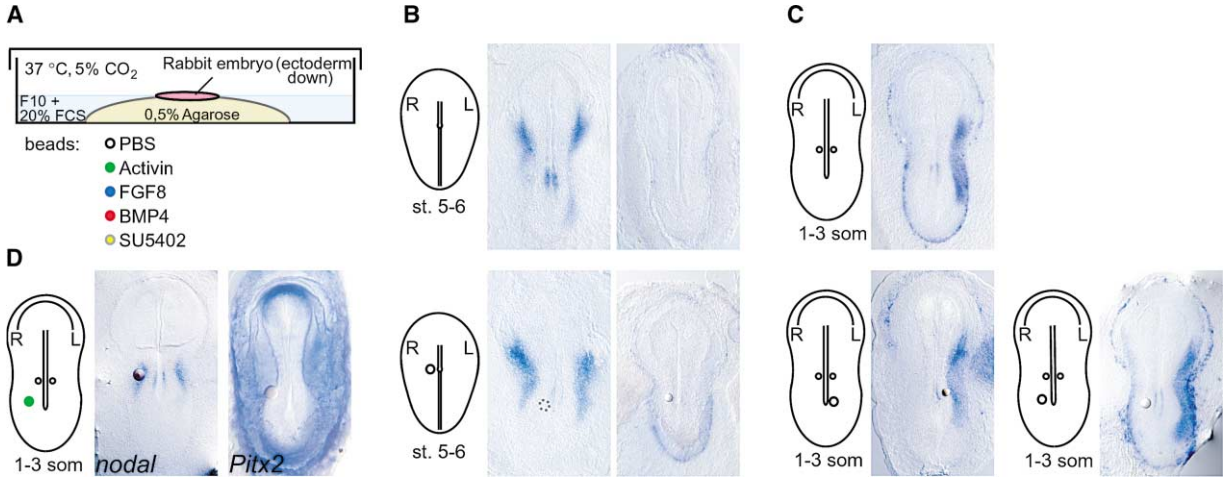

Figure 2. Activin Induces *nodal* and *Pitx2* on the Right Side

(A) A schematic diagram of the experimental design. The color code indicates the use of beads in the experiments shown in Figures 2–6. For details, see the text.

(B and C) Establishment of culture conditions. Following treatment and culture, embryos were processed for whole-mount in situ hybridization with a *nodal*-specific probe. (B) Culture of presomite rabbit embryos resulted in loss of asymmetric *nodal* expression in controls with (lower panel) or without (upper panel) application of PBS-soaked beads. (C) At the 1- to 3-somite stage, asymmetric *nodal* expression was retained in cultures of untreated embryos (upper panel) and following placement of PBS-soaked beads either on the left or right side of the node (lower panel).

(D) Placement of an Activin-soaked bead into the right LPM resulted in ectopic *nodal* (left embryo) and *Pitx2* (right embryo) expression. The schematic diagrams indicate the experimental design.

Repression of Asymmetric Gene Expression by FGF8

To analyze if FGF8 induces ectopic *nodal* expression, beads were placed on the right side of the node at the 1- to 3-somite stage. No induction of right-sided *nodal* (20/20) or *Pitx2* (6/6) transcription was observed (Table 2; Figure 3A). Thus, unlike in mouse [11], FGF8 did not act as an inducer of the left-sided Nodal cascade in cultured rabbit embryos. We next asked whether, as in chick [12], FGF8 represses *nodal* and *Pitx2*. When FGF8-soaked beads (1 mg/ml) were introduced on the left side of the node at the 2- to 3-somite stage, however, both genes were expressed as in wild-type unmanipulated embryos (Figure 3B; Table 2). The biological activity of the FGF8 used in these experiments was verified in *Xenopus* animal cap explant cultures (not shown).

Gain-of-function experiments in chick were performed at stage 4/5 [12]. We therefore wondered whether experimental repression of *nodal*/*Pitx2* was restricted to presomite-stage embryos. When FGF8 beads were placed on the left side of the node at stages 5 and 6, in the vast majority of cases *nodal* was not expressed in the left LPM (31/36; Table 2; Figures 3C and 3E). In a few cases (4/36), residual *nodal* transcripts were found on the left side, but signals were clearly absent around the bead (Figure 3C, left embryo). About one third (14/36) of embryos cultured with a left FGF8 bead at stages 5 and 6 revealed *nodal* mRNA signals on the right side (Figure 3C, left embryo; Table 2). This ectopic expression of *nodal* was not due to FGF8, as control cultures at stages 5 and 6 resulted in bilateral *nodal* expression in about 30%–50% of the cases (Figure 2B; Table 1). When FGF8 beads were placed on the right side of the node at stages 5 and 6, right-sided *nodal* expression was never observed, while the normal expression in the left LPM was retained in 7/21 embryos (Figures 3D and 3E; Table 2). This inverse correlation between FGF8 bead placement and *nodal* transcription is summarized in Fig-

Table 1. Activin Induces *nodal* and *Pitx2* in Cultured Rabbit Embryos

Bead	Stage	l/r	Gene	n	Left	Bilateral	None	Right
–	5 and 6	–	*nodal*	23	3 (13)	13 (57)	7 (30)	–
PBS	5 and 6	l	*nodal*	39	5 (13)	10 (26)	16 (41)	8 (20)
PBS	5 and 6	r	*nodal*	14	3 (21)	5 (36)	6 (43)	–
–	1- to 3-somite	–	*nodal*	11	10 (91)	1 (9)	–	–
PBS	1- to 3-somite	l	*nodal*	13	11 (85)	2 (15)	–	–
PBS	1- to 3-somite	r	*nodal*	14	12 (86)	2 (14)	–	–
Activin	1- to 3-somite	r	*nodal*	18	8 (44)	10 (56)	–	–
Activin	1- to 3-somite	r	*Pitx2*	18	9 (50)	9 (50)	–	–

Embryos were cultured with or without (–) beads soaked in PBS or human recombinant Activin A (800 U/ml) until they reached the 3- to 6-somite stage, and they were analyzed for marker gene expression by whole-mount in situ hybridization. The numbers (n) of embryos in each row were compiled from three to six independent experiments. The numbers in parentheses indicate percentages.

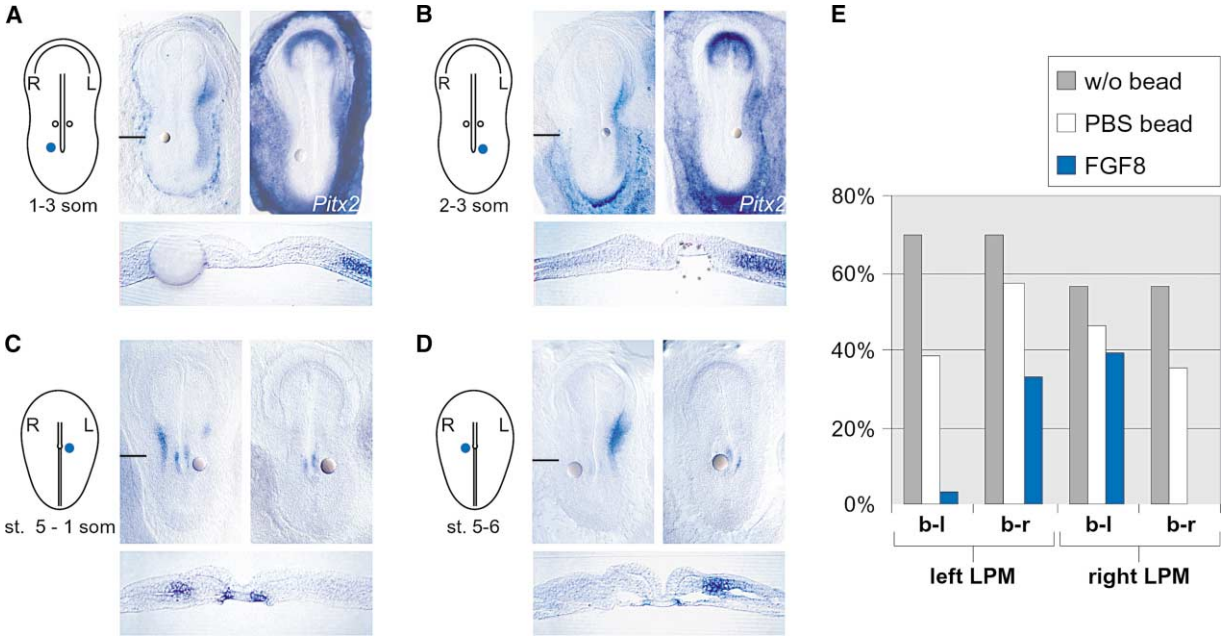

Figure 3. FGF8 Represses *nodal* in Presomite Rabbit Embryos

FGF8-soaked beads (1 mg/ml) were placed on the left or right side of the node of presomite or 1- to 3-somite stage rabbit embryos, cultured, and processed for whole-mount in situ hybridization.

(A) No induction of *nodal* and *Pitx2* following application of right-sided beads at the 1- to 3-somite stage.

(B) No repression of *nodal* and *Pitx2* following application of right-sided beads at the 2- to 3-somite stage.

(C and D) No expression of *nodal* on the side of FGF8 bead placement in presomite and 1-somite stage embryos. Planes of sections, which demonstrate normal development of cultured embryos, are indicated. Schematic diagrams indicate stages of embryos and placement of beads.

(E) Quantification of *nodal* expression patterns in the left and right LPM of cultured presomite rabbit embryos (Tables 1 and 2). b-l, left bead; b-r, right bead. Please note the inverse correlation between *nodal* transcription and FGF8 bead application.

ure 3E. Differences between control PBS and FGF8 beads were statistically highly significant (p values of <0.001 for left-sided bead applications, and 0.007 for right-sided bead applications; Figure 3E). Control bead placement had some effect on *nodal* expression in a side-specific manner as well (p values of 0.002 for left beads, and 0.028 for right beads; Figure 3E), perhaps by affecting the transfer of laterality cues from the midline to the LPM. Effects of PBS and FGF8 beads on *nodal* expression on the contralateral sides were not significant (p values of 0.047–0.184; Figure 3E).

To determine more precisely the time window of FGF8-mediated repression of *nodal* expression, embryos in which the first pair of somites was about to

Table 2. FGF8 Represses, and the FGFR1 Inhibitor SU5402 Induces, *nodal* and *Pitx2* in Rabbit Embryo Cultures

Bead	Dose	Stage	l/r	Gene	n	Left	Bilateral	None	Right
FGF8	1 mg/ml	5 and 6	l	*nodal*	36	1 (3)	4[a] (11)	21 (58)	10 (28)
FGF8	1 mg/ml	5 and 6	r	*nodal*	21	7 (33)	–	14 (67)	–
FGF8	1 mg/ml	0- to 1-somite	l	*nodal*	6	3[a] (50)	–	3 (50)	–
FGF8	1 mg/ml	2- to 3-somite	l	*nodal*	7	7 (100)	–	–	–
FGF8	1 mg/ml	2- to 3-somite	l	*Pitx2*	7	7 (100)	–	–	–
FGF8	1 mg/ml	1- to 3-somite	r	*nodal*	20	20 (100)	–	–	–
FGF8	1 mg/ml	1- to 3-somite	r	*Pitx2*	6	6 (100)	–	–	–
FGF8	3 mg/ml	1- to 2-somite	l	*nodal*	7	4[a] (57)	–	3 (43)	–
FGF8	3 mg/ml	2- to 3-somite	l	*nodal*	10	10 (100)	–	–	–
SU5402	1 mg/ml	1- to 2-somite	l	*nodal*	6	5 (83)	–	1 (17)	–
SU5402	1 mg/ml	1- to 2-somite	r	*nodal*	22	5 (23)	17 (77)	–	–
SU5402	1 mg/ml	1- to 2-somite	r	*Pitx2*	2	–	2 (100)	–	–
SU5402	1 mg/ml	3- to 4-somite	r	*nodal*	3	3 (100)	–	–	–

Beads soaked in mouse recombinant FGF8b or the FGFR1 inhibitor SU5402 were inserted at the indicated stages on the left (l) or right (r) side of the node, and embryos were cultured until they reached the 3- to 6- somite stage and were analyzed for marker gene expression by whole-mount in situ hybridization. The numbers (n) of embryos in each row were compiled from three to six independent experiments. The numbers in parentheses indicate percentages.

[a] Expression on the left side was observed; however, the signal was repressed around the bead, as shown in the embryo in Figure 3C (left embryo).

form or had just appeared (0- to 1-somite stage) were treated with left-sided FGF8 beads. At this stage, FGF8 was still able to repress *nodal* transcription, resulting either in complete absence of staining in the left LPM or in a clear repression around the bead (Table 2). To exclude the possibility that insufficient FGF8 was applied to repress *nodal* at later stages, higher doses (3 mg/ml) were attempted. In this set of experiments, the time window of repression was slightly extended to a stage when the second pair of somites was just about to form (1- to 2-somite stage; Table 2). At later time points (2- to 3-somite stage), however, no effect on *nodal* expression in the left LPM was observed (Table 2). Because of limited solubility of lyophylized FGF8, higher doses could not be tried. Histological analysis of cultured embryos confirmed that beads did not affect normal embryonic development (Figure 3). These experiments showed that FGF8 did not induce ectopic expression of left-asymmetric marker genes in the rabbit; that, in presomite-stage embryo cultures, FGF8 was incompatible with *nodal* expression; and that the time window of FGF8-mediated repression of *nodal* transcription in the left LPM was closing in a dose-dependent manner with the appearance of the second pair of somites.

These gain-of-function cultures strongly suggested that FGF8 functions as a right determinant in rabbit embryos. Repression of endogenous *nodal* transcription could only be demonstrated at stages at which L-R cues were still labile (Figure 2B; Table 1). Ectopic, culture-induced residual artificial right-sided *nodal* expression of 1- to 3-somite stage embryos, which was found in about 15% of control cultures (Figure 2C; Table 1), however, was quantitatively repressed in embryos cultured in the presence of a right-sided FGF8 bead (Figure 3D; Table 2). This observation made us wonder whether the left-sided gene cascade could be repressed at all in 1- to 3-somite embryos in which the positive Nodal signaling feedback loop could be well underway.

In Cultured Rabbit Embryos, BMP4 Induces the Nodal Cascade

BMP growth factors were described as potent inhibitors of *nodal* and *Pitx2* expression in the left LPM in chick [19, 20]. We therefore introduced beads soaked in human recombinant BMP4 on the left side of the node in early-somite rabbit embryos. Embryos were cultured until they reached 3–6 somites and were analyzed for marker gene expression. These experiments are summarized in Table S1 (see the Supplementary Material available with this article online), and representative embryos are shown in Figure 4. Downregulation of the *nodal* cascade genes was never observed in embryos treated with BMP4. Two unexpected BMP4 effects on the L-R cascade were detected, however: bilateral gene expression of *nodal* and *Pitx2* (Figure 4B), and induction of ectopic mRNA transcription in the intermediate and paraxial mesoderm and the underlying endoderm (Figures 4A–4C). In extreme cases, the entire mesodermal cell layer, with the exception of the nascent primitive streak mesoderm and the head mesenchyme, was positive for *nodal* transcripts, while ectopic signals were not found in neuroectoderm and midline tissue (Figures 4A, 4A′, 4B, and 4B′ and data not shown). Contrary to the broadening of the endogenous expression domain on the left side, induction on the right side was confined to the LPM tissue (Figures 4B and 4B′). The two effects were observed independently or in the same specimen, and they occurred in about 50% of treated embryos (Table S1). In addition, BMP4 effects were dosedependent. In most experiments, BMP4 was used at a concentration of 100 μg/ml. While 50 μg/ml was equally efficient (not shown), induction was rarely observed at 10 μg/ml (not shown). In four cultures, a BMP4 bead (100 μg/ml) was inserted on the right side of the node (not shown). Again, both effects were observed, bilateral expression of *nodal* (3/4) and induction in intermediate and paraxial mesendoderm (3/4). These results strongly suggest that, at early-somite stages, misexpression of BMP4 induces the Nodal cascade. As the experiments in chick, which resulted in repression of *nodal* and *Pitx2*, were performed at earlier stages and used 10-fold higher growth factor concentrations [19, 20], we wondered if BMP4 would repress marker genes in presomite embryos. When beads were applied on the left side of the node between stages 5 and 6, again, repression was not observed. Many of the stage-5 cultures could not be evaluated, however, as embryos frequently developed abnormally. At late stage 6, as in early-somite embryos, BMP4 positively regulated the Nodal cascade. The inductive potential of BMP4 was most pronounced between stage 6 and the 1-somite stage, i.e., when the first pair of somites formed, and decreased in 2- and 3-somite embryos. Taken together, these experiments demonstrate that BMP4 in rabbit embryo cultures positively regulates the Nodal cassette, contrary to results obtained in chick [19, 20].

The induction of bilateral marker gene expression at a stage at which, in control cultures, laterality cues were fixed in most cases (Table 1), was unexpected. We wondered if the midline was affected in such embryos, because, in mouse, loss of *lefty1* resulted in bilateral expression of *nodal* and *Pitx2* [21–23], and, in chick, it was recently shown that BMP4 acts as a repressor of midline *lefty* [19, 24]. We therefore analyzed *lefty* expression in rabbit embryos cultured at the 1- to 3-somite stage in the presence of a left-sided BMP4 bead (100 μg/ml). Our probe did not discriminate between *lefty1*, which, in mouse and chick, predominantly is expressed in the left prospective floor plate, and *lefty2*, which is transcribed in the left LPM [20, 25]. Three effects were distinguishable. As described above for *nodal* and *Pitx2*, bilateral LPM expression was observed, and the domain of *lefty* in the left LPM was broadened, resulting in a fusion of the two *lefty* expression domains in manipulated embryos (Figures 4E and 4E′; Table S1). In addition, midline *lefty* was repressed in BMP4-treated cultures (Figures 4D and 4E), while, in control embryos treated with a PBS bead, both expression domains of *lefty* were indistinguishable from uncultured embryos at the same stage (Figure 4F and data not shown). In summary, these experiments demonstrated that, in rabbit embryos, BMP4 did not repress asymmetric gene activity, but led to a broadening of the endogenous expression domains; BMP4 repressed midline *lefty*; and BMP4 induced bilateral *nodal*, *Pitx2*, and *lefty* expression in the LPM.

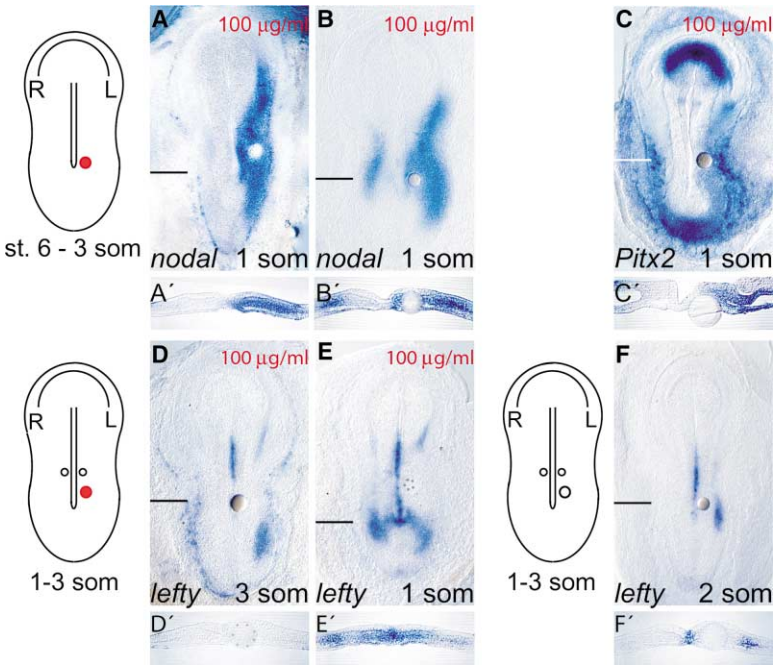

Figure 4. Positive Regulation of the Nodal Cassette by BMP4

(A–C) Induction of ectopic (A and B) *nodal* and (C and D) *Pitx2* expression following placement of a left-sided bead soaked in 100 μg/ml human recombinant BMP4. (A′–C′) Induction was observed in the intermediate and paraxial mesoderm and the underlying endoderm.

(D and E) BMP4 effects on *lefty* expression in cultured rabbit embryos. (E and E′) Medial extension of LPM *lefty* expression such that the LPM and midline domains fused at the node. (D and D′) Repression of midline *lefty* by BMP4. (F and F′) Normal *lefty* mRNA localization after application of a control PBS bead between the node and LPM on the left side. Please note that the *lefty* probe detected both *lefty1* (midline) and *lefty2* (left LPM).

Repression of BMP4-Induced Ectopic *nodal* Transcription by FGF8

The induction of ectopic *nodal* transcription by BMP4 afforded the opportunity of testing whether FGF8 was able to affect this induction. Two beads were applied simultaneously on the left side of 1-somite embryos, one soaked in 10, 50, or 100 μg/ml BMP4 in the immediate vicinity of the node, and a FGF8 bead (1 mg/ml) placed in a posterior-lateral position (Figure 5). Analysis of *nodal* expression in cultured embryos revealed a clear downregulation around the FGF8 bead (Figures 5A–5C). While in embryos that received a single BMP4 bead, induction of ectopic *nodal* was clearly seen to orginate from the bead (Figures 4A, 4A′, 4B, and 4B′), repression of *nodal* in double-bead cultures extended to and beyond the BMP4 bead (Figures 5A–5C). No repression of *nodal* was observed when BMP4 and a PBS control bead were applied (Figures 5C and 5D). Taken together, these data showed that, in gain-of-function experiments, FGF8 led to a downregulation of the endogenous left-asymmetric expression of *nodal* and *Pitx2* in the LPM.

Induction of Bilateral *nodal* and *Pitx2* by Interference with FGF8 Signaling on the Right Side

To assess the endogenous role of FGF signaling in the transfer of the left-asymmetric signal from the midline to the LPM, we performed loss-of-function experiments. FGF8 presumably signals through the FGF receptor 1 (FGFR1) [14]. The drug SU5402, which was previously used to block FGF8 function in cultured vertebrate embryos, interferes with receptor autophosphorylation and substrate phosphorylation by binding to the tyrosine kinase domain of FGFR1 [26]. Beads soaked in 1 mg/ml SU5402 were placed on the left and right side of the node at the 1- to 2-somite stage. Embryos were cultured to the 3- to 6-somite stage and were analyzed for marker gene expression. Left-sided bead application had no effect on *nodal* mRNA transcription (Table 2 and data not shown). Bilateral expression of *nodal* and *Pitx2* was seen in most embryos treated on the right side (Figures 6A and 6B; Table 2). Right-sided ectopic expression in all cases mimicked the endogenous expression on the contralateral side (Figures 5A′ and 5B′). At the 3- to 4-somite stage, competence for induction was lost (Table 2 and data not shown). These results strongly suggest that, in rabbit embryos, FGF8 functions asymmetrically on the right side to inhibit expression of the left-asymmetric Nodal cascade.

Discussion

Asymmetric Right-Sided Function of FGF8 in the Rabbit

Our experiments in rabbit confirm data in chick, which describe FGF8 as right determinant, but contradict the left-sided role of FGF8 in mouse. This unexpected failure to reproduce the mouse results in rabbit was not due to a lack of competence of the right LPM, as Activin, which, like Nodal, signals through Activin receptors and was previously used to mimic nodal signaling in frog and chick embryos [20, 27, 28], was able to induce the Nodal cassette ectopically on the right side (Figure 2D).

A clear correlation between placement of a FGF8 bead and absence of *nodal* transcription in presomitic embryos was observed irrespective of the side of bead application (Table 2; Figure 3E). The repression of *nodal* by FGF8 was confirmed in late-neurula embryos (from stage 6 to the 1-somite stage) in which ectopic expression of *nodal* was induced by BMP4 (Figures 4A and 4B). The ability of FGF8 to repress *nodal* transcription in the left LPM was lost in a dose-dependent manner between the appearance of the first and second pair of somites (Table 2 and data not shown). Interestingly, up

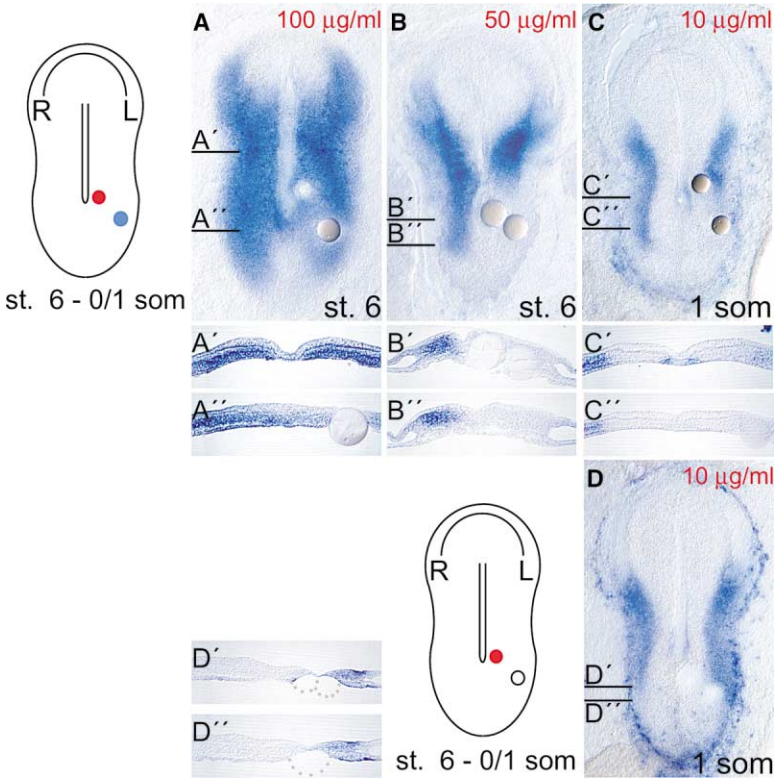

Figure 5. FGF8 Represses Ectopic *nodal* Transcription Induced by BMP4

Two beads were introduced in 1-somite embryos: a BMP4 bead at (A) 100, (B) 50, or (C and D) 10 µg/ml on the left side of the node, and a FGF8 bead (1 mg/ml) in a posterior-lateral position. Embryos were cultured to the 3- to 6-somite stage and were analyzed for *nodal* transcription.

(A–C) Repression of ectopic and endogenous *nodal* expression by FGF8.

(D) No repression of *nodal* in cultures treated with BMP4- and PBS-soaked beads.

Note that, in the (D, D′, D″) control embryos, the *nodal* signal extended to the PBS bead, (A–C) while the area around the FGF8 bead was clearly free of *nodal* transcripts in BMP4/FGF8 double cultures.

to around the same time point, L-R patterning can be inverted by artificial fluid flow in mouse embryo cultures [9], indicating that the L-R labile period is conserved in mouse and rabbit. The closure of the time window for repression in rabbit at early somitogenesis could be due to a loss of competence for FGF8 signal transduction. Alternatively, the positive feedback loop of Nodal signaling on *nodal* transcription might prevent efficient interference after the onset of endogenous *nodal* expression. The latter reasoning is supported by the finding that FGF8 was able to efficiently repress ectopic, culture-induced, right-sided *nodal* transcription (Figure 3A; Tables 1 and 2), i.e., in a scenario of simultaneous induction (culture) and repression (FGF8 bead application). An asymmetric right-sided role of endogenous FGF8 was further supported by the inhibitor experiments, in which inhibition of signal transduction on the right side resulted in ectopic induction of the Nodal cascade (Figure 6; Table 2). In summary, these gain- and loss-of-function experiments demonstrate that FGF8 acts as a right determinant in L-R axis formation in the rabbit.

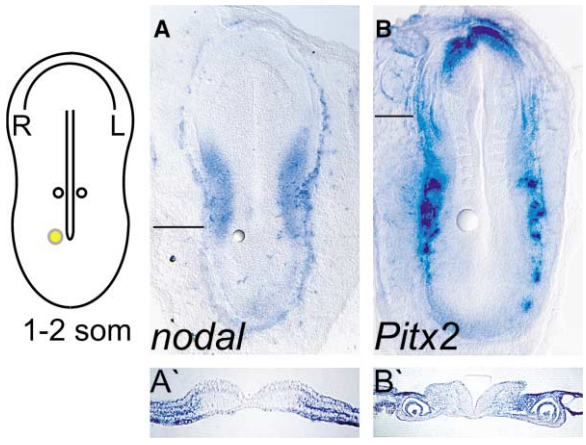

Figure 6. Repression of FGFR1 Signaling Induced Bilateral *nodal* Transcription

(A and B) Beads soaked in the FGFR1 inhibitor SU5402 were placed on the posterior right side of the node at the 1- to 3-somite stage; embryos were cultured to the 4- to 5-somite stage and were processed for whole-mount in situ hybridization with probes specific for (A) *nodal* or (B) *Pitx2*. (A′and B′) Note that ectopic induction on the right side was confined to the exact same domain where endogenous *nodal* transcripts were found on the left side.

In chick, the asymmetric expression of FGF8 at the posterior right side of the node has led to the suggestion that mesodermal cells, which leave the node and migrate to the LPM on the right side, would receive the FGF8 signal, resulting in repression of the Nodal cassette, while cells on the left side would not be influenced by FGF8 and would express the left-asymmetric marker genes [12]. In rabbit, as in mouse [13], we did not find any evidence of asymmetric mRNA expression of *fgf8* (Figure 1A). Furthermore, other genes with asymmetric expression patterns at or around the node in chick, such as *shh*, *ptc*, *HNF3*β, *BMP4*, *ActRIIB*, and *Act*β*B* [17, 24, 29–31], were found symmetrically in the rabbit (Figures 1B and 1C and data not shown). The chick so far represents the only vertebrate embryo with asymmetric gene expression patterns at the organizer. Morphological asymmetries of the chick node were first described in 1929 by Wetzel [32] (see also [33]). Asymmetric gene expression at the chick node, therefore, might simply

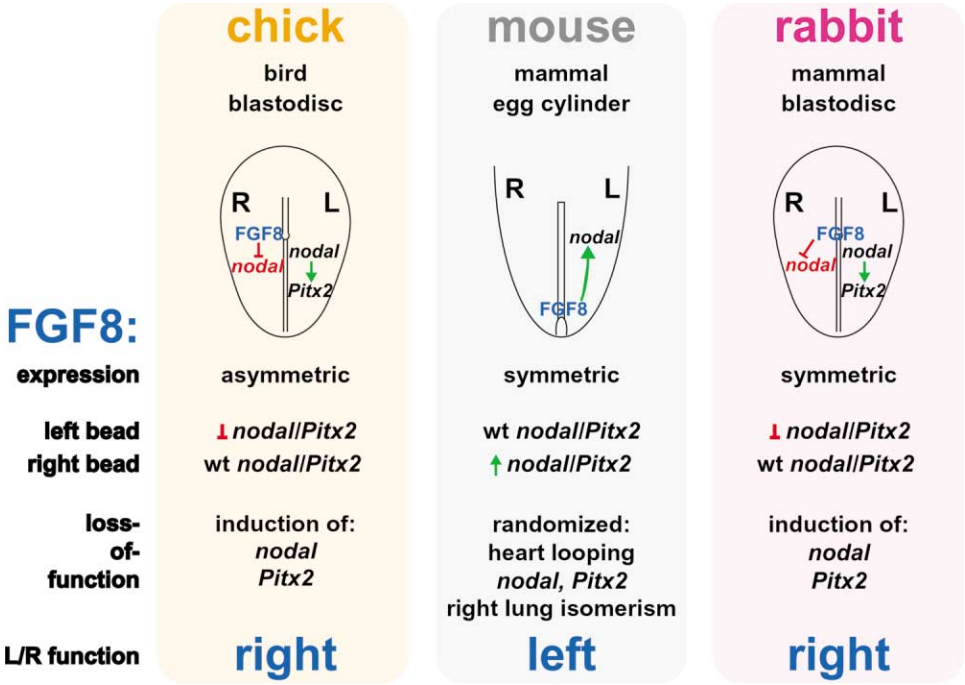

Figure 7. FGF8 Function in L-R Axis Pattern Formation in Rabbit, Mouse, and Chick

In rabbit and chick blastodiscs, FGF8 represses the nodal cascade on the right side, while, in the mouse, egg cylinder FGF8 functions as a left determinant and is required for induction of *nodal*. Comparison of gene expression patterns, gain-, and loss-of-function experiments.

reflect an underlying structural asymmetry and not per se imply asymmetric gene function.

BMP4 Acts as an Inducer of the Nodal Cassette in the Rabbit

The finding that a left BMP4 bead resulted in ectopic expression of *nodal* and *Pitx2* came totally unexpected, as BMP growth factors in chick were described as potent inhibitors of *nodal* transcription [19, 20]. Side-specific ectopic BMP expression has also been shown to inhibit asymmetric *nodal* expression in *Xenopus* [34]. Our experiments differed from the comparable chick studies in two aspects. First, we used 10- to 100-fold lower BMP4 concentrations (10–100 μg/ml versus 1 mg/ml in [19, 20]), indicating that different BMP levels could have differential effects on *nodal* transcription. Alternatively, the higher doses used in chick might induce apoptosis, as it was shown that BMP4 induces cell death in a dose-dependent manner [35]. Second, rabbit embryos were treated between stage 6 and the 3-somite stage, whereas some of the chick experiments were performed at earlier stages [20]. BMP thus might have different effects at different time points. Additional evidence that the BMP effects we report here may represent a physiological response was obtained in chick, in which Schlange et al. [36] demonstrated that expression of the Nodal cofactor *cryptic* was dependent on BMP signaling. Genetic experiments in zebrafish and mouse have shown that *nodal* transcription in the LPM does not occur in the absence of *cryptic/oep* [37, 38]. And finally, a positive effect of BMP on *nodal* expression was recently also found in chick [39, 40].

Two BMP4 effects were distinguishable in treated embryos: broadening of the endogenous expression domains of *nodal*, *lefty*, and *Pitx2* on the left side (Figures 4A–4C and 4E), and induction of ectopic right-sided expression (Figures 4B and 4E and data not shown). In light of the recent demonstration that Nodal acts as a long-range morphogen in zebrafish [41], the broadening could be explained by induction of a competence factor in paraxial and intermediate tissue that renders cells receptive to Nodal signals emanating from the notochordal and/or lateral plate. It is tempting to speculate that *cryptic* qualifies for this function, as BMP induces ectopic *cryptic* in chick [36].

Induction of bilateral *nodal*, *lefty*, and *Pitx2* was found in most cases when BMP4 beads were placed close to the midline (Figures 4B and 4E and data nor shown). In contrast to the broadening on the left, expression on the right side was confined to the LPM (Figure 4B' and data not shown), indicating that BMP4 affected the barrier function of the midline rather than exerting a long-range effect across the midline. We indeed found repression of midline *lefty* close to the bead (Figures 4D and 4E), and this repression is a BMP4 effect previously described in chick [19, 24]. In mouse mutants, loss of midline *lefty* is always accompanied by bilateral expression of the Nodal cassette [42].

Different L-R Function of FGF8 in Mouse and Rabbit

Most surprisingly, this study revealed fundamental differences between mouse and rabbit with respect to FGF8 function in L-R axis formation (Figure 7). Expres-

sion patterns in both species are virtually indistinguishable; in particular, unlike in chick [12], no asymmetries were found at the node. Asymmetric function of FGF8, thus, is independent of primary asymmetry in mRNA localization. Furthermore, there has been no switch from right-sided to left-sided FGF8 function in the course of evolution from birds to mammals, because, in gain- and loss-of-function experiments, FGF8 functions identically in chick and rabbit.

The most intriguing and consistent difference between mouse on one hand and chick and rabbit on the other hand appears to be the architecture (topology) of the gastrula/neurula embryo. Rabbit embryos, like chick and most other mammals, including human embryos, develop via a flat blastodisc. Rodents, in contrast, assume a cup-shaped, cylindrical appearance. How could these anatomical differences affect events downstream of FGF8 signaling?

An attractive scenario that one could envisage is that FGF8 signaling affects vectorial transport through gap junctions within the epithelial ectodermal cell layer. A role for FGF in gap junction-mediated intercellular communication was recently described in chick lens cultures [43], in which FGF signaling controls the opening status of existing gap junctional communications (GJC). In the chick, limb bud FGF4/8 in addition induced transcription of *connexin 43* [44], further supporting a function of FGF signaling in the control of intercellular transport of small molecules. In a blastodisc, GJC couple cells on either side of the primitive streak; however, the left and right sides are only connected in front of the node. In the egg cylinder, cells on both sides of the streak form a continous cell sheath in which individual cells are able to communicate via GJC throughout. Experiments in chick and *Xenopus* have demonstrated the involvement of GJCs in L-R axis formation [45, 46]. For example, injection of a dominant-negative mutant of *connexin 43* randomizes organ situs in *Xenopus* [45]. In contrast, a knockout mouse for *connexin 43* had no effect on left-right asymmetry [47]. Most interestingly, in humans, *connexin 43* mutations were described in laterality patients, indicating different functions of GJC in human and mouse [48].

Conclusions

We provide compelling evidence for a right-asymmetric function of FGF8 in the rabbit. Our data suggest that taxonomical differences among the various classes of the vertebrates (birds versus mammals) are less important than topological differences between different gastrula/neurula-stage embryos (blastodisc versus egg cylinder). As human embryos develop via a flat blastodisc as well, the rabbit has the potential to become a relevant second mammalian model system for axis formation and early patterning events.

Experimental Procedures

PCR Cloning of Rabbit Gene Probes

For primer design, mouse and human cDNA and/or genomic sequences were aligned. Typically 4–6 primers were selected per gene and were used in different combinations. PCR products of the correct size were cloned and sequenced on both strands. Successful conditions were as follows: *FGF8*, 5′-ATGGGCAGCCCCCGCTCCG CGCTGA-3′, 3′-CCCATCAACTCCTTGAGCTTCGCG-5′, 40 cycles at 95°C, 62°C, 72°C, resulting in amplification of a 590-bp fragment, corresponding to nucleotides 174–763 of the mouse cDNA (accession number: AF474181); *nodal*, 5′-CAGAACTGGACITTCACITTTGA CTT-3′, 3′-ATICGTAACATGACGAAICCCAT-5′, 40 cycles at 95°C, 55°C, 72°C, resulting in amplification of a 635-bp fragment, corresponding to nucleotides 541–1169 of the mouse cDNA (accession number: AF474180); and *Pitx2*, 5′-GA(GT)GA(CT)CC(GC)TC(CT)AAG AAGAAG-3′, 3′-ACGAA(CA)CGAAA(CG)TCAGAGTCC-5′, 40 cycles at 95°C, 60°C, 72°C, resulting in amplification of a 641-bp fragment, corresponding to nucleotides 222–862 of the mouse cDNA (accession number: AF474182).

Embryo Culture and Manipulation

Pregnant rabbits (New Zealand White) were purchased from Harlan-Winkelmann. Embryos were dissected from uteri in sterile PBS at room temperature, and they were placed ectoderm-side down on a clot of agarose (0.5% in PBS) in a 5 cm Petri-dish in Ham's F10 medium supplemented with FCS (20%), such that only the extraembryonic portion of the embryo was submerged in medium (semi-dry culture). Cultures were incubated at 37°C/5% CO_2 in a Gasboy C40 incubator (Labotect) for 4–24 hr.

Beads were applied under a dissecting microscope by using a pulled-out pasteur pipette. For treatment with PBS, FGF8, Activin, and BMP4, heparinized acrylic beads were used (Sigma). Beads were washed in PBS and were incubated for 1 hr in PBS/0.1% BSA in the respective growth factor (FGF8: mouse recombinant FGF8b, [R&D Systems, lot No. YH02 and YH012071], 1 and 3 mg/ml; BMP4: human recombinant [R&D Systems], 10–100 μg/ml). The FGF receptor 1 inhibitor SU5402 (Calbiochem, Bad Soden, Germany) was applied on AG 1-X2 Resin beads (Biorad) at a concentration of 1 mg/ml. When embryos reached the 3- to 5-somite stage, cultures were stopped by fixation in 4% PFA for 1 hr at room temperature or overnight at 4°C.

In Situ Hybridization and Histology

Nonradioactive whole-mount in situ hybridization of wild-type and cultured rabbit embryos with digoxigenin-labeled probes was performed following standard methodology. For histological examination, embryos were embedded in gelatine/albumin and were sectioned at 30 μm with a vibratome.

Supplementary Material

Supplementary Material including a table in which the inducing effects of BMP4 on the nodal cascade genes are summarized is available at http://images.cellpress.com/supmat/supmatin.htm.

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