Shh induces right-sided marker gene expression in chick embryos from stage 4 up to the 1-2 somite stage

To exclude the possibility that the observed differences between rabbit and chick embryos are due to an impaired functionality of the Shh protein used, the chick culture experiments were repeated using the same protein as in the rabbit experiments and the settings described (Levin et al. 1995, Monsoro-Burq and Le Douarin, 2001). Beads soaked either in a BSA solution as control or in Shh protein (1µg/µl) were placed onto the right side of the embryo next to Hensen's node. This was done in embryos ranging from stage 4 to 1-2 somites and the cultures were allowed to develop to the 4-6 somite stage for the assessment of *Nodal* expression. If embryos had reached stages older than the 6 somite stage a *Pitx2* probe was used to evaluate results.

In untreated control cultures only left-sided *Nodal* and *Pitx2* expression was observed (n=8, data not shown). Cultures in which a bead soaked in BSA control solution was implanted on the right side of Hensen's node also yielded left-sided expression of marker genes in a high number of cases, namely in 83.3% (n=12; Fig. 13, A, B). Only 2 embryos showed bilateral expression after treatment with a BSA control bead (16.7%; Fig. 13, A). However, if beads soaked in Shh were placed on the right side of Hensen's node most of the embryos expressed *Nodal* and *Pitx2* in both lateral plates as well as on both sides of the notochord showing a clear induction on the right side in 84.6% (n=13; Fig. 13A, C). This induction could also be observed in embryos that received the Shh bead after stage 4, namely up to the 1-2 somite stage, which has not been described before. In only 15.4% of cases the implanted Shh bead had no effect on marker gene expression, as the embryos showed normal left-sided expression after culture (n=2; Fig. 13A). The change in marker gene expression after treatment with a Shh loaded bead was statistically very highly significant (p=0.001). Taken together, this shows that the Shh protein used was highly efficient in chick embryos and that the differences between chick and rabbit are not due to the protein used.

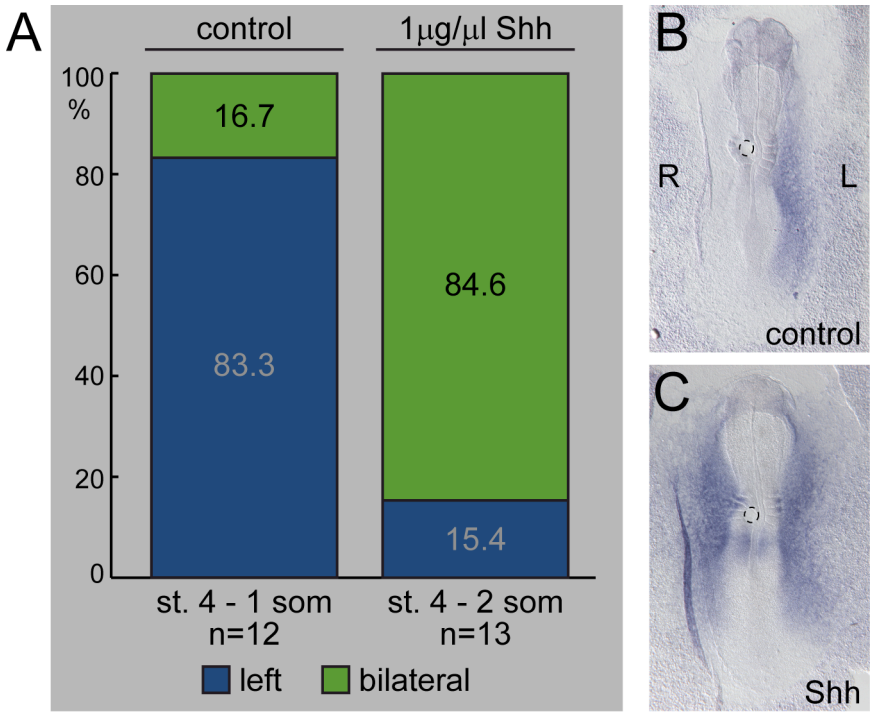

Fig. 13 Shh induces right-sided marker gene expression in chick embryos. (**A**) Diagram showing relative numbers of embryos cultured with either a BSA control bead or a bead loaded with Shh protein placed on the right side of Hensen's node with left-sided (blue) or bilateral (green) marker gene expression. Note that bilateral expression was statistically very highly significantly increased in embryos cultured in the presence of Shh (p=0.001). (**B**, **C**) Representative embryos cultured with a BSA bead and left-sided *Nodal* expression (**B**) and a SHH bead showing bilateral *Nodal* expression (**C**). Dashed circles highlight bead positions after culture. Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.

Shh loss-of-function in rabbit embryos: cyclopamine effectively suppresses Shh signalling

Cyclopamine, a teratogenic alkaloid isolated from the corn lily *Veratrum californicum* (Keeler and Binns, 1968), has been shown to suppress the response of vertebrate target tissues to Shh by directly binding to its signal transducer Smoothened (Chen et al., 2002). Thereby it can lead to cephalic malformations, including cyclopia in the most severe cases. In this study cyclopamine was used to perform Shh loss-of-function experiments. Therefore it had to be assured that cyclopamine was able to suppress Shh signalling in rabbit embryos. As Patched is not only the receptor but also a target gene of Shh signalling its expression can be used to assay pathway

activity. In order to test the ability of cyclopamine to suppress Shh signalling, a rabbit embryo of the 3 somite stage was cultured in the presence of 80µM cyclopamine in the culture medium and evaluated for *Ptc* expression afterwards. Two other 3 somite stage embryos were used as controls, one was left untreated and the other was cultured in the presence of the same amount of ethanol that was used as solvent for cyclopamine. As depicted in Fig. 14 both of those embryos showed the same high levels of *Ptc* expression in its normal expression domains: the axial midline consisting of notochordal plate and floor plate, the somites and lateral plates. The cyclopamine-treated embryo (Fig. 14C), however, showed severely reduced *Ptc* expression compared to control embryos (A, B). Especially notochordal plate and floor plate as the main sources of Shh showed almost no *Ptc* expression (Fig. 14C´). All embryos were cultured and left in the *in situ* hybridisation staining solution for exactly the same period of time. In summary, this indicates that cyclopamine is able to suppress Shh signalling in rabbit embryos.

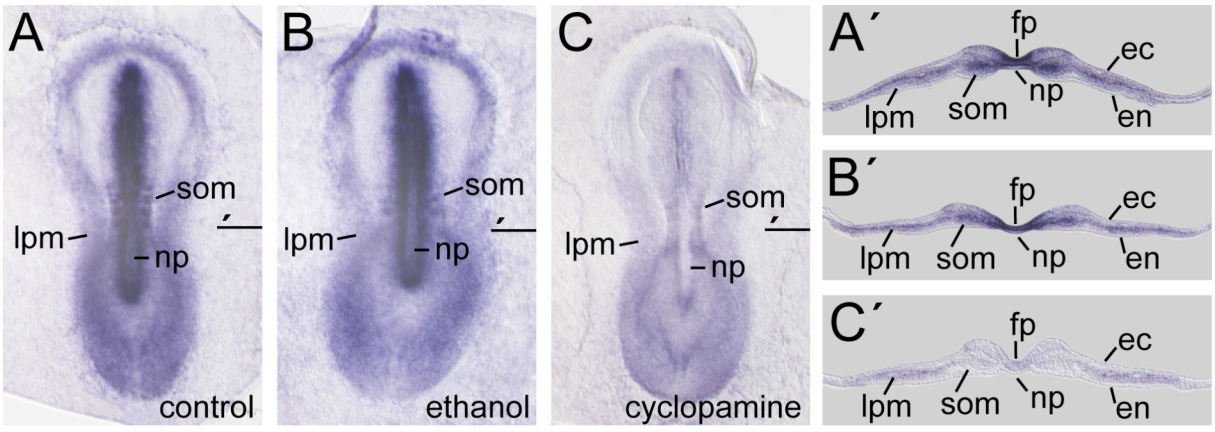

Fig. 14 Cyclopamine effectively suppresses Shh signalling. Embryos were either cultured in the presence or absence of 80µM cyclopamine and then assessed for the expression of *Patched* as an indicator for pathway activity. (**A**) Untreated control embryo and (**B**) embryo cultured in the presence of the same amount of ethanol as used as solvent for cyclopamine showing the same high levels of *Ptc* expression. (**C**) Embryo treated with 80µM cyclopamine in the culture medium showing reduced levels of *Ptc* expression. (**A´-C´**) Transversal section of embryos shown in (**A-C)** at the levels indicated. Note the reduced expression of Ptc in the notochordal plate (np) and floor plate (fp). (ec) ectoderm, (lpm) lateral plate mesoderm, (en) endoderm, (som) somite. Embryos are shown in ventral view with anterior to the top.

Inhibition of Shh signalling causes bilateral marker gene expression

It has been reported in various contexts that the loss of Shh signalling impairs the establishment of the left-right axis. In chick embryos for example the reciprocal approach to the experiments shown earlier revealed that a bead loaded with anti-Shh antibody implanted on the left side of Hensen's node was able to block left-sided *Nodal* expression (Pagán-Westphal and Tabin, 1998). The *Shh* mutant mouse, however, also shows laterality defects like alterations in heart looping and randomisation of embryonic turning but the expression of left-right marker genes turned out to be bilateral (Tsukui et al., 1999). As the expression of *Lefty1* is lost in *Shh*$^{-/-}$ embryos the observed laterality defects might be due to the loss of the midline barrier function of *Lefty1*. Whereas in *Smo* knockout mice as well as in *Shh/Ihh* compound mutants expression of *Nodal* and *Pitx2* is lost analogous to the chick experiments (Zhang et al., 2001).

To examine the effect the inhibition of Shh signalling has on laterality determination in rabbit, embryos were incubated with cyclopamine in the culture medium at a concentration of 80µM. As a control the same amount of the solvent ethanol was used. Embryos explanted during this experiment ranged from stage 5 to 2 somites and were cultured until they had reached the 3-6 somite stage for the assessment of *Nodal* expression. If embryos had reached stages older than the 6 somite stage a *Pitx2* probe was used to evaluate the results. Embryos cultured in the presence of ethanol showed left-sided marker gene expression in 72.2% of cases (n=13; Fig. 15A, B). Less frequently bilateral expression (22.2%, n=4; Fig. 15A) was observed and only one embryo failed to initiate expression completely (5.6%; Fig. 15A).

However, when embryos were cultured in the presence of cyclopamine this changed dramatically. Only 34.6% of embryos expressed *Nodal* or *Pitx2* on the left side (n=9; Fig. 15A) whereas the majority of embryos showed expression on both sides (57.7%, n=15; Fig. 15A, C). The remaining two embryos lacked marker gene expression (7.7%; Fig. 15A). This increase of bilateral expression in embryos treated with cyclopamine was statistically significant ($p<0.05$).

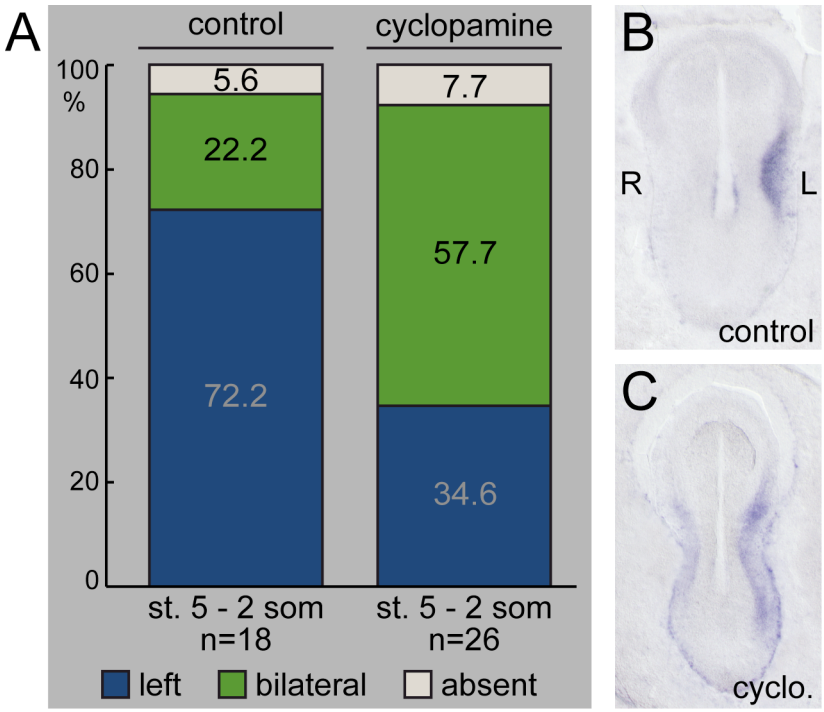

Fig. 15 Inhibition of Shh signalling causes bilateral marker gene expression. (**A**) Diagram showing relative numbers of embryos cultured either in the presence of 80µM cyclopamine or in the same amount of the solvent ethanol as a control with left-sided (blue), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture. Note that the increase in bilateral expression in embryos cultured with cyclopamine was statistically significant ($p<0.05$). (**B**, **C**) Representative embryos cultured in the presence of ethanol showing left-sided *Nodal* expression (**B**) and in the presence of cyclopamine showing bilateral *Nodal* expression (**C**). Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.

These results indicate that cyclopamine did not block Shh signalling completely in the concentration used (see also Fig. 14) as this most likely would have resulted in a loss of marker gene expression like in the chick experiments or the *Smo* knockout mice mentioned above. Instead, the observed effect resembles the phenotype of *Shh* mutant mice, where the loss of midline barrier and residual Hh signalling activity lead to bilateral marker gene expression as well (Tsukui et al., 1999).

Inhibition of Shh signalling leads to loss of midline barrier

The experiment described above indicated that the resulting bilateral marker gene expression was due to a loss of midline barrier. This barrier is thought to be established in the floor plate by the Nodal antagonist *Lefty*, which is induced by *Nodal* expression in the LPM (Meno et al., 1998; Yamamoto et al., 2003).

In order to test if a loss of midline barrier might be the reason for the bilateral expression of marker genes in rabbit embryos as well, a second set of cyclopamine experiments was performed. Again, embryos ranging from stage 5 to 2 somites were explanted and either treated with 80µM cyclopamine or the same amount of ethanol as the solvent. For the evaluation of results a probe was used that recognises both *Lefty1* and *Lefty2*. All embryos cultured in the presence of ethanol (n=6) showed the normal expression pattern, i.e. the left side of the floor plate (*Lefty1*) and two domains in the anterior and posterior part of the left lateral plate mesoderm (*Lefty2*) respectively (Fig. 16A). In embryos cultured in the presence of cyclopamine the *Lefty1* domain in the left part of the floor plate was almost always lost (Fig. 16B-D). Only two out of 12 embryos displayed patchy remnants (not shown). The expression of *Lefty2* in the left LPM also becomes bilateral in *Shh* knockout mice (Tsukui et al., 1999). Surprisingly, this occurred in only one embryo cultured with cyclopamine (Fig. 16C). Instead, 58.3% of embryos displayed left-sided *Lefty2* expression (n=7; Fig. 16B) and in 33.3% the expression domain was lost completely (n=4; Fig. 16D). To analyze if floor plate cells were even specified in cyclopamine treated embryos histological sections were made. These sections revealed that the floor plate was indeed absent in embryos cultured with cyclopamine becoming evident by the morphology of the cells. In cyclopamine treated embryos these cells did not show the normal columnar shape with an apical constriction seen in ethanol treated embryos (Fig. 16A´-D´´). As a consequence the neural folds of cyclopamine treated embryos adopted a wider U shaped form rather than the wild type V shape seen in control embryos (Fig. 16A´-D´´).

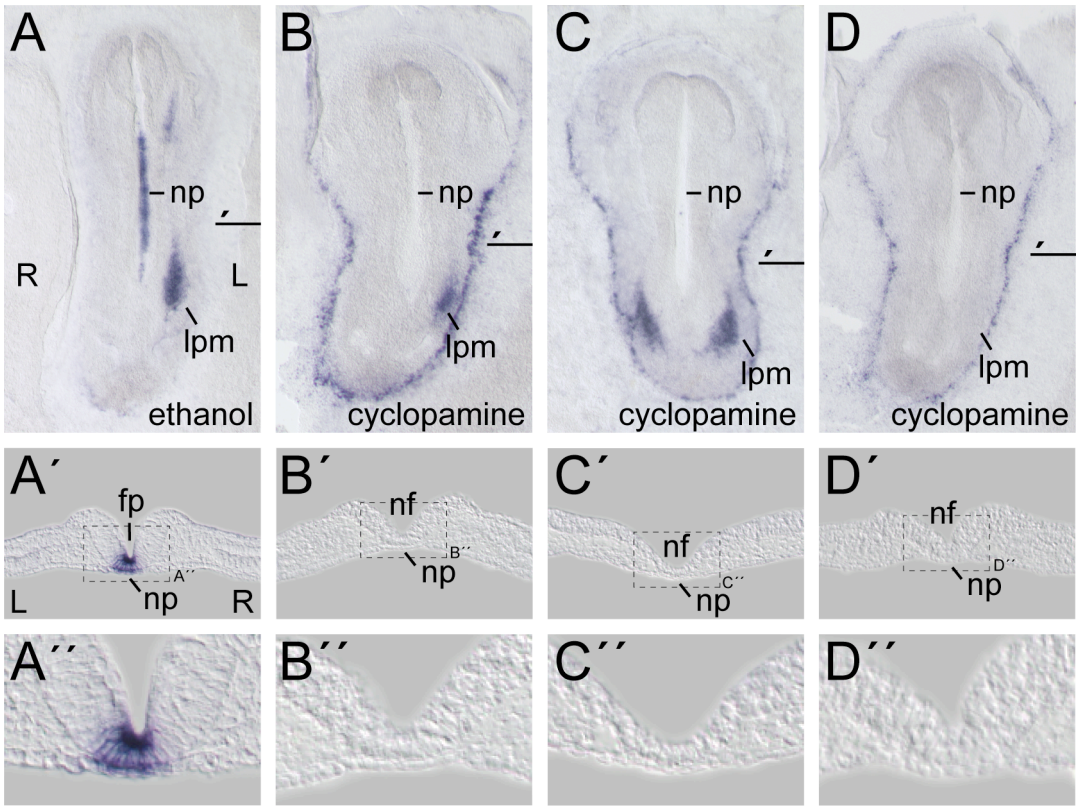

Fig. 16 Inhibition of Shh signalling leads to loss of midline barrier. (**A**) Representative embryo cultured in the presence of ethanol as control and assessed for *Lefty* expression after *in vitro* culture. *Lefty* is expressed in its normal domains, i.e. the left part of the floor plate (fp) and in the anterior as well as the posterior part of the left lateral plate mesoderm (lpm). (**A´**) Histological section at the level indicated in (**A**) showing *Lefty* expression in floor plate cells. (**A´´**) Higher magnification of the area indicated in (**A´**). Note the columnar-formed floor plate cells expressing *Lefty*. (**B**-**D**) Representative embryos incubated with 80µM cyclopamine and assessed for *Lefty* expression after *in vitro* culture showing no expression in the floor plate and either left-sided (**B**), bilateral (**C**) or absent expression (**D**) in the posterior LPM. (**B´**-**D´**) histological sections at the level indicated in (**B**-**D**). (**B´´**-**D´´**) Higher magnification of areas indicated in (**B**-**D**). Note the malformed floor plate cells in comparison with (**A´´**). Embryos are shown in ventral view with anterior to the top. (nf) neural folds, (np) notochordal plate, (R) right, (L) left.

Taken together this suggested that the inhibition of Shh signalling by cyclopamine led to a failure to specify a floor plate as is also the case in *Shh*- mice (Chiang et al. 1996). Moreover, this indicated that the bilateral expression of LR marker genes after cyclopamine treatment was due to a loss of floor plate cells and therefore midline barrier function normally exerted by *Lefty*.

FGF8 signalling in the rabbit embryo

In zebrafish, FGF8 signalling has been implicated in the set up of cilia-driven flow in Kupffer's vesicle whereas in rabbit earlier work indicated a role in the relay of LR cues downstream of the flow (Neugebauer et al., 2008; Feistel and Blum, 2008). These contradictory roles were examined further in the rabbit embryo in the third part of this thesis work.

Systemic inhibition of FGF8 signalling causes loss of marker gene expression prior to the 2 somite stage

SU5402, a compound specifically interfering with the catalytic centre of FGF-receptor 1 (FGFR1) and therefore disrupting FGF8 signal transduction, can be used to experimentally manipulate FGF8 signalling (Mohammadi et al., 1997). Earlier work in rabbit embryos showed that unilateral inhibition of FGF8 signalling by application of a bead loaded with SU5402 in embryos of the 1-2 somite stage is able to eliminate the repressive function of FGF8 and thereby inducing ectopic nodal expression (Fischer et al., 2002). Later, this mechanism was shown to depend on gap junctional communication downstream of cilia-driven leftward flow (Feistel and Blum, 2008). In zebrafish embryos, however, systemic treatment with SU5402 led to an ectopic induction of marker gene expression as well, but this was shown to be due to shorter cilia in Kupffer's vesicle and thus disruption of flow (Neugebauer et al., 2008).
When rabbit embryos before the 2 somite stage were cultured in the presence of 20-60μM SU5402 in the culture medium and assessed for marker gene expression after *in vitro* culture they mostly developed aberrant marker gene expression. Surprisingly, most embryos lacked expression (51.9%, n=14; Fig. 17A, C). Only 14.8% (n=4; Fig. 17A) of embryos treated with SU5402 displayed expression on both sides. Normal left-sided marker gene expression occurred in 33.3% of cases (n=9; Fig. 17A). DMSO, serving as solvent for SU5402 was used as control and embryos were cultured in the presence of the same amount of DMSO as was used for SU5402 incubations. Embryos taken into culture before the 2 somite stage and treated with

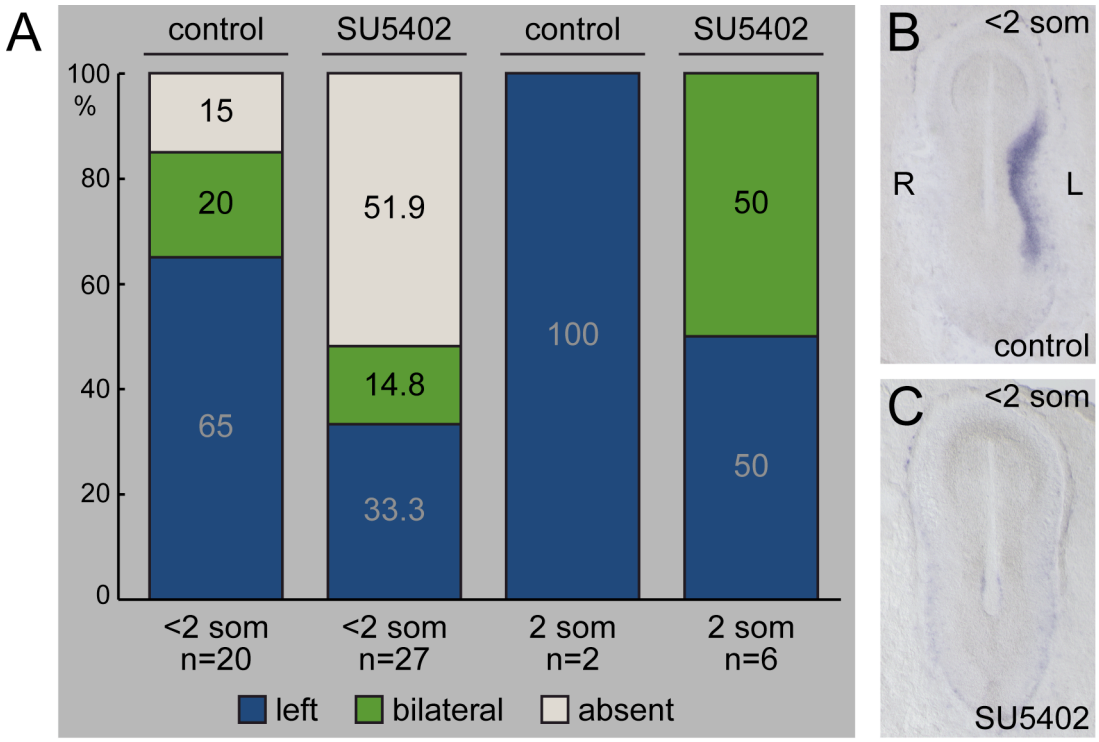

Fig. 17 Inhibition of FGF8 signalling causes loss of marker gene expression in embryos cultured prior to the 2 somite stage. (**A**) Diagram showing relative numbers of embryos explanted before or during the 2 somite stage and cultured either in the presence of 20-60µM SU5402 or the same amount of the solvent DMSO as a control with left-sided (blue), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture. Note that the loss of marker gene expression in SU5402 incubated embryos was statistically highly significant ($p<0.01$). (**B**, **C**) Representative embryos cultured with DMSO showing left-sided Nodal expression (**B**) and cultured in the presence of SU5402 with absent Nodal expression (**C**). Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.

DMSO showed normal left-sided marker gene expression in 65% of cases (n=13; Fig. 17A, B) while both sides showed or lacked expression in 20% and 15%, respectively (n=4 and n=3; Fig. 17A). The increased absence of marker gene expression recorded in embryos incubated with SU5402 was statistically highly significant ($p<0.01$). When embryos were taken into culture at the 2 somite stage lack of *Nodal* or *Pitx2* expression never occurred. Instead, embryos showed bilateral expression in 50% of cases (n=3; Fig. 17A) or displayed normal left-sided expression (n=3; Fig. 17A) whereas DMSO-treated embryos only showed left-sided expression (n=2; Fig. 17A). Despite the low number of embryos this might indicate that at the 2 somite stage the systemic treatment has the same effect as the unilateral bead

placement. The data obtained in earlier stages, however, hint at a second function for FGF8 signalling.

Cilia-driven leftward flow is not affected by systemic inhibition of FGF8 signalling

To test if altered gene expression observed upon systemic treatment with SU5402 is causally related to changes in the dynamics of leftward flow, embryos from the experiment described above were subjected to flow analysis when they had reached the 3-5 somite stage. After *in vitro* culture they were incubated in culture medium containing fluorescent latex beads (200nm diameter) and examined using fluorescence microscopy. The obtained films were analyzed as described earlier (see also *Methods* section). After each individual film had been analyzed the data were merged for embryos that 1) were incubated with DMSO and showed left-sided expression, 2) were treated with SU5402 and still showed left-sided expression, 3) displayed bilateral or absent expression after SU5402 treatment or 4) had received a bead loaded with FGF8 onto the left side of the PNC and lacked marker gene expression. The merged films of 4 DMSO-treated embryos with left-sided expression showed a rho value of 0.49 and a direction toward the posterior/left as in untreated control cultures, which had a rho value of 0.5 (Fig. 18A, B). Three embryos cultured in the presence of SU5402 that showed left-sided marker gene expression exhibited a merged rho of 0.49 as well (Fig. 18C). However, merged films of 6 embryos incubated with SU5402 that had bilateral or no expression of marker genes did not show grossly altered flow dynamics (average rho value of 0.43 and direction toward the left and left/posterior; Fig. 18D). Likewise, the merged films of 2 embryos with a FGF8-loaded bead implanted onto the left side of the PNC and hence no marker gene expression displayed a rho value of 0.46 (Fig. 18E). Individual rho values of all films evaluated are depicted in supplementary Fig. 1. Since all rho values analyzed in this experiment were within the range of untreated control cultures, cilia-driven leftward flow was considered unaffected by treatment with agonists or antagonists of the FGF8 signalling pathway.

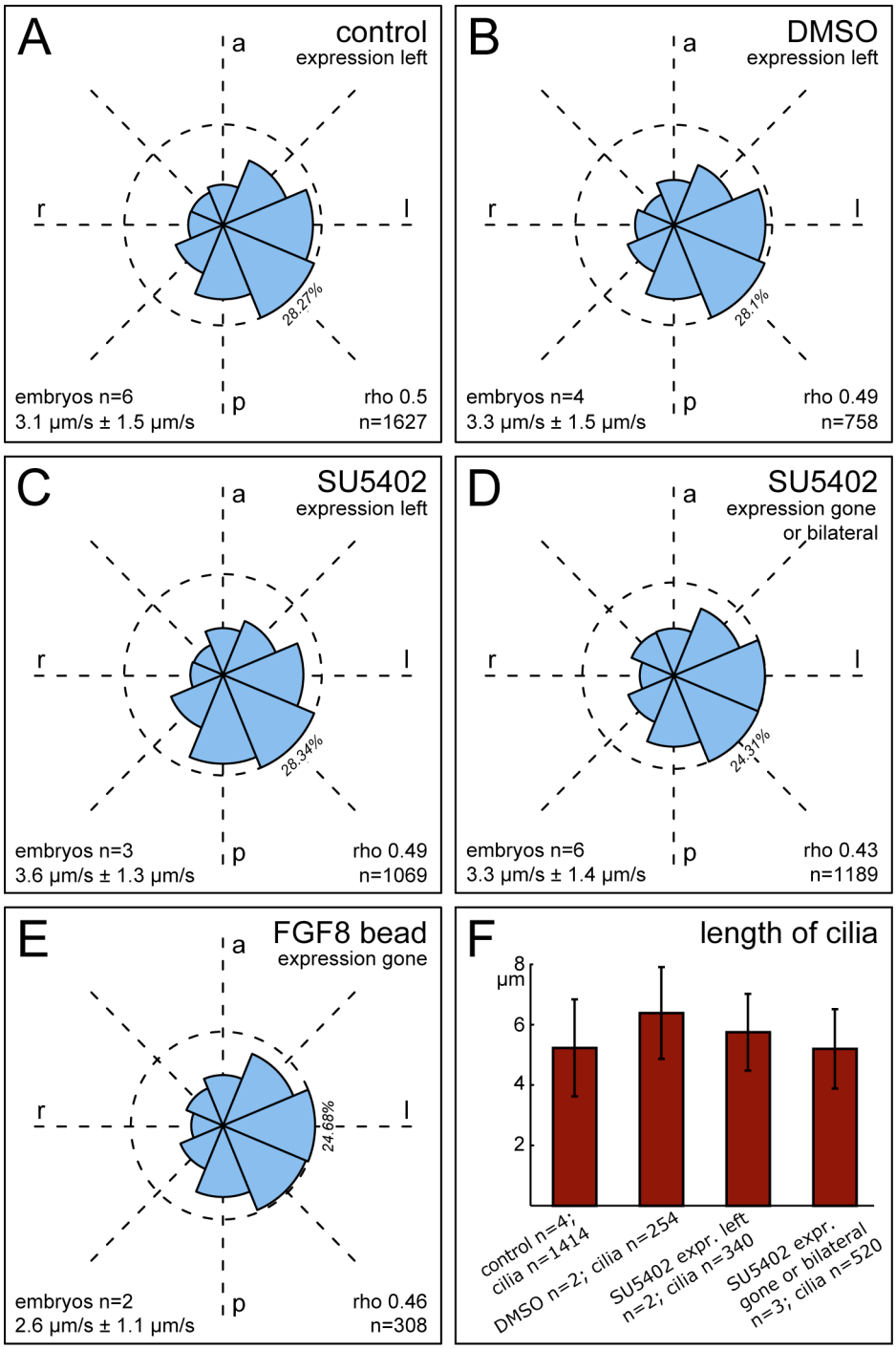

Fig. 18 Cilia-driven leftward flow is not affected by SU5402 treatment. (**A**) Merged flow measurements of 6 untreated control embryos with a rho value of 0.5. (**B**) 4 DMSO treated embryos with left-sided marker gene expression show a merged flow with a rho value of 0.49. (**C**, **D**) Embryos cultured in the presence of 20-60µM SU5402. Merged flow measurements of three embryos still displaying left-sided expression with a rho value of 0.49 (**C**) and 6 embryos with altered marker gene expression (gone or bilateral) due to the SU5402 treatment with a rho value of 0.43 (**D**). (**E**) Flow measurements of 2 embryos with an FGF8 loaded bead implanted on the left side of the PNC and therefore absent marker gene expression displaying a rho value of 0.46. Note that the flow of all

groups analyzed had a direction toward the left or left/posterior. n represents the number of particles analyzed for each group and the mean velocity of the flow in µm/s is stated. Results of analysis are shown in ventral views, anterior to the top. (a) anterior, (p) posterior, (r) right, (l) left. (**F**) Diagram indicating the average length of cilia measured for the different treatment groups. (n) number of embryos.

Additionally, the length of cilia was measured after immunohistochemistry with an antibody against acetylated tubulin, marking the entire axoneme of the cilium (Fig. 18F). Surprisingly, the 254 cilia measured in two DMSO treated embryos averaged a length of 6.4µm, which is longer than the cilia of untreated control cultures averaging 5.2µm (1414 cilia of 4 embryos). Embryos incubated with SU5402
but left-sided marker gene expression harboured slightly longer cilia as well (5.75µm, 340 cilia of two embryos) and incubated embryos with no or bilateral expression displayed cilia with the same average length as untreated control cultures (520 cilia of three embryos). As the differing cilia lengths did not alter flow characteristics, this seemed to be within the normal range of ring-cultured embryos. Taken together, these results thus indicated that FGF8 function is not required for setting up the ciliated epithelium and the subsequent cilia-driven leftward fluid flow in rabbit embryos.

FGF8 signalling is epistatic to cilia-driven leftward flow

As the experiment described above indicated that the inhibition of FGF8 signalling had no influence on cilia-driven fluid flow, an epistasis experiment was performed to test if FGF8 signalling functions independent of flow. Therefore embryos ranging from stage 5 to 2 somites were cultured with a bead soaked in SU5402 implanted into the right side and a bead loaded with 1 µg/µl FGF8 into the left side of the PNC. This setting was then covered with culture medium containing 1.5% methylcellulose to block leftward flow across the PNC. If FGF8 signalling would work under these circumstances the FGF8 bead should be able to repress marker gene expression in the left lateral plate whereas the SU5402 bead should release the repression of the

endogenous FGF8 and therefore allow marker gene expression in the right lateral plate. Embryos were scored for *Nodal* expression when they had reached the 3-6 somite stage. If embryos had already developed 6-8 somites a probe against *Pitx2* was used. Normal left-sided expression was found in only 6.9% of embryos (n=2; Fig. 19A) whereas right-sided expression was detected in 37.9% of cases (n=11; Fig. 19A, B). Bilateral expression was found in 10.9% (n=3) and both lateral plates lacked marker gene expression in 44.8% (n=13; Fig. 19A, C). These results were compared to the scenario in which only the flow was blocked with 1.5% methylcellulose in the culture medium (cf. Fig. 7). In both settings normal left-sided expression occurred

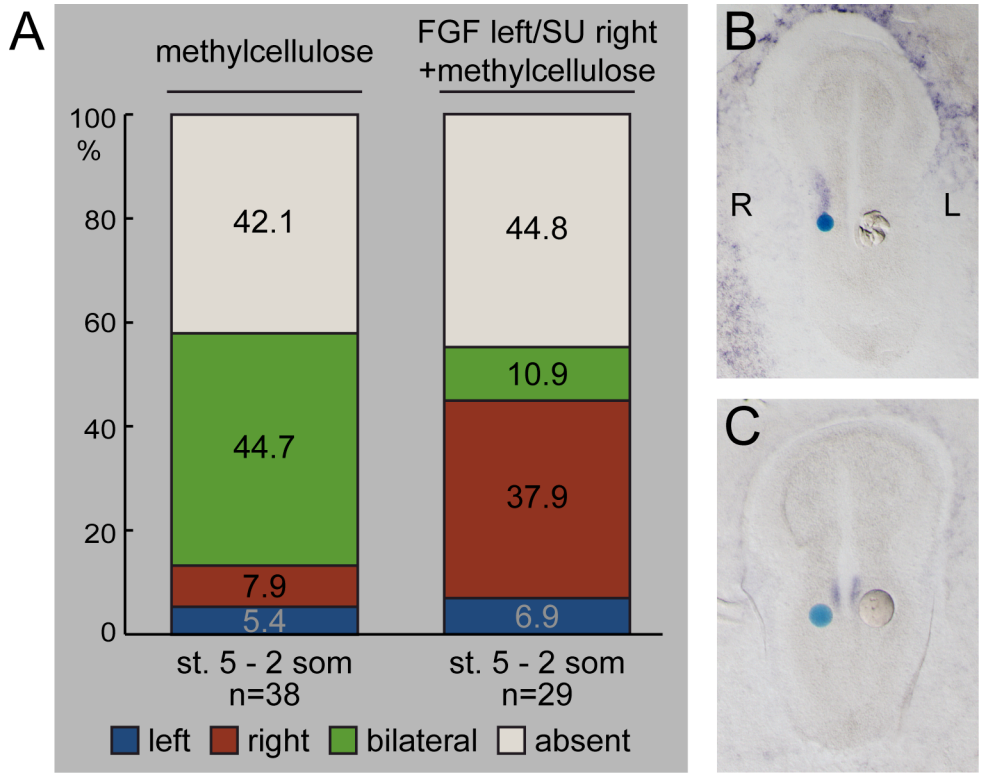

Fig. 19 FGF8 signalling functions independent of ciliary-driven fluid flow. (**A**) Diagram showing relative numbers of embryos cultured either with 1.5% methylcellulose in the culture medium or with a SU5402 loaded bead implanted on the right side of the PNC, a bead soaked in FGF8 implanted on the left side of the PNC and 1.5% methylcellulose in the culture medium with left-sided (blue), right-sided (red), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture. Note that right-sided expression in embryos cultured with both beads and methylcellulose was elevated statistically highly significant ($p<0.01$). (**B**, **C**) Representative embryos showing right-sided (**B**) and absent *Nodal* expression (**C**) after bead implantation and treatment with methylcellulose (SU5402 bead in blue, FGF8 bead in white). Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.

only very rarely, i.e. in 5.4% and 6.9 % of cases (Fig. 19A). Also the ratio of embryos showing absent marker gene expression was almost unaltered with 42.1% of methylcellulose treated embryos and 44.8% of embryos that had received the two different beads and methylcellulose (Fig. 19A). But with 37.9% the proportion of embryos with right-sided expression was conspicuously higher than in methylcellulose treated embryos (7.9%; Fig. 19A). This increase in right-sided expression was statistically highly significant ($p<0.01$). Taken together these data show that even though the viscosity of the methylcellulose blocked cilia-driven leftward flow, FGF8 signalling was able to exert its function. As the proportion of expression in the left lateral plate dropped from 50% in methylcellulose-treated embryos to 17.2% in response to the repressive effect of the FGF8 bead.

Taken together, these results indicate a dual function of FGF8 signalling in the development of LR asymmetry: firstly, before the 2 somite stage, FGF8 signalling seems to be required to render the lateral plate competent for the Nodal signalling cascade. Secondly, during the 2 somite stage, it is required for the relay of LR cues. However, the establishment of a functional PNC and cilia-driven leftward flow is not affected by the loss of FGF8 signalling.