## Expression of *Hedgehog-interacting protein* (*Hip*) in rabbit embryos

Hip is a vertebrate-specific, membrane-bound glycoprotein that has been shown to be transcriptionally activated in response to Hedgehog signalling. It acts in parallel to the receptor Patched (Ptc) to attenuate Hh signalling by directly binding to Hhs with a similar affinity as Ptc (Chuang and McMahon, 1999; Chuang et al., 2003; Jeong and McMahon, 2005). It therefore represents a second layer of the Hh pathway's negative feedback mechanism, exclusive to vertebrates.

In the mouse, *Hip* is expressed in cells immediately adjacent to *Shh* expressing cells overlapping in part with the expression of the Hh receptor *Ptc* (Chuang and McMahon, 1999). In *Xenopus Hip* expression is found in or near *Shh*-positive areas as well (Cornesse et al., 2005). Zebrafish embryos however, are supplied with maternal *Hip* mRNA. In later stages *Hip* is expressed adjacent to *Shh* expressing cells of the axial midline and the developing somites (Ochi et al., 2006).

The expression pattern of *Hip* was analyzed in rabbit embryos of defined stages by means of whole mount *in situ* hybridisation using a specific antisense probe. At stage 4b, when the primitive streak was fully elongated and Hensen's node became apparent, *Hip* was expressed rostral and lateral to the node narrowing down to more and more lateral parts of the embryo in the posterior half (Fig. 10A, A´). Transversal sections showed the restriction of signal to mesodermal and endodermal cells yet the node itself was free of *Hip* transcripts (Fig. 10A´). This expression domain was maintained during elongation of the notochordal process at stage 5 (Fig. 10B). When the notochordal process epithelialized into a plate and displaced the endoderm ventrally during stage 6, *Hip* expression changed dramatically. Only a thin strip of cells on each side of the notochordal plate expressed small amounts of *Hip* at this stage (Fig. 10C, C´). This strip was confined caudally to about the level of the first forming somite and faded off anteriorly. It consisted mostly of mesodermal cells directly adjacent to notochordal cells but to a lesser extent, the underlying endodermal cells transcribed *Hip* as well (Fig. 10C´). During the following somite stages *Hip* expression could be detected in the condensing somites (Fig. 10D-F, D´´, F´´). First, the highest level of expression was confined to the most recent developing

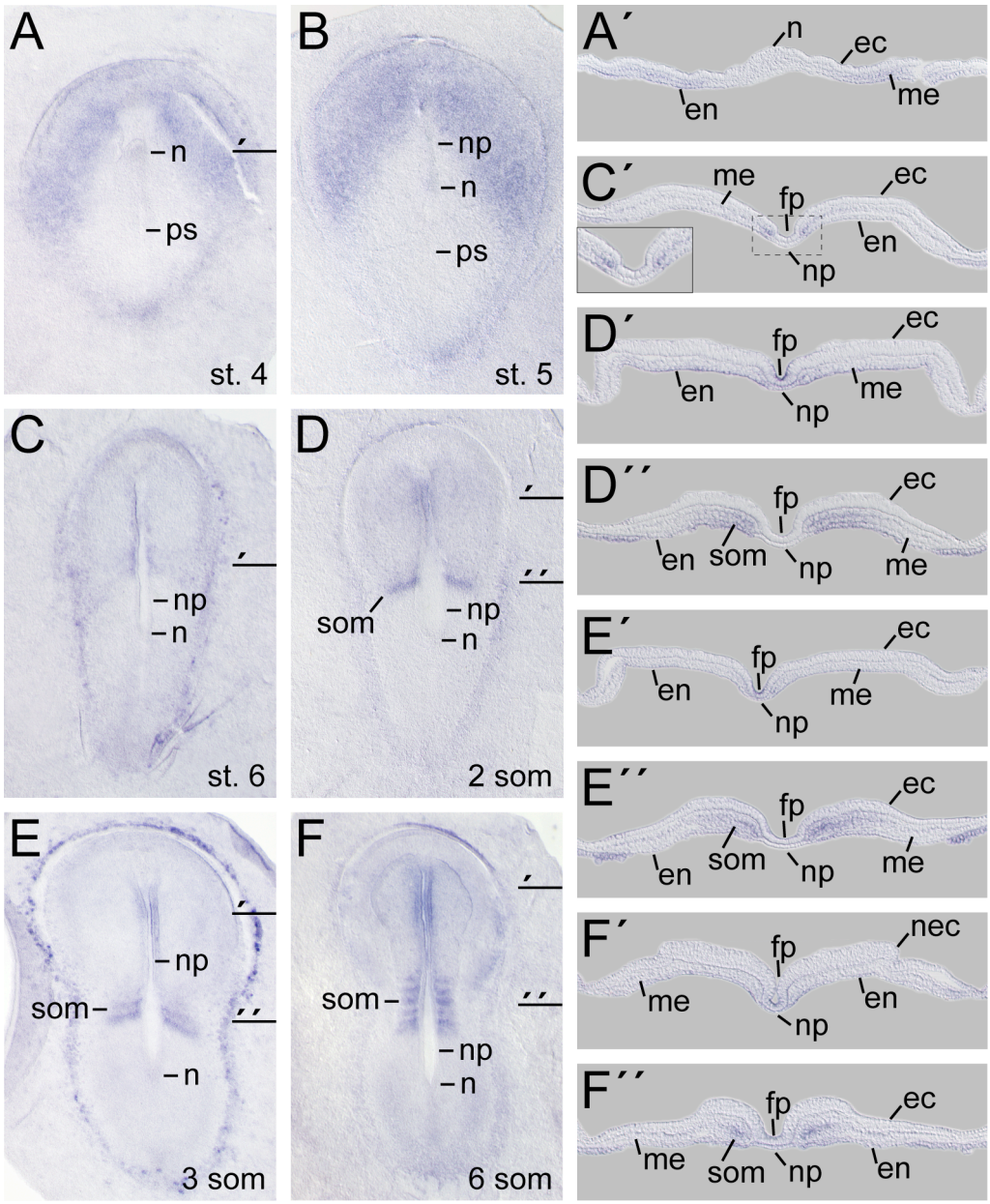


**Fig. 10 Expression of *Hedgehog-interacting protein* (*Hip*) in rabbit gastrula and neurula stage embryos**. Whole mount *in situ* hybridisation of defined stages using a specific antisense probe against *Hip*. At stage (st.) 4 and 5 (**A**, **B**) *Hip* was expressed rostral and lateral of Hensen's node (n). The node itself as well as the posterior part of the embryo remained free of transcripts. During stage 6 (**C**, **C´**) *Hip* expression narrowed to a thin strip of mesodermal (me) and endodermal (en) cells (see inset in **C´** for higher magnification) adjacent to the epithelialized axial midline consisting of floor plate (fp) and notochordal plate (np). During the following somite stages (**D**-**F**) *Hip* expression could be detected within the somites (som, D´´, E´´, F´´). Rostral of the somites floor plate and notochordal plate cells expressed *Hip* as well as mesodermal and endodermal cells flanking them (**D´**, **E´**, **F´**). Note that the endoderm still was positive for *Hip* transcripts beneath the somites where notochord and floor plate had terminated *Hip* expression. (ps) primitive streak, (ec) ectoderm. Embryos are shown in ventral views with anterior to the top.

somite, but until the 6 somite stage all somites showed similar signal strength (Fig. 10D-F). Rostral to the somites low levels of *Hip* were expressed in the head region of somite stage embryos (Fig. 10D-F). This domain comprised cells of the floor plate and the notochordal plate as well as mesodermal cells immediately lateral of the notochordal plate. Additionally, the underlying endodermal cells showed *Hip* expression that faded off laterally (Fig. 10D´, E´, F´). Expression in floor plate and notochordal plate declined caudally and could no longer be detected at the level of the somites, whereas expression in the endodermal cells stayed on underlying the somitic mesoderm (Fig. 10D´´, E´´, F´´).
Taken together, this shows that in the rabbit *Hip* is expressed in or near *Shh*-positive tissues as well.

## Expression of *Growth/differentiation factor 1* (*Gdf1*) in rabbit embryos

Gdf1 belongs to the transforming growth factor-β (Tgf-β) superfamily of secreted growth factors (Lee, 1990). Like Nodal, it signals through Activin receptors and requires the presence of co-receptors of the EGF-CFC type (Cheng et al., 2003). It has been proposed that Hh signalling is responsible for the activation of Gdf1 in a paranotochordal expression domain and the subsequent induction of left-side determinants (Zhang et al., 2001).
In the mouse *Gdf1* is expressed throughout the embryo proper in early stages of development. Later, expression is confined to the ventral neural tube and intermediate as well as lateral plate mesoderm. Additionally, *Gdf1* is expressed in a domain framing the posterior notochord reminiscent of the paranotochordal expression of *Nodal* (Rankin et al., 2000).
*Gdf1* expression in rabbit embryos of defined stages was determined using whole mount *in situ* hybridisation with a specific antisense probe. During stage 4, *Gdf1* was expressed circumferentially in the embryo proper (Fig. 11A, A´). Transverse sections showed that transcripts were equally distributed among the germ layers, ectoderm, endoderm as well as the nascent mesoderm (Fig. 11A´). Along with the elongation of

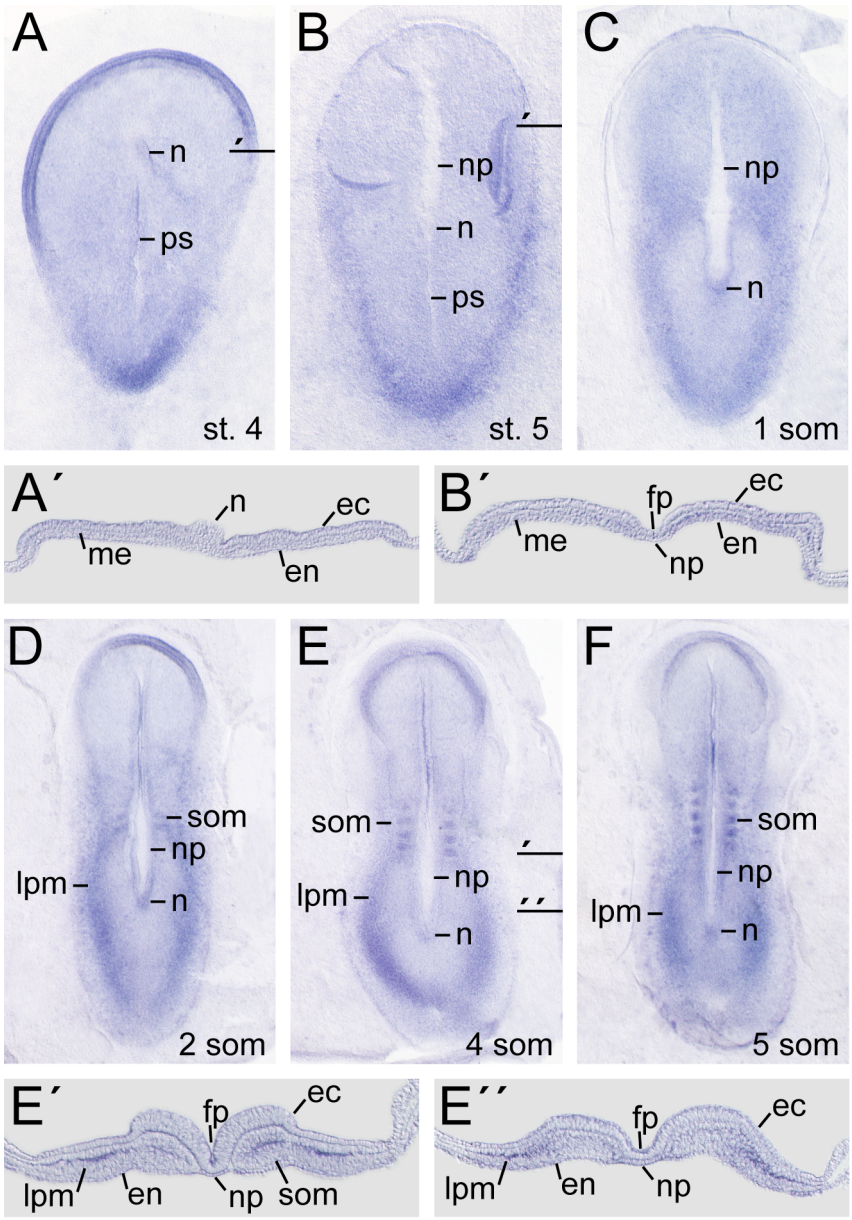


**Fig. 11 Expression of *Growth/differentiation factor 1* (*Gdf1*) in rabbit gastrula and neurula stages**. Whole mount *in situ* hybridisation of defined stages using a specific antisense probe against *Gdf1*. At stage (st.) 4 and 5 (**A**, **B**, **A´**) *Gdf1* is expressed ubiquitously in all three germ layers, ectoderm (ec), mesoderm (me) and endoderm (en). Note that the lighter appearing axial midline structures (notochordal plate, np, and floor plate, fp) also expressed *Gdf1* at similar levels (**B**-**F**, **B´**-**E´´**). During somite stages *Gdf1* transcripts became more regionalised with higher expression levels in the posterior lateral plate mesoderm (lpm) and the condensed somites (som, C-F, E´, E´´). Embryos are shown in ventral views with anterior to the top.

the notochordal process during stage 5 and the subsequent epithelialisation of the midline cells into notochordal plate and floor plate, *Gdf1* expression seemed to be almost absent in these cells in views of whole-mount embryos (Fig. 11B-F). Transversal sections, however, revealed ubiquitous expression of *Gdf1* in the axial midline showing that the translucent appearance was due to the thin bilaminar arrangement of notochordal and floor plate cells (Fig. 11B´-E´´). During the process of somitogenesis when somites begin to condense from paraxial presomitic mesoderm on both sides of the axial midline *Gdf1* expression became more regionalised. Apart from the ubiquitous expression in the whole embryo *Gdf1* transcripts became up-regulated in the posterior lateral plate mesoderm (Fig. 11C-F, E´, E´´). Higher levels of expression could also be detected in the condensed somites (Fig. 11C-F, E´). The paranotochordal expression domain described in the mouse was never discernible in the corresponding stages of rabbit embryos (Fig. 11C-F).
Besides the missing paranotochordal expression domain, *Gdf1* is expressed in rabbit embryos as described for the mouse.

Taken together, all Hedgehog signalling marker genes analyzed in this study are expressed in rabbit embryos largely as shown in other vertebrate model organisms.

## The role of Shh during the establishment of laterality in rabbit embryos

In order to investigate the function of Shh signalling with respect to the determination of the left-right axis, gain- and loss-of-function approaches were employed. These experiments were carried out in cultured rabbit and chick embryos, which were scored for marker gene expression by whole mount *in situ* hybridisation after *in vitro* culture.

## Shh induces right-sided marker gene expression only during the 2 somite stage in rabbit embryos

Consequently to its left asymmetric expression in Hensen's node of chick embryos, *Shh* has a left inductive function on the Nodal signalling cascade in this species (Levin et al., 1995). This has been shown by ectopical placement of Shh expressing cells onto the right side of Hensen's node of stage 4 chick embryos from where Shh is able to induce *Nodal* expression in the lateral plate mesoderm of the right side. In these experiments, paranotochordal expression of *Nodal* is induced as well on the right side where it is normally expressed only later in chick embryos (Levin et al., 1995). In order to examine the function of Shh in a mammalian model organism that can be stably cultured from early stages onwards, this experiment was repeated with stage 5 to 2 somite stage rabbit embryos. Embryos were taken into culture and beads loaded either with 1µg/µl Shh or BSA as a control were implanted on the right side of the PNC. Embryos were assessed for *Nodal* or *Pitx2* expression by *in situ* hybridisation when they had reached the 3-6 or 6-8 somite stage, respectively. Surprisingly, Shh did not induce right-sided marker gene expression in the same way as in chick embryos. In rabbit embryos taken into culture prior to the 2 somite stage 88.2% showed left-sided marker gene expression although a Shh bead was implanted into the right side (n=15; Fig. 12A, C). The remaining two embryos lacked marker gene expression altogether (Fig. 12A). Embryos that had received a BSA bead displayed normal left-sided expression in 73.1% of cases (n=19; Fig. 12A, B). Unlike embryos treated with a Shh bead, BSA treated embryos showed bilateral marker gene expression in 19.2% (n=5; Fig. 12A). In one embryo even right-sided expression was detected and another one lacked expression (Fig. 12A). This proportion of disturbed marker gene expression in embryos with BSA beads, however, is statistically not significant ($p>0.05$). When embryos were explanted at the 2 somite stage and a BSA bead was implanted into the right side bilateral marker gene expression was observed in only 11.1% of cases (n=1; Fig. 12A). The remaining 88.9% showed normal left-sided expression (n=8; Fig. 12A, D). When 2 somite stage embryos were cultured with a Shh bead implanted into the right side the proportion of bilateral expression events raised to 42.1% (n=8; Fig. 12A, E) whereas

the remainder of embryos displayed left-sided expression (57.9%, n=11; Fig. 12A). Unfortunately, this increase in bilateral expression of marker genes is statistically not significant because of the low number of BSA bead treated embryos.

Taken together this experiment strongly suggests that Shh signalling functions differently in rabbit than in chick. As cilia-driven flow becomes stable at the 2 somite stage this might indicate that Shh works in conjunction with the flow in rabbit embryos.

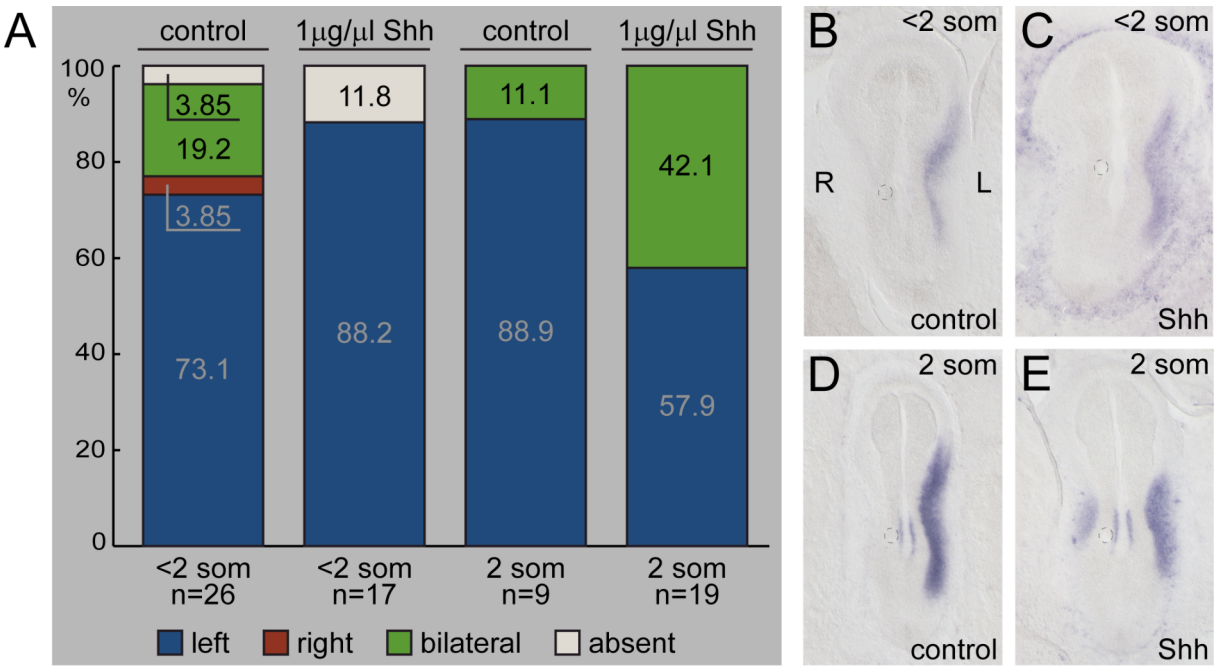


**Fig. 12 Shh induces right-sided marker gene expression only at the 2 somite stage in rabbit embryos.** (**A**) Diagram showing relative numbers of embryos explanted before or during the 2 somite stage and cultured with either a BSA control bead or a bead loaded with Shh protein placed on the right side of the PNC with left-sided (blue), right-sided (red), bilateral (green) or lack of marker gene expression (white) after *in vitro* culture. (**B**, **C**) Representative embryos explanted before the 2 somite stage cultured with a BSA bead and left-sided *Nodal* expression (**B**) and a Shh bead showing left *Nodal* expression (**C**). (**D**, **E**) Representative embryos explanted during the 2 somite stage cultured with a BSA bead and left-sided *Nodal* expression (**D**) and a Shh bead showing bilateral *Nodal* expression (**E**). Dashed circles highlight bead positions after culture. Embryos are shown in ventral view with anterior to the top. (R) right, (L) left.