Results

Establishment of a new *in vitro* culture method: the ring culture

A semi-dry culture system for rabbit embryos has been established in previous work (Fischer et al., 2002; Viebahn et al., 1995). Although most embryos cultured with this method develop relatively normal they show altered asymmetric marker gene expression. In the work presented here a new culture system was developed assuring normal asymmetric marker gene expression in most cases also in presomite stages.

Reproducing the *in vivo* conditions more closely in ring cultures

In vivo the 7.5 to 8.0 day old rabbit embryo lies with its ectoderm down on the uterus mucosa. At this implantation site of the embryo the uterus shows a bulging, which is fluid-filled. Therefore the embryo is covered with fluid on its endodermal surface during these stages of development. In the semi-dry or standard culture the embryo is placed ectoderm down on an agarose mound resembling *in vivo* conditions (Fig. 2A and *Methods* section). But in order to keep the embryo in place during the course of culture the level of surrounding medium has to be adjusted so that it just reaches the extraembryonic tissue. In this way, the embryo is still moistened by a thin liquid film but no considerable body of fluid is covering the embryo like *in vivo*.

To resemble the *in vivo* situation more closely a new technique was designed in which 300µm thick plastic rings were placed upon the extraembryonic tissue framing the embryo. In those so-called ring cultures (Fig. 2B) a drop of medium supplied the embryo with fluid above its endodermal surface. Aside from providing the embryo

with additional medium the ring also applied a certain amount of tension to the embryonic and extraembryonic tissues, which might enhance development of the embryo in ring cultures as well.

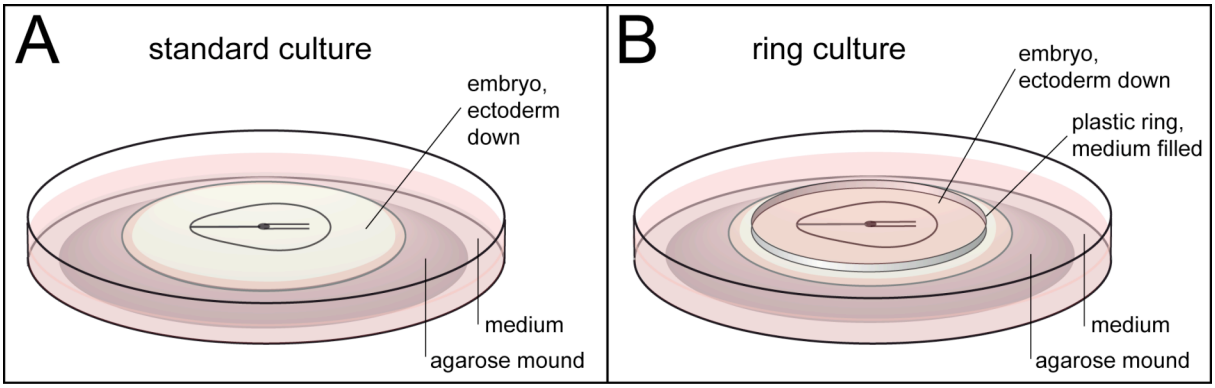

Fig. 2 Schematic representation of culture methods. (**A**) Standard or semi-dry culture. Note that the level of medium just reaches the extraembryonic tissue of the embryo. (**B**) Ring culture. Here the medium filled plastic ring supplies the embryo with both fluid above its endodermal surface as well as tension.

Expression of asymmetric marker genes in untreated standard and ring cultures

Using the semi-dry standard culture technique untreated embryos were incubated as described in the *Methods* section and afterwards assessed for asymmetric marker gene expression by whole mount *in situ* hybridisation. Standard control cultures yielded mostly normal morphology as judged by the appearance of axial mesodermal structures, the progression of somitogenesis, the development of the heart anlage and the overall shape of the embryo although they appeared smaller and thicker than wild type embryos. As described, these embryos showed disturbed marker gene expression despite their relatively normal development (Fischer et al., 2002, Feistel, 2006). Embryos taken into culture from stage 4b up to the 2 somite stage (n=62) were assessed for *Nodal* expression by *in situ* hybridisation when they had reached the 3-6 somite stage. If embryos had already developed 6-8 somites a probe against *Pitx2* was used. However, normal left-sided expression of either of these marker

genes was found in only 21.2% of cases (n=14; Fig. 3), whereas most embryos showed bilateral expression (51.5%, n=34; Fig. 3). Less frequently, either right-sided *Nodal* or *Pitx2* expression was observed (9.1%, n=6; Fig. 3) or both lateral plates lacked expression completely (18.2%, n=12; Fig. 3). As was also shown in previous work (Feistel, 2006) most cases of left-sided marker gene expression comprised embryos that were explanted at the 2 somite stage where 5 out of 7 embryos displayed normal left-sided expression.

To test if the conditions of the ring culture method are more suitable with respect to the expression of left-right marker genes embryos of the same stage range (4b-2 somites) were incubated as stated in the method section and evaluated for marker gene expression by whole mount *in situ* hybridisation. Concerning the morphological landmarks mentioned above the embryos cultured with a medium-filled ring also

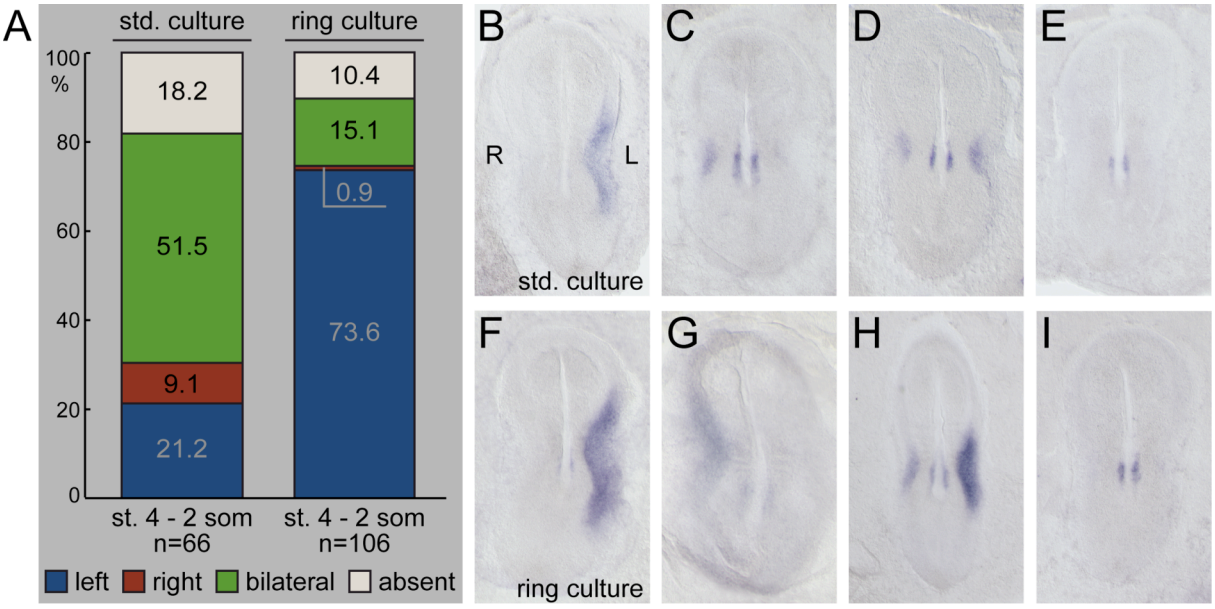

Fig. 3 Expression of marker genes in untreated standard and ring cultures. (**A**) Diagram showing relative numbers of embryos cultured with either method explanted from st. 4 to the 2 somite stage with left-sided (blue), right-sided (red), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture. Note that left-sided expression was statistically very highly significantly elevated in ring cultures ($p<0.001$). (**B-E**) Representative examples of embryos cultured with the standard method showing left-sided (**B**), right-sided (**C**), bilateral (**D**) or lack of marker gene expression (**E**). (**F-I**) Representative examples of embryos cultured with the ring method showing left-sided (**F**), right-sided (**G**), bilateral (**H**) or lack of marker gene expression (**I**). Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.

developed relatively normal albeit looking more wild type-like compared to standard-cultured embryos. Also their temporal development seemed to be faster with a stage 5 embryo reaching the 3-4 somite stage after about 12 hours in culture whereas in standard cultures this took about 14 hours. When scored for expression of *Nodal* or *Pitx2* normal left-sided expression could be detected in 73.6% of the 106 embryos examined (n=78; Fig. 3). This increase of incidences of expression in the left lateral plate was statistically very highly significant ($p<0.001$; all p-values calculated using the χ^2-Test). However, bilateral expression of marker genes occurred in only 15.1% of cases (n=16; Fig. 3) and 10.4% of embryos lacked *Nodal* or *Pitx2* transcripts completely (n=11; Fig. 3). Right-sided expression was observed in only one embryo (0.9%; Fig. 3) taken into culture at stage 4b.

Fig. 4 shows the expression of asymmetric marker genes separately for the different stages of embryos explanted and cultured with the ring technique. If embryos were taken into culture at stage 4b where axial mesodermal structures have not yet developed, stable expression of *Nodal* or *Pitx2* after culture in a high percentage of embryos could not be achieved. Here, only 43.75% of the embryos examined (n=16) showed expression in the left lateral plate (n=7; Fig. 4) while the remaining embryos either showed bilateral (25%, n=4; Fig. 4), right-sided (6.25%, n=1; Fig. 4) or no expression of marker genes (25%, n=4; Fig. 4). Also, many embryos explanted at that stage did not develop normally even after long periods of culture as judged by morphological landmarks like axial mesodermal structures and somites. However, if stage 5 embryos were taken into culture (n=47) in which axial mesodermal structures had already started to develop the percentage of normal left-sided expression increased to 70.2% (n=33; Fig. 4). Both lateral plates showed expression in 17% (n=8; Fig. 4) or lacked transcripts completely in 12.8% of cases (n=6; Fig. 4). Right-sided marker gene expression was never observed in embryos explanted from stage 5 onwards. In stage 6 embryos however, left-sided expression of marker genes could be detected in 100% (n=17; Fig. 4). Peculiarly, this figure dropped again in embryos taken into culture at the 1 somite stage. Here, 83.4% (n=10) showed expression in the left lateral plate, whereas in two embryos expression was altered (bilateral n=1, absent n=1, 8.3% respectively; Fig. 4). In embryos explanted at the 2 somite stage

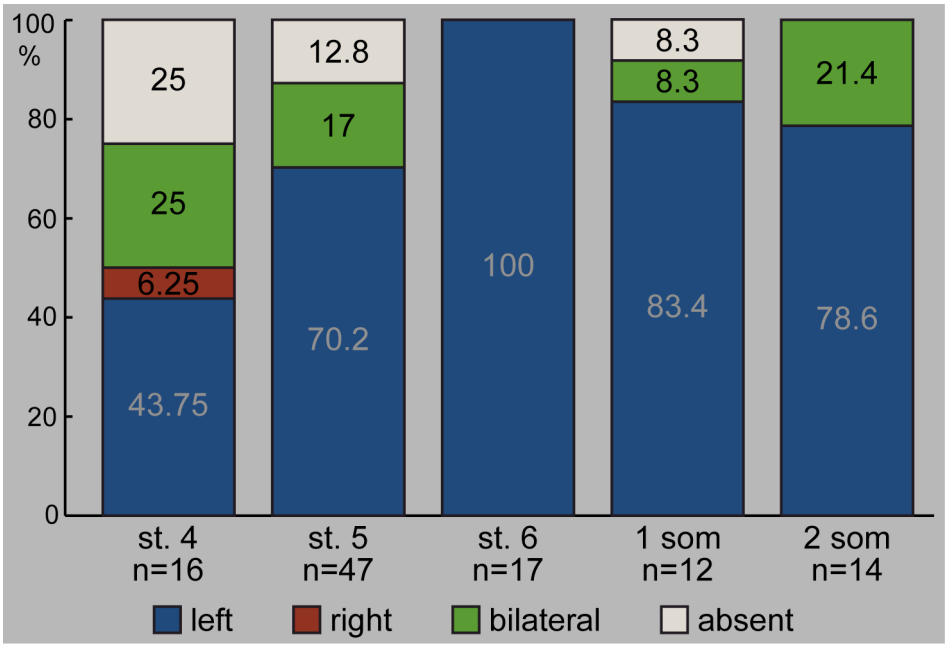

Fig. 4 Stage specific marker gene expression of embryos cultured with the ring method. Diagram showing relative numbers of embryos with left-sided (blue), right-sided (red), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture.

lack of marker gene expression never occurred but 21.4% (n=3; Fig. 4) of embryos displayed bilateral expression. Yet, in 78.6% of cases expression occurred normally on the left side (n=11; Fig. 4). Taken together these data show that normal left-sided marker gene expression could be achieved to a greater extend with the ring culture method even in embryos taken into culture at presomite stages.

Because stage 4 embryos still showed relatively high incidences of altered marker gene expression when cultured with the ring culture method, only embryos from stage 5 onwards were used for any of the following functional experiments.

Development of the notochordal plate in standard and ring cultures

Mutations specifically affecting PNC or KV morphogenesis in mouse or zebrafish are associated with tail malformations and LR defects, indicating its requirement for the establishment of laterality (Herrmann and Kispert, 1994; Nonaka et al., 1998; Amack

and Yost, 2004; Beckers et al., 2007; Amack et al., 2007). Hence, to further investigate the cause of disturbed marker-gene expression in standard cultures versus mostly normal marker-gene expression in ring cultures a scanning electron microscopy (SEM) study was conducted in which the notochordal plates and PNCs of wild type, standard- and ring-cultured embryos were examined in greater detail. The embryos analyzed were of the 3-4 somite stage as this is when the PNC has fully emerged from its initial hypoblast/endodermal cover, cilia have lengthened to about 3-4µm and are polarized to the posterior part of the cells in rabbit embryos (Feistel and Blum, 2006).

The notochordal plate and the posterior widening of the PNC were easily discernible in embryos cultured with both methods although in some cases of standard cultured embryos the notochordal plate had failed to emerge from the hypoblast/endodermal cover (not shown). Fig. 5A-C shows an overview of the ventral midlines of the different types of embryos. It becomes apparent that the embryo cultured with the standard method did not grow to the dimensions of the wild type embryo although it had reached the 3 somite stage as well. The embryo cultured with a ring also did not quite reach the normal dimensions of the corresponding wild type embryo, but the amount of tension that the ring provides to the tissue allowed for a more wild type-like development of the notochordal plate and PNC. Both cultured embryos were explanted during stage 5 when prechordal mesoderm and the first notochordal cells had already exited the node. It has been shown before that rabbit PNC cilia lengthen to 3-4µm at the 3-4 somite stage (Feistel and Blum, 2006). Therefore, the length of cilia of standard and ring cultured embryos was measured in SEM pictures as well as in immunohistochemical (IHC) stainings for acetylated tubulin. For the standard culture a total of 512 cilia in 4 embryos were measured and the mean length averaged 4.1µm. In 4 ring-cultured embryos a total of 1414 cilia could be measured with an average length of 5.2µm. That PNC monocilia surprisingly grew longer in ring-cultured embryos can be seen in Fig. 5C´ in relation to A´´ and B´´. It also has been shown that a posteriorly tilted localisation of the cilium relative to the cell surface is fundamental in order to produce a directed leftward fluid flow (Cartwright et al., 2004; Nonaka et al., 2005; Okada et al., 2005). In the wild type embryo 74% of

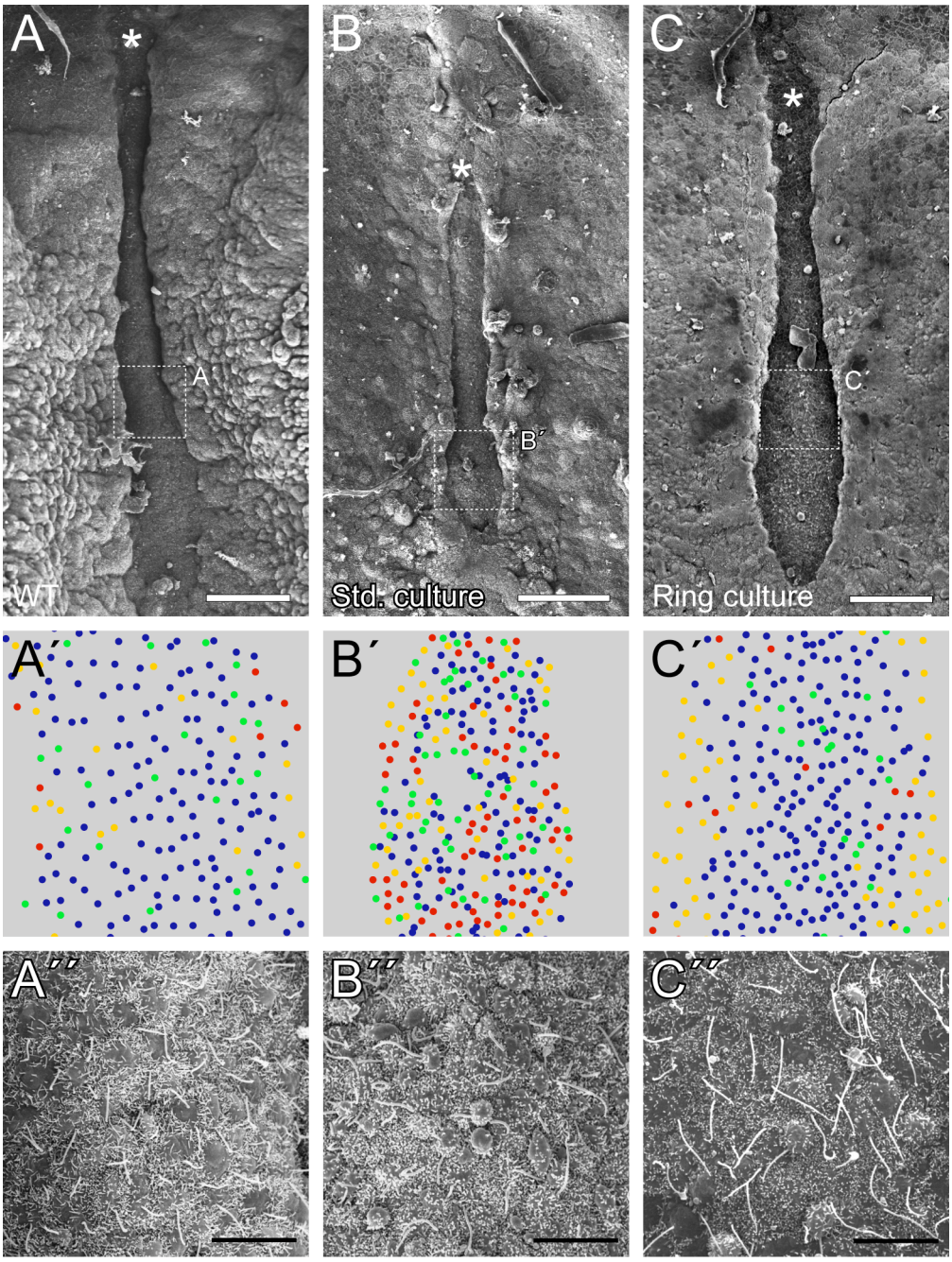

Fig. 5 Development of the notochordal plate in wild type, standard- and ring-cultured embryos. Scanning electron micrographs of a wild type embryo (**A**, WT) fixed at the 3-4 somite stage, a standard-cultured embryo (**B**) and a ring-cultured embryo (**C**). The cultured embryos were explanted at stage 5 and stopped at the 3-4 somite stage. The posterior widening of the PNC is clearly visible in all three embryos. Asterisks in (**A**-**C**) mark prechordal plate mesoderm. Scale bars in (**A**-**C**) represent 100µm. (**A´**-**C´**) Position of the ciliary base relative to the cell surface. Depicted are the regions marked in (**A**-**C**). Cells lacking cilia are indicated by red dots, posteriorly localized cilia by blue, central cilia by yellow, and cilia with unclear insertion by green dots. Note that in (**B´**) only 40% of cilia are located posterior. (**A´´**-**C´´**) Details in higher magnification within the regions indicated in (**A**-**C**). Scale bars represent 10µm. Note that in (**C´´**) cilia are longer than in (**A´´**, **B´´**). Embryos are shown in ventral views, anterior to the top.

cilia were localised to the posterior pole of the cell (Fig. 5A´). With 68% of posteriorly localised cilia the ring-cultured embryo nearly reached wild type levels (Fig. 5B´), whereas in the standard-cultured embryo only 40% of the cilia matched this criterion (Fig. 5C´). The remainder of cells either had no cilium, were centrally positioned or their insertion was unclear.

Taken together these data show that the development of the notochordal plate and PNC is impaired in standard cultured embryos and despite the relatively normal development of ring-cultured embryos cilia surprisingly show an increase in length.

Analysis of cilia-driven leftward flow in standard and ring cultures

The impaired development of the notochordal plate of early embryos cultured using the standard method suggested that, as a consequence, cilia-driven leftward flow might be affected in these embryos. In order to explore this possibility the flow of wild type embryos as well as embryos cultured with both methods was analyzed.

Embryos were incubated in culture medium containing fluorescent latex beads and filmed using fluorescence microscopy. The obtained films were analyzed using the open source programme ImageJ (http://rsb.info.nih.gov/ij/) and the plugin “ParticleTracker” (Sbalzarini and Koumoutsakos, 2005) as well as a custom-made program (GTT, gradient time trail) in statistical R (http://www.r-project.org/; R Development Core Team, 2008) as described in Weber, 2006 and Schweickert et al., 2007 and modified by Weber as described in the *Methods* section. Briefly, based on the file calculated by the “Particle tracker” a bezier fit curve was generated for each particle trail. Afterwards the flow was calculated by excluding trails that 1) did not occur in at least 10 consecutive frames and 2) had a rho value of <0.6 (empirically determined), i.e. trails that showed random movement. In this context rho represents the mean resultant length of a vector showing the mean direction of the analyzed trails calculated for their bezier fit curves, where rho=0 represents completely random direction and rho=1 represents exactly straight trails aiming in the same direction. For visualisation of time information and movement, the trails were differentially coloured

over time ranging from green (time=0s) via yellow to red (time=25s) and merged into one frame. To visualize the directionality (rho value) of individual movies or merged data of a given group the resultant length of all trails was plotted in form of a windrose histogram subdivided into groups of 45° (Fig. 6A´´-F). In the case of rabbit embryos owing to the topology of the PNC individual masks had to be introduced to the films in order to select the area of the PNC where particles were actually moved by cilia, i.e. where the flow is in the plane of focus and to exclude the very strong backflow further above.

In total 4 wild type embryos ranging from the 2-3 somite to 4 somite stage were filmed and analyzed. As shown before a robust fluid flow across the PNC toward the left side could be detected (Fig. 6A-A´´, D). The mean velocity reached 2.4µm/s and the directionality (rho) averaged 0.59. With the same settings 6 standard-cultured embryos were examined. After filming they were fixed and subjected to *in situ* hybridisation. In these embryos marker gene expression was bilateral in two cases, absent in three and right-sided in one case. When the flow of these embryos was analyzed it did not show very high directionality (Fig. 6B-B´´, E). Mostly it pointed toward the posterior of the embryo or had no direction at all. In average the flow of standard-cultured embryos had a rho of 0.2 and a velocity of 2.5µm/s. With the ring culture method 9 embryos were filmed and analyzed when they had reached the 3-5 somite stage as well. After *in situ* hybridisation 6 out of the 9 embryos displayed left-sided marker gene expression. The flow of those 6 embryos reached a mean rho of 0.5, which was nearly wild type like (Fig. 6C-C´´, F). It also showed directionality towards the left side although it was tilted a little to the posterior. Surprisingly the velocity reached 3.1µm/s and was thus higher than in wild type embryos. Two out of three remaining ring-cultured embryos showed no marker gene expression while in one embryo bilateral expression was detected. In two of those embryos the flow also was affected displaying a rho in the range of standard cultured embryos (0.35 and 0.37) and direction toward posterior. The embryo lacking marker gene expression exhibited a very robust leftward flow with a rho of 0.71, possibly indicating that left-sided gene expression had just not started yet.

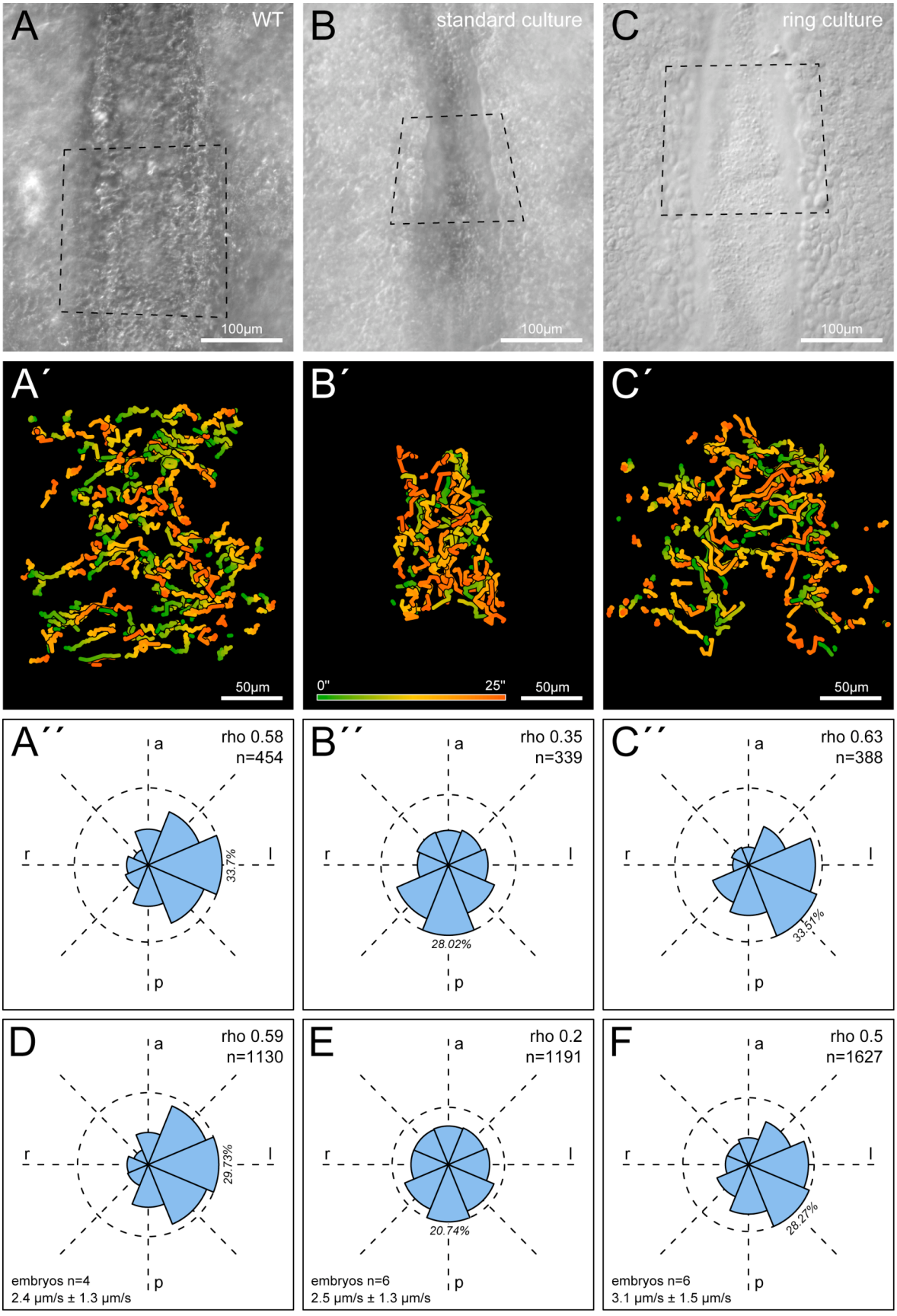
A
WT
100µm
B
standard culture
100µm
C
ring culture
100µm
A´
50µm
B´
0"
25"
50µm
C´
50µm
A´´
rho 0.58
n=454
a
r
l
p
33.7%
B´´
rho 0.35
n=339
a
r
l
p
28.02%
C´´
rho 0.63
n=388
a
r
l
p
33.51%
D
rho 0.59
n=1130
a
r
l
p
29.73%
embryos n=4
2.4 µm/s ± 1.3 µm/s
E
rho 0.2
n=1191
a
r
l
p
20.74%
embryos n=6
2.5 µm/s ± 1.3 µm/s
F
rho 0.5
n=1627
a
r
l
p
28.27%
embryos n=6
3.1 µm/s ± 1.5 µm/s

Fig. 6 Analysis of cilia-driven fluid flow in wild type, standard- and ring-cultured embryos. Bright-field view of the posterior notochordal plate (PNC) of a 3 somite wild type embryo (WT, **A**), a standard-cultured embryo (**B**) and a ring-cultured embryo (**C**). Cultured embryos were explanted at stage 5 and filmed at the 3-4 somite stage. (**A´**-**C´**) GTT analysis of particle movement in the regions indicated in (**A**-**C**). The colour gradient in (**B´**) represents 25s ranging from green (t=0s) via yellow to red (t=25s). Note that the particle trails of the standard-cultured embryo in (**B´**) do not show a direction to the left. For films depicted in (**A´**-**C´**) see supplementary CD. (**A´´**-**C´´**) Plotted resultant lengths of trails (=rho, see also main text) shown in (**A´**-**C´**) in form of a windrose histogram in groups of 45°. n represents the number of particles analyzed in each film. Note that in the wild type embryo most particles are moved to the left, whereas in the standard-cultured embryo they are directed mostly to the posterior and in the ring-cultured embryo again to the left but tilted to the posterior. (**D**-**F**) Merged resultant lengths of trails (=rho) of 4 wild type embryos (**D**) 6 standard-cultured (**E**) and 6 ring-cultured embryos (**F**). The rho values and number of particles (n) analyzed for the merged films are stated as well as the mean velocity of the flow in µm/s. Embryos and results of analysis are shown in ventral views, anterior to the top. (a) anterior, (p) posterior, (r) right, (l) left.

Supplementary Fig. 1 shows an overview of the rho values of all embryos analyzed. Wild type flow started at a rho of just above 0.5 and reached to nearly 0.7. Standard-cultured embryos, however, never reached a rho of 0.4 with values ranging from 0.09 up to 0.36. Ring-cultured embryos with left-sided marker gene expression covered a wide range of rho values starting from as low as 0.37 to even 0.75.

Taken together, these data show that in standard cultured embryos cilia-driven leftward fluid flow was disturbed, whereas the ring culture facilitated leftward flow in most cases.

Inhibition of cilia-driven leftward flow in ring cultures causes laterality defects

In mice and zebrafish, genetic studies showed that cilia-driven fluid flow was necessary and sufficient to initiate the Nodal signalling cascade in the left lateral plate mesoderm (LPM; Nonaka et al., 1998; Okada et al., 1999; Nonaka et al. 2005; Essner et al., 2005; Hirokawa et al., 2006). Furthermore, elegant experiments in *Xenopus* demonstrated that inhibition of the flow by injection of 1.5% methylcellulose into the archenteron resulted in laterality defects (Schweickert et al., 2007).

In order to test if cilia-driven fluid flow is also required for the correct establishment of the left-right axis in rabbit, embryos were cultured in the presence of methylcellulose. Therefore, culture medium containing 1.5% of methylcellulose was used giving the

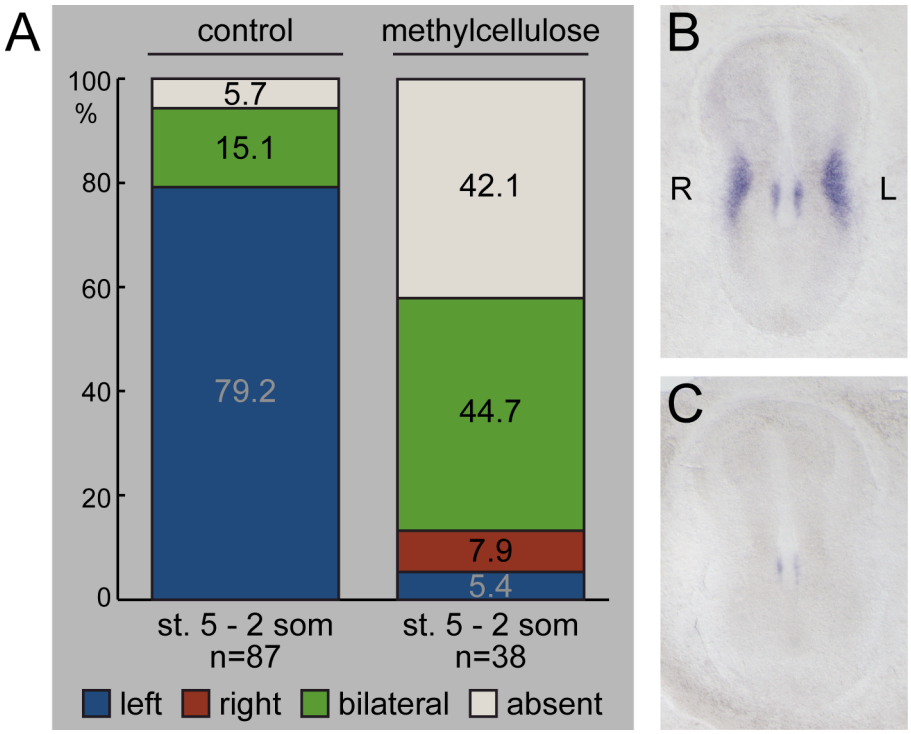

Fig. 7 Inhibition of flow with 1.5% methylcellulose causes alterations in L-R marker gene expression. (**A**) Diagram showing relative numbers of embryos cultured with either normal culture medium (control) or 1.5% methylcellulose in culture medium with left-sided (blue), right-sided (red), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture. Note that normal left-sided expression was statistically very highly significantly reduced in methylcellulose treated embryos ($p<0.001$). (**B**, **C**) Representative embryos cultured in the presence of methylcellulose with bilateral (**B**) and absent *nodal* expression (**C**). Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.

medium a much higher viscosity. Embryos ranging from stage 5 to 2 somites were then cultured either in the presence of normal medium as a control or the rings were filled with the methylcellulose-containing medium. This treatment yielded overall normal morphological development as compared to control ring-cultures. In addition, the temporal development of the explanted embryos did not seem to be impaired by the higher viscosity of the medium. After embryos had reached the 4 to 8 somite stage, they were assessed for marker gene expression by *in situ* hybridisation. The results showed that the treatment with methylcellulose increased the proportion of embryos with laterality defects with a statistically very high significance ($p<0.001$). Namely, in only two out of the 38 embryos cultured in the presence of methylcellulose, marker gene expression could be detected exclusively on the left

side (5.4%; Fig. 7). Most of the embryos, however, showed either expression in both lateral plates (n=17, 44.7%; Fig. 7) or failed to initiate expression completely (n=16, 42.1%; Fig. 7). In control cultures this occurred in only 15.1% (n=12; Fig. 7) and 5.7% of cases (n=7; Fig. 7) respectively. In rare cases right-sided expression could be observed in methyl-cellulose treated embryos (7.9%, n=3; Fig. 7), which was never seen in control cultures of stage 5 to 2 somite embryos.
These experiments demonstrate that cilia-driven fluid flow was required for the establishment of laterality in rabbit embryos as in most cases methylcellulose treatment resulted in altered marker gene expression representing the fate of either left isomerism (bilateral expression) of right isomerism (no expression).

Taken together these data show that in standard-cultured embryos the development of the notochordal plate and PNC was impaired leading to disturbed cilia-driven leftward fluid flow. Additionally, it could be shown that leftward flow per se was essential for LR axis development in rabbit embryos.