Sonic hedgehog (Shh) signalling in the rabbit embryo

In the first part of this thesis work the physical properties of cilia-driven leftward flow were characterised in the rabbit embryo. Since its discovery in the mouse it was postulated that a LR determinant could be transported via the flow toward the left side. As Shh seems to be a good candidate for this, with its signalling also shown to be dependant on cilia in the mouse, Shh signalling was examined more closely in rabbit embryos in the following part the present study (Tanaka et al., 2005; Huangfu and Anderson, 2006).

Expression of Hedgehog (Hh) signalling marker genes

In order to study the Hedgehog signalling pathway in rabbit in greater detail, fragments of several marker genes were cloned by RT-PCR (for sequences see *Supplementary Fig. 2*) and their temporal and spatial expression pattern was analyzed in defined embryonic stages (see *Introduction* section) using whole mount *in situ* hybridisation. *Shh* itself was already cloned in previous work and described to be expressed in Hensen's node, the notochordal plate as well as the overlying floor plate. In addition to axial midline structures, Shh transcripts were also found in the endoderm of somite stage embryos (Fischer et al., 2002).

Expression of *Indian hedgehog* (*Ihh*) in rabbit embryos

Indian hedgehog is one of three vertebrate homologs of *Drosophila* Hedgehog that were first cloned in the mouse (Echelard et al., 1993). Like *Drosophila* Hedgehog, Ihh is a secreted signalling peptide, which undergoes autoprocessing and dual lipid modification (Mann and Beachy, 2004). It has been shown that Ihh is mainly involved in cartilage and bone morphogenesis by regulating the growth and differentiation of chondrocytes (Vortkamp et al., 1996; St-Jacques et al., 1999; Karp et al., 2000) but much earlier expression was also reported. Here, *Ihh* was described to be expressed

in the visceral endoderm of the yolksac (Bitgood and McMahon, 1995) as well as the node and PNC of 7.75-8.0 dpc old mice where it acts redundantly with Shh (Zhang et al., 2001).

In stage 4b rabbit embryos, when Hensen's node was discernible as a marked thickening at the anterior end of the primitive streak, *Ihh* was expressed circumferentially in the extraembryonic endoderm (Fig. 8A) leaving the embryo proper almost free of transcripts. Only in the most lateral (Fig. 8A´) and posterior parts (Fig. 8A´´) of the embryo, expression could be detected in endodermal cells in transverse sections. Hensen's node itself was free of *Ihh* transcripts at this stage (Fig. 8A´). During stage 5, when the notochordal process started to elongate rostral of Hensen's node, a weak signal of *Ihh* transcription was detected in the ventral cells of Hensen's node (Fig. 8B, B´). The expression in Hensen's node persisted during stage 6 when the notochordal process epithelialized and displaced the endoderm ventrally but expression also started to extend into the posterior part of the notochordal plate (Fig. 8C, C´). To a lesser extent the overlying floor plate cells showed expression as revealed in transversal sections (Fig. 8C´). The extraembryonic endoderm continued to express *Ihh* during the following stages. However, the expression domain in the lateral and posterior proportions of embryonic endoderm became more pronounced in later stages (Fig. 8C) until at around the 3 somite stage a continuous band of lateral endoderm circumferentially expressed *Ihh* (Fig. 8E). A sagittal midline section at the 3 somite stage revealed the precise dimensions of this *Ihh* expressing band of endodermal cells, being relatively narrow in the anterior but broader in the posterior part of the embryo where it nearly reached Hensen's node (Fig. 8E´). Also the strong expression in the ventral layers of Hensen's node, i.e. the cells from which the notochordal plate arises, and the declining signal in the notochordal as well as the floor plate cells became apparent. The notochordal expression domain extended approximately to the level of the latest developing somite during the examined somite stages (Fig. 8E, F). At the 6 somite stage the entire endoderm at the level of the notochordal domain expressed *Ihh,* thus underlying the paraxial and intermediate as well as the lateral plate mesoderm (Fig. 8F, F´´´). Also endodermal cells beneath the condensed somitic mesoderm became

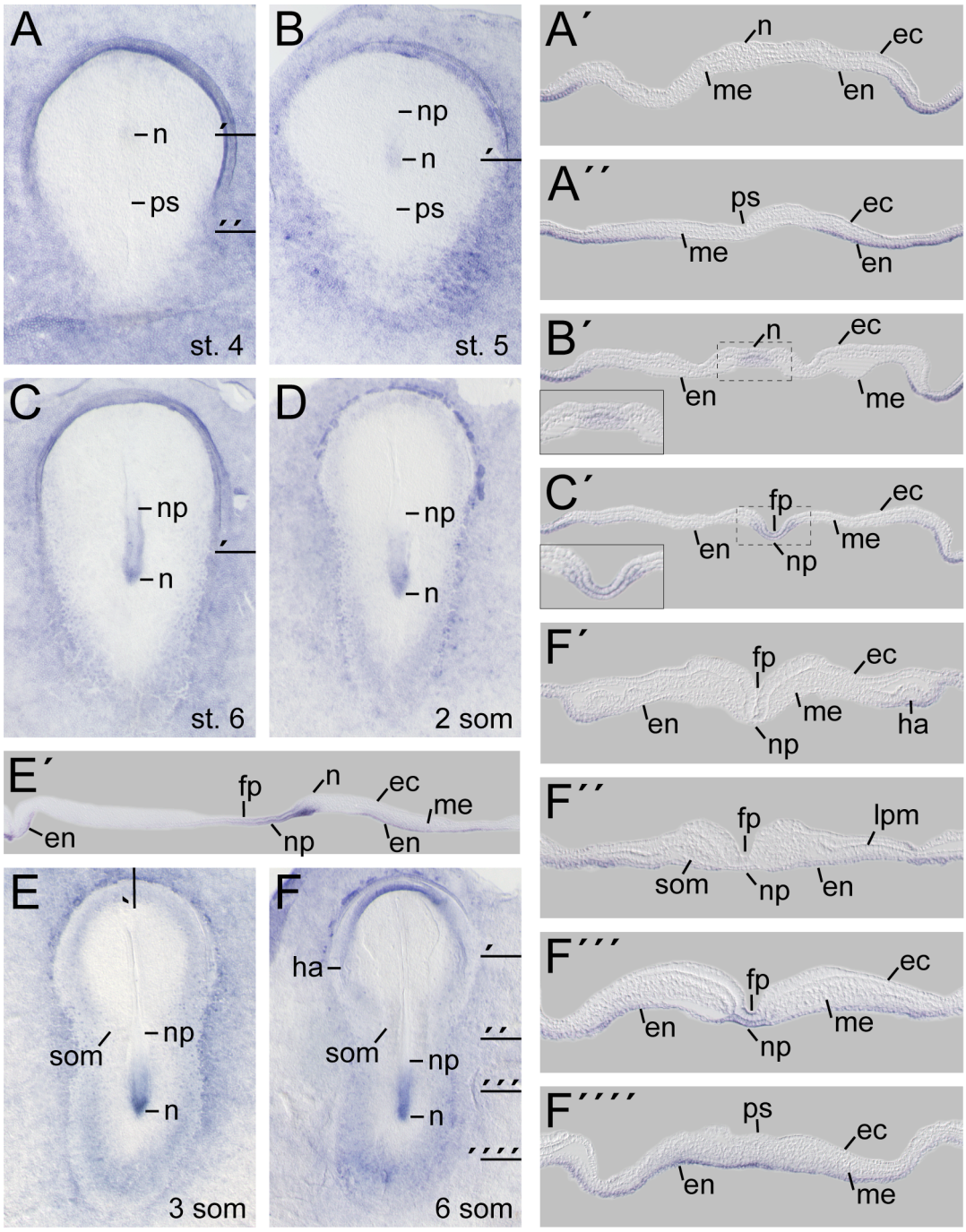

Fig. 8 Expression of *Indian hedgehog* (*Ihh*) in rabbit gastrula and neurula stage embryos. Whole mount *in situ* hybridisation of defined stages using a specific antisense probe against *Ihh*. At stage (st.) 4 (**A**) extraembryonic endoderm and the most lateral embryonic endoderm (en) expressed *Ihh* (**A´**, **A´´**) as well as in later stages (**B**-**F**, **B´**-**F´´´**). At st. 5 (**B**) Hensen's node (n) became also positive for *Ihh* transcripts (**B´**). Inset in (**B´**) shows the node in higher magnification. This expression persisted during later stages (**C**-**F**). Note that only the ventral layers of the node expressed *Ihh* (**B´**, **E´**). From stage 6 (**C**) onwards expression extended into the bilaminar midline mesoderm consisting of the notochordal plate (np) and floor plate (fp) (**C´**, **D**-**F**, **F´´´**). Inset in (**C´**) shows midline mesoderm in higher magnification. Around the 3 somite stage the lateral endodermal cells expressing *Ihh* formed a continuous band (**E**, **E´**). At the 6 somite stage expression could be detected in the entire endoderm at

the level of the posterior notochord as well as the somites (som). (ps) primitive streak, (me) mesoderm, (lpm) lateral plate mesoderm, (ec) ectoderm, (ha) heart anlage. Embryos are shown in ventral views with anterior to the top.

positive for *Ihh* transcripts although the midline expression had already ceased at this level (Fig. 8F, F´´). Rostral of the somites the expression domain narrowed down again to the most lateral endodermal cells comprising also those cells underlying the bilaterally developing heart anlage (Fig. 8F, F´). Expression in the ectoderm or in mesoderm apart from the notochordal plate was never observed (Fig. 8A´-F´´´´).
In summary, this analysis shows that *Indian hedgehog* in the rabbit blastodisc is largely expressed in the same domains as described in mouse embryos although the expression in endodermal cells has not been described yet.

Desert hedgehog (*Dhh*) is not expressed in rabbit gastrula/neurula embryos

Desert hedgehog is the third vertebrate homolog of *Drosophila* Hedgehog, which is actually more closely related to *Drosophila* Hedgehog than Sonic or Indian hedgehog (Echelard et al. 1993). It has been described to be expressed in testes and is essential for testis development by regulating both early and late phase of spermatogenesis (Bitgood et al. 1996). Additionally, *Dhh* is required for the development of peripheral nerve sheaths (Parmantier et al. 1999).
In order to investigate if *Dhh* also has unrecognised earlier expression patterns whole mount *in situ* hybridisation with rabbit embryos of defined stages was performed. Therefore, a specific antisense probe against *Dhh* was used but no expression of *desert hedgehog* was detected in the embryonic stages examined (data not shown).

Expression of *Smoothened* (*Smo*) in rabbit embryos

Smoothened, a seven-transmembrane receptor-like protein is the only responsible transducer of the Hedgehog signalling pathway in *Drosophila*, mouse and zebrafish (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Varga et al., 2001; Zhang et al., 2001). It has been shown that zebrafish embryos are already supplied with maternal *Smoothened* mRNA. Later, the embryo expresses *Smoothened* widely with higher levels in the head and tail regions (Chen et al., 2001). In mice however, *Smoothened* is strongly expressed throughout the whole embryo (Zhang et al., 2001), which is also the case in chick embryos (Aglyamova and Agarwala, 2007).

A specific antisense probe against *Smoothened* was used to determine its expression in rabbit gastrula and neurula stages through whole mount *in situ* hybridisation. In all stages examined in this study a strong ubiquitous expression of *Smoothened* was detected (Fig. 9). During stage 4 and 5 Hensen's node, located at the anterior end of the primitive streak, showed lower levels of expression as compared to other cells of the embryo (Fig. 9A, B, A´). Otherwise transcripts were detected throughout the whole embryo and in all three germ layers, ectoderm, mesoderm and endoderm respectively (Fig. 9A, B, A´, A´´). In views of whole-mount embryos from stage 6 to the 4 somite stage (Fig. 9C-F) the axial midline mesoderm appeared almost free from *Smoothened* expression. Transversal sections (Fig. 9D´, F´) though revealed that this was due to the very thin bilaminar arrangement of the notochordal plate and the floor plate rendering the axial mesoderm more translucent. Cells of the notochordal plate and the floor plate actually showed expression at the same level as the surrounding tissue. During somite stages, along with the condensation of somitic mesoderm and the process of neurulation, embryos adopted a waisted shape. Apart from the ubiquitous expression in the whole embryo also in somite stages, slightly increased expression levels were detected in lateral plate mesoderm as well as the condensed somites (Fig. 9E, F, F´).

Smoothened expression in rabbit embryos thus showed the same characteristics as described in other vertebrate species examined to date.

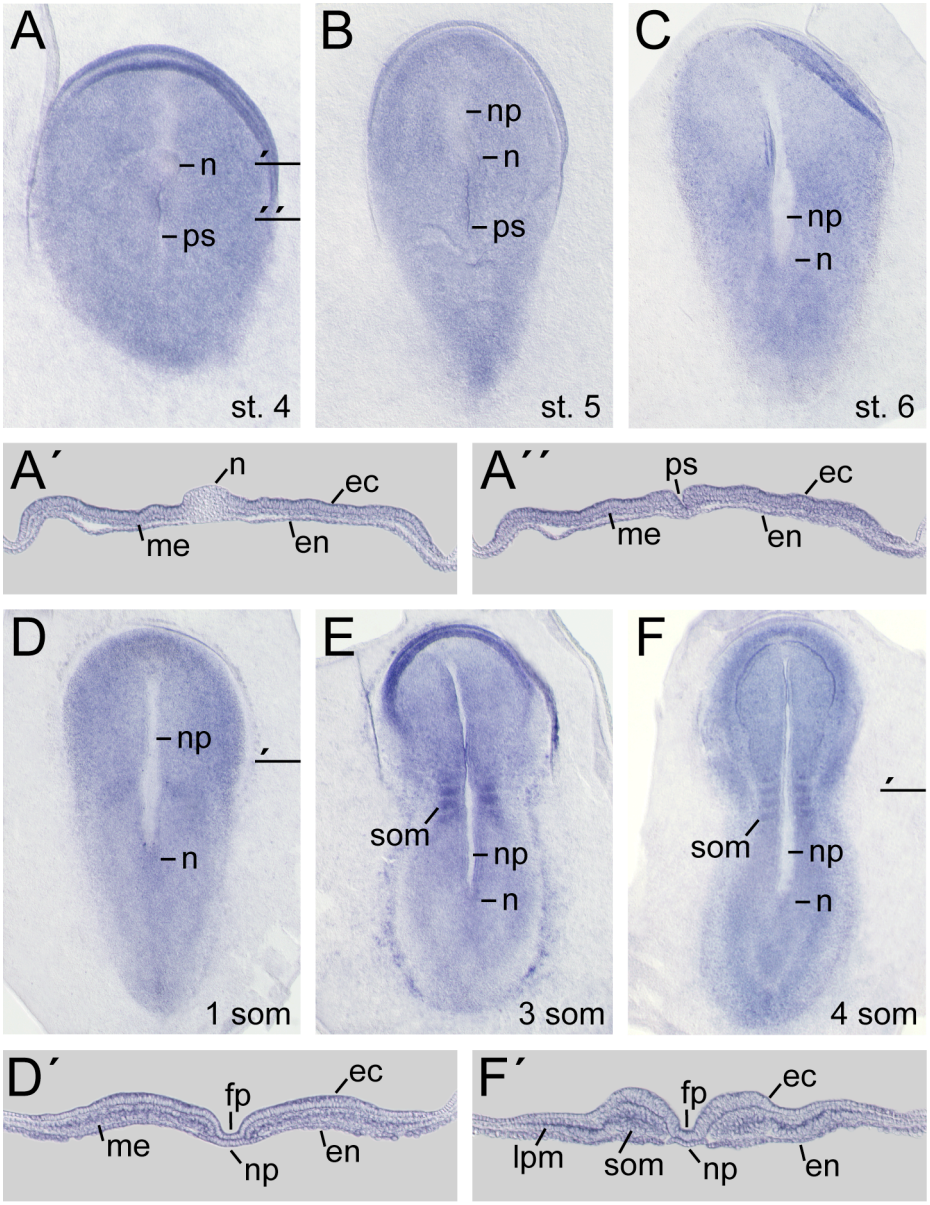

Fig. 9 Expression of *Smoothened* (*Smo*) in rabbit gastrula and neurula stage embryos. Whole mount *in situ* hybridisation of defined stages using a specific antisense probe against *Smo*. Ubiquitous expression of *Smo* in all stages analyzed (**A**-**F**). At stage (st.) 4 and 5 (**A**, **B**) lower levels of signal were detected in Hensen's node (n) (**A´**). Apart from this, transversal sections revealed smo expression in all three germ layers, (ec) ectoderm, (me) mesoderm and (en) endoderm (**A´´**-**F´**). Despite the translucent appearance of the bilaminar, epithelialized axial midline, notochordal plate (np) and floor plate (fp) cells expressed *smo* equally to surrounding tissues (**D´**-**F´**). During somite stages (**E**, **F**) stronger signal was detected in the lateral plate mesoderm (lpm) and condensed somites (som). (ps) primitive streak. Embryos are shown in ventral views with anterior to the top.