Establishment of a new *in vitro* Culture System and functional Analysis of Sonic Hedgehog and FGF8 in the Determination of Laterality in the Rabbit Embryo

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Abstract

Cilia-driven leftward flow plays a pivotal role in the determination of left-right (LR) asymmetry. In mammals, this extracellular fluid flow is produced by motile monocilia situated on the posterior notochordal plate (PNC). The PNC is homologous to superficial mesoderm derived structures of other species like the gastrocoel roof plate (GRP) in frog and Kupffer's vesicle (KV) in fish. Directional fluid flow created at these structures subsequently leads to the initiation of the left-specifying Nodal signalling cascade in the left lateral plate (LPM). The rabbit develops via a flat blastodisc phase representing the archetypical mode of mammalian embryogenesis. These specific advantages of the rabbit were employed in this study to further examine the role of two central determinants of laterality, namely Sonic hedgehog (Shh) and FGF8, and also extended by the design of a new *in vitro* culture technique. In this new method, the so-called ring culture, a medium-filled plastic ring was placed upon the extraembryonic tissue of the explanted embryo. In contrast to the semi-dry standard culture method, this setting corresponded more to the *in vivo* conditions of gastrulating/neurulating rabbit embryos in the uterus. It therefore facilitated stable development of laterality in most cases also when presomite stages were taken into culture. Subsequent analysis showed that this was due to improved development of the PNC. Conversely, in standard-cultured embryos showing altered LR marker gene expression, maturation of the PNC was impaired leading to a disturbance of leftward flow. This study also provided evidence that cilia-driven leftward flow is indispensable for the determination of laterality in rabbit embryos. When the flow was blocked during culture by methylcellulose-containing medium embryos displayed altered LR marker gene expression in a very high proportion.

The unilateral gain-of-function of Shh revealed important differences between rabbit and chick embryos. In rabbit, Shh induced right-sided marker gene expression only in the 2 somite stage, whereas in chick this inductive effect lasted from stage 4 until up to the 1-2 somite stage. This indicated that in rabbit Shh works in conjunction with the flow, which has not been described up to now in chick. The systemic inhibition of Shh signalling by cyclopamine led to bilateral expression of LR marker genes in rabbit.

This was due to disruption of the floor plate and therefore the loss of the restrictive midline barrier function of *Lefty* expression as described in Shh mutant mice.

FGF8 has a right-sided repressive function in the rabbit implicated in the transfer of laterality cues. In the present study it could be shown that this repressive effect is epistatic to cilia-driven leftward flow, because it also functioned when the flow was blocked. The systemic inhibition of FGF8 signalling with SU5402 caused loss of LR marker gene expression prior to the 2 somite stage but did not influence ciliogenesis or the setup of cilia-driven leftward flow. Taken together, this suggested a dual function for FGF8 signalling: First, it is needed to confer competence to the lateral plate and second, during the 2 somite stage, it is needed for the transfer of LR cues.

Zusammenfassung

Bei der Entstehung der links-rechts (LR) Asymmetrie spielt ein von Cilien getriebener, linksgerichteter Flüssigkeitsstrom (CLF) eine entscheidende Rolle. In Säugern wird dieser extrazelluläre Flüssigkeitsstrom von motilen Monocilien auf der posterioren Notochordplatte (PNC) erzeugt. Das PNC ist homolog zu den ebenfalls aus superfiziellem Mesoderm hervorgehenden Strukturen anderer Spezies wie der Dachplatte des Gastrocoels (GRP) der Amphibien und dem Kupfferschen Vesikel (KV) des Fisches. Der in diesen Strukturen entstehende CLF führt schließlich zur Aktivierung der links-spezifizierenden Nodal-Signalkaskade im linken Seitenplatten-mesoderm (LPM). Das Kaninchen entwickelt sich über ein flaches Keimscheibenstadium und repräsentiert somit den ursprünglichen Modus der Säugerentwicklung. Diese besonderen Vorteile des Kaninchens wurden in der vorliegenden Arbeit genutzt, um die Rolle zweier wichtiger Lateralitätsdeterminanten, Sonic hedgehog (Shh) und FGF8, weiter zu untersuchen. Außerdem wurden die experimentellen Möglichleiten durch die Entwicklung einer neuen *in vitro* Kulturmethode erweitert. Bei dieser so genannten Ringkulturmethode wurde ein mit Medium gefüllter Plastikring auf das extraembryonale Gewebe des explantierten Embryos gelegt. Im Gegensatz zur halbtrockenen Standardkulturmethode entspricht

dies mehr den *in vivo* Gegebenheiten eines Kaninchen Embryos im Uterus. Dies ermöglichte daher meist eine stabile Entwicklung der Lateralität auch bei der Kultivierung von präsomitischen Stadien. Nachfolgende Untersuchungen zeigten, dass dies mit einer verbesserten Entwicklung des PNC zusammen hing. Umgekehrt war bei Standardkultivierten Embryonen, die veränderte LR Markergenexpression zeigten, die Entwicklung des PNC und nachfolgend des CLF beeinträchtigt. In der vorliegenden Arbeit konnte ebenfalls gezeigt werden, dass der von Cilien getriebene Flüssigkeitsstrom unverzichtbar für die Entstehung von Asymmetrie in Kaninchenembryonen ist. Wurde der CLF während der Kultivierung durch methylcellulosehaltiges Medium blockiert, so zeigte ein großer Anteil der so behandelten Embryonen anschließend veränderte Markergenexpression.

Der unilaterale Funktionsgewinn von Shh konnte wichtige Unterschiede zwischen Kaninchen- und Hühnerembryonen aufzeigen. Im Kaninchen wurde nur während des 2-Somiten-Stadiums rechtsseitige Markergenexpression durch Shh induziert, wohingegen im Huhn dieser induktive Effekt von Stadium 4 bis bis hin zum 1-2 Somiten-Stadium zu beobachten war. Dies legte nahe, dass Shh im Kaninchen in Verbindung mit dem CLF wirkte, der bisher im Huhn nicht beschrieben wurde. Die systemische Inhibition des Shh Signalwegs verursachte bilaterale Expression von LR Markergenen im Kaninchen. Dies konnte auf eine Störung der neuralen Bodenplatte und damit auf ein Fehlen der restriktiven *Lefty* Expression zurückgeführt werden, wie bereits für Shh mutante Mäuse beschrieben.

FGF8 besitzt eine rechtsseitige repressive Wirkung im Kaninchen, die am Transfer von Lateralitätssignalen beteiligt ist. In der vorliegenden Arbeit konnte nun gezeigt werden, dass dieser repressive Effekt epistatisch zum CLF ist, da der Effekt erhalten blieb auch wenn dieser blockiert war. Die systemische Inhibition des FGF8 Signalwegs durch SU5402 bewirkte den Verlust von LR Markergenexpression in Embryonen vor dem 2-Somiten-Stadium, beeinflusste die Ciliogenese bzw. die Entstehung des CLF jedoch nicht. Zusammenfassend deutete dies auf eine duale Funktion des FGF8 Signalweges hin: Es wird zuerst für die Übertragung von Kompetenz auf die Seitenplatten sowie später, im 2-Somiten-Stadium, für den Transfer von Lateralitätssignalen gebraucht.

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Introduction

Development of left-right asymmetry

The exterior body plan of vertebrates is characterised by the bilateral symmetry of external structures common to all members of the taxon of bilateria. However, there are numerous interior left-right asymmetries in the arrangement of internal organs. In humans, for example, the apex of the heart points to the left, stomach, pancreas and spleen also locate to the left side whereas liver and gallbladder come to lie on the right side. Even the seemingly bilaterally symmetric lung has a diverging number of lobes with three lobes on the right and two lobes on the left side. This remarkably conserved asymmetric alignment of inner organs is established during embryogenesis with the first morphological evidence of directionality being the rightward bend of the heart tube. Eventually, all organ primordia follow complex patterns of loops and turns that result in their conserved asymmetric positioning called *situs solitus*. The complete mirror-image arrangement of organs is referred to as *situs inversus*, which occurs with a varying prevalence among different populations, but is less than 1 in 10.000. However, there are also laterality defects other than complete *situs inversus*. First, disturbed determination of laterality can lead to heterotaxia, which results in a failure to achieve left-right asymmetry at the level of individual organs. In a second class of conditions known as left or right isomerism, the identity of one side is imposed onto the other, which for some organs can lead to loss or duplication.

Several insights into how the left-right axis is determined have been gained from the detailed description and genetic analysis of human syndromes with distinct laterality defects. However, studies using mouse models for laterality are by now the main source of information about the establishment of asymmetry during embryogenesis.

Other well-established vertebrate model organisms such as *Xenopus*, zebrafish and chicken have been employed successfully to investigate LR axis development as well. Generally, this led to the identification of 4 steps into which the process of laterality determination can be divided:

1) The breakage of bilateral symmetry
2) The asymmetric transfer of cues to the left side
3) Initiation of the conserved Nodal signalling cascade
4) Asymmetric morphogenesis of organ primordia

While many of the described mechanisms seem to be generally applicable to the process of asymmetric organ placement among these species, diverging or even contradictory results have been gained regarding the early events of LR axis development. One possible explanation for this might be found in the major differences concerning early embryonic architecture (reviewed by Hamada et al., 2002; Hirokawa et al., 2006; Hamada, 2008)

The rabbit as a mammalian model organism

The relatively big and flat blastodisc of the chicken embryo combined with its easy accessibility made the chick a widely used model organism. As development via such a flat disc-shaped embryo also represents the typical mammalian mode of development the chicken as a bird is in many aspects analogous to gastrulating/neurulating mammalian embryos, whereas in the general mammalian model organism, the mouse, embryos adopt an untypical cup-shaped 'egg-cylinder' appearance, which is only found in rodents. The rabbit as a mammal, which develops via a flat blastodisc can hence be used to study archetypical mammalian embryogenesis as it is conserved in humans as well.

Due to the resemblance of early embryos the staging system for rabbit embryos is based on the Hamburger-Hamilton (HH) stages for chicken embryos (Fig. 1; Hamburger and Hamilton, 1992; Blum et al., 2007). The process of gastrulation starts

at stage 3 with the formation of the primitive streak when the first cells from the epiblast migrate through the streak in order to create mesoderm and replace the hypoblast with definitive endoderm. The pear-shaped stage 4 embryo is characterised by a fully elongated primitive streak and a discernible thickening at its anterior end, Hensen's node. With the exit of prechordal and the following notochordal cells from the node during stage 5, axial mesodermal structures are established and the embryo elongates anteriorwards. When the notochordal plate finally exceeds the primitive streak in length and the node consequently is located in the posterior half of the blastodisc the embryo represents stage 6. During the following somite stages the embryo adopts a waisted appearance. Unlike the HH system the somite stages in rabbit are consecutively numbered according to the number of somites formed.
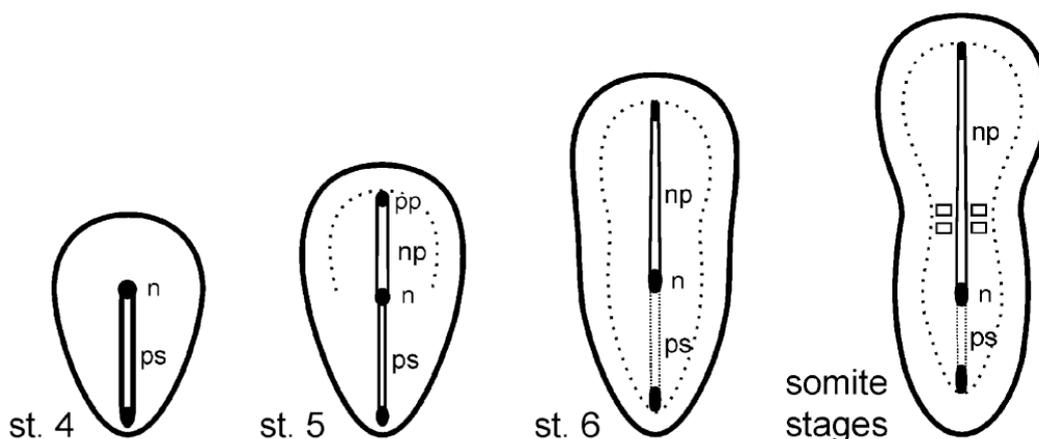
Fig 1 Staging system of rabbit gastrula/neurula embryos. Between 7.0 and 8.0 dpc, the rabbit embryo develops from stage (st.) 4 (characterised by the presence of Hensen's node, n), via stage 5 (notochordal plate, np, and prechordal plate, pp) and stage 6 (elongated notochordal plate and headfold) to somitogenesis. Ps, primitive streak. (Reproduced with permission from Blum et al., 2007).

Nodal expression during gastrulation

Regardless of the divergent modes of early development there is, furthermore, a highly conserved set of factors among vertebrates preceding asymmetric organ morphogenesis. This precisely orchestrated sequence of gene expression events is

called "Nodal signalling cascade" and operates mainly in the left lateral plate mesoderm (LPM) of neurula stage embryos.

In rabbit, mouse and chick, *Nodal* is first expressed in the primitive streak, the site of gastrulation, as well as in the organizer, the node or Hensen's node (Zhou et al., 1993; Levin et al., 1995; Blum et al., 2007). In *Xenopus*, transcripts of *Nodal*-related genes are found in equivalent structures, the circumblastoporal collar and the blastoporal lip (Jones et al., 1995). The same applies for zebrafish embryos where *Nodal* homologs are expressed circumferentially in the germ ring and embryonic shield (Rebagliati et al., 1998). It has been shown that during gastrulation Nodal is required in these structures for the formation of mesoderm and definitive endoderm (reviewed by Schier and Shen, 2000). While the first cells leave the organizer to form midline mesodermal structures *Nodal* expression is terminated in streak and organizer equivalents.

Soon after, *Nodal* transcripts reappear in a domain framing the posterior aspect of the notochordal plate (PNC) in mouse and rabbit embryos. Similarly, this domain can be found around the homologous structures in zebrafish and *Xenopus*, Kupffer's vesicle (KV) and the gastrcoel roof plate (GRP) respectively (Blum et al., 2007). Since the PNC in the mouse was previously referred to as the "Node" this is where the name "*Nodal*" came from (Zhou et al., 1993). This paranotochordal domain of *Nodal* expression is bilateral in mammals, *Xenopus* and zebrafish and precedes expression and initiation of the cascade in the left LPM (Lowe et al., 1996; Long et al., 2003). In the chick, however, the morphological asymmetry of Hensen's node precedes *Nodal* transcription and the paranotochordal expression first occurs unilaterally on the left becoming bilateral only after the initiation of left LPM expression (Levin et al., 1995; Dathe et al., 2002).

Data from transgenic mice showed that the paranotochordal domain of *Nodal* expression is crucial for the initiation of asymmetric gene expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003). When *Nodal* expression was selectively removed from the paranotochordal domain embryos failed to express *Nodal* in the left LPM (Brennan et al., 2002) whereas the reconstitution of the domain was sufficient to restore asymmetric *Nodal* expression (Saijoh et al., 2003).

Nodal signal transduction and regulation

Nodal belongs to the TGFβ superfamily of secreted growth factors, which typically signal as dimers. The dimerised ligand can transduce signal through a heterodimeric receptor complex of type I (Alk4 and Alk7) and type II (ActRIIa and ActRIIb) receptors of the serin/threonine kinase type. Nodal also requires, unlike other TGFβ members, the presence of an accessory receptor of the EGF-CFC type (such as Cryptic and Cripto) to facilitate binding to the receptor. Once bound to the receptor the pathway is activated by the autophosphorylation of the cytoplasmatic domains of the receptor and the subsequent phosphorylation of the intracellular transducer Smad2. In turn, Smad2 forms a nuclear complex with Smad4 and the transcription factor Foxh1 to activate downstream target gene expression (Whitman and Mercola, 2001; Massague, 2003). As *Nodal* itself is one of these target genes a positive feedback loop on its own transcription is created.

Asymmetric expression of *Nodal* is mainly regulated by one of two identified enhancers called ASE, which is located in the first intron of the *Nodal* gene. The ASE is necessary and sufficient for asymmetric LPM expression and was shown to contain Foxh1 binding sites revealing its Nodal responsiveness (Norris et al., 2002). The second enhancer named LSE for left side-specific enhancer is located about 4kb upstream of the transcription start site. It is Nodal-responsive as well but seems to be dispensable for L/R patterning as $Nodal^{\Delta LSE/\Delta LSE}$ mice show no laterality defects (Saijoh et al., 2005). Additionally, a paranotochordal domain specific enhancer for *Nodal* expression was identified 9.5kb upstream of the transcription start site, named NDE (Adachi et al., 1999; Norris and Robertson, 1999). Unlike enhancers driving asymmetric expression, the NDE is not Nodal responsive (Saijoh et al., 2003), indicating that the maintenance of *Nodal* expression at the posterior notochord (PNC) is governed by a factor other than *Nodal* itself. This could also be confirmed by *cryptic* knock-out mice (Gaio et al., 1999) where embryos lack the asymmetric LPM expression but the paranotochordal domain of *Nodal* is unchanged. The knockdown of the *crypic* homolog in Xenopus, *Xcr2*, by morpholino antisense oligonucleotides embryos also had the same effect (Onuma et al., 2006) leading to the conclusion that

the EGF-CFC co-receptor is necessary for Nodal signal transduction in the LPM but not for the paranotochordal domain.

The conserved Nodal signalling cascade

As indicated above, even before the first morphological sign asymmetry can be detected within the embryo on a molecular level. Here, *Nodal* is the first gene to be transcribed unilaterally in the left lateral plate mesoderm (LPM). It encodes a secreted molecule belonging to the transforming growth factor beta (TGFβ) superfamily of growth factors. In rabbit, mouse and chick, *Nodal* is expressed in the left LPM from the 2-3 somite stage onwards (Levin et al., 1995; Lowe et al., 1996; Fischer et al., 2002). In *Xenopus* respectively, *Nodal* can first be detected in the left LPM at stage 19/20 as well as in 10-12 somite stage zebrafish embryos (Rebagliati et al., 1998; Ohi and Wright, 2007).

In the left LPM *Nodal* transcription on the one hand positively feeds back upon itself whereby a fast rostro-caudal expansion of its expression is achieved. On the other hand, Nodal signalling targets the transcription of two genes important for the regulation and maintenance of the cascade, namely *Lefty2* and *Pitx2*. The untypical TGFβ family member Lefty acts as a feedback inhibitor of Nodal signalling as it competes for receptor as well as co-receptor binding (Schier and Shen, 2000). The expression of *Lefty* temporally and spatially follows *Nodal* expression leading to an effective attenuation of Nodal activity (Meno et al., 1999; Cheng et al., 2000). The transcription of the other target gene, the homeobox transcription factor Pitx2, however is not terminated by Lefty. Therefore it remains transcriptionally active in the left lateral plate mesoderm mediating first cues to asymmetric organ morphogenesis (Logan et al., 1998; Ryan et al., 1998; Campione et al., 1999).

Cilia and the determination of laterality

Cilia are present on almost all types of vertebrate cells. There, they can either transduce a multitude of sensory stimuli as non-motile primary cilia or move fluid past cells as motile ones. In recent years one ciliated structure in particular has drawn attention as its function seems to be specialised on the determination of laterality: the mammalian PNC and its homologs KV and GRP in fish and frog, respectively.

Ciliary architecture and assembly

Cilia are ancient, microtubule-based organelles conserved in eukaryotes ranging from the single-celled green algae, *Chlamydomonas*, to humans. At the apical surface of the cell, a cilium extends from the basal body, a modified centrosome, and projects into the extracellular space. The plasma membrane of the cilium is continuous with the remainder of the cell. The axonemal structure as the inner framework, common to all cilia consists of a circular arrangement of nine microtubule doublets with a vast variety of accessory proteins attached (reviewed by Satir and Christensen, 2007). The force for motility, for example, is provided by dynein motors anchored to the microtubule scaffold. Here, the inner and outer arm dynein complexes perform a sliding movement between neighbouring doublets and as the tubules are all anchored to the basal body their shearing results in the bending of the cilium (Wemmer and Marshall, 2004). However, all proteins necessary for ciliary axoneme assembly and maintenance are moved bidirectionally along the axoneme by a motor-protein driven process called intraflagellar transport (IFT). Because axonemal microtubules are oriented with their plus-ends to the tip of the cilium, and motor-proteins only move in one direction, the IFT process depends on two types of motors. Namely, the heterotrimeric Kinesin-II-complex for anterograde and Dynein-Ib for retrograde movement of the two raft-like IFT particle complexes, IFTA (linked to retrograde transport) and IFTB (linked to anterograde transport) which, in turn,

provide multiple binding sites for the large number of cargo that has to be delivered to the distal tip of the cilium (reviewed by Scholey 2008).

The PNC/GRP/KV as transient ciliated structures

In the mouse it was described that there were motile cilia on the notochordal plate (Sulik et al., 1994) but that they were vital for the specification of left-right asymmetry became clear only later when it was demonstrated that knockout mice, mutant for the Kif3b subunit of the heterotrimeric Kinesin-II-complex, lacked cilia and displayed laterality defects (Nonaka et al., 1998). In this ground-breaking study it was also shown that if cilia are present they are not only motile but are also able to produce a vectorial leftward flow of extracellular fluid. Unfortunately, the posterior widening of the murine notochordal plate where the leftward fluid flow occurred was misnamed as the "node" reflecting the assumption that it represented the equivalent of the embryonic organizer in chick and rabbit, called Hensen's node (Beddington et al., 1992). Consequently, the fluid motion produced by these cilia was termed "Nodal Flow". A comparative approach however demonstrated that the indentation at the distal tip of the mouse egg cylinder represents the posterior most aspect of the notochordal plate, termed PNC, rather than the embryonic organizer (Blum et al., 2007). The equivalent structures of rabbit, zebrafish and frog PNC, Kupffer's vesicle (KV) and gastrocoel roof plate (GRP) are all characterised by the following criteria: they consist of epithelialized mesodermal cells each carrying a monocilium (Sulik et al., 1994; Shook et al., 2004; Essner et al., 2005), they can be located by the bilateral paranotochordal *Nodal* expression (Blum et al., 2007) and are involved in the determination of laterality by setting up a leftward directed fluid flow (Nonaka et al., 1998; Kramer-Zucker et al., 2005; Hirokawa et al., 2006; Schweickert et al., 2007). The PNC/KV/GRP comes to lay rostral of the organizer and stands in continuity with the notochord. Therefore mutations affecting PNC or KV morphogenesis or function cause laterality defects but do not disturb the two main body axis (Herrmann and

Kispert, 1994; Nonaka et al., 1998; Amack and Yost, 2004; Beckers et al., 2007; Amack et al., 2007).

Cilia-driven leftward flow at the PNC/GRP/KV

Since its discovery in 1998 cilia-driven leftward fluid flow has been further characterised and several models for its role in the determination of laterality have been put forward. First, it could be demonstrated that the cilia of the spontaneous mouse mutant *iv* (*inversus viscerum*), which shows randomisation of LR determination were immotile and thus did not produce a fluid flow (Okada et al., 1999). The left-right dynein gene (*lrd*) mutated in these mice codes for an axonemal dynein heavy chain, which as a motor protein is essential for motility of cilia (Supp et al., 1997). In another classic mouse model for LR defects, *inv* (*inversion of embryonic turning*), cilia show motility but seem only capable to produce abnormal, weak and turbulent fluid flow (Okada et al., 1999; Okada et al., 2005). The function of Inversin, the gene product of *Inv* with respect to ciliary motility is still unclear. In an elegant experiment it was shown that the expression of LR marker genes could be reversed by applying an inverted fluid flow across the PNC of wild type embryos. Conversely, randomised marker gene expression of *iv* mice was rescued by the application of an artificial fluid flow toward the left side (Nonaka et al., 2002).

These findings led to a first straight forward chemosensoric model proposing that the directional fluid flow would generate a concentration gradient of a morphogen such as Shh, FGF8 or Nodal itself since they all are expressed in or near the PNC (Nonaka et al., 1998; Nonaka et al., 2002). Supporting this notion it could indeed be shown that added molecules of an appropriate size are transported toward the left side by the intrinsic fluid flow (Okada et al., 2005). Instead of the identification of one transported morphogen it was demonstrated that FGF signalling triggered the release of membrane sheathed objects termed "nodal vesicular parcels" (NVP) carrying Shh and retinoic acid that are transported toward the left via the fluid flow (Tanaka et al. 2005).

Prerequisites for leftward fluid flow formation

As mentioned above the inner scaffold of a cilium consists of 9 outer microtubule doublets. Motility of cilia is in part controlled by the central pair apparatus (CP), which is represented by two single but tethered microtubules. The typical beat pattern of such 9+2 cilia is a planar and biphasic whip-like one: the fast power stroke downright pushes the surrounding fluid whereas the slow recovery stroke does not move the fluid due to friction along the cell membrane. As cilia of the 9+0 type lack the central pair of microtubules, it is not surprising that most of these cilia are immotile and were considered to be of sensory nature. PNC cilia of the mouse, however, have been shown to be motile and of the 9+0 type (Sulik et al., 1994). Presumably due to the lack of the regulatory CP, they perform conical rotation movements rather than planar beating (Nonaka et al., 2002; Nonaka et al., 2005; Okada et al., 2005). Interestingly, Kupffer's vesicle cilia in zebrafish display 9+2 axonemes (Kramer-Zucker et al., 2005) and in rabbit even three different axonemes were found, 9+0, 9+2 and a novel 9+4 type (Feistel and Blum, 2006) all performing rotational beat patterns. Recently, CPs were also observed in mouse PNC cilia (Caspary et al., 2007).

How simple clockwise rotation could evoke directional movement of fluid was first mathematically modelled by Cartwright et al. 2004. This study showed that PNC cilia would need to be tilted toward the posterior of the cell in order to produce a linear leftward fluid flow. This was indeed the case in mouse and rabbit embryos (Nonaka et al., 2005; Okada et al., 2005). The posterior position of the cilium coupled with the convex shape of epithelial PNC cells thus results in an effective (leftward) half of the rotation farthest from the cell surface and an ineffective half "sweeping" the cell surface generating a unidirectional linear fluid flow to the left (Brokaw, 2005; Nonaka et al., 2005).

Evolutionary conservation of the cilia-driven fluid flow module

Although the left and right side of the body are already defined by the alignment of the anterior-posterior (AP) and dorso-ventral (DV) axis an asymmetric left-right axis has to be specifically determined with respect to the other body axis. Even before the first asymmetrically expressed genes were described Brown and Wolpert (1990) proposed a theoretical model involving a chiral "F-molecule", which could assign LR asymmetry simply by aligning its arms along the DV and AP axis. They also suggested that this "F-molecule" might be associated with an oriented microtubule cytoskeleton. As microtubules possess a chiral structure PNC cilia indeed fulfil the criteria for the "F-molecule" and thus for the symmetry breaking event: the AP and DV axis are represented by the posterior tilt of the cilia and the site of their protrusion, respectively. Therefore, PNC cilia create a leftward-directed flow by implementing the pre-existing positional cues and their own chirality (Hamada, 2008). The finding that cilia are also present in structures homologous to the mouse PNC (Essner et al., 2002) in other vertebrate species allowed the assumption that directional fluid flow could be conserved in other species as well. This turned out to be the case as fluid flow was not only shown in Kupffer's vesicle of zebrafish and medaka (Essner et al., 2002; Okada et al., 2005) but also in rabbit (Okada et al., 2005) and *Xenopus* embryos (Schweickert et al., 2007). In humans, syndromes like Kartagener's or Bardet-Biedl are genetic disorders affecting the integrity of cilia, which are also associated with laterality defects, further prove the conserved connection between cilia and LR asymmetry (Afzelius, 1976; Beales, 2005). On the other hand, a ciliated structure homologous to the PNC has not been reported in chick up to now. Also pig embryos do not seem to have a ciliated PNC homolog (Feistel, 2006). Curiously, embryos of both species display a morphologically asymmetric Hensen's node and the paranotochordal domain of *Nodal* is expressed unilaterally.

The role of Shh and FGF8 in left-right asymmetry

Both Shh and FGF8 are powerful secreted growth factors implicated in a multitude of processes during development including the determination of laterality. Their function was examined in various model organisms sometimes yielding diverging or even contradictory results.

Hh signal transduction

Hedgehog (*Hh*) was initially identified in *Drosophila* as a gene affecting the patterning of the larval cuticle (Nüsslein-Volhard and Wieschaus, 1980). In vertebrates, multiple homologs were characterised shortly thereafter named *sonic* (*Shh*), *indian* (*Ihh*) and *desert hedgehog* (*Dhh*) in mouse (Echelard et al., 1993). Their expression in a number of embryonic organising centres explicitly highlighted the importance of these secreted signalling molecules (Echelard et al., 1993; Krauss et al., 1993), which were also demonstrated to function as morphogens that are able to alter cell patterning in a concentration dependent manner (Heemskerk and DiNardo, 1994; Riddle et al., 1993; Briscoe and Ericson, 1999).

In the sending cell Hh proteins undergo autoprocessing and lipid modification producing a dually modified signalling peptide. Signal transduction in the receiving cell is then triggered by binding of Hh ligand to its receptor Patched (Ptc), which in the absence of Hh blocks the activity of Smoothened (Smo), the main signal transducer. Hence, binding of Hh to Ptc leads to a release of repression of Smo activity, which in turn results in the activation of downstream transcription factors, Ci in *Drosophila* and the three Gli homologs in vertebrates (reviewed by Jia and Jiang, 2006; Ingham and McMahon, 2001).

The role of Shh in laterality determination

First evidence that Shh is involved in the establishment of laterality came from a study using chick embryos. Here, *Shh* is expressed asymmetrically on the left side of Hensen's node and if placed ectopically onto the right side it was able to induce *Nodal* expression. Conversely, blocking of Shh signalling using a specific antibody resulted in a loss of *Nodal* expression in the left lateral plate (Levin et al., 1995). These findings lead to the assignment of a left inductive function for Shh.

On the contrary, *Shh* is expressed symmetrically in the organizer and notochord in mouse as well as in rabbit, *Xenopus* and zebrafish (Fischer et al., 2002, Collignon et al., 1996; Sampath et al., 1997; Schilling et al., 1999). Nonetheless, *Shh* knock out mice are characterised by laterality defects like left pulmonary isomerism, randomisation of embryonic turning and bilateral expression of marker genes (Tsukui et al., 1999). The finding of bilateral marker gene expression was unexpected in the light of the experiments in chick. However, as homozygous *Shh* mutant mice lack a functional floor plate, expression in both lateral plates could be due to a secondary loss of repressive *Lefty* function. In mice mutant for the Shh signalling mediator *Smo* as well as in *Shh/Ihh* compound mutants asymmetric marker gene expression is lost and embryos display linear heart tubes, cyclopia and fail to turn. This, on the one hand, demonstrated functional redundancy between Shh and Ihh and, on the other, suggested a left inductive role for Hh signalling in the mouse as well, although this seems not to be as direct as in chick but involves the control of the paranotochordal expression of *Nodal* via the TGFβ growth factor *Gdf1* (Zhang et al. 2001). In *Xenopus* and zebrafish the misexpression of Shh also led to ectopic activation of marker gene expression on the right side and disturbance of situs development (Sampath et al., 1997; Schilling et al., 1999).

The involvement of cilia in Hh signalling

The connection between cilia and Hh signalling first arose in 2003 when a study was published showing that mutations in IFT genes as well as motor proteins cause phenotypes suggestive of defective Hh signalling (Huangfu et al., 2003). In particular mutations in the complex B IFT genes *Ift172, Ift88/Polaris* and the Kinesin motor *Kif3a* did not only show randomisation of laterality associated with the loss of PNC cilia but also defects in limb patterning and neural tube development. In the neural tubes of these embryos a set of ventral cell types normally specified by Shh were missing, clearly indicating that the IFT machinery has an essential role in Shh signal transduction. Subsequent studies revealed that mice mutant for several other IFT, dynein or even basal body genes show a similar loss of Shh-dependant ventral neural cell types and that the IFT mutations affect Shh signalling downstream of Ptc (Huangfu and Anderson, 2005; Liu et al., 2005; Houde et al., 2006; Vierkotten et al., 2007). How ciliary IFT is integrated into Hh signalling became clear by the demonstration that Smo becomes enriched on cilia upon Shh exposure (Corbit et al., 2005) and several other pathway components, namely the Gli transcription factors, Ptc and Supressor of fused (Sufu), an essential negative regulator, also localise to cilia (Haycraft et al., 2005, Rohatgi et al., 2007).

Apart from the mouse studies described above *Xenopus* Hh signalling seems to require intact cilia as well (Park et al., 2006) whereas in *Drosophila* mutations that disrupt cilia do not affect Hh signalling. Thus, the otherwise highly conserved signalling pathway only became coupled to cilia at some stage in the deuterostome lineage.

The FGF8 signalling pathway

FGF8 is a secreted growth factor belonging to the large group of fibroblast growth factors initially identified as stimulators of fibroblast and epithelial cell growth. FGFs are involved in the regulation of many developmental processes including patterning,

morphogenesis, cell proliferation or migration. The transduction of FGF8 signalling is initiated by binding of FGF8 to the FGF receptor 1 (FGFR1), a receptor-tyrosine-kinase. Upon binding of FGF8 the receptor dimerises and gets transactivated through phosphorylation. The main signal transduction then leads via the Ras-MAPK/ERK pathway to the activation of specific transcription factors of the Ets family and finally to cell type- and context-specific responses (reviewed by Thisse and Thisse, 2005).

The function of FGF8 in left-right asymmetry

For FGF8 in laterality determination diverging roles have been reported in different model organisms. FGF8$^{neo/-}$ compound heterozygous mice, for example, lack *Nodal* expression in the left lateral plate and if placed ectopically on the right side of wild type mice FGF8 induces *Nodal* expression oh the right (Meyers and Martin, 1999). In contrast to the inductive function in the mouse FGF8 acts as a repressive determinant in chick where beads soaked in FGF8 placed on the left side of Hensen's node effectively suppress the activation of the Nodal cascade (Boettger et al., 1999). Previous work using the rabbit as a model system for archetypical mammalian development established a right-sided repressive function for FGF8 as well (Fischer et al., 2002). Placement of beads soaked in FGF8 on the left side of the PNC abrogated left-sided marker gene expression whereas the inhibition of FGF8 signalling on the right side lead to ectopic activation of the cascade. Furthermore, a subsequent study revealed that the inhibition of Nodal by FGF8 requires intercellular communication via Gap junctions (GJC) whereas the unilateral attenuation of GJC in later stages allows for the release of FGF8 mediated repression and therefore asymmetric Nodal expression (Feistel and Blum, 2008). Yet another mode for FGF8 signalling in LR asymmetry seems to operate in zebrafish embryos (Neugebauer et al., 2008). Here, blocking of FGF8 signalling negatively regulates ciliogenesis resulting in shorter cilia in various tissues including Kupffer's vesicle. This consequently impairs cilia-driven fluid flow and leads to bilateral marker gene expression.

Aim of this work

In previous work, a culture method for early somite stage rabbit embryos has already been established assuring normal left-sided marker gene expression in most cases after *in vitro* culture. Yet for some experiments it is desirable to use presomite stage embryos as well. Therefore the first aim of this thesis was to develop a culture method allowing normal expression of asymmetric marker genes also in presomite stage rabbit embryos.

Furthermore the role of two central determinants of laterality, namely Shh and FGF8 should be examined further using descriptive as well as functional approaches to address the following questions:

- How are Shh signalling marker genes expressed in the rabbit blastodisc?
- Does Shh have an inductive function on the Nodal signalling cascade in the rabbit as well?
- Does the loss-of Shh function influence the development of LR asymmetry?
- Is FGF8 signalling epistatic to cilia-driven fluid flow?