PRIMARY CILIA AND COORDINATION OF SIGNALING PATHWAYS IN HEART DEVELOPMENT AND TISSUE HOMEOSTASIS

PhD thesis by

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The papers included in this thesis are:


In the thesis, these articles are referred to by their number in brackets, the rest by the first author and year.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AVC</td>
<td>Atrioventricular canal</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CHD</td>
<td>Congenital heart disease</td>
</tr>
<tr>
<td>CNC</td>
<td>Cardiac neural crest</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert hedgehog</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EC</td>
<td>Embryonal carcinoma</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EMT</td>
<td>Endocardial epithelial-mesenchymal transformation</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FHF</td>
<td>First heart field (also known as primary heart field)</td>
</tr>
<tr>
<td>Gata4</td>
<td>GATA binding protein 4</td>
</tr>
<tr>
<td>Gli</td>
<td>Glioma transcription factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin staining</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IFM</td>
<td>Immunofluorescence microscopy</td>
</tr>
<tr>
<td>IFT</td>
<td>Intraflagellar transport</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>IVS</td>
<td>Interventricular septum</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun aminoterminal kinase</td>
</tr>
<tr>
<td>Kif</td>
<td>Kinesin superfamily protein</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LR</td>
<td>Left/Right</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>Mchr1</td>
<td>Melanin-concentrating hormone receptor 1</td>
</tr>
<tr>
<td>mESC</td>
<td>Mouse embryonic stem cells</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubuli</td>
</tr>
<tr>
<td>Nkx2-5</td>
<td>NK2 transcription factor related, locus 5</td>
</tr>
<tr>
<td>OFT</td>
<td>Outflow tract</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericentriolar material</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity pathway</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet-derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic kidney disease</td>
</tr>
<tr>
<td>Pkd1</td>
<td>Polycystin 1</td>
</tr>
<tr>
<td>Pkd2</td>
<td>Polycystin 2</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>Ptc</td>
<td>Patched</td>
</tr>
<tr>
<td>Q-RT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo-nucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>SA</td>
<td>Sinuatrial</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SHF</td>
<td>Second heart field</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>Sst3R</td>
<td>Somatostatin Sst 3 receptor</td>
</tr>
<tr>
<td>Sufu</td>
<td>Suppressor of fused</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tg737orm;</td>
<td>Oak Ridge polycystic kidney mouse with defect in gene Tg737 (encoding Polaris)</td>
</tr>
<tr>
<td>(TGF)-Beta</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless/INT</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
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ABSTRACT

This thesis focuses on cilia and their sensory function in the mammalian organism. In particular, the Hedgehog (Hh) signaling pathway functions via the primary cilium and plays a unique role in development, differentiation, cancer and possibly in stem cell fate. Defects in primary cilia assembly or function are tightly coupled to developmental disorders and diseases in mammals termed “ciliopathies”.

The primary objective of this thesis was to investigate the role the primary cilium in coordinating Hh signaling in stem cell differentiation and heart development in the mouse. We show that human embryonic stem cells (hESC) and mouse embryonal carcinoma stem cells (P19.CL6 EC cells) have primary cilia that display ciliary localization of the essential Hh proteins; Gli2, Ptc1 and Smo. Inhibition of the Hh pathway by KAAD-cyclopamine in P19.CL6 cells hinder formation of synchronously beating clusters of cardiomyocytes. Knockdown of the primary cilium in P19.CL6 EC cells by nucleofection with plasmids expressing Ift20 and Ift88 siRNA significantly reduced the appearance of beating cardiomyocyte clusters thereby mimicking the effect of cyclopamine treatment. In vivo experiments revealed that mouse E11.5 Ift88−/− null mutants (which have no primary cilia) have severe endocardial cushion defects, decreased trabeculation and increased pericardial space along with shortened and malformed cardiac outflow tract. These observations suggest that primary cilia coordinate Hh signaling in stem cell differentiation and cardiogenesis. In support of this, preliminary chimera mouse studies showed that primary cilia are important for heart development. This was judged by the distribution of enzymatically tagged wt and Ift88−/− ES cells in the developing heart at E8.5, where only the wt cells are localized to the heart chambers. This signifies that primary cilia are needed for the formation of the heart chambers.

The secondary thesis objective was to investigate the role of progesterone signaling in the female reproduction organs in addition to the role of primary cilia in human pancreatic development and cancer. The findings of the progesterone receptor in the lower half of the motile cilia in the oviduct, suggest a sensory role of motile cilia in progesterone signaling where they might coordinate post ovulatory events. In tissue sections of the developing human pancreas we found up to 20µm long primary cilia projecting into the duct lumen of the exocrine duct, which have increased ciliary localization of Gli2 and Smo after initiation of fetal development, i.e., at weeks 14 and 18. In contrast, ciliary localization of these Hh components was absent at the embryonic stage of development, i.e., at week 7.5. This suggests a role of primary cilia in coordinating Hh signaling in human pancreatic development and postnatal tissue homeostasis. In cultures of human pancreatic duct adenocarcinoma cell lines PANC-1 and CFPAC-1, Ptc in addition to Gli2 and Smo localize to primary cilia. These findings are consistent with the idea that the primary cilium continues to coordinate Hh signaling in cells derived from the mature pancreas. The fact that the Hh signaling pathway is active in the CFPAC-1 and PANC-1 cell lines without Hh stimulation suggests that ciliary Hh signaling plays a potential role in tumorigenesis.

In conclusion, this thesis supports the idea that both motile and primary cilia are critical organelles in the coordination of developmental processes and tissue function.
ABSTRACT IN DANISH – DANSK RESUME

Denne PhD afhandling har fokus på ciler og deres sensoriske funktion hos pattedyr. Mere specifikt spiller Hedgehog (Hh) signaleringsvejen, der virker via det primære cillum, en afgørende rolle for udvikling, differentiation, cancer og muligvis også stamcelle vedligeholdelse. Defekt ciledannelse eller ciliefunktion er tæt knyttet til udviklingsdefekter og sygdomme der betegnes "ciliopatier".


Ydermere støtter præliminære forsøg med chimeramus hypotesen, at det primære cillum er vigtigt for hjerteudviklingen. Dette er bedømt ud fra distribueringen af enzymatisk mærkede vildtype og Ift88−/− ES celler, hvor vildtypecellerne lokaliserer til hjertekamrene, som stort set er fri for mutant ES celler. Dette betyder at det primære cillum er nødvendig for dannelsen af hjertekamrene.

Den sekundære opgave i denne afhandling var at undersøge rollen af progestosteronsignalering i de kvindelige reproduktionssorganer samt rollen af det primære cillum i udviklingen af human pankreas og cancer. Tilstedeværelsen af progestosteronreceptoren i den nedre halvdel af de bevægelige ciler i æggelederne indikerer en mulig sensorisk rolle for de bevægelige ciler i progestosteronsignaleringen som koordinator for postovulatoriske begivenheder. I undersøgelserne med den humane pankreas, fandt vi, at den udviklende eksokrine dukt danner primære ciler på op til 20µm, der projicerer ud i duktlumen fra duktepithelet både under embryonal (uge 7.5) og føtal udvikling (uger 14 og 18). Analyserne med vævssnittdiogene viste endvidere, at niveauet af Hh-komponenterne, Gli2 og Smo, kraftigt stiger under den føtale udvikling og er fraværende under den embryonale udvikling. Denne forøgelse i cilar Hh signalering foreslår en rolle for det primære cillum i koordineringen af Hh signalering i modning af det eksokrine duktsystem. Ydermere ses der i kulturer af humane pankreasdukt adenocarcinoma celllinier, PACN-1 og CFPC.-1, at både Ptc, Smo og Gli2 lokaliserer kraftigt til de primære ciler og at Hh signalering er kraftigt opreguleret i cancercellerne i fravær af Hh-stimulering. Disse resultater tyder på, at ciler Hh-signalering kan spille en rolle i tumordannelse.

Afhandlingens resultater støtter konklusionen, at både motile og primære ciler spiller en afgørende rolle som et sensorisk organel under udvikling og i vævsfunktion.


CHAPTER 1 - THESIS OBJECTIVES

1.1 INTRODUCTORY REMARKS

Primary cilia are solitary organelles which are organized in a 9+0 microtubule axonemal ultra structure that project from the centrosomal mother centriole at the surface of stem cells and most growth-arrested cells in our body (Satir & Christensen, 2008). Primary cilia are sensory organelles that coordinate a series of signal transduction pathways to control developmental processes, tissue homeostasis and behavioral responses (Singla & Reiter, 2006; Satir & Christensen, 2008; Berbari et al., 2009). The physiological importance of primary cilia is underscored by an ever-growing list of diseases and developmental disorders ('ciliopathies') associated with defective primary cilia, e.g. cystic kidney and liver diseases, retinal degeneration, abnormalities in neural tube closure and patterning, heart defects, skeletal and left-right patterning defects, hydrocephalus, obesity and cancer (Kuehn et al., 2007; Kennedy et al., 2007; Mans et al., 2008; Michaud & Yoder, 2006; Plotnikova et al., 2008; Wong et al., 2009; Han et al. 2009; Slough et al., 2008; reviewed in; Davenport and Yoder, 2005; Christensen et al., 2008; Pan J, 2008; Lehman et al., 2008; Berbari et al., 2009; Veland et al., 2009). Although some of the overall pathways are known, our understanding of the detailed mechanisms by which the cilium controls cell organization and function is still rudimentary.

This thesis gives novel insights into the function of primary cilia in stem cell differentiation and in coordinating the complex events taking place in the early heart development. The work was carried out in part by investigating the role of the primary cilium in coordinating Hedgehog (Hh) signaling and in promoting the differentiation of mouse embryonal carcinoma (EC) stem cells (P19.11.6) into beating cardiomyocytes, and in part by investigating defects in heart development in Ift88−/− mouse embryos, which have defects in ciliary assembly. Further, preliminary data obtained with chimera mice studies with Ift88−/− stem cells support the thesis hypothesis that primary cilia are critical in heart development. As a second objective, this thesis also presents novel findings on the role of primary cilia in coordinating Hh signaling in human pancreatic development and postnatal tissue homeostasis as well as the potential role in progesterone signaling in motile cilia of the human and mouse oviduct in coordinating post ovulatory events. A more detailed description of my thesis objectives is listed in chapters 1.2 and 1.3.

Chapter 2 of this thesis is an introduction to primary cilia, ciliary signaling mechanisms in health and disease, the murine heart physiology, heart development, stem cells, female reproductive organs and the pancreas, which serve as background for the chapters 3 and 4 that discuss the primary and secondary objectives in the thesis respectively. The introduction contains unpublished data and observations made during the work period.

1.2 THESIS PRIMARY OBJECTIVE

Heart development, which includes the formation of the cardiac crescent, linear heart tube, heart looping, chamber formation and septation/maturation of the young heart, is regulated by a series of various signaling pathways that mediate or interact with progenitor cells to expand, migrate, differentiate and ultimately integrate into the forming heart. The signaling pathways in heart development include Hedgehog (Hh), Wingless/INT (Wnt), fibroblast growth factor (FGF), transforming growth factor (TGF)-beta, platelet-derived growth factor (PDGF) and bone morphogenic protein (BMP) signaling.
The main objective of this thesis was to investigate the role the primary cilium in coordinating Hh signaling in stem cell differentiation and heart development in the mouse. Initially, primary cilia were characterized by immunofluorescence microscopy (IFM) and electron microscopy (EM) analysis in cultures of human embryonic stem cells (hESC), and we show that primary cilia are associated with regulation of Hh signaling in these cells [3].

The focus of my thesis was then to investigate the function of the primary cilium in coordinating Hh signaling and differentiation of P19.CL6 EC stem cells into beating clusters of cardiomyocytes. In this work, a full characterization of how P19.CL6 EC stem cells in cultures differentiate under normal conditions was carried out before experiments on ciliary knockdown and inhibitory chemicals on signaling pathways could be tested. This included studies on heart transcription factors, stem cell markers and morphological analysis on the clustering cardiomyocytes using western blot (WB), quantitative real-time-PCR (Q-RT-PCR) and immunofluorescence microscopy (IFM) analysis. Electroporation with plasmids expressing siRNAs against Ift88 and Ift20 was used to knock down key proteins in ciliogenesis in order to analyze the significance of the primary cilium in early heart development in vitro. Furthermore, the role of primary cilia in heart development was analyzed in vivo. This was carried out partly by investigating heart defects in \textit{Ift88}/- mice that lack or have severely stunted primary cilia, and partly by studying the development of the heart in chimera mice with injected wild type (wt) and \textit{Ift88}/- mouse embryonic stem cells (mESC) in order to clarify whether primary cilia are required for heart development. In parallel to the P19.CL6 cardiomyocyte differentiation experiments, a few preliminary studies on \textit{Ift88}/- and wt ES cell differentiation into cardiomyocytes were conducted to get a broader perspective on the role of the primary cilium in cardiomyogenesis.

List of the cell types and animals used in the primary thesis objective:

- Human embryonic stem cells (hESC) (Chapter 7: [3]).
- Mouse embryonal carcinoma (mEC) stem cells (P19.CL6) (Chapter 7: [4-5]).
- \textit{Ift88}/- and wt mouse embryonic stem cells (mESC) (preliminary data, not shown).
- E11.5 wt and \textit{Ift88}/- mouse embryos (Chapter 7: [4]).
- Mouse Chimera with \textit{Ift88}/- and wt ES injects (preliminary data shown in Chapter 3: section 3.4).

1.3 Thesis secondary objective

The secondary objective of my thesis emerged because of collaboration with two students, Sonja K. Brorsen and Stefan C. Teilmann who worked on the sensory function of cilia in the pancreas and in the female reproductive organs in the laboratories of Drs. Søren T. Christensen, Kjeld Møllgård and Anne Grete Byskov. The work with Sonja K. Brorsen focused on the function of primary cilia in Hh signaling during development of the exocrine duct of the human pancreas and how aberrant Hh signaling may be associated with primary cilia in pancreatic cancer. This work was carried out partly by performing IFM analysis on tissue sections of the developing human pancreas and partly by IFM and WB analysis of human pancreatic duct adenocarcinoma cell lines. The work with Stefan C. Teilmann included IFM and WB analysis on the expression and localization of progesterone receptors in human and mouse female reproductive organs with special focus on changes in the localization of the receptors to motile cilia of the oviduct upon ovulation in the mouse. This work would help understand how post ovulatory responses are coordinated in the oviduct and how motile cilia could play a part of this regulation.

List of the cell types and tissues used in the thesis secondary objectives and related articles:

- Human pancreatic duct adenocarcinoma PANC-1 and CFPAC-1 cell lines and NIH3T3 cells (Chapter 7: [2]).
- Tissue sections from 7.5-week-old human embryos and 14- and 18-week-old human fetuses [2].
- Tissue sections from human and mouse oviduct and ovary (Chapter 7: [1]).
CHAPTER 2 - INTRODUCTION AND BACKGROUND

2.1 CILIARY STRUCTURES AND FUNCTIONS

Cilia are membrane-bounded, centriole-derived projections from the cell surface that contain a microtubule (MT) cytoskeleton, the ciliary axoneme, surrounded by a ciliary membrane (Satir and Christensen, 2007) (Figure 1). The microtubule cytoskeleton of the cilium, the axoneme, grows from and continues the ninefold symmetry of the centriole that becomes a ciliary basal body. Ciliary axonemes are formed with two major patterns: 9+2, in which the nine doublet microtubules surround a central pair of singlet microtubules, and 9+0 cilia, in which the central pair is missing. Most often, 9+2 cilia are motile; motility being regulated by axonemal inner and outer arm dyneins that coordinate ciliary beat frequency and form, respectively (Brokaw & Kamiya, 1987; Satir 1998; Christensen et al., 2001). In contrast, 9+0 cilia usually lack axonemal dynein arms and are consequently non-motile (Satir and Christensen, 2008). Nodal cilia, like primary cilia also possess 9+0 axonemes, but nodal cilia have dynein arms with LR (left/right) dynein (Supp et al., 1999) and are motile, generating a leftward flow across the node required for establishment of the left–right asymmetry axis (Hirokawa et al., 2006). Motile 9+2 cilia are found in a wide range of organisms spanning from single celled organisms to humans, in which they are found lining the airway epithelium (Jeffery & Reid, 1975), the brain ventricles (Cathcart & Worthington, 1964), the ependyma/choroid plexus (Wodarczyk et al., 2009) and the oviduct epithelium (Boisvieux-Ulrich et al., 1989). In ciliates, e.g. Tetrahymena thermophila, the motile cilia are primarily used for swimming and to collect food particles from the surrounding environment. Mammalian 9+2 motile cilia may also function as a sensory organelles (Christensen et al., 2007) such as in the oviduct (Teilmann & Christensen, 2005; [1]) and in the airway epithelium,
where motile cilia possess sensory bitter taste receptors (Shah et al., 2009). In contrast to motile 9+2 cilia, primary cilia are solitary organelles that project from the centrosomal mother centriole at the surface of stem cells and most growth-arrested cells in our body (Satir and Christensen, 2007) (Figure 2). Further, primary cilia lack radial spokes and the central sheath surrounding the central microtubule pair in 9+2 motile cilia (Satir and Christensen, 2008). As will be discussed in the below, primary cilia are thought to function as unique mechano-, osmo- and chemosensory organelles, which enable the cells to interact and communicate with the surrounding environment via the primary cilium to control cell cycle entry, migration and differentiation during development and in tissue homeostasis.

![Table Examples of mammalian tissues and cells with primary cilium](http://www.bowserlab.org/primarycilia/cilialist.html)

**Figure 2.** The figure shows examples of mammalian tissues and cell types that have a primary cilium. For a more thorough list see the reference.

### 2.1.1 CILIARY ASSEMBLY AND MAINTENANCE

Both primary and motile cilia are assembled and maintained via a highly conserved process called intraflagellar transport (IFT) (reviewed in; Rosenbaum & Witman, 2002; Pedersen & Rosenbaum, 2008) first discovered in the green algae *Chlamydomonas reinhardtii* by Kozminski and co-workers in 1993 (Kozminski et al., 1993). Transmembrane proteins as well as axonemal components are transported in vesicles via the Golgi-complex along microtubules using the cytoplasmic dynein 1 motor to the base of the cilium (see figure 3). It is proposed that Ift20 (a complex B particle) and GMAP210 (a golgin anchor protein) function at the Golgi-complex to sort proteins into vesicles destined for the cilium, where Ift20 reside in the vesicles and GMAP210 stays in the Golgi-complex. At the base of the cilium, Ift20 on the vesicles interacts with the Ift54 (a subunit of IFT complex B) to form the complete IFT complex (Follit et al., 2009) however knockdown of the Ift20 gene reduces ciliary assembly without affecting Golgi structure (Follit et al., 2006). Ift20 was shown to coordinate Wnt signaling and cell proliferation required for proper positioning of the centrosome in non-dividing cells and for correct orientation of the mitotic spindle in kidney collecting duct epithelium cells (Jonassen et al., 2008).
The complete IFT complexes with the cargo destined for the cilium rendezvous at the base of the cilium where they connect with ciliary motor proteins. At the “ciliary necklace”, only proteins (or protein complexes) with a ciliary targeting motif can enter the zone or “pore complex” created by the transition fibers (Gilula & Satir, 1972; Rosenbaum & Witman, 2002). The anterograde transport of protein/IFT complexes is mediated by kinesin-II along the ciliary axoneme to the ciliary tip along with inactive cytoplasmic dynein 2. In addition to kinesin-2, motor proteins belonging to other kinesin families may contribute to ciliary structural and functional diversity (reviewed in; Scholey, 2008). At the ciliary tip, turnover products are switched over to cytoplasmic dynein 2 for retrograde IFT transport back to the basal body region to re-enter the cytosol. Irf88, a subunit of the IFT particle complex B, is required for both anterograde and retrograde IFT (Pazour et al., 2000; Murcia et al., 2000; Haycraft et al., 2001; Taulman et al., 2001; Yoder et al., 2002b; Lucker et al., 2005). Kif3 motors (comprising of Kif3a and Kif3b subunits) are a functionally diverse subgroup of the kinesin super family, characterized by an NH2-terminal motor domain (N-IV class) and forms a complex with the non-motor protein KAP3. Together they are responsible for MT-based anterograde transport to membranous organelles including cilia (Yamazaki et al., 1995; Hirokawa, 1998; Harauzchi et al., 2006). A way to stop ciliogenesis and thereby ciliary functions is by using knockout or knockdown of IFT particles. Two well-known IFT particles that have been used to disrupt ciliary assembly, includes Ift88 (Pazour et al., 2000; Murcia et al., 2000; Haycraft et al., 2001; Taulman et al., 2001; Yoder et al., 2002b; Lucker et al., 2005; Schneider et al., 2005) and Ift20 (Follit et al., 2006; 2008; 2009) that leave no or severely stunted cilia.

2.1.2 Introduction to ciliary signaling pathways and ciliopathies

The ciliary membrane consists of a bilayer lipid membrane that is continuous with the plasma membrane of the cell body, but which contains a different complement of membrane receptors and ion channels. As outlined in the above it is now evident that primary cilia play a major role in coordinating a series of signal transduction pathways in cell cycle entry, migratory responses and differential processes. These pathways include Hedgehog (Hh), Wingless/INT (Wnt), neuronal and purinergic receptors as well as signaling through the transient receptor potential (TRP) ion channels, receptor tyrosine kinases (RTK) and extracellular matrix communication (12).
Since primary cilia are critical in regulation of signaling pathways in behavioral responses and cellular processes during development and in tissue homeostasis, lack of normal functioning primary cilia causes various diseases now commonly known as ciliopathies. These include cystic kidney and liver diseases, retinal degeneration, abnormalities in neural tube closure and patterning, heart defects, skeletal and LR patterning defects, hydrocephalus, obesity and cancer (Kuehn et al., 2007; Kennedy et al., 2007; Mans et al., 2008; Michaud & Yoder, 2006; Plotnikova et al., 2008; Wong et al., 2009; Han et al., 2009; Slough et al., 2008; reviewed in; Davenport & Yoder, 2005; Christensen et al., 2008; Pan, 2008; Lehman et al., 2008; Berbari et al., 2009; Veland et al., 2009).

One of the first diseases to be related to dysfunctional primary cilia, was polycystic kidney disease (PKD) found in mice mutated in the gene encoding the Ift88/Tg737/Polaris protein in the Oak Ridge Polycystic Kidney mouse (ORPK mouse, or currently designated Ift88Tg737Rpw), (Moyer et al., 1994; Pazour et al., 2002; Yoder et al., 2002a; Pazour et al., 2004; Lehman et al., 2008). In Chlamydomonas, Ift88 mutants showed defective ciliogenesis, and it was established that cilia of the mouse kidney were also abnormally short or missing, which suggested that PKD might be a ciliary disease (Pazour et al., 2000). The Tg737ORPK mouse was induced by insertional mutagenesis integrated into an intron near the 3’ end of the Tg737 gene thereby partially disrupting the expression and function of the Ift88 protein. The hypomorphetic allele of Ift88 in the ORPK mouse makes this mouse a good model to study the role of primary cilia since the animals are viable into young adulthood compared to the Ift88-/null
mice (Ift88tm1Rpw, Ift88tm1.1Bky, and Ift88fxo), which are embryonic lethal around the beginning of organogenesis (Lehman et al., 2008). The core phenotypes of the Tg737ORPK mouse was originally described as a triad of the following: scruffy fur, severe growth retardation, and preaxial polydactyly on all limbs (Moyer et al., 1994). The Tg737ORPK mouse revealed another very significant phenotype which became the best known phenotype, the cystic renal phenotype which resembles that of human autosomal recessive polycystic kidney disease, which is characterized by extensive cystic enlargement of both kidneys that fail to concentrate the urine (see figure 5). This experimental mouse was also the first mammalian model to establish a connection between ciliary dysfunction and cystic kidney disease (Pazour et al., 2000; 2002; Taulman et al., 2001). Loss of cilia function in the Tg737ORPK mice also revealed additional phenotypes such as hepatic and pancreatic ductal abnormalities and cysts, retinal degeneration, skeletal defects, cerebellar hypo-plasia, hydrocephalus, respiratory defects, infertility, situs inversus and heart defects (Moyer et al., 1994; Pazour et al., 2002a; Cano et al., 2004; Banizs et al., 2005; Zhang et al., 2005; Chizhikov et al., 2007; Haycraft et al., 2007; Hildebrandt & Otto, 2005; Hildebrandt & Zhou, 2007).

Since primary cilia are involved in a wide range of signaling pathways controlling and coordinating cellular responses new ciliopathies are frequently added to the list. In embryogenesis, signaling through the primary cilium is necessary for normal development in e.g. PDGF-R and Hh signaling pathways, probably because such signaling regulates the balance between cell division, polarity, migration, differentiation and apoptosis for many tissues (Schneider et al., 2005; Rohatgi et al., 2007; reviewed; Singla & Reiter, 2006; Michaud & Yoder, 2006; Christensen et al., 2007; Christensen & Ott, 2007). More specifically in cell migration, the primary cilium was proposed to function as a cellular GPS that orients towards the leading edge of the cell and in parallel to the migration path (Christensen et al., 2008). In terms of PDGF-Rαα signaling, PDGF-Rα is translocated to the cilium where activation of the receptor by homodimerization with its specific ligand, PDGF-AA, induces the activation of the Mek1/2-Erk1/2 and Akt pathways in the cilium or centrosomal region to control changes in cytoskeletal proteins partly via activation of the Na+/H+ exchanger, NHE1, at the leading edge (Schneider et al., 2005; 2009a; 2009b). In Ift88/- null fibroblasts without primary cilia, chemotaxis towards PDGF-AA is blocked, leaving the cells blindfolded to coordinate their migration in early wound healing in vivo.

### 2.1.3 Hedgehog signaling and primary cilia in developmental processes

In mammals, Hh signaling is induced by three different ligands, Indian (Ihh), Desert (Dhh) and Sonic hedgehog (Shh). The Hh signaling pathway controls and maintains many steps in development and several studies have revealed that dysfunctional Hh signaling results in a wide range of developmental disorders (reviewed in; Wong & Reiter, 2008; Simpson et al., 2009; Veland et al., 2009). Some examples where Hedgehog signaling is important for proper development are in LR asymmetry (Tsukui et al., 1999), skeletogenesis and digit patterning in the limbs (Johnson et al., 1994; Gouttenoire et al., 2007; Haycraft et al., 2007; Bastida et al., 2009), neural tube...
formation (Gorivodsky et al., 2008), cerebellar development (Chizhikov et al., 2007; Spassky et al., 2008), mammary gland development, ovarian function (Johnson et al., 2008) and development of the lung (Belusci et al., 1997; Rutter et al., 2009), the heart (Washington Smoak et al., 2005; [4]) and the pancreas ([3]; Bailey et al., 2009). Besides coordinating development, Hh signaling plays a pivotal role in cancer formation and generation of tumors. Indirect activation of Hh signaling in a subset of epithelial cancers; e.g. colon, pancreatic, and ovarian cancer can promote tumor growth by activating Hh signaling in the surrounding stroma, which then provides a more favorable environment for the developing tumors. This is why the Hh signaling pathways is a therapeutic target in cancer where manipulation of the Hh pathway potentially can delay or cure cancers. The Hh pathway is already being used in therapy and preclinical studies in addition to clinical trials, which are underway in a range of malignancies (reviewed in; Theunissen & Sauvage et al., 2009; O'Toole et al., 2009). The primary cilium is associated with regulation of Hh signaling and is also present on human tumors e.g. in basal cell carcinomas (BCCs) which are frequently ciliated. Removal of the primary cilium in these tumors strongly inhibited BCC-like tumors induced by an activated form of Smoothened. On the other hand, removal of cilia accelerated tumors induced by activated Gli2. Somehow, there is a dual role for primary cilia controlling Hh signaling which can then either mediate or suppress tumorigenesis depending on the oncogenic initiating event (Wong et al., 2009).

The general mechanism by which Hh works in vertebrates, is by the binding of the Hh ligand to the transmembrane receptor patched (Ptc) which thereby abolishes the inhibitory effect of Ptc on Smoothened (Smo), a seven-transmembrane receptor. Complete loss of Ptc activity turns the Hh pathway fully on even in the absence of Hh ligands (Ingham & McMahon, 2001). Following the loss of Ptc activity, Smo is able to transduce a signal via Gli transcription factors to the nucleus that initiate expression of Hh target genes. The activity of Smo is essential for any response either to Hh or to loss of Ptc activity, which indicate that Smo acts downstream of Ptc (reviewed by; Kalderon, 2005; Varjosalo & Taipale, 2008). There exist three Gli transcription factors, Gli1-3, where Gli1 functions as a constitutive activator (Hynes et al., 1997; Ruiz I Altaba, 1999; Liu et al., 2005). In contrast, Gli2 and Gli3 have an N-terminal transcriptional repressor domain and a C-terminal transcription activator domain. The proteolytic events that switch between the activating and repressing form of Gli2 and Gli3 are controlled by Smo (Huangfu et al., 2006; Pan et al., 2006). Hh signaling plays a critical role in establishing the LR asymmetry axis and proper heart tube looping during gastrulation, as well as maintaining the adult coronary vasculature and survival of small coronary arteries and capillaries (Lavine et al., 2008). The LR axis is initiated at the Hensen’s node of the mouse at E7.75 where two populations of nodal cilia coexist (McGrath et al., 2003); 1) the first are motile cilia with a mixture of 9+2 and 9+0 cilia containing the outer arm dyneins, called left–right dynein (lrd), which generate a left-ward fluid flow at the node (Supp et al., 1997; Caspary et al., 2007; review; Basu & Brueckner, 2008), 2) the second are non-motile cilia with a 9+0 microtubule architecture that are located around the edge of the node, which functions as mechano sensors and/or chemo sensors via the cation channel polycystin-2 in the ciliary membrane. The non-motile cilia respond to the nodal flow generated by the motile cilia which initiate a Ca^2+ response in the cells at the left border of the node (McGrath et al., 2003). Within the Hensen’s node, LR asymmetry is initiated by asymmetric expression of activinβB that inhibits Shh expression in the right portion of the node and thereby allowing its expression in the left. Here it diffuses into the adjacent lateral plate mesoderm and induces Nodal expression (Wagner & Siddiqui, 2007). Consequently, Shh mutants show severe effects on cell survival in the pharyngeal arch and neural crest, in addition to reduced size of the right ventricle (RV) and out flow tract (OFT) and delayed Nkx2.5 expression and heart development, thus suggesting direct effects of Shh on the second heart field (SHF) specification, proliferation or deployment (Zhang et al., 2001).

The primary cilium has been proposed to act as a key regulator of Hh signaling (Kovacs et al., 2008; for reviews; Eggenschwiler & Anderson, 2007; Christensen & Ott, 2007; Wong & Reiter, 2008). In many cell types the essential Hh signaling components Gli2, Gli3, and Smo localize to the primary cilium and transports actively together with the IFT complexes, e.g. in fibroblasts (Haycraft et al., 2005; Rohatgi et al., 2007; 2009), epithelial cells in renal tubules (Harris & Torres, 2008) and the exocrine duct of the pancreas [2] as well as in human embryonic stem cells ([3]; Breunig et al., 2008). In these cells the Smo and Ptc was found to enter and leave the
cilium upon stimulation with Hh ligand, see figure 6. The binding of ligands to Ptc in the cilium may activate the Hh pathway by removal of Ptc from the ciliary compartment and in that process, allowing Smo to enter the cilium and hereby coordinating the proteolytic events of the Gli2 and Gli3 transcription factors (Rohatgi et al., 2007; Wong & Reiter, 2008). In these events it has been proposed that the primary cilium may function as a switch by which the cells can regulate Hh signaling during development and tissue homeostasis (Corbit et al., 2005; Rohatgi et al., 2007). Suppressor of fused (Sufu) is a major negative regulator of Hh signaling in vertebrates (Cooper et al., 2005; Svard et al., 2006) and is taking part in the regulation of protein levels of full-length Gli transcription factors. Sufu has been found to localize to the primary cilium and in the nucleus/ cytosol (Haycraft et al., 2005), where it directly interact with the Gli transcription factors (Dunaeva et al., 2003). A possible hypothesis was that Sufu could regulate Gli proteolysis and generation of activator forms in the cilium in coordination with Smo, but recent data suggest that the regulation of Gli protein levels by Sufu is cilium-independent. The generation of Gli activator forms might still be a cilium dependent process that is regulated by a Smo mediated mechanism, but where Sufu controls ubiquitination of Gli proteins via the speckle-type POZ protein, Spop. This is a new role of mammalian Sufu in controlling Gli protein stability that is important for understanding ciliary Hh signaling and how it is regulated (Jia et al., 2009; Chen et al., 2009; Ruel & Thérond, 2009).

Figure 6. Ciliary Hh signaling mechanisms. The binding of Hh to Ptc1 in the cilium abolishes inhibition of Smo. Smo enters the cillum in contrast to Ptc1 leaving the cilium for degradation in the cytoplasm. With Smo active in the cilium it has been proposed that it may coordinate the proteolytic events that favor the Gli2 and Gli3 full length activator forms. The Gli2-3A then translocate to the nucleus and initiate transcription of Hh response genes (Ptc1 and Gli1). In mammals, Smo is thought to inhibit Suppressor of Fused (Sufu), a negative regulator of the Hh pathway, leading to activation of target-genes through the Gli transcription factors.

2.2 STEM CELLS

Stem cell research is a very important field of study with the purpose of gathering information on how to use stem cells as a therapeutic tool in regenerative medicine and as a model of human development. Stem cell transplantations are seen as a possible cure for Alzheimer's disease, cancer, neurodegenerative disorders and in regeneration of the heart in patients with myocardial infarction, which is characterized by irreversible loss of cardiomyocytes leading to heart failure (Guillaume & Zhang, 2008; Song et al., 2009). The use of hESCs in differentiation experiments in vitro will help identifying new gene targets for drugs in tissue regeneration therapies. However, many key elements in stem cell signaling and differentiation are still not known and will need to be clarified before a wide spread use of stem cells can be trusted in regenerative medicine. The Geron Corporation is the first company in the world given clearance (Jan. 23rd -2009) for clinical trials on humans with
hESC derived cells, where spinal cord injuries can be treated with oligodendrocyte progenitor cells injected into the lesion.

Stem cells are found in most multi-cellular organisms and are characterized by the ability to self-renew through mitotic cell division and differentiate into any cell type (Smith, 2001). Embryonic stem cells (ESC) are pluripotent, which mean that they have the capacity to generate derivatives of all the three embryonic germ layers: the ectoderm, mesoderm and endoderm. The ectoderm contribute to the central nervous system, the lens of the eye, the ganglia and nerves, pigment cells, head connective tissues, the epidermis, hair, and mammary glands. The mesoderm forms skeletal muscle, bone, connective tissue, the heart, blood, and the spleen. The endoderm forms the gut and lung tissue, the liver, pancreas, the urinary bladder, the thyroid and more (Chandros et al., 2001). Pluripotent stem cells occupy the inner cell mass of the early blastocyst during embryonic development (Lensch, 2009), see figure 7.

The internationally recognized gene markers to characterize hESCs for determining if the cells are in an undifferentiated state are: **NANOG, TDGF, POU5F1, GABRB3, GDF3** and **DNMT3B**. No hESC lines reported of today have tested negative for these six markers (Adewumi et al., 2007), provided by the International Stem Cell Initiative, ISCI. In mESC three important transcription factors have been identified for regulating pluripotency, namely Oct4, Sox2 and Nanog. All of these transcription factors are highly expressed in the inner cell mass, epiblast and in undifferentiated mESC (Pesce & Scholer, 2001; Niwa, 2007). Null mutations of each of the three genes cause early embryonic lethality due to the inability to maintain cells in a pluripotent state (Nichols et al., 1998; Avilion et al., 2003; Mitsui et al., 2003). Oct4 by itself, induces differentiation of ES cells through Cdx2 and eomesodermin if the expression of Oct4 is reduced by 50% (Niwa et al., 2000; 2005), and Sox2 RNAi silencing results in ES cell differentiation into multiple lineages (Ivanova et al., 2006). Sox2 can also interact synergistically with Oct4 in vitro to activate Oct–Sox enhancers, which in turn can regulate Nanog, Oct4 and Sox2 themselves (Masui et al., 2007). It is therefore important that Nanog, Oct4 and Sox2 are closely regulated since changes in their expression rates have dire consequences for controlling stem cell pluripotency and developmental processes (Niwa et al., 2000). Recent work has shown that stem cells possess primary cilia with signaling molecules and receptors that may be critical for stem cell renewal and differentiation ([3]: Awan et al., 2009). The following sections 2.2.1 and 2.2.2 describes in more detail the features of stem cells in developmental research.
2.2.1 EMBRYONIC STEM CELLS

It took 17 years after the first isolation of mouse ES cells before James Thomson and co-workers derived hESC from donated blastocysts from couples undergoing treatment for infertility (Thomson et al., 1998). The method used was almost the same as was used for isolating mESC. The trophectoderm (trophoblast, group of cells that produces no embryonic structures) was removed by immunosurgery, where the inner cell mass (ICM) was plated onto mouse embryonic fibroblasts to act as a feeder layer. Several groups had tried this approach but the culture media from the mESC protocol resulted in differentiation and not the derivation of stable pluripotent cell lines (Bongso et al., 1994). Some experiments with ES cell lines from two non-human primates: the rhesus monkey and the common marmoset (Thomson et al., 1995; 1996), gave the necessary experience to adjust the culture conditions to produce stable undifferentiated human pluripotent ES cells. mESC are different in many aspects compared to primate ES cells, particularly in their morphology and their ability to withstand dissociation into single cells (Pera et al., 1999).

Human ESC are karyotypically normal and have the capability to maintain the developmental potential to contribute to all of the three germ layers even after extended undifferentiated proliferation (Amit et al., 2000). After the first successful attempt to isolate stable hESCs (Thomson et al., 1998), others derived them from the morula and the blastocyst stage embryos (Stojkovic et al., 2004; Strelchenko et al., 2004), followed later by isolation from single blastomeres (Klimanskaya et al., 2006), and parthenogenetic embryos (unfertilized human eggs). (Lin et al., 2007; Mai et al., 2007; Revazova et al., 2007). It is still not known whether pluripotent cell lines derived from these various sources have any consistent developmental differences or whether they have an equivalent potential (Yu & Thomson, 2008).

Pluripotent stem cells are not present at all times in the developing embryo, since they rapidly differentiate into more specialized somatic cells. The first mESC lines were extracted from the ICM of a mouse blastocyst and then cultured on a mitotically inactivated fibroblast feeder layer with serum. These culturing conditions were adapted from the mESC cultures in vitro (Evans & Kaufman, 1981; Martin, 1981). ES cell cultures that are clonally derived from a single ES cell could then differentiate into a wide variety of cell types in vitro and form teratocarcinomas when injected into mice (Martin, 1981). In contrast to mouse embryonal carcinoma cells (mEC), mESC can differentiate into a variety of tissues in chimeras at high frequency, including germ cells, which give the possibility of introducing modifications to the mouse germ line (Bradley et al., 1984; Robertson, 1986). To culture mESCs several methods have been used. One is to culture the ES cells on feeder layers as described above; another is to culture them in conditioned media that are able to sustain the ES cells without growing them on feeder cells. This led to the identification of leukemia inhibitory factor (LIF), a cytokine that is a key factor to sustain ES cells in an undifferentiated state (Smith et al., 1988; Williams et al., 1988).

2.2.2 EMBRYONAL CARCINOMA (EC) CELLS

Embryonal carcinoma (EC) cells comprise a special class of tumor cells which have the ability to change phenotype from malignant into non-malignant via cellular differentiation. EC cells are derived from teratocarcinomas which is where the field of pluripotent stem cells arose from in the 1950s. Teratocarcinomas are found in the testes of mice and humans that occur from defective germ cells (Stevens & Little, 1954; van der Heyden et al., 2003). In 1964, Kleinsmith and Pierce showed that single EC cells are capable of self-renewal and differentiation into multiple cell lineages and hereby establishing the existence of pluripotent stem cells (Kleinsmith & Pierce, 1964). This provided the intellectual basis for more advanced studies of both mouse and human ES cells and was also the first experimental demonstration of a cancer stem cell (Yu & Thomson, 2008). In the 1970s, stable mouse EC cell lines could be cultured in vitro and used for studies in development that could not be carried out with intact mammalian embryos (Kahan & Ephrussi, 1970). On the other hand, most EC cell lines have limited developmental potential and contribute poorly to chimera mice studies (see section 3.4), properly due to the accumulation of genetic changes during teratocarcinomas formation and growth (Atkin et al., 1974). But still mouse EC cells, compared to human EC cells, are more useful because the human EC cells are highly aneuploid (have abnormal number of chromosomes), which might explain why they can’t differentiate.
into a wide range of somatic cell types, which limits the use for studies of human development in vitro (Yu & Thomson, 2008; Kennedy et al., 2009).

The P19 cell line, a murine EC cell, is an undifferentiated stem cell that originates from a teratocarcinoma (Martin, 1980). As a stem cell, it is able to differentiate into all three germ layers by culturing the cells in suspension with several chemical inducers. With addition of high concentrations of retinoic acid (RA, 0.1-1µM), the cells can differentiate into neurons and glia (McBurney et al., 1982) or with low concentrations of RA (1-10nM) or dimethyl sulfoxide (DMSO) (0.5-1%) the cells can differentiate into cardiac and skeletal myocytes (McBurney et al., 1982; Edwards et al., 1982). Because of the multipotential abilities of P19 cells, this cell line is an often used model system to study early heart differentiation in vitro. To improve on the P19 cells ability to differentiate into cardiomyocytes, a clonal line was isolated from the P19 cells, called CL6 (Habara-Ohkubo, 1996). This P19.CL6 sub line efficiently differentiates into cardiac muscle with the addition of 1% DMSO in adherent culture (Habara-Ohkubo, 1996). Unlike the P19 cells that depend on aggregate formation in suspension (Campione-Piccardo, 1985), the CL6 cells can be cultured without aggregation and feeder cells. How the CL6 cells effectively differentiate into beating muscle in adherent rather than suspension culture is unclear, but aggregate structures that resemble embryoid bodies are observed during the multilayer sheet formation during the differentiation into cardiomyocytes (Habara-Ohkubo, 1996). Although CL6 cells are thought to be morphologically similar to P19 cells, only CL6 cells express the mesodermal marker gene Brachyury but not the stage-specific embryonic antigen-1, SSEA-1, which is a cell surface embryonic antigen whose loss of expression characterizes the differentiation of murine EC cells (Habara-Ohkubo, 1996; Uchida et al., 2007). Moreover, CL6 cells differentiate into neurons at a much lower frequency than P19 cells, which is why it was suggested that the CL6 cells are not committed to the mesoderm but represent a developmental stage closer to differentiated cardiomyocytes than P19 cells (Habara-Ohkubo, 1996). Further, P19.CL6 cells are quite sturdy and are easily electroporated in siRNA knockdown experiments. For these reasons, we used cultures of the P19.CL6 EC cell line to study the role of the primary cilium in cardiogenesis.

2.3 HEART DEVELOPMENT

The heart is among the most studied of all organs but also the one most susceptible to disease. Early heart development in vertebrates is a complex process initiated in embryos shortly after gastrulation, where cardiomyocyte progenitor cells aggregate and become allocated from the mesodermal population which migrate and organize into the cardiac crescent. Hereafter the cardiac crescent will develop into a beating linear heart tube, the first functional organ of the developing embryo, as a result of migration and fusion along the ventral midline of the precursor cells from the cardiac crescent (Sucov, 1998; Nemer, 2008). The heart is not only composed of muscle cells but also contain a wide range of cell types such as atrial/ventricular cardiac myocytes, conduction system cells, smooth muscle/endothelial cells of the coronary arteries and veins, valvular components, endocardial cells and connective tissue (Laugwitz et al., 2008). Three major sources of heart cell precursors have been identified in the mouse embryo: the cardiogenic mesoderm, the proepicardial organ and the cardiac neural crest. These three sources represent a distinct pool of progenitor cells where the cardiogenic mesoderm gives rise to the linear heart tube and the myocardium in the ventricular and atrial chambers, see figure 8. The proepicardial organ and the cardiac neural crest gives rise to the epicardial mantle, which later contributes to the coronary vasculature and to the vascular smooth muscle of the aortic arch, ductus arteriosus respectively (Laugwitz et al., 2008). It is critical that regulation of these different cell progenitors is under the strict control so that the correct cell lineages differentiate at the correct time and in the correct location (Bruneau, 2008). Many signal transduction systems are implicated as essential coordinators of early cardiogenesis, including Hh, Wnt, BMP, FGF, PDGF and (TGF)-beta signaling pathways (Washington Smoak et al., 2005; Kwon et al., 2008; Hirata et al., 2007; Rochais et al., 2009; van Wijk et al., 2007). These signaling pathways
control multiple genes that are expressed throughout the cardiomyocyte population prior to the fusion of the linear heart tube and remain expressed hereafter.

Figure 8. Schematic diagram illustrating the origin and lineage relationships of cardiac cell types in mouse development. A: Three sources contribute in heart development with progenitor cells during cardiac morphogenesis in the mouse: the cardiogenic mesoderm (red), the cardiac neural crest (CNC, purple) and the proepicardial organ (yellow). Early in development at E7.5, progenitor cells of the cardiogenic mesoderm are recognizable under the head folds (HFs) of the embryo, which then move ventrally to the midline (ML) and form the linear heart tube and finally the four chambers of the heart. After the looping of the heart tube at E8.5, cardiac neural crest progenitors migrate from the dorsal neural tube at E10.5 to engulf the aortic arch (AA) arteries and further contribute to the vascular smooth muscle cells of the outflow tract (OFT). Simultaneously the proepicardial organ precursors contact the surface of the developing heart and give rise to the epicardial mantle (yellow area around the heart at E10.5) and contribute later to the coronary vasculature. Abbreviations: PhA, pharyngeal arches; PLA, primitive left atrium; PRA, primitive right atrium; LV, left ventricle; RV, right ventricle. B: A display of cardiac cell types that arise through the lineage differentiation of the three embryonic precursor pools. The contribution of the proepicardium to the smooth muscle cells of the coronary system and to the mesenchymal cells of the heart is well accepted, the origin of the endothelial lineage in the coronary vasculature is still controversial. Modified from Laugwitz et al., 2008.

Congenital heart disease (CHD) is a common infant morbidity and arises from abnormal heart development during embryogenesis. CHD is reported to have 6 to 8 incidences per 1000 live births and approximately accounts for 3% of all infant deaths and 46% of deaths from congenital malformations. Further, cyanotic heart defects (a group-type of CHD where the patient appears blue “cyanotic”, due to deoxygenated blood bypassing the lungs and entering the systemic circulation) occur in about 6 per 100,000 live births in the United States. Cyanotic heart defects can be caused by right-to-left or bidirectional shunting, or mal-positioning of the great arteries. Also, the frequency of CHD in premature infants is 12.5 per 1000 live births (Sadowski, 2009). Stem cell regeneration of cardiac tissue may be a therapeutic tool in the future to save a large number of patients suffering from myocardial infarction. In order to transform stem cell therapy from idea to clinical use a lot of basic
knowledge is needed on how the heart signaling pathways interact, coordinate, initiate and maintain the developing/adult heart.

2.3.1 Heart fields and developmental stages

The cardiac crescent originates from cells in the cardiogenic mesoderm and is one of the earliest steps in cardiogenesis. The cardiogenic mesoderm consists of two populations or heart fields of cardiac precursor cells that contribute to different parts of the heart. The first heart field (FHF, the earliest), is located in the anterior splanchnic mesoderm, which primarily gives rise to the cardiac crescent, as well as to the linear heart tube and to parts of the atrial chambers and the left ventricular region later in development. The second heart field (SHF, also known as the anterior heart field) lies anterior and dorsal to the linear heart tube and is derived from the pharyngeal mesoderm medial to the cardiac crescent. Cells from this second heart lineage are added to the developing heart tube and give rise to the outflow tract, the right ventricular region and the main parts of the atrial tissue, see figure 9 (reviewed by Buckingham et al., 2005; Laugwitz et al., 2008).

The myocardium was thought to be derived from a single source of cells until recently. The identification of a second source of myocardial cells that contribute to the cardiac chambers has modified the classical view of heart formation. The SHF was first discovered in the chick (Mjaatvedt et al., 2001; Waldo et al., 2001; reviewed in Buckingham et al., 2005) and then in mouse (Kelly et al., 2001). In the mouse it was shown that a second source of myocardial cells in the pharyngeal mesoderm contributes to the outflow tract myocardium at the arterial pole of the heart. These cells initially lie medially to the cardiac crescent before assuming a position that is dorsal and anterior to the heart tube (Kelly et al., 2001). Later by using lineage-tracing CRE-LOXP recombination system experiments, results showed that the heart tube derived from the FHF may predominantly provide a scaffold upon which cells from the SHF migrate to and build the requisite cardiac chambers at the later stages in heart development (Meihac et al., 2004; Brown et al., 2004; Xu et al., 2004). The SHF is further subdivided into a number of lineage pools (Buckingham et al., 2005), which contribute either to anterior structures (such as the

Figure 9. Schematic diagram illustrating the early steps in heart development and with key transcription factors activation points at the different stages. The diagrams of the heart development are shown in ventral views. At the earliest stages of heart formation (cardiac crescent), two pools of cardiac precursors exist. The first heart field (FHF, in pinkish colour) contributes to the LV, and the second heart field (SHF, in bluish colour) contributes to the right ventricle (RV) and later to the outflow tract (OT), sinus venosus (SV), and left and right atria (LA and RA, respectively). Abbreviations: V, ventricle; A, Atria; PA, pulmonary artery; Ao, Aorta. Bottom half of the diagram show when the transcription factors are turned on. Figure modified from Bruneau, 2008 and Nemer, 2008.
OFT) or posterior components (such as the atria). These findings may explain how mutations associated with CHD, by only affecting specific cell lineages within the SHF result in defects in specific heart structures (Bruneau, 2008).

The initial steps to build a fully functional four chambered heart starts with ventral movement of cells from the cardiac crescent which combine into a linear heart tube (DeHaan, 1965) that consist of an interior layer of endocardial cells and an exterior layer of myocardial cells. At the linear heart tube stage, the heartbeat is initiated (Srivastava, 2006) and transcription factors initiate distinct segmental precursors of the OFT, atria, and ventricles (Srivastava & Olson, 2000). The heart tube continuously grows by division of myocardial cells and by the addition of cells to both poles of the heart (Buckingham et al., 2005). Around E9 in mouse development (heart looping stage), the outflow region swings to the right as the heart adopts a spiral form, which realigns the future ventricles into a left-right juxtaposition. The inflow portion of the heart moves in an anterior and dorsal direction such that the inflow and outflow complexes converge. The crude heart then undergoes considerable remodelling where the most highly proliferative cardiomyocytes are located along the outer surface of the heart, also termed the compact myocardium, which then thickens and becomes the myocardial wall (Sedmera & McQuinn, 2008). On the inside the cardiomyocytes organizes into trabeculae, a sponge-like layer of myocytes and finger like projections thought to enhance oxygen and nutrient exchange in the absence of a coronary circulation (Franco et al., 2006). Polarised growth of myocardial cells forms in a highly defined region, called the interventricular septum (IVS), which will divide the ventricles, encompassing the junction of future left and right ventricles. The sinuatrial (SA) and atrioventricular (AVC) nodes form within slow-conducting myogenic tissue of the inflow tract where the SA node becomes the cardiac pacemaker (Nanot & Douarin, 1977). In the AVC, endocardial cushions are the precursors of the tricuspid and mitral valves, while in the OFT they form a scaffold for the aorticpulmonary septum which divides the OFT into the aorta and pulmonary artery and forms the aortic and pulmonary valves. During the growth process of the cardiac epithelium another distinct cell lineage, the migrating cardiac neural crest cells, populate the heart through the outflow channel and contribute to the septation of the OFT into distinct vessels of the aortic and pulmonary arteries (reviewed in; Hutson & Kirby, 2007; Bruneau, 2008).

2.4 SIGNALING PATHWAYS IN HEART DEVELOPMENT

Hedgehog, Wnt, BMP, FGF, PDGFR and (TGF)-beta signaling coordinate heart development in part by activating essential early heart genes such as GATA binding protein 4 (Gata4), NK2 transcription factor related, locus 5 (Nkx2-5), myocyte enhancer factor 2C (Mef2C), cardiac actin, and desmin (Lyons, 1994). The Gata family transcription factors are zinc-finger proteins that play important roles in heart formation, e.g. in cardiac muscle and heart tube development at the ventral midline (Grepin et al., 1994; Kuo et al., 1997). In vertebrates, three Gata genes exist (Gata4-6), which are expressed in the heart (Molkentin, 2000; Molkentin et al., 2000a). Gata4-/null mice embryos have bilateral heart tubes (cardia bifida) and a reduced number of cardiac myocytes (Kuo et al., 1997; Molkentin et al., 1997), where Gata5-/null mutants are viable (Molkentin et al., 2000b). Never the less, homozygous Gata4-/null embryonic stem cells are able to differentiate into contractile myocytes in chimeric embryos, which suggests that the cardia bifida phenotype is related to an endoderm deficiency (Narita et al., 1997). Heart development studies in vitro show that Gata4 expression precedes that of Nkx2-5 which is also one of the earlier heart transcription factors, which are important for proper heart development and cardiomyocyte differentiation (Grepin et al., 1997). Nkx2-5 is a transcription factor with a homeobox domain, which is highly expressed in cells of both the FHF and SHF (Stanley et al., 2002) and continuously during cardiac development throughout adulthood (Lints et al., 1993). In mice, Nkx2-5 is required for terminal differentiation of cardiac myocytes and the expression is clearly crucial for the normal growth of the embryonic myocardium, which is visible in the poorly developed myocardium of mice lacking Nkx2-5. These mice do not grow beyond the earliest
stages of heart looping (Lyons et al., 1995; Tanaka et al., 1999) and show left ventricular and conduction system defects (Yamagishi et al., 2002; Jay et al., 2004). In cardiomyocyte differentiation, the Myocyte enhancer factor 2c (Mef2c) act as a cofactor for Gata proteins (Morin et al., 2000) during the cardiac, skeletal, and smooth muscle development (Skerjanc et al., 1998).

Positive inducers of cardiogenesis are BMP, FGF, Shh and Wnt-JNK (also known as the Wnt-polarity pathway, Wnt11), which are expressed in the mesoderm, endoderm and ectoderm. Inhibitory signals include Wnt ligands expressed in dorsal neural tube (Wnt-3a and Wnt-8c) via β-catenin, and anti-BMPs expressed in the axial tissues e.g. Noggin in the notochord (reviewed in; Brand, 2003; Wagner & Siddiqui, 2007), see figure 10. Collectively these positive and negative signals drive mesodermal cells to the cardiogenic cell lineage, presumably by inducing the expression of cardiogenic transcription factor genes (Wagner & Siddiqui, 2007; Rochais et al., 2009).
CHAPTER 3 – PRIMARY CILIA IN STEM CELL DIFFERENTIATION AND CARDOI GENESIS

3.1 Introductory Remarks

Without a doubt, intensive research in the last decade has revealed that the primary cilium plays a critical role in a wide range of developmental processes in mammals (reviewed in; Lehmann et al., 2008; Satir & Christensen, 2008; Veland et al., 2009, Berbari et al., 2009). This thesis presents new data that support the conclusion that primary cilia are critical organelles in heart development and stem cell fate. This chapter will discuss and summarize the novel data from the primary objective articles and will round up with some conclusions. In addition, some new preliminary data will be presented and taken into consideration in the Discussion. The articles for the primary objective are found in chapter 7 in the following sections: Collaborative work with Aashir Awan on hESCs and ciliary Hh signaling (Chapter 7: [3]) and Heart development in P19.CL6 cells and cardiomyocyte differentiation (Chapter 7: [4-5]).

3.2 Primary Cilia with Functional Hh Signaling in Human Embryonic Stem Cells

In our paper [3] we demonstrate for the first time that hESC in cultures form primary cilia with the characteristic 9+0 axoneme as evidenced by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and IFM analysis [3]. Further, we show that key components in Hh signaling, including Smo, Ptc and Gli2 localize to hESC primary cilia, and that stimulation with the Smo agonist, SAG, promotes the concerted movement of patched out of, and smoothened into, the primary cilium, in accordance with the hypothesis that primary cilium functions as a cellular switch in turning the Hh signaling pathway on and off (Christensen & Ott, 2007). In addition, SAG promotes the increased expression of Ptc1 and Gli1, which are the two immediate response genes upon Hh pathway activation [3]. These results support the conclusion that primary cilia are involved in the regulation and coordination of the first steps of hESC differentiation, and/or the maintenance of the undifferentiated state/self-renewal. Since hESCs hold promise for the treatment of many diseases and provide an excellent system for studying mechanisms involved in early human development, these findings provide the groundwork to determine specific aspects of early differentiation controlled by the machinery of primary cilia. This knowledge may ultimately reveal pathways for manipulation of hESC differentiation into specific cell and tissue lineages.

3.3 Primary Cilia and Hh Signaling in Stem Cell Differentiation and Cardiogenesis

Recently, Slough et al., (2008) showed that the embryo heart at E9.5 Kif3a-/- mice have abnormal heart development, indicating that primary cilia could coordinate processes in cardiac morphogenesis. However, since Kif3 family proteins regulate cellular processes in mammalian cells that are not necessarily related to the primary cilium (Teng et al., 2005; Haraguchi et al., 2006; Corbit et al., 2008), there was a need for a more
thorough investigation on the role of the primary cilium in early cardiogenesis and Hh signaling, which is critical in cardiomyocyte differentiation. In our paper [4] we demonstrate that primary cilia play a critical role in coordinating Hh signaling and cardiomyogenesis in P19.CL6 EC cells. Further, we show that E11.5 old embryos of the Ift88tm1Rpw (Ift88-/- null) mice, which form no cilia, have many different heart defects, supporting the conclusion that cardiac primary cilia are critical in early heart development, partly via coordination of Hh signaling.

To sum up on the mouse P19.CL6 EC differentiation studies [4], we showed that P19.CL6 stem cells form primary cilia, which have ciliary Hh components such as Ptc1, Smo and Gli2. This is the first discovery of primary cilia in this cell line. The mouse P19.CL6 EC cell line is of pluripotent lineage as evidenced by expression of the stem cell markers Sox2 and Oct4. Moreover, inhibition of the Hh pathway by KAAD-cyclopamine blocked DMSO-induced differentiation of P19.CL6 cells into beating clusters of cardiomyocytes by restraining the expression and nuclear localization of the heart transcription factors Gata4 and Nkx2-5. In addition, KAAD-cyclopamine inhibited Hh signaling in P19.CL6 cells, confirmed by a failed up-regulation of Gli1 and Ptc1 mRNA expression and nuclear localization of Gli1, Gli2 and Gli3. The Gli2-repressor protein levels was increased in the KAAD-cyclopamine treated cells in contrast to the DMSO-induced control cells, which had higher levels of full length Gli2 that may function as the activator form. These results suggest that Hh signaling is required for differentiation of P19.CL6 cells into cardiomyocytes, and that Hh signaling may be associated with primary cilia in P19.CL6 EC cells. However, recent data showed that treatment with KAAD-cyclopamine during aggregation in P19 EC cells does not inhibit the general up regulation of Gata4, BMP4, Brachyury T, Meox1 and Gli2 during cardiomyogenesis, but merely delays it (Gianakopoulos & Skerjanc, 2009). This interesting difference might be explained by the culturing conditions by aggregation in the P19 cells, which is supposed to initiate mesoderm induction. Somehow, P19 cells manage to initiate the early cardiomyogenesis in absence of Hh signaling, possibly by Wnt signaling pathways that may compensate and activate Gli2. This has been observed in P19 cells where Wnt3a induces skeletal myogenesis in aggregated P19 cells, which is then followed by up-regulation of Gli2 (Petropoulos & Skerjanc, 2002). The Wnt/β-catenin pathway is critical in regulation of cardiogenesis where precise timing is an important factor to coordinate specific cellular responses. Studies in mouse and zebrafish embryos, as well as in embryonic stem cells clearly demonstrate that the Wnt/β-catenin pathway plays distinct, even opposing, roles during various stages of cardiac development (Tzahor, 2007). Whether the P19.CL6 cells have already undergone mesoderm induction compared to the P19 cells is not known. However, the P19.CL6 cells are a sub clone from the original P19 cells and should be more prone to cardiomyocyte differentiation. Even though the P19.CL6 cells primarily differentiate into cardiomyocytes, they are not only a mesoderm driven cell line since they can also produce neurons, which are ectoderm derived cells, see figure 11.

To answer the question whether primary cilia are important in Hh signaling for driving P19.CL6 cells down the cardiogenic pathway, we used the nucleofector technique described in detail in chapter 7, article
The primary cilium was knocked down by Ift88 and Ift20 siRNA which both are IFT complex B proteins required for functional IFT and delivery of ciliary membrane proteins from the Golgi complex to the cilium respectively (Pazour et al., 2000; Lucker et al., 2005; Follit et al., 2006; 2009). Knockdown of Ift88 in P19.CL6 cells resulted in a reduced frequency of ciliated cells to about 30% and inhibited the expression levels of Gata4 and Nlx2-5 to about 40% of mock controls. Consequently the number of beating cardiomyocytes was reduced as well as the nuclear localization of Gata4 at day 12. The Ift88 siRNA transfected cells were Sox2 positive, indicating that knock down of the cilium maintains cells in their undifferentiated state which do not undergo apoptosis or differentiate into other cell lineages. Using a combination of both Ift88 and Ift20 siRNA we further reduced the number of ciliated cells and resulted in an additional decrease in the number of beating clusters of cardiomyocytes along with a more pronounced decrease in mRNA expression levels of Gata4 and Nkx2-5 and protein levels of Gata4, Nlx2-5 and α-actinin. The knockdown of Ift88 and Ift20 in P19.CL6 cells mimics the inhibitory response on Ptc1 and Gli1 expression levels as seen with KAAD-cyclopamine treatment at day 5 of differentiation. These data lead to the hypothesis that differentiation of P19.CL6 cells into cardiomyocytes is coordinated by the primary cilium and partly by regulation of Hh signaling. To test if this hypothesis was correct we performed microscopy analysis on the Ift88−/− mice. These mice display at E11.5 severe endocardial cushion defects, decreased trabeculation and increased pericardial space along with malformations of the OFT. Many of these phenotypes are observed in the Pkd2−/− and Kif3a−/− mice but not in the lrd−/− embryos, which indicate that the malformations of the heart are not due to defective left-right asymmetry, which is coordinated by the nodal cilia (Slough et al., 2008). The OFT malformations in our Ift88−/− embryos was not observed in Slough et al., (2008), possibly due to the time difference in embryonic development since they sacked the mice at E9.5 compared to our E11.5, or the sheer difference of the Kif3a−/− versus Ift88−/− cilia derived mice. Gli2 localizes to both primary cilia in the developing heart of wt embryos and in P19.CL6 cells, as in contradiction to Ift88−/− embryos where this localization is disrupted. Interestingly, the OFT phenotype of Ift88−/− embryos resembles the phenotype of Shh−/− mice embryos (Washington Smoak et al., 2005; Goddeeris et al., 2007), suggesting that primary cilia mediate Hh signaling responsible for correct OFT development and potentially in development of the other cardiac structures that are malformed in the Ift88−/− embryos. These results support the hypothesis, that the primary cilium is critical for proper development of the mammalian heart.

3.4 Heart development studied in Chimera Mice

The first embryonic mouse chimera was generated by aggregating two eight-celled embryos (Tarkowski, 1961). The result was a normal-sized mouse with tissue that was a mix of cells from both embryos. Chimera mice can also be generated by injecting foreign pluripotent ES cells into a mouse blastocyst, which allows the ES cells to differentiate into all tissue types. Homologous recombination of ES cells (Doetschman et al., 1988; Thomas & Capecchi, 1989) is a powerful tool to engineer and generate designer chimera mice that are mutated in genes of interest, where the effect of genetic changes can be analyzed (Tam & Rossant, 2003). Today, chimera embryos can be generated by injecting enzymatically tagged wt and mutant ES cells into a wt blastocyst. In order to investigate the function of the primary cilium in heart development in more detail, we performed analysis on chimera mice in further collaboration with Professors Bradley K. Yoder and Robert Kesterson from the University of Alabama at Birmingham, USA. Embryos were harvested at the preferred time points in mouse development and stained to visualize where mutant or wt ES cells contribute in specific tissue/organ of the chimera embryo. Figure 13 shows an example of enzymatically tagged wt and mutant Ift88−/− mouse ES cells that are cultured on feeder fibroblasts. Cultures like these can be trypsinized and separated into single cells followed by differential attachment culturing to separate the ES cells from the feeders. The single ES cells can then be collected and injected into mouse blastocysts. To assess the in vivo significance and relevance of the in vitro P19.CL6 data, we conducted chimera embryo studies generated by the method described above. The chimera mice were created by using the mouse ES cells shown in figure 12, which were injected into blastocysts. The mouse embryos were harvested at various time points in embryonic development and stained for wt and Ift88−/−.
ES cell contribution. To distinguish the contribution of the wt from the mutant \textit{Ift88}/- mESCs, the β-galactosidase gene reporter was incorporated into the wt cells (which stains cells blue), while the bacterial alkaline phosphatase reporter was used for the \textit{Ift88}/- mESC (which stain cilia mutant cells red). With this approach, tissues where primary cilia function is required should reveal no contribution of \textit{Ift88}/- cilia mutant cells. The distribution of cells was determined using whole mount embryos in addition to paraffin and cryofreeze sections of the embryos.

\textbf{Figure 12.} Light microscope images of enzymatically tagged mouse ES cells growing on fibroblast feeders and of E8.5 chimera mouse embryos. \textbf{Top row, from left to right:} wt mouse ES (\textit{Ift}zap) colonies that contain a \textit{LacZ} gene reporter that express the β-galactosidase enzyme that is thereby able to cleave X-gal substrate and consequently stain wt cells blue (inserted image: IFM with DAPI and anti-hnn showing a primary cilia: arrow). Hnn is an allele of Art13b, a small GTPase of the Art/Arl family, and the Art13b protein predominantly localizes to cilia (Caspary et al., 2007). Embryo showing only wt ES cell contribution after β-galactosidase and alkaline phosphatase assay with 8-10 injected cells of both wt (blue) and \textit{Ift88}/- (red) mESC. The arrows points at the ventricle region of the early heart. \textbf{Bottom row from left to right:} mutant \textit{Ift88}/- mESC colonies that contain a bacterial alkaline phosphatase gene reporter that stain \textit{Ift88}/- cells red in response to \textit{Ift88} deletion (inserted image: Immunofluorescence microscopy (IFM) with DAPI and anti-Hennin (hnn) showing missing primary cilia). Embryo showing only mutant \textit{Ift88}/- ES cell contribution after β-galactosidase and alkaline phosphatase assay with 8-10 injected cells of wt and \textit{Ift88}/- mESC. The arrows points at the ventricle region of the early heart. C. A. Clement, unpublished data made in collaboration with Bradley K, Yoder, Nickolas Berbari and Robert Kesterson).

In these preliminary studies, \textit{Ift88}/- ESCs (red) fail to contribute to the development of the heart chambers, in contrast to differentiated wt ESCs (blue), which clearly localize to the heart region (figure 13); wt contribution is additionally seen in other parts of the developing embryo such as the brain. On the other hand, the mutant \textit{Ift88}/- ESC contribution localize to the tissue surrounding the heart chambers (body wall), whereas the internal regions such as the out flow tract and atria are practically free of mutant \textit{Ift88}/- ESC. Although conducted on a small number of embryos at this point, the results support our hypothesis that primary cilia are important for cardiogenesis.
3.5 PRIMARY OBJECTIVE CONCLUSIONS AND PERSPECTIVES

At several points in cardiomyogenic development multiple signaling pathways and their downstream effector molecules crosstalk and overlap. This complicates the matter in understanding the specific mechanisms that determines when, where and how stem cells initiate differentiation, migration and proliferation in early heart development. In figure 14, I have given a brief overview of the signaling pathways in cardiogenesis to describe where there could be possible crosstalk between the individual pathways in heart development. Some examples of signaling pathways that overlap are the Hh and Wnt pathway, which were proposed to be coordinated by the primary cilium. The essential Hh signaling components Gli2, Gli3, and Smo localize to the primary cilium, in various cell types including fibroblasts (Haycraft et al., 2005; Rohatgi et al., 2007), where Wnt signaling is divided up into three distinct pathways that has been proposed to work as a network of interacting rather than individual pathways (Kestler & Kuhl, 2008). The primary cilium and basal body have been proposed to act as regulator in both the non-canonical and canonical Wnt pathways due to the ciliary/basal body localization of essential proteins like PCP (planar cell polarity) protein inversin (Morgan et al., 2002), Vangl-2 (Ross et al., 2005) in addition to members of the degradation complex Glycogen synthase kinase 3 beta, GSK-3β, (Wilson & Lefebvre, 2004) and Adenomatous Polyposis Coli, APC (Corbit et al., 2008).

Wnt signaling is affected in ORPK mice which show abnormal cyst formation in the pancreas where the cysts and dilated ducts have an increased cytosolic localization of β-catenin in addition to an increased expression of Tcf/Lef, which activates transcription of Wnt target genes (Willert & Nusse, 1998; Roose & Clevers, 1999; Cano et al., 2004; Zhang et al., 2005). In neural tube development inhibition of Shh via the Gli3 repressor inhibit the canonical Wnt-mediated transcriptional activation by physical interaction with the carboxy-terminal domain of β-catenin (Ulloa et al, 2007). However, the primary cilium does not seem to be essential for the canonical Wnt signaling pathway demonstrated in recent findings in the mouse embryo and mouse embryonic fibroblasts (Ocbina et al., 2009). The Wnt signaling appears intact in the absence of primary cilia in \textit{Ift88} and \textit{Ift172} knockout mice or in the anterograde motor Kif3a and retrograde motor Dync2h1 knockouts (Ocbina et al., 2009). These data contradict previous findings, suggesting that IFT proteins and especially Kif3a have specific roles in

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**Figure 13.** Chimera embryo A: Light microscope image of E8.5 chimera mouse embryo after β-galactosidase and alkaline phosphatase assay with 8-10 injected cells of both wt (blue) and Ift88−/− (red) mESCs, (dotted line: heart region). B: Light microscope image of a frontal cut 10µm cryofreeze section of the mouse embryo viewed in (A): Abbreviations; A: atrium, OFT: outflow tract, B: future brain, BW: body wall, closed arrow: alk. phos. staining of the tissue surrounding the heart, open arrow: LacZ staining of the heart chambers. C. A. Clement, unpublished data.
Figure 14: Brief overview of signaling pathways in cardiogenesis. A: Ciliary Hh and Wnt signaling pathways. With Hh ligand present the Gli transcription factor activators can control timely activation of early heart transcription factors. The non-Canonical Wnt signaling pathway favors cardiogenesis through degradation of beta-catenin. Wnt 11 has been observed to induce cardiomyogenesis (Flaherty & Dawn, 2008). In terms of Hh signaling, binding of ligands to Ptc in the cilium activate the Hh pathway by removal of Ptc from the cilium in a process that is associated with ciliary enrichment of Smo. The red arrow indicate a possible crosstalk between the Gli2 activator and the Wnt signaling pathways. B: Bone morphogenetic protein (BMP) and transforming growth factor (TGF)-beta signaling pathways function in formation of the endocardial cushion tissue development. The cushion tissue is formed in the outflow tract and in the atrioventricular regions during cardiogenesis (Yamagishi et al., 2009). Whether the two pathways work via the primary cilium is not yet understood. The red arrow indicates strong crosstalk between the two pathways. C: Platelet-derived growth factor receptor (PDGF-R) and fibroblast growth factor receptor (FGF-R) signaling pathways are also involved in cardiogenesis. PDGF-R signaling plays a role in the migration of epicardial cells that form the coronary artery and myocardium (Mellgren et al., 2008). FGF-R signaling regulates the early heart transcription factors Gata5 and TBX6 and TBX16 (Neugebauer et al., 2009). Little is known about FGF signaling in the primary cillum in contrast to PDGF-R signaling that has been documented to work through the cillum (Schneider et al., 2005; 2009a). The red arrow indicates strong crosstalk between the two pathways. D: Examples of potential signaling crosstalk between Wnt, BMP and Hh signaling pathways in embryonic development. Lack of Hh signaling was observed to delay BMP4 signaling in P19 cardiac progenitor cells (Gianakopulos & Skerjanec, 2009). Furthermore, Gli3-repressor activity was shown to negatively regulate Wnt/beta-catenin signaling (Ulloa et al., 2007). The red arrow indicates possible crosstalk between the individual signaling pathways, although we still know very little as to the potential association with the primary cilium.
regulation of the canonical Wnt pathway (Gerdes et al., 2007; Gerdes & Katsanis, 2008; Corbit et al., 2008). In addition, zebrafish studies show that oval mutants (ovl;ift88) lack primary cilia but still have normal canonical and non-canonical Wnt signaling but show defects in Hh signaling (Huang & Schier, 2009). A possible hypothesis is that the Wnt signaling pathway is intact because the basal body adapts the role of the primary cilium and compensate for the absence of cilia. Consequently, it is plausible that the ciliary axoneme and the basal body are two distinct signaling organelles with separable functions, where the ciliary axoneme is required for Hh signal transduction and the basal body might be essential for the Wnt signaling response. How the Wnt and Hh signaling pathway crosstalk in the primary cilium or not is not fully understood. Of the other cardiogenic pathways in figure 14, both BMP and (TGF)-beta signaling pathways function in the formation of the endocardial epithelial-mesenchymal transformation (EMT), which is a critical process in endocardial cushion tissue development. The cushion tissue is formed in the outflow tract and in the atrioventricular regions during cardiogenesis (Yamagishi et al., 2009). Whether the two pathways work via the primary cilium is not yet known, however the findings that Hh and BMP signaling crosstalk in cardiomyocyte formation in P19 cells (Gianakopoulos & Skerjanc, 2009), might indicate a possible ciliary mechanism for the BMP signaling pathway. The PDGF-R and FGF-R signaling pathways also play a role in cardiogenesis. PDGF-R signaling plays a role in the migration of epicardial cells that form the coronary artery and myocardium (Mallgren et al., 2008), where FGF-R signaling regulate the early heart transcription factors Gata5, TBX6 and TBX16 which are proposed to play a role for regulating ciliary length (Neugebauer et al., 2009). Little is still known about FGF signaling in the primary cilium where PDGF-R signaling previously has been shown to regulate ciliary PDGF-Ralpha signaling that control tissue homeostasis, migration and mitogenic signaling pathways in fibroblasts (Schneider et al., 2005; 2009a; 2009b).

The complexity in early heart development makes it difficult to assess how individual signaling pathways function because of the possible crosstalk. Future in vitro experiments on Ift88-/- mESCs, P19 and P19.CL6 EC cells with focus on the Wnt, Hh, FGF, PDGF and BMP signaling pathways should help determine how the individual pathways interact with one and other. In some cases, siRNA may have off target effects and may not induce a complete loss of function. In order to eliminate these concerns, more experiments with Ift88-/- mESCs and wt as well as rescued mutant ES cells would clarify these possible off target effects. Further and in contrast to P19.CL6 cells, mESCs are true pluripotent stem cells that will give more clear information on the function of the primary cilium in the earliest steps of cell fate determination and prior to formation of cardiac progenitor cells. Copying the same characterization done on P19.CL6 EC cells onto the Ift88-/- and wt mESCs will reveal interesting knowledge on how the early heart development progress in mESCs and possibly how Wnt and Hh signaling function in these cells, with and without inhibitors like KAAD-cyclopamine. Preliminary data in the P19.CL6 differentiation studies reveal fluctuations of Ptc1 and Smo in and out of the cilium around the forming clusters of cardiomyocytes which is something that would be interesting to study in detail [4]. This might give more insight on how the Hh signaling pathway is regulated locally in cardiogenesis. Additionally, more in vivo studies are needed with chimera Ift88-/- ESC lines and Ift88-/- mice followed by histological sectioning to pinpoint where the primary cilia are needed in embryonic development, in stem cell positioning and differentiation during the various stages of heart development from E8.0 old embryos and in adult mice. Moreover, immunofluorescence microscopy-3D reconstruction and in situ hybridization analysis would identify ciliary signaling components during in vivo heart development, and reveal how defects in ciliary assembly affect the level of their activation in Ift88-/- null embryos.
CHAPTER 4 – SENSORY CILIA IN THE PANCREAS AND REPRODUCTIVE ORGANS

4.1 INTRODUCTORY REMARKS

This chapter will discuss and summarize the data from the secondary objective articles that are found in chapter 7 in the following sections: Collaborative work with Stefan Teilmann on progesterone receptor localization and expression in the female reproductive organs (Chapter 7: [1]) and on pancreatic development in humans in addition to cancer cell lines (Chapter 7: [2]).

4.2 HEDGEHOG SIGNALING IN PANCREATIC DEVELOPMENT AND CANCER

Precise and timely Hh signaling is required for proper regulation and development of the pancreas, but even in the mature adult tissue, Hh signaling take part in the general maintenance of the pancreatic tissue (Hebrok et al., 2000). Therefore, mal-regulation of the Hh signaling pathway results in a series of diseases, including annular pancreas, diabetes mellitus, chronic pancreatitis and pancreatic cancer (Lau et al., 2006). The role of the primary cilium in pancreatic development and cancer via the Hh signaling pathway is not fully known and needs to be investigated further before treatments are effective enough to cure cases with pancreatic cancer.

4.2.1 PRIMARY CILIA AND HEDGEHOG SIGNALING IN PANCREATIC DEVELOPMENT

In the early mouse development, the pancreas initiates its development around E8.0 and is situated in the anterior midgut region of the endoderm epithelium. The pancreas is formed by two primary tissues, the exocrine compartment, which contains acinar and ductal cells, and the endocrine compartment with cells that localize to the islet of Langerhans structure. The exocrine acinar cells compose the majority of the mature organ and produce enzymes that drain into the intestinal tract through the ductal tissue. The islets of Langerhans are imbedded within the exocrine tissue, where they produce important hormones that regulate blood glucose levels. These islets contain four different cell types, the glucagon producing α–cells, somatostatin producing δ–cells, insulin producing β–cells and the pancreatic polypeptide producing PP–cells. Hence the main function of the pancreas is to produce enzymes and secrete hormones that aid digestion and controls blood glucose homeostasis (Slack, 1995).

As discussed in [2] a number of previous investigations have indicated a link between primary cilia and development of the pancreas in mice. In addition to kidney defects, the loss of primary cilia in the Tg737ORPK mouse causes a series of abnormalities in the pancreas, such as extensive cyst formation in ducts (Cano et al., 2004; Zhang et al., 2005). This may indicate a possible functional similarity between cilia in kidney and pancreatic duct systems. Cells of both exocrine and endocrine systems in the pancreas possess primary cilia, including islet cells and the ducts, but not in the acini (Kodama, 1983; Ashizawa et al., 1997; Cano et al., 2004; 2006; Zhang et al., 2005). In the Tg737ORPK mouse pancreas abnormalities begin with dilations of the ducts in late gestation, which after birth are accompanied by extensive formation of large, interconnected cysts as well as apoptosis and vacuolization of acini. In the dilated ducts and cysts PC-2 is mislocalized to the intracellular compartments. These changes are reminiscent of chronic pancreatitis, supporting the speculation that primary cilia of ducts play an essential role in the development of the pancreas (Cano et al., 2004; Zhang et al., 2005). As
will be discussed below, primary cilia may also play a major role in coordinating Hh signaling during human pancreatic development and aberrant Hh signaling in pancreatic cancer may be associated with defects in ciliary assembly in pancreatic adenocarcinoma cell lines.

Hh signaling appears to play multiple roles during mouse embryonic pancreatic development. During the early stages of gut formation in mice, expression of both Shh and Ihh genes is found throughout the endoderm epithelium (Bitgood & McMahon, 1995; Aubin et al., 2002), even though both genes are absent from the early endodermal area specified to become pancreas (Hebrok et al., 1999; Kim & Melton, 1998). In situ hybridization experiments have shown that Ptc1 expression is found in the mesenchyme adjacent to, but missing from, the pancreas anlage in E9.5 old embryos (Apelqvist et al., 1997). This difference in Hh signaling may ensure the correct establishment of organ boundaries. Subsequently Hh signaling is activated to promote proliferation and maturation of the tissue, as observed at embryonic day E13.5 where the developing pancreas expresses several Hh genes such as Ihh, Dhh, Hhip and Ptc1 (Kawahira et al., 2005; Lau et al., 2006; Cano et al., 2007; van den Brink, 2007). In the adult pancreas both Ptc1 and Smo has been observed in the islet and ductal cells, which indicate that Hh signaling is present and active during later stages of pancreas development and in the mature organ (Hebrok et al., 2000; Kawahira et al., 2003). To sum up on the collaborative work with Sonja Brorsen [2], we found primary cilia projecting into the pancreas duct lumen. These primary cilia are up to 20µm long and show increased Smo and Gli2 localization when the embryos enter the fetal stages of development at weeks 14 and 18, compared to the 7.5 week old embryos in the embryonic stage. Contrarily, the nuclear and cytosolic expression levels of the repressor form of Gli3 decreases as the embryos enter the fetal stages, suggesting an increased Hh activity. These changes in localization correlate with known activity of the Hh pathway during pancreas development. The primary cilium may be the critical organelle that coordinates pancreatic development to promote maturation of the tissue and function in tissue homeostasis in adult individuals. Loss of primary cilia function in the Tg737ORPK mouse further strengthens the hypothesis that primary cilia are key regulators of pancreatic development, since these mice show severe pancreatic abnormalities including extensive cyst formation in the ducts (Cano et al., 2004; Zhang et al., 2005).

### 4.2.2 Primary Cilia and Hedgehog Signaling in Pancreatic Cancer

Pancreatic cancer is a severe disease that is diagnosed in 33.000 patients annually in the USA alone, worldwide it is estimated to cause more than 200.000 deaths each year (Parker et al., 2003). Notch, Hh, and Wnt signaling pathways play an important role in multiple tissues during development and are for most part turned off in adult somatic cells, including the exocrine pancreas. Abnormal transcriptional activation of these pathways has been reported in both human and mouse models of pancreatic neoplasia. Aberrant activation of the Hh signaling pathway has been reported in pancreatic intraepithelial neoplasia (PanIN) (Miyamoto et al., 2003; Berman et al., 2003; Thayer et al., 2003; Zeng et al., 2006). In addition, activation of the Hh pathway in a human pancreatic ductal epithelial cell line resulted in up-regulation of extra-pancreatic foregut markers observed in the early PanIN lesions (Prasad et al., 2005), which normally are not present in normal ductal epithelium (Koorstra et al., 2008).

In the collaborative work with Sonja Brorsen [2] we further investigated the role of primary cilia in pancreatic adenocarcinoma cell lines, CFPAC-1 and PANC-1, which are isolated from metastatic and primary tumors, respectively (Schoumacher et al., 1990; Lieber et al., 1975). Initially, we show that CFPAC-1 and PANC-1 cells have long primary cilia, up to 20µm long, which have ciliary localization of Ptc, Gli2 and Smo. This localization is consistent with the idea that the primary cilium continues to coordinate Hh signaling in cells derived from the mature pancreas. Aberrant Hh signaling in these two cancer cell lines may be associated with the autonomous activation of the signaling pathway in the cilium, judged by the relative high levels of Smo and low levels of Ptc in the cilium. Furthermore, the high expression of full-length form of Gli2 in the cilium and low levels of Gli3 repressor in the nucleus suggest even further that there is a high activation level of Hh signaling pathway. The fact that Hh signaling is highly active in the CFPAC-1 and PANC-1 cell lines suggests that ciliary Hh signaling
plays a potential role in tumorigenesis. However these results proposes that a certain level of Hh signaling is required for proper organ formation, as observed in gain of function studies that have demonstrated that deregulation of Hh signaling activity results in critical changes of pancreas morphogenesis and function (Lau et al., 2006).

4.3 Sensory motile cilia in the oviduct

The mouse ovary is enclosed in a thin epithelial bursa. The inner surface epithelium of the bursa is continuous with the ovarian surface epithelium in the areas around the hilus or ovarian stalk. It encloses the ovary and the upper part of the oviduct, the infundibulum. Ovulated oocytes are prevented from escaping into the peritoneum by the bursa and are led to the opening of the oviduct (salpinx), which collects the oocytes. The mouse oviduct has a coiled appearance and can be divided into four parts (Nielsson & Reinius, 1969), see figure 15. In the ovary the follicles begin to grow soon after birth and continue until the pool of follicles is depleted (Peters et al., 1975). Initiation of follicle growth begins with mitotic activity of the granulosa cells surrounding the oocyte, which also increases in volume (Pedersen & Peters, 1968). In the late follicular development, multiple layers of granulosa cells develop around the oocyte where fluid begins to accumulate in the space around the oocyte (called antrum). A wide range of signaling pathways and hormones control the follicular development including progesterone. Ovulation is controlled by the luteinizing hormone, LH, which induces luteinization of granulosa cells that particularly increases the expression of progesterone and its receptor (PR), (Shimada & Terada, 2002; Park & Mayo, 1991). Progesterone is an important local regulator of ovulation, lutenization, oviductal gamete transport and implantation. Additionally progesterone mediates various effects in the female reproductive organs through its cognate nuclear receptors, PR-A and PR-B which often are co-expressed within the same cells, e.g. in granulosa cells of pre-ovulatory follicles. The two receptor types although co-expressed have distinct roles and show different phenotypes in knockout mice. In PR-A/- mice ovulation is critically impaired and the implantation is no longer possible, demonstrating that only PR-A is obligatory for female fertility, in contrast to PR-B/- mice that has defective mammary gland development (Mulac-Jericevic et al, 2000).

Motile and primary cilia are found in abundance in the oviduct. In the ovaries, primary cilia are particularly found on the granulosa cells in the antral follicles, which have been shown to have the TRP ion channel, polycystin-1 and -2 localizing to the primary cilia (Teilmann et al., 2005). Furthermore, the angiopoietin receptors such as the Tie-1 and Tie-2 receptor tyrosine
kinases localize to motile cilia of the oviduct. Tie-2 specifically localize to primary cilia of the surface epithelium of the ovary, bursa and extra-ovarian rete ducts as well as to plasma membranes of the ovarian theca and endothelial cells (Teilmann & Christensen, 2005). In the oviduct ciliated epithelial cells of both adult human and mice, revealed progesterone localization to the lower half of the motile cilia, whereas the nuclei were not stained or otherwise only faintly. It is possible that ciliary progesterone receptors in the oviduct play a role in progesterone signaling after ovulation, possibly via non-genomic events [1]. The presence of progesterone/Tie receptors in addition to polycystins in the cilia population of the female reproductive organs, support the hypothesis that cilia both motile and primary, play an important sensory role in coordinating and regulating hormonal and reproductive events.

To sum up on the collaborative work with Stefan we found PR localization in the lower half of the motile cilia in the oviduct. The ciliated cells with PR localization did not show PR localizing to the nuclei or if the case, only very faintly. In the pubertal mice the localization of PR was increased in the cilia, in contrast to the primary granulosa cell cilia, which lacked PR staining at all stages. Since progesterone is a regulator of ciliary beat frequency, we suggest that ciliary PR directly modulates the ciliated oviduct epithelium by operating as a fast means to sense and relay changes in the levels of progesterone in the oviduct, such as those induced through release of follicular fluid at ovulation or released by the oocyte cumulus complex. In this scenario, ciliary beat frequency may be regulated directly by progesterone via ciliary receptors to control uptake and/or transport of the oocyte cumulus complex. Additionally, the findings of polycystins 1 and 2 as well as Tie receptors to motile cilia in the oviduct further support the hypothesis that cilia of the female reproductive organs play a significant sensory role in relaying physiochemical changes from the extracellular environment to epithelial cells of the oviduct and ovary (Teilmann et al., 2005; Teilmann & Christensen, 2005).

4.4 Secondary objective conclusions and perspectives

Motile cilia in the mouse and human oviduct revealed progesterone receptor localization in the lower half of the cilia. This localization was increased in pubertal mice, which suggest a possible role for the cilia in progesterone signaling after ovulation. Hedgehog signaling also plays a significant role in development of the pancreas in mammals. Ciliary localization of Gli2 and Smo in both cultures of human pancreatic duct adenocarcinoma cell lines and in duct epithelial tissue indicates that Hh signaling is a strong regulator in pancreatic development, which may also be responsible for tumorigenesis. Tight regulation of the Hh pathway in the embryo, fetus and adult is critical judged by the observed changes from the 7.5 weeks old embryos to the 14 and 18 weeks old fetuses. These findings support the hypothesis that primary as well as motile cilia play significant roles in cell signaling to maintain tissue homeostasis and control differentiation, in addition to coordinate developmental events.
5.1 Thesis conclusions

This thesis presents data to support the conclusion that primary cilia as well as motile cilia in the oviduct are critical organelles in coordinating signaling transduction pathways during development and in tissue homeostasis. Several of the studies presented have focused on the Hh signaling pathway, which is an important pathway in heart development and differentiation. Aberrant Hh signaling can lead to cancer if not properly coordinated, however very little is still known about the Hh signaling pathway and how it is coordinated \textit{in vivo} during heart development and in the adult. The connection between cilia and various human diseases has clearly demonstrated the importance of cilia. Many functions in cilia assembly and maintenance are still not known and will need to be investigated to determine how diseases arise from ciliary defects. A future challenge will be to further improve our understanding of the ciliary signaling pathways and how receptors in addition to signaling molecules work via the primary cilium, which impinges on cellular responses and gene expression. Especially in heart development and stem cell research, the primary cilium may play a significant role in regulating cellular processes that can be used as effective therapeutic tools against cancer and in regeneration of damaged tissue in the near future.
CHAPTER 6 – REFERENCES


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CHAPTER 7 – ARTICLES [1-5]
Expression and localization of the progesterone receptor in mouse and human reproductive organs

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Abstract

The effects of gonadotropins on progesterone receptor (PR) expression and localization in the mouse oviduct, uterus, and ovary was examined. In the oviduct ciliated epithelial cells of adult mice and human revealed a unique PR localization to the lower half of the motile cilia whereas the nuclei were unstained or faintly stained. Pubertal female mice were further studied by confocal laser scanning microscopy and western blotting before and after injection with FSH and LH followed by human chorionic gonadotropin (hCG) injection after a 48-h period. PR immunolocalization to the oviduct cilia was greatly increased in pubertal mice upon hCG stimulation. In neighboring goblet cells, the PR staining was confined to the nuclei. Nuclear PR localization was evident in epithelial cells of the uterus as well as in a fraction of stromal and muscle cells. Staining intensity and number of stained cells was not affected by hormone stimulation. In the ovary, weak PR immunolocalization was observed in unprimed animals but increased significantly after hCG stimulation. In granulosa cells of preovulatory follicles PR was exclusively observed in mural cells, whereas cumulus cells remained negative. At all stages examined, primary granulosa cell cilia lacked PR staining. SDS-PAGE and western blotting analysis of tissues from oviduct, uterus, and ovary confirmed antibody specificity, and identified two bands corresponding to the PR isoforms PR-A and PR-B. Upon hCG stimulation, a new band cross-reacting with anti-PR emerged above the PR-A form in oviduct fractions, suggesting LH-induced phosphorylation of PR-A. We suggest that ciliary PR in the oviduct plays a role in progesterone signaling after ovulation, possibly via non-genomic events. These novel findings warrant further studies of oviduct and postovulatory signaling events and suggest a sensory role for oviduct cilia in the process of oocyte transport/fertilization.


Introduction

Many reproductive functions in mammals are regulated by the ovarian steroids estradiol and progesterone (P). P is an important local regulator of critical reproductive events such as ovulation, luteinization, gamete transport within the oviduct, and implantation. P mediates various effects in the female reproductive organs through its cognate nuclear receptors, PR-A and PR-B, which often are co-expressed within the same cells, e.g., in granulosa cells of preovulatory follicles (Hild-Petito et al. 1988, Sheridan et al. 1989, Park & Mayo 1991, Gava et al. 2004). Both the PR forms are ligand-activated transcription factors that upon ligand binding undergo phosphorylational and conformational changes (Weigel 1996, Clemm et al. 2000). The importance of the two PR isoforms has been demonstrated in PR null mice (PRKO), which are anovulatory and unable to properly respond to exogenous gonadotropins (Lydon et al. 1995). Mice deficient in either PR-A (PRAKO) or PR-B (PRBKO) have different phenotypes, demonstrating a complex and tissue-specific interplay between the two receptor forms. In PRAKO mice, ovulation is severely hindered and implantation impossible showing that only PR-A is obligatory for mouse female fertility (Mulac-Jericevic et al. 2000).

Cyclic changes of P and estradiol control homeostasis of the oviduct epithelium as well as formation and beat frequency of the cilia (Brenner 1969, Wessel et al. 2004), although little is known about the underlying regulatory mechanisms. Present research has positioned cilia as key sensory organelles in human health and reproduction (Satir & Christensen 2006), and increasing evidence suggests a role for cilia in sensing in the female reproductive organs. We have previously described the unique localization of signal components to both motile cilia of the oviduct and to primary cilia of ovarian and extraovarian tissues of the mouse, and signaling through these cilia were suggested to play a role in follicular maturation, ovulation, and gamete transport. In the overy,
the Ca\textsuperscript{2+}-selective transient receptor potential (TRP) ion channels localize to primary cilia of granulosa cells of antral follicles and to motile cilia of the oviduct (Teilmann et al. 2005). In addition, receptor tyrosine kinases, angiopoietin receptors, localize to cell type-specific primary cilia of the reproductive organs and to the tip of motile cilia in the oviduct (Teilmann & Christensen 2005).

In the present study, we analyzed murine PR in oviduct, uterus, and ovary by immunolocalization and western blotting techniques upon follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) stimulation. Using specific antibodies against PR, we were able to show that hCG radically upregulates PR in nuclei and cytosol of mural granulosa cells of the preovulatory follicles and in motile cilia of the oviduct. These results indicate the discovery of a novel function of PR in reproductive biology, in which ciliary beat frequency may be regulated directly by progesterone via ciliary receptors.

Materials and Methods

Animals and tissues

Pubertal female C57Bl/6j mice (21–23 days old, \( \sim 14 \) g) were injected intraperitoneally with 30 IU gonadotropins (Menopur: 15 IU FSH and 15 IU luteinizing hormone (LH) (Ferring Pharmaceuticals, Copenhagen, Denmark) followed by 5 IU menotropin (hCG) (Pregnyl, Organon AS, Skovlund, Denmark) injection 48 h later. Mice were killed by cervical dislocation 48 h after FSH or 6 h after hCG. Non-stimulated mice of coeval age were used as controls. Oviducts, uteri, and ovaries were immediately removed and processed for immunohistochemistry (IHC) or western blot and SDS-PAGE analysis. Furthermore, lung, heart, and reproductive organs of 3-month-old female mice in estrous were used as control tissues. Ovaries from three 30-days-old Wistar rats were collected in PBS rinsed from fat and extraovarian tissue, fixed in 4% paraformaldehyde, and processed for IHC. Animal experiments were conducted in accordance with EU guidelines and approved by the Danish Ministry of Justice, Animal Ethics Committee no. 2003/561-713 (A G B).

Human material

Human material was used after ethical approval was obtained by the ethics committee for Copenhagen and Frederiksberg no. KF 01-170/99, and after informed consent was obtained from each patient following the guidelines in the Declaration of Helsinki.

Immunohistochemistry

After fixation, oviduct, uterus, and ovary were embedded in paraffin and cut in 8 \( \mu \)m thick sections that were collected on microscope slides (SuperFrost/Plus, Menzel Gläser, Germany). Importantly, we found what appeared to be time- and temperature-dependent degradation of specific PR epitopes in the tissue sections examined, leading to decreased or absent staining intensity. Multiple sections from ovaries from three different animals in each group were therefore always stored at 4 °C and used within a week after cutting. Sections were deparaffinized, rehydrated, and rinsed in PBS. For antigen retrieval, slides were boiled in citric acid buffer (0.01 M, pH 6), and incubated for 15 min in PBS (pH 6.5) containing 5% (w/v) BSA and 1% (v/v) preimmune goat serum (Dako, Glostrup, Denmark). Sections were incubated with one of the following primary antibodies diluted in PBS containing 5% (w/v) BSA and 0.02% (w/v) Na\textsubscript{3}N\textsubscript{2} overnight at 4 °C: diagnostic grade rabbit monoclonal PR antibody (1:300, Clone SP2, LabVision, Westinghouse Drive, Fremont, CA, USA) directed against an epitope corresponding to amino acid (aa) sequence 410–516 in mouse PR and to aa sequence 412–526 in human PR was a gift from AH Diagnostics, Aarhus, Denmark. Mouse monoclonal PR antibody (1:300, Clone Ab-4, NeoMarkers, LabVision) directed against the N-terminal region of PR, i.e. aa sequence 1–557 in mouse PR and aa sequence 1–566 in human PR. Mouse monoclonal anti-acetylated \( \alpha \)-tubulin (1:3000, Cat no. T6793, Sigma-Aldrich) for localization of cilia and cytosolic network of acetylated microtubules (Alieva et al. 1999). Non-specific binding of the PR antibody was evaluated by substitution with preimmune rabbit IgG (Dako) with the same concentration as primary antibody. Primary antibodies were detected by 1-h incubation at room temperature with species-specific Alexa Fluor anti-IgG F(ab\textsuperscript{‘})\textsubscript{2} secondary antibody (5 \( \mu \)g/ml, Molecular Probes, Eugene, OR, USA) and counterstained with propidium iodide (1 \( \mu \)g/ml) or TO-PRO-3 iodide (2 \( \mu \)g/ml Molecular Probes) in PBS for 8 min. After washing, slides were mounted in 1:1 (v/v) glycerol/ PBS with 2% (w/v) Na\textsubscript{3}N\textsubscript{2} and sealed with nail polish. A series of sections as well as isolated and fixed single cell preparations (see below) were double labeled with anti-acetylated \( \alpha \)-tubulin and anti-PR.

Tissue and immunofluorescence analysis

The three parts of the oviduct studied were divided into the following separate groups: fimbriae (the cranial part), ampulla (the middle part), and isthmus (the caudal part). For single cell analysis, the cranial and middle part of the oviduct were collected in PBS with Ca\textsuperscript{2+}/Mg\textsuperscript{2+} and cut into small pieces to expose the ciliated epithelium. Then the tissues were placed in ice-cold incubation buffer containing 0.25 M sucrose, 0.02 M HEPES, 2 mM EDTA, and 25 mM KCl supplemented with freshly made phenylmethylsulfonyl fluoride (1 mM) and N-ethylmaleimide (10 mM). For the next 50 min, the sample was kept on ice and vortexed intermittently. Pieces of oviduct were removed and an equal volume of incubation buffer was added. After 60 s of incubation, the material was vortexed and spun down (500 g for 10 min). The pellet (containing ciliated cortices, single cells and nuclei) was fixed and loaded onto glass cover slips for immunohistochemical analysis. Ovarian follicles were categorized according to Pedersen & Peters (1968) and antral follicles were classified as atretic when > 5 granulosa cells
were pyknotic, corresponding to the stage one atresia described (Byskov 1974) and was excluded from analysis. Stained sections and isolated cells were observed on an IX70 confocal laser scanning microscope (Olympus, Tokyo) with a Krypton/Argon laser using a 60× oil immersion objective (NA:1.25) and a 40× air objective (NA:0.85), both equipped with appropriate Normarski optics. Care was taken to avoid bleedthrough between channels, and at the beginning of each evaluation, image settings was optimized so that it contained the maximum number of gray levels, and during subsequent image acquisition all settings (laser power, photomultiplier tube gain and offset) were kept constant so that the images could be compared.

SDS-PAGE and western blot analysis

In the isolation of oviduct infundibulum samples, care was taken only to include the outer cranial part in order to minimize non-ciliated epithelium from the sample. Ovaries and oviducts were cleaned from fat and connective tissues before protein isolation. Protein was extracted using a common protocol (VanSlyke & Musil 2001). The protein concentrations were estimated using a BCA protein kit (Pierce Biotechnology, Rockford, IL, USA), and proteins were resolved by gel electrophoresis under denaturing and reducing conditions and electrophoretically transferred to nitrocellulose membranes as previously described (Christensen et al. 2001). The membranes were incubated with anti-PR (1:300) and anti-β-tubulin (1:300; Cat no. T4026, Sigma-Aldrich) and antibody cross-reactivities were identified with species-specific alkaline phosphatase-coupled secondary antibodies (1:1200, Jackson Laboratory, Bar Harbor, ME, USA) followed by developing with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (KPL, Cessna Court, Gaithersburg, Maryland, USA).

Statistical analysis

Band intensities of PR proteins in western blot analysis were measured using UN-SCAN-IT Version 5.1 (Silk Scientific, Inc., Orem, Utah, USA). Data are presented as mean values ± S.E.M. from a minimum of three individual experiments, in which tissue homogenates were obtained from a minimum of six animals. Significant differences in the level of PR expression between non-stimulated and hormone-stimulated mice were estimated using a two-tailed paired t-test. For all statistical evaluations, P values <0.05, <0.01, and <0.001 were considered statistically significant, very significant, and extremely significant respectively.

Results

Localization and expression of PR in the ovary upon hormone stimulation of pubertal mouse

In the untreated pubertal ovary anti-PR (SP2) weakly localized to theca and granulosa cells (Fig. 1A and D), and a slight nuclear anti-PR (SP2) staining was observed in the interstitial tissue. Forty-eight hours after FSH a subpopulation of theca cells around large antral follicles (stage 6) began to show nuclear PR immunoreactivity (SP2), whereas the PR signal seemed reduced in a subpopulation of granulosa cells of any follicle stage (Fig. 1B and E). Six hours after hCG, nuclei of many theca cells in the large preovulatory follicles (stage 7) were anti-PR (SP2) positive (Fig. 1C). At this stage, follicular granulosa cells are divided into two distinct populations: mural granulosa cells facing the theca cell layer and cumulus granulosa cells facing the oocyte. Clearly, hCG stimulation dramatically increased the level of anti-PR (SP2) immunofluorescence of mural granulosa cells, whereas cumulus cells remained PR negative (Fig. 1F). To further characterize PR localization in the granulosa cells of hCG-stimulated ovaries, we used anti-acetylated α-tubulin to detect primary cilia in mural and cumulus granulosa cells in stage 7 follicles. Most non-dividing mural and cumulus granulosa cells had a primary cilium that was often presented into the antrum (Fig. 1G). In double labelings of anti-acetylated α-tubulin and anti-PR in granulosa cells, nuclear and cytosolic PR expression did not correlate with the presence of a cilium and no detectable co-localization between acetylated α-tubulin and PR was observed (Fig. 1G). As a further control on PR localization in the ovary, tissue sections of mice were subjected to co-localization analysis with anti-PR (clone Ab-4) that recognizes the entire N-terminal region of PR (Fig. 1H). It is seen that SP2 and Ab-4 co-localize to mural granulosa cells in hCG-stimulated ovaries, confirming specific immunoreactivity to PR in this cell population.

A strong immunofluorescent signal in the cytoplasm of mouse oocytes from all follicle stages including atresia was observed with Anti-PR (SP2; Fig. 1A–F). However, no immunofluorescent signal was detected with this antibody in oocytes from either rat or human (Fig. 1I). Importantly, the Ab4 antibody did not label oocytes from either rat or mouse (Fig. 1I). Since, unspecific immunohistochemical staining of oocytes is a well known problem, these observations suggest that excessive oocyte staining can be considered a phenomenon restricted to that particular antibody when used on mouse tissue. Although we do not know whether this signal represents a true receptor population within the oocytes, it is most likely an artifact and has not been investigated further.

Using western blot analysis anti-PR (SP2) specifically recognized two PR forms of approximately 115 and 83 kDa, corresponding to the B- and A-form respectively. In the ovary protein fraction of the non-stimulated mice both PR forms could be detected at a low level (Fig. 1J). After 48 h with FSH, the level of PR–A was significantly reduced to about 40% compared with that of the non-stimulated mice (Fig. 1J and K). In contrast, 6 h after hCG, PR–A and PR–B were present at a level about 19- and 8-fold higher than in the non-stimulated mice respectively (Fig. 1J and L). In some experiments, we also observed a protein migrating in SDS-PAGE as a 60 kDa protein, which was recognized by anti-PR (SP2) and upregulated upon hCG stimulation (data not shown).
Localization of PR in the oviduct and uterus of adult mice

Localization and expression of PR isoforms in the oviduct and uterus were initially examined in tissues of adult mice (Fig. 2). In all parts of the oviduct, nuclear PR immunoreactivity was detected in a fraction of the stromal cells and the luminal non-ciliated epithelial cells. In ciliated epithelial cells, anti-PR (SP2) uniquely localized to the cilia, whereas the antibody exclusively localized to the nucleus in non-ciliated glandular goblet epithelial cells (Fig. 2A). In the uterus, anti-PR (SP2) localized to nuclei of epithelial, stromal, and muscle cells (Fig. 2B). In the stroma and muscle layers, PR staining had a mosaic-like pattern with...
Figure 2 Immunolocalization of progesterone receptor in tissue sections of oviduct (A) and uterus (B) of adult C57Bl/6J female mice. Cilia are marked with arrow heads and nuclei are stained with propidium iodide (red). Progesterone receptor is localized with monoclonal rabbit anti-PR (SP2; green). Scale bars: 50 μm. (A') Close up of the mouse oviduct epithelium showing ciliated and goblet cells. Cilia are marked with arrow heads. Nuclei are stained with propidium iodide (red) and progesterone receptor is localized with monoclonal rabbit anti-PR (SP2; green). Scale bar: 10 μm. (C) SDS-PAGE and western blot analysis with anti-PR (SP2) showing the expression of progesterone receptor forms, PR-A (ca. 83 kDa) and PR-B (ca. 115 kDa) in extracts of oviduct, uterus, lung, and heart of adult mice. Molecular markers (kDa) are shown to the left. (D) Immunolocalization of anti-PR (SP2) in a tissue section of ciliated lung epithelium from mouse (arrow heads indicate cilia). Nuclei are stained with propidium iodide (red). Scale bar: 50 μm. (E) Ciliary immunolocalization of anti-PR (SP2; green) in the mouse oviduct in a tissue section (upper row; scale bar: 5 μm) and in an isolated ciliated epithelial cell (lower row; scale bar: 1 μm). Cilia are detected with anti-acetylated α-tubulin (red) and marked with arrow heads. Nuclei are detected with TO-PRO (blue). Nuclear localization of anti-PR is indicated with asterisks. (F) Immunolocalization of anti-PR (SP2; green) in a tissue section of ciliated epithelial cells from adult human oviduct (arrow heads indicate cilia). Nuclei are stained with propidium iodide (red). Scale bar: 5 μm. (G and H) Immunolocalization of anti-PR (Ab-4; green) in tissue sections of ciliated epithelial cells from adult mouse (arrow heads indicate cilia). Nuclei are stained with propidium iodide (red). Scale bars: 25 μm (G) and 5 μm (H). (I) Co-immunolocalization of anti-PR (Ab-4; red) and anti-Pr (SP2; green) in a tissue section of ciliated epithelial cells from adult mouse (arrow heads indicate cilia). Nuclei are stained with propidium iodide (red) and nuclear localization of anti-PR is indicated with asterisks.
neighboring cells having PR-positive or negative nuclei. SDS-PAGE and western blot analysis showed that anti-PR (SP2) recognized PR-A and PR-B isoforms in isolated tissues of both oviduct and uterus (Fig. 2C). PR expression was not detected in lung extracts (Fig. 2C) and no PR immunoreactivity was detected in lung cilia and epithelial cells (Fig. 2D). This confirms specificity of PR to cilia of the oviduct. Further, PR was not detected in heart extracts (Fig. 2C).

The subcellular localization and expression level of PR in the oviduct was investigated by high resolution confocal laser scanning microscopy. Double labeling of anti-acetylated α-tubulin and anti-PR (SP2) in tissue sections and isolated cells from the oviduct epithelium of hCG-stimulated mice showed that PR was confined to the lower region of the cilia and confirmed that nuclear PR is mostly limited to goblet cells in the epithelium (Fig. 2E). A similar pattern of immunolocalization was observed in human oviduct epithelium (Fig. 2F). As a further control, PR localization in the mouse oviduct was investigated using anti-PR (clone Ab-4) raised against the entire N-terminal region of PR. This antibody localized in a similar fashion as SP2, i.e. it localized to the nuclei of stromal cells, to the nuclei of goblet cells, and to the lower part and at the base of the cilia in ciliated epithelial cells (Fig. 1G–I). Importantly, we observed that tissue sections kept at room temperature for several days show no or very little ciliary PR localization, whereas localization to nuclei remains intact. This may mean that ciliary PR is subjected to rapid degradation if sections are not stored properly.

In order to analyze ciliary localization of PR in more detail, we performed a lateral inspection of anti-PR (SP2) fluorescence along the length of the cilia in tissue sections of oviduct epithelial cells in hCG-stimulated mice. We used the differential interference contrast (DIC) signal as well as a vertical intensity plot of PR fluorescence and acetylated α-tubulin fluorescence that mark individual cilia along the surface of the cells (Fig. 3). The lateral inspection showed that PR localization was restricted to the lower half of the ciliary area (Fig. 3A), and that this localization was specifically assigned to individual cilia (Fig. 3B). These results show that PR in ciliated epithelial cells localizes to the lower half of the cilia and not to microvilli, which are positioned at the base between individual cilia.

**Localization and expression of PR in the oviduct and uterus upon hormone stimulation of pubertal mice**

In the untreated pubertal oviduct, PR (SP2) predominantly localized to stromal cell nuclei, whereas ciliary localization...

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**Figure 3** Subciliary immunolocalization of progesterone receptor (green) in tissue sections of oviduct of adult C57Bl/6j female mice. (A) Lateral fluorescence intensity profile of PR immunofluorescence (SP2) along the length of the cilia of the infundibulum (white bar). DIC profile was used to define the base and the tip of the cillum. (B) Vertical fluorescence intensity profile of PR immunofluorescence (SP2) along the cell surface (white bar). Anti-acetylated α-tubulin (red) was used to define the cilia (arrow heads) along the epithelial surface.
was weak (Fig. 4A). Upon 48 h FSH stimulation nuclear PR immunoreactivity was increased in the stromal cell nuclei of the ampulla and isthmus, whereas this was less pronounced in stromal cells of the infundibulum (not shown). Six hours after hCG, the stromal as well as the non–ciliated epithelial cell nuclei PR immunoreexpression was increased in all regions of the oviduct with the weakest relative staining intensity observed in the infundibulum (Fig. 4B). At this time, ciliary PR localization was heavily increased along the entire oviduct; this was also associated with a minor nuclear PR staining in the ciliated epithelial cells of the ampulla (Fig. 4B). The increase in ciliary PR along the oviduct was predominantly confined to the lower half of the cilia (Fig. 4C). Upon elicitation of ovulation 16 h after hCG treatment, PR was still absent in mural granulosa cells but present at a high level in the cilia of the oviduct (Fig. 4D). The figure shows a close up of the cumulus cells in close proximity with the cilia of the oviduct. These results support the conclusion that ciliary PR is important during transport of the cumulus–oocyte complex, since cumulus cells secrete progesterone to the oviductal fluid.

In protein samples from whole infundibulum tissue, PR–A and PR–B immunoreactivity could be demonstrated with anti–PR (SP2) in western blot analysis in both non–stimulated and hormone–stimulated mice (Fig. 4E). However, the level of these proteins increased significantly upon hormonal stimulation, such that the levels of PR–A and PR–B increased about twofold after 48 h of FSH stimulation, and three- and fourfold respectively after an additional 6 h treatment of hCG (Fig. 4E and F). Further, after 6 h of hCG, we observed the appearance of a distinct and major PR–immunoreactive protein band migrating just above PR–A (Fig. 4E). The level of this band was increased about 25-fold upon hCG treatment (Fig. 4F). It was also observed that the increase in the level of PR–B upon hormonal stimulation was associated with a slight migration shift such that the protein migrated at a higher molecular mass (Fig. 4E).

In the uterus, the luminal epithelium PR localization was less intense after 48 h of FSH, although after 6 h of hCG, the nuclear epithelial cell staining slightly intensified (data not shown). Uterus stroma and muscle cell nuclei PR staining was comparable in all groups examined. Western blot analysis of protein samples from uterus indicated that the levels of PR–A and PR–B are not significantly altered upon hormonal stimulation (Fig. 4H and G), although the PR–A protein band appeared slightly more diffuse after hCG.

**Discussion**

In the mouse oviduct, we show here for the first time a unique PR localization to motile cilia of the epithelial cells. The biological significance of this finding is strengthened by the fact that the PR staining intensity increased upon gonadotropin stimulation of pubertal mice. The gonadotropin–primed mouse has close similarities with the preovulatory cyclic mouse and the staining pattern of PR in the fallopian tube, ovary, and uterus in the present study is largely comparable (Gava et al. 2004). However, previous studies failed to identify PR in ciliated cells of the oviduct and it was speculated that effects on ciliary activity were mediated by goblet or stroma cells positive for PR (Okada et al. 2003). Our findings on ciliary PR suggest a novel function of PR that could aid in the regulation of ciliary activity and function of oviduct epithelium.

The observed PR localization is confined to the lower half and at the base of the cilium, suggesting that ciliary activity regulated by P is mediated through effector molecules that specifically localize to this part of the cilium. Fliegauf et al. (2005) showed that outer arm dynein (OAD) heavy chains regulating ciliary beat frequency in human respiratory cilia and sperm flagella may be regionally and differentially distributed along the axoneme, indicating that regulated beat frequency is controlled by regional localization of OAD heavy chains. Controlled ciliary beating is essential for proper pickup and transport of the ovulated cumulus–oocyte complex (COC), although the identity of extracellular signals that regulate beat frequency and form are not clear. It has been shown that estrogen accelerates and P decelerates oviduct egg transport (Mahmood et al. 1998, Orihuela & Croatto 2001, Orihuela et al. 2001) and antiprogestins added to natural cyclic rats accelerates ovum transport and results in prematurity arrival to the uterus (Fuentesalba et al. 1987). More recently, Wessel et al. (2004) used explants of bovine oviduct to show that P regulates ciliary beat frequency by a fast, non-genomic hormonal interaction. Ciliary beat frequency in the different parts of the oviduct is regulated by Ca$^{2+}$ (Verdugo 1980), and ciliary beat frequency changes during the natural cycle and during pregnancy (Lyons et al. 2002). We suggest that ciliary PR directly modulates the ciliated oviduct epithelium by operating as a fast means to sense and relay changes in the levels of P in the oviduct, such as those induced through release of follicular fluid at ovulation or released by COCs. Cumulus cells from ovulated COCs are known to produce and secrete large amounts of P (Vanderhyden & Macdonald 1998), and thus we speculate that these signaling pathways involve specific Ca$^{2+}$–regulated functions in these cells. Recent studies of the mouse oviduct have shown that the Ca$^{2+}$–selective TRP ion channel, polycystin–2, and the Ca$^{2+}$–binding receptor protein, polycystin–1, are highly upregulated in the cilia along the entire oviduct upon hCG stimulation (Teilmann et al. 2005). In addition, the Ca$^{2+}$–permeable cation channel gated by thermal and osmotic stimuli, TRP vanilloid 4 (TRPV4), localizes to a subpopulation of motile cilia on epithelial cells of the ampulla and isthmus (Teilmann et al. 2005), and the TRPV4 channel was suggested to be involved in the coupling of fluid viscosity changes to oviduct epithelial ciliary activity (Andrade et al. 2005). Together, these findings favor a model where ciliated oviduct epithelial cells perceive signals from the extracellular milieu in a previously unappreciated manner. Thus, ciliary signaling components such as membrane–associated P receptors and TRP ion channels
may act in concert to co-ordinate uptake, transport, and fertilization of the gamete.

Western blotting analysis of isolated infundibulum suggests that PR-A is the principal PR form undergoing transcriptional and/or posttranslational changes in this part of the oviduct. Cyclic changes in PR localization in the uterus and oviduct can easily be appreciated by immunohistochemistry during the estrous cycle, but when analyzed by immunoblotting only small changes in total PR expression are observed (Ohta et al. 1993, Gava et al. 2004). Changes in the relative amount and distribution of the two PR isoforms can alter the signaling capacity dramatically and both PR isoforms undergo phosphorylation upon ligand binding that results in increased sensitivity to changes in the levels of P (Lange 2004). Phosphorylation of PR may also facilitate subcellular relocalization (Qiu & Lange 2003, Lange 2004). We suggest that the PR band observed migrating just above PR-A in SDS-PAGE analysis of oviduct protein fractions, and emerging specifically after hCG stimulation, may represent a phosphorylated and sensitized/activated form of PR-A as described previously (Sheridan et al. 1989, Denner et al. 1990, Poletti et al. 1993, Takimoto & Horwitz 1993).

We also investigated the expression and localization of PR in the ovary and confirmed previous findings that both nuclear and cytoplasmic PR localization in theca and granulosa cells of large preovulatory follicles greatly increases upon administration of LH (Iwai et al. 1991, Park & Mayo 1991, Robker et al. 2000, Jo et al. 2002). PR mRNA expression was suggested to be confined to mural granulosa cell compartment (Conneely et al. 2003) and no PR expression was reported in isolated mouse cumulus cells immediately after isolation (Conneely et al. 2001). In agreement with these studies, we find that PR localization in preovulatory follicles is restricted to mural granulosa cells. In our study, luteinizing granulosa cells displayed less staining than non-luteinized granulosa cells 6 h after hCG. Induction of PR isoforms in granulosa cells upon the preovulatory gonadotropin surge have been reported in bovine (Jo et al. 1993, Iwai et al. 1994, Iwai et al. 1990, Suzuki et al. 1994). Although species differences clearly exist, some PR expression in granulosa cells appears to sustain in the corpus luteum. Our western blot analysis of ovary protein fractions supported the immunohistochemical findings in that PR-A and PR-B forms are highly upregulated in ovaries in mice after hCG. Further, we observed a shift in molecular mass for PR-A in western blot analysis of ovary extracts upon hCG treatment, but this shift was less prominent compared with that observed in the ovary. Most interestingly, the protein level of ovarian PR-A is reduced in mice treated with FSH and prior to stimulation with hCG compared with the non-stimulated mice. To our knowledge, these observations are the first to show a downregulation of ovarian PR specifically upon FSH stimulation, suggesting additional roles of PR in preovulatory follicle development.

The mechanism by which ovarian PR is suppressed by FSH remains to be determined.

It is now clear that P in many cell types may act via non-genomic pathways (Luconi et al. 2002, Peluso et al. 2003, Wessel et al. 2004) and in non-luteinized granulosa cells P stimulation rapidly increases intracellular levels of Ca$^{2+}$ via membrane receptors (Machelon et al. 1996). The increased non-nuclear PR localization in granulosa cells of preovulatory follicles could therefore represent membrane-bound PR forms that act through Ca$^{2+}$ signaling and possibly in coordination with Ca$^{2+}$ channels (Teilmann et al. 2005) and Ca$^{2+}$ currents (Assem et al. 2002). In rat ovaries, P was suggested to inhibit granulosa cell apoptosis through the binding and activation of a 60 kDa membrane protein that functions as a low-affinity, high-capacity receptor for P (Peluso et al. 2001, Peluso 2004). It remains an open question whether the ca. 60 kDa anti-PR immunoreactive protein observed in some western blot analysis of granulosa cells in preovulatory follicles (data not shown) represents a membrane-bound PR form.

In conclusion, PR principally localizes to the lower half and at the base of the cilium in ciliated oviduct epithelium, whereas non-ciliated epithelial cells primarily show nuclear receptor localization. In contrast, ciliated mural granulosa cells uniquely express cytosolic and nuclear PR. Further, cell-specific localization and expression of PR are highly regulated

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**Figure 4** Immunolocalization of progesterone receptor with anti-PR (SP2; green) in oviduct tissue sections before and after gonadotropin stimulation of pubertal female C57Bl/6J mice. (A) Control ovary (no stimulation; T=0); (B) Forty-eight hours after FSH stimulation and 6 h after additional stimulation with hCG (T=48+6). Cilia are marked with arrow heads and nuclei are detected with propidium iodide (red). Scale bars: 25 μm. (C) Close up of the ciliated oviduct epithelium of stimulated mice (T=48+6). Cilia are detected with anti-acetylated α-tubulin (red, arrow heads) and nuclei were detected with TO-PRO (blue). Scale bar: 5 μm. (D) Forty-eight hours after FSH stimulation and 16 h after additional stimulation with hCG (T=48+16). Cilia are marked with arrow heads, cumulus granulosa cells from ovulated ovary are marked with asterisk and nuclei are detected with propidium iodide (red). Scale bars: 25 μm. (E) SDS-PAGE and western blot analysis with anti-PR showing the expression of progesterone receptor forms, PR-A (ca. 83 kDa) and PR-B (ca. 115 kDa), in oviduct before and after gonadotropin stimulation. Molecular markers (kDa) are shown to the left. (F) Relative levels of PR-A lower protein band (open bars), PR-A upper protein band (hatched bars), and PR-B (solid bars) in oviduct after 48 h FSH (T=48) and 6 h of HCG (T=48+6) compared with pubertal controls (T=0). (G) SDS-PAGE and western blot analysis with anti-PR showing the expression of progesterone receptor forms, PR-A (ca. 83 kDa) and PR-B (ca. 115 kDa), in uterine before and after gonadotropin stimulation. Molecular markers (kDa) are shown to the left. (H) Relative levels of PR-A (open bars) and PR-B (solid bars) protein bands in uterus after 48 h FSH (T=48) and 6 h HCG (T=48+6) compared with pubertal controls (T=0). (I and H) Error bars indicate standard errors from three separate experiments. Significant changes in PR levels are marked with one, two and three asterisks for P<0.05, P<0.01, and P<0.001 respectively.
by gonadotropins, in which P released around the time of ovulation may act directly through the ciliated oviduct epithelium to immediate receptivity of the ovulated eggs.

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Characterization of Primary Cilia and Hedgehog Signaling During Development of the Human Pancreas and in Human Pancreatic Duct Cancer Cell Lines

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Hedgehog (Hh) signaling controls pancreatic development and homeostasis; aberrant Hh signaling is associated with several pancreatic diseases. Here we investigated the link between Hh signaling and primary cilia in the human developing pancreatic ducts and in cultures of human pancreatic duct adenocarcinoma cell lines, PANC-1 and CFPAC-1. We show that the onset of Hh signaling from human embryogenesis to fetal development is associated with accumulation of Hh signaling components Smo and Gli2 in duct primary cilia and a reduction of Gli3 in the duct epithelium. Smo, Ptc, and Gli2 localized to primary cilia of PANC-1 and CFPAC-1 cells, which may maintain high levels of nonstimulated Hh pathway activity. These findings indicate that primary cilia are involved in pancreatic development and postnatal tissue homeostasis. Developmental Dynamics 237:2039–2052, 2008. © 2008 Wiley-Liss, Inc.

Key words: primary cilia; pancreas; exocrine duct; development; cancer; Hedgehog signaling; Smoothened; Patched; Gli2; Gli3

INTRODUCTION

Primary cilia are microtubule-based organelles that project into the extracellular environment from the mother centriole of most quiescent cells in the human body. Recent research has demonstrated that cilia function as sensory units that coordinate a variety of signal transduction pathways essential for embryonic and postnatal development as well as tissue homeostasis in the adult body (Christensen et al., 2007; Eggenschwiler and Anderson, 2007; Kiprilov et al., 2008). These pathways include Hedgehog (Hh), Wnt, and platelet-derived growth factor receptor (PDGFR) signaling as well as Ca2+-signaling by means of TRP ion channels. Furthermore, primary cilia may control behavioral responses, such as occurs in the central nervous system where neuronal cilia function in a pathway that controls satiety responses (Davenport et al., 2007). Consequently, defects in ciliary assembly lead to a plethora of diseases and disorders, including kidney, liver and pancreatic cysts, blindness, obesity, diabetes, developmental disorders, and other human diseases now collectively referred to as ciliopathies (Badano et al., 2006; Satir and Christensen, 2007; Yoder, 2007; Fliegauf et al., 2007).

Primary cilia on the epithelium of renal tubules control tissue homeostasis probably by acting as mechanosensors that monitor the composition and flow rate of urine in the nephron (Praetorius and Spring, 2003; Yoder, 2007). Obstruction of this flow-sensing response in, for example, the IFT88Tg737Rpw

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mouse (hereafter referred to as Tg737orpk), causes disruption of tissue organization and cyst formation. The Tg737orpk mouse has a mutation in the Tg737/IFT88 gene that encodes a subunit of the IFT particle complex B required for ciliary assembly (Pazour et al., 2000; Yoder et al., 2002a; Rosenbaum and Witman, 2002; Pedersen et al., 2008). Among the ciliary signaling modules involved in renal tissue homeostasis are the TRP channel polycystin 2 (PC-2) and polycystin-1 (PC-1), which are disrupted in human ADPKD (Yoder et al., 2002b; Pazour et al., 2002; Nauli et al., 2003). Bending of the ciliary axoneme due to fluid movement has been shown to induce a Ca$^{2+}$-response (Praetorius and Spring, 2001), which is dependent on PC-1 and PC-2. This calcium response is thought to be important in detecting fluid movement for maintaining tissue function, because mutations leading to its loss result in cyst development (Delmas et al., 2004; Liu et al., 2005b; Kötting, 2007; Weimbs, 2007; Yoder, 2007). In addition, in the absence of flow PC-1 may be proteolytically processed (Chauvet et al., 2004), leading to the nuclear translocation of the transcription factor STAT6 and co-activator P100 (Low et al., 2006). Furthermore, defects in ciliary assembly may result in up-regulation of the canonical Wnt pathway and impair the ability of non-canonical Wnt signals to suppress the canonical pathway (Gerdes et al., 2007; Corbit et al., 2008). Altered regulation of the Wnt signaling pathway in the kidney has been associated with uncontrolled cell proliferation and differentiation and was found to result in cystic kidney disease (Cano et al., 2004; Merkel et al., 2007).

In addition to kidney defects, the loss of primary cilia in the Tg737orpk mouse causes a series of abnormalities in the pancreas, including extensive cyst formation in ducts (Cano et al., 2004; Zhang et al., 2005). This may indicate a functional similarity between cilia in kidney and pancreatic duct systems. Cells of both exocrine and endocrine systems in the pancreas possess primary cilia, including islet cells and the ducts, but apparently not the acini (Kodama, 1983; Ashizawa et al., 1997; Cano et al., 2004, 2006; Zhang et al., 2005). Pancreas abnormalities in the Tg737orpk mouse begin with dilations of the pancreatic ducts in late gestation, which after birth are accompanied by extensive formation of large, interconnected cysts as well as apoptosis and vacuolization of acini. These changes are reminiscent of chronic pancreatitis, supporting the speculation that primary cilia of ducts play an essential role in the development of the pancreas (Cano et al., 2004; Zhang et al., 2005). In the dilated ducts and cysts, PC-2 is mislocalized to intracellular compartments, the cytosolic localization of β-catenin is increased and there is an increased expression of Tcf/Lef transcription factors. This finding suggests that, as in the kidney, there is an alteration in the Wnt signaling pathway (Cano et al., 2004).

Another signaling pathway essential for pancreatic development and tissue homeostasis is the Hedgehog (Hh) pathway. Hh regulates cell proliferation and differentiation in numerous embryonic tissues and Shh is expressed in many regions of the embryo where it functions as a key organizer of tissue morphogenesis (Odent et al., 1999). One embryonic tissue where the initial absence of Shh signaling is required for development is in the pancreatic anlage, because ectopic expression of Shh leads to transformation of pancreatic mesoderm into intestinal mesenchyme (Apelqvist et al., 1997). In addition to being an important inductive signal in embryonic development, Hh signaling is required in homeostasis of mature tissues and is also implicated in many human cancers, including endodermal derived carcinomas of the esophagus, stomach, and pancreas (Beachy et al., 2004) as well as neurodegenerative disorders (Bak et al., 2003). Although the mechanism is not completely understood, it has been shown that Hh signaling is coordinated by the primary cilia (e.g., Huangfu et al., 2003; Corbit et al., 2005; Huangfu and Anderson, 2005; May et al., 2005; Haycraft et al., 2005; Liu et al., 2005a; Koyama et al., 2007; Vierkoten et al., 2007; Ruiz-Perez et al., 2007; Caspary et al., 2007; Rohatgi et al., 2007; Kiprilov et al., 2008). Regulation of the Hh pathway is complex with Hh responsive Gli transcription factors having either activator or repressor functions that depend on IFT proteins.
that localization of Hh signaling components is regulated during human pancreatic development with Gli2 and Smo accumulating in the cilium at later stages while the level of nuclear and cytosolic Gli3 expression is reduced. These changes in localization correlate with known activity of the Hh pathway during pancreas development. We demonstrate that components of the Hh pathway also localize to the cilium in CFPAC-1 and PANC-1 cells and that these cells may maintain high levels of nonstimulated Hh pathway activity. These findings indicate that Hh signaling coordinated by the primary cilium in the pancreas is essential for normal pancreatic development as well as postnatal tissue function.

RESULTS

Early Development of the Human Pancreas

To investigate the presence of primary cilia in human pancreatic progenitor cells of the initial duct epithelium we analyzed 3-μm-thick tissue sections from 7.5-week-old embryos and from 14- and 18-week-old fetuses. Hema-toxylin and eosin (HE) staining of the pancreatic tissue and identification of the developing ducts at these stages are presented in Figure 1A–C. In 5- to 6-week-old embryos a dorsal and a ventral out pocketing of the foregut duodenal endoderm result in the formation of separate dorsal and ventral pancreatic primordia. During the seventh embryonic week a fusion of these ducts takes place followed by a rapid expansion and branching of the ductal epithelium (Fig. 1A). Pancreatic progenitor cells in this epithelium engage in two separate differentiation programs at distinct developmental stages. At the first stage from week 8 to 10, the endocrine progenitor cells appear as cell clusters budding from the central ductal epithelium. These buds form the islets of Langerhans in the central pancreas at week 12, and at midgestation, the endocrine compartment is established. At a much later stage, the exocrine progenitors are responsible for the differentiation and expansion of the exocrine acinar epithelium, which is mainly a late fetal or early perinatal event. Figure 1B shows a section of the ductal system from a 14-week-old fetus (Jackerott et al., 2006) with initial endocrine progenitors budded from the ductal epithelium, and Figure 1C shows a section from an 18-week-old fetus with endocrine progenitors budded from the ductal epithelium as well as acinar progenitors surrounding the ductal epithelium.

Characterization of Primary Cilia in Ducts of the Developing Human Pancreas

To identify primary cilia, we used two markers for cilia (antiacetylated α-tubulin (ac.et. tb) and anti-detyrosinated α-tubulin (glu-tub)) in immunofluorescence microscopy (IF) analysis and found that epithelial cells in ducts at all stages form primary cilia that either project into the duct lumen, form spiral-like structures or align parallel to the lumen surface (Fig. 1D–H). The cilia varied in lengths from approximately 5 to 20 μm, and in some ducts long cilia seemed to form contact or bridge with cilia from neighboring epithelial cells or from cells on the opposite side of the duct (Fig. 1E,F). At the late stage of embryonic development (week 7.5), long acetylated-tubulin structures often projected from epithelial cells in a “star”-like configuration by pointing into the site of the developing duct (Fig. 1F and inset). Both short and long primary cilia were observed during all three developmental stages. Long cilia were predominantly observed in week 7.5, possibly due to the prevalence of small intercalated and intralobular type ducts that do have longer cilia (Kodama, 1983). The formation and orientation of primary cilia in the human developing ducts is similar to that reported for the pancreas in the rat and mouse (e.g., Kodama, 1983; Hidaka et al., 1995; Ashizawa et al., 1997; Cano et al., 2004; Zhang et al., 2005).

Development of the Human Pancreas Correlates With Ciliary Localization of Hh Signaling Components in Duct Epithelial Cells

It was previously shown that the graded response to Hh-signaling controls pancreatic organogenesis in the mouse, where Hh signaling is at low levels during early embryonic stages to ensure the correct establishment of organ boundaries and tissue architecture. Hh signaling is then activated at later developmental stages to promote proliferation and maturation of the tissue (Kawahira et al., 2005; Lau et al., 2006; Cano et al., 2007; van den Brink, 2007). In Hh signaling, Gli2 in vivo acts primarily as a transcriptional activator, whereas Gli3 mainly works as a transcriptional repressor to control development. To address the potential role of primary cilia in human pancreatic development, we investigated the expression and cellular distribution of Smo, Gli2, and Gli3 in developing pancreatic ducts by IF analysis. In other cell types, such as fibroblasts (Rohatgi et al., 2007) and human embryonic stem cells, hESC (Kiprilov et al., 2008), Smo becomes concentrated while Ptc levels decrease in primary cilia upon stimulation with a Hh ligand, which may result in activation or deactivation of Gli transcription factors, possibly in the cilium (Huangfu and Anderson, 2006; Christensen and Ott, 2007). We found that Gli3 (Fig. 2A) and Gli2 (Fig. 2B) are absent from primary cilia in pancreatic ducts of embryos (week 7.5), but are concentrated in the cilia of ducts of early fetuses (week 14), and even more so in 18-week-old fetuses. We then investigated the localization of Gli3 in IF analysis using two different Gli3 antibodies that were raised against amino acids 1-280 of Gli3 (Gli3-H-280) and against a peptide mapping at the N-terminus of Gli3 (Gli3-N-19). Both antibodies showed a decrease in staining intensity and cellular distribution of Gli3 during development. At week 7.5, Gli3 strongly localized to duct epithelial cells and was present in the nuclei and in an intense punctate pattern in the cytosol of duct epithelial cells. In contrast, in ducts of 14- and 18-week-old fetuses, Gli3 staining was reduced (Fig. 2C–E). As a control, a blocking peptide against anti-Gli3-N-19 decreased localization of the antibody to the duct epithelium (Fig. 2D). These results could reflect activation of the pathway because Hh is known to repress Gli3 expression (Marigo et al., 1996; Büscher and Rüther, 1998; Schweitzer et al., 2000).
Characterization of Primary Cilia in Cultures of Human Pancreatic Duct Cell Lines

CFPAC-1 and PANC-1 are human pancreatic ductal adenocarcinoma cell lines, which form adherent epithelial monolayers in culture (Fig. 3A,B) and are often used as model cell lines for studying ion transport and signaling in human pancreas (Novak et al., 2008). PANC-1, derived from epithelial carcinoma, forms a monolayer of heterogeneous duct cells also with some cells growing on top of each other. Actin is mainly arranged in the cortex and lamellipodia (Fig. 3B). CFPAC-1 cells, derived from a patient with cystic fibrosis containing deletion in Phe-508 in CFTR, appear homogenous and on glass surface they form a monolayer interrupted by some void spaces. In most CFPAC-1 cells actin is concentrated in well-organized stress fibers and no subcortical actin network is apparent (Fig. 3A). Also the cytosolic network of microtubules seems dispersed throughout the cells, as also observed by Hollande et al. (2005). The epithelial-like cytoskeletal

Fig. 1. Characterization of primary cilia in human pancreatic progenitor cells of the initial ductal epithelium. A–C: Hematoxylin and eosin (HE) staining of sections of the human developing pancreas from 7.5-week-old embryos (A), 14-week (B), and 18-week-old (C) fetuses. Lower panels show higher magnification images of the developing ducts. d, ducts; mPD, main pancreatic duct; PI, pancreatic islets budding from the duct (d); D, duodenum. D–H: Immunofluorescence microscopy (IF) analysis of primary cilia in the developing ducts at the stages presented in (A–C) by using two markers for primary cilia (arrows; anti-acetylated α-tubulin, acet. tb, red; and anti-detyrosinated α-tubulin, glu-tub, green). Nuclei were stained with DAPI (blue, 4',6-diamidine-2-phenylidole-dihydrochloride). Shifted overlays (E) shows the combined staining with acet. tb and glu-tb, where the images for each antibody are slightly shifted from one another. Scale bars = 50 μm in A–D, 10 μm in E,G,H, 20 μm in F.
arrangement of PANC-1 cells may aid the targeting and function of adenosine receptors, which regulate ion transport (Novak et al., 2008). The distribution of these receptors, carbonic anhydrase, and cytoskeleton arrangement is defective in CFPAC-1 cells (Novak et al., 2008).

To study the time course of primary cilia formation, the cells were analyzed by IF microscopy both in cultures with 10% serum (at 50% and 100% confluency) and following serum starvation of confluent cultures in medium with 0.5% serum for periods of 48 and 72 hr (Fig. 3G). At 50% confluency and in the presence of 10% serum PANC-1 cells are most often in interphase growth and mitosis, such that very few cells are ciliated. At 100% confluency, approximately 4% of the cells had formed single primary cilia, presumably as a consequence of cellular contact inhibition of growth. After 48 and 72 hr of serum starvation, the frequency of ciliated cells increased to approximately 12 and 46%, respectively, and cilia had lengths of approximately 10 – 20 μm (Fig. 3D, left panel, and 3G). Similar results on appearance of cilia were obtained with cultures of CFPAC-1 cells; these cells, however, started to lose contact with the polylysine-coated glass coverslips at 72 hr of serum starvation. The relatively low percentage of ciliated cells in pancreatic cell lines is in sharp contrast to that of other cell types such as cultured fibroblast, in which more than 95% of the cells are ciliated after 24 hr of serum starvation (Schneider et al., 2005). The low frequency of ciliated cells in the adenocarcinoma cell lines could be related to their cancerous origin because cells that have an increased rate of growth more rarely enter growth arrest, which is required for assembly and maintenance of the primary cilium (Satir and Christensen, 2007). Indeed, IF analysis revealed many mitotic and dividing cells

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**Fig. 2.** Expression of Hedgehog signal components in human pancreatic progenitor cells of the initial ductal epithelial cells and their primary cilia from 7.5-week-old embryos and 14-week and 18-week-old fetuses. A–E: Localization of Smo (green) in 7.5-week-old embryos and 18-week-old fetuses (A), and localization of Gli2 (H-300; green, B), Gli3 (H-280, green, C), and Gli3 (N-19, green, D, E) in 7.5-week-old embryos and 14-week and 18-week-old fetuses. Primary cilia (arrows) were localized with acet. tb (red) and nuclei were stained with DAPI (blue, 4',6-diamidine-2-phenylidole-dihydrochloride). d, ducts. Scale bars = 10 μm.
in the CFPAC-1 and PANC-1 cultures as evidenced by the presence of mitotic spindles and midbodies (Fig. 3C,D, right panels). As in other cell types, the primary cilia emanate from the centrosomes (labeled by anti-Pctn, Fig. 3E,F left panels) and, in particular, from one of the two centrioles (labeled by anti-centrin, Fig. 3E,F right panels), presumably the mother centriole, which also functions as the basal body (Satir and Christensen, 2007). PANC-1 cells grew longer cilia (10–20 μm) compared with CFPAC-1 cells (5–15 μm), where in some cases the short cilia projected vertically into the medium, visualized as “dots” in the IF analysis (Fig. 3C, middle panel).

Localization of Ptc and Smo to Primary Cilia of Human Pancreatic Duct Cell Lines

To evaluate whether Hh-signaling and primary cilia might be linked to development of cancer in adult pancreas, we next investigated whether components of the Hh signaling system are present in CFPAC-1 and PANC-1 primary cilia. Initially, we demonstrate by IF analysis that as in other cell types, Ptc and Smo localized to primary cilia of both cell lines (Fig. 4A,F). As a control, we exogenously expressed Smo from a YFP-Smo construct and show that it localized to the cilia of CFPAC-1 cells (Fig. 4G). The Ptc antibody recognized Ptc as a single protein band in Western blot analysis (Fig. 4B, upper panel), and this band was removed in the presence of anti-Ptc blocking peptide (Fig. 4C).
Expression of Ptc at the mRNA level was previously shown to be up-regulated as a consequence of the autonomous operation of an active Hh signaling process in pancreatic cancer cells in which Ptc mRNA in PANC-1 cells is up-regulated at a higher level than that of CFPAC-1 cells (Berman et al., 2003; Thayer et al., 2003). Consistent with these earlier findings, we show that the protein level of Ptc in PANC-1 was higher than that in CFPAC-1 cells (Fig. 4B, upper and lower panels). As further controls for antibody specificity, we show that Ptc localized to primary cilia of wild-type mouse embryonic fibroblasts (wt MEFs) but not to mutant Ptc+/- MEFs (Fig. 4D) as previously demonstrated by Ro- hatgi et al. (2007). Then we stimulated wt MEFs with the Smo agonist, SAG, that activates Hh-signaling (Chen et al., 2002) to analyze changes in protein levels of Ptc, which is up-regulated with these earlier findings, we show that the presence of the primary cilium (wt MEFs) but not to mutant Ptc+/- MEFs (Fig. 4D) as previously demonstrated by Ro- hatgi et al. (2007). Then we stimulated wt MEFs with the Smo agonist, SAG, that activates Hh-signaling (Chen et al., 2002) to analyze changes in protein levels of Ptc, which is up-regulated in response to activation of the Hh pathway. As shown in Figure 4E, the Ptc is present at a low level in wt MEFs in the absence of SAG, whereas it increased after Hh pathway activation. This increase was dependent on the presence of the primary cilium, because the level of Ptc was kept at a low level in the presence of SAG in MEFs derived from the Tg737orpk mouse (Fig. 4E). These results support the idea that the cillum is required for activation of the Hh pathway, and that aberrant Hh signaling in pancreatic cancer may be associated with increased Hh signaling by means of the primary cilium.

Another observation was that Ptc localized only weakly to the primary cilium (Fig. 4A), while Smo localized very strongly and in a punctate pattern in the cilium of the pancreatic duct cancer cells (Fig. 4F). This is in sharp contrast to that observed in cultures of normal cells such as MEFs and hESCs, where Ptc is highly concentrated in the cilium and Smo is mostly absent from the cilium in non-stimulated cells (Rohatgi et al., 2007; Kiprilov et al., 2008). Stimulation of the pancreatic duct cells with SAG did not further increase ciliary localization of Smo in CFPAC-1 cells (Fig. 4H), supporting the conclusion that the Hh pathway is maintained at a high and autonomous level in these cells.

Localization of Gli Transcription Factors to Primary Cilia of Human Pancreatic Duct Cell Lines

Gli2 and Gli3 are the primary transcription factors that are being regulated in Hh signaling. The full-length transcription factors mainly function as transcriptional activators, but in the absence of the Hh signaling they may undergo proteolytic processing and function as transcriptional repressors (Wang et al., 2000; Littingtung et al., 2002; Pan et al., 2006). To investigate the expression and localization of Gli2 in growth-arrested PANC-1 and CF-PAC-1 cells we used three different antibodies raised against amino acids 841-1140 mapping near the C-terminus of Gli2 (Gli2-H-300), a peptide mapping near the N-terminus of Gli2 (Gli2-N-20), and a peptide mapping within an internal region of Gli2 (Gli2-G-20). All three antibodies localized uniquely to the primary cilium and most often as an enrichment at the tip of the cilium (Fig. 5A,B, left panels), similar to what has been observed in primary cilia of the murine limb bud and hESC (Haycraft et al., 2005; Kiprilov et al., 2008). Ciliary localization was abolished in the presence of blocking peptides to the two peptide antibodies (Fig. 5B, right panels). Also, localization of Gli2 to the ciliary tip was confirmed by transfection and overexpression of a green fluorescent protein (GFP) -Gli2 construct in NIH3T3 fibroblasts (Fig. 5C). To confirm antibody specificity, we performed Western blotting analysis with the peptide antibodies in the absence and in the presence of blocking peptides. As shown in Figure 5D, Gli2-N-20 specifically recognized both full-length (ca. 170 kDa) and processed (ca. 70 kDa) forms of Gli2 that function as activator (Gli2(FL)) and repressor (Gli2(R)) forms in Hh signaling, respectively (Pan et al., 2006). In contrast, Gli2-G-20 recognized only Gli2(FL) (Fig. 5E). This finding suggests that the activator forms of Gli2 are present in the primary cilium and that ciliary Gli2 may be part of the Hh signaling machinery that is up-regulated in pancreatic cancer cells.

We then analyzed lysates from CF-PAC-1 cells by Western blotting analysis using the Gli3 antibodies, Gli3-N-19 and Gli3-H-280. Gli3-N-19 recognized both full-length (Gli3(FL) at ca. 180 kDa) and processed forms (Gli3(R) at ca. 80 kDa) of Gli3 (Fig. 5F,G). In contrast, Gli3-H-280 predominantly recognized the processed version of Gli3 (Fig. 5F), supporting the conclusion that this antibody mainly identifies the repressor form of Gli3 under the experimental conditions carried out in this work. These data further support the assumption that the observed decrease in staining intensity and cellular distribution of Gli3 in the epithelium of the pancreatic ducts during fetal development (Fig. 2) is due to the increased activation of the Hh signaling that favors the processing of transcriptional activators of the Hh pathway. Based on these observations, it was therefore interesting to investigate the expression and localization of Gli3(R) in pancreatic cancer cells upon stimulation of the Hh pathway using Gli3-H-280 in IP analysis. As seen in Figure 5H, Gli3-H-280 stained CF-PAC-1 cells very weakly in both the absence and in the presence of SAG. In contrast, Gli3-H-280 strongly stained the nucleus as well as small cytoplasmic puncta in NIH3T3 cells and this staining was largely absent in the presence of SAG (Fig. 5I). Furthermore, we used transfected NIH3T3 cells with GFP constructs of either the GFP-Gli3(R) and Gli3(FL) to evaluate the cellular localization of activator and repressor forms of Gli3. Similar to that seen in nonstimulated NIH3T3 cells with Gli3-H-280 (Fig. 5I) and previously in mouse limb bud mesenchyme cells, we found that GFP-Gli3(R) localized predominantly to the nucleus and to small cytosolic puncta, but not to the primary cilium (Fig. 5J, left panels). In contrast, GFP-Gli3(FL) seemed to localize to the primary cilium with minimal localization in the nucleus and without cytosolic puncta (Fig. 5J, right panels). These results are in line with our conclusion that Gli3-H-280 recognizes the repressor form of Gli3, which is concentrated in the nucleus in the absence of Hh signaling, and that Gli3-repressor forms are expressed at a low level in CF-PAC-1 cells.
Localization of Patched (Ptc) and Smoothened (Smo) in cultures of quiescent human pancreatic adenocarcinoma cells. Primary cilia are marked with arrows and were localized with acetyl tb (red). A: Localization of Ptc (green) in PANC-1 cells (upper panels) and CFPAC-1 cells (lower panels). B: The upper panel shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE, and Western blotting analysis of anti-Ptc cross-reactivity to proteins in CFPAC-1 and PANC-1 cells. The lower panel shows protein relative levels of Ptc in CFPAC-1 and PANC-1 cells. Values are given relative to the level of Ptc in CFPAC-1 cells (n = 3). C: Western blotting analysis of anti-Ptc cross-reactivity in the absence and in the presence of blocking peptide in PANC-1 cells. D: Localization of Ptc (green) to the primary cilium in wild-type mouse embryonic fibroblasts (wt MEFs, upper panels) and in mutant Ptc−/− MEFs (lower panels). E: Protein levels of Ptc before and after stimulation with SAG for 24 hr in quiescent wt and Tg737™ MEFs. F: Localization of Smo (green) in PANC-1 cells (upper panels) and CFPAC-1 cells (lower panels). G: Localization of YFP-Smo (green) in CFPAC-1 cells. Scale bars = 10 μm.
DISCUSSION

Recent research has shown that primary cilia coordinate signal pathways, which are essential in mammalian development, tissue homeostasis and behavioral responses. Accordingly, defective primary cilia assembly and maintenance lead to a plethora of diseases and disorders, now collectively referred to as ciliopathies (Badano et al., 2006; Fliegaf et al., 2007; Satir and Christensen, 2007; Yoder, 2007). Previous reports have shown that loss of primary cilia in the Tg737orpk mouse causes a series of developmental defects in exocrine and endocrine tissues of the pancreas, including extensive cyst formation in ducts (Can et al., 2004; Zhang et al., 2005). However, the function of primary cilia in pancreatic organogenesis and adult tissue homeostasis is largely unknown. In this report, we demonstrate that essential components of the Hedgehog (Hh) signaling pathway localize to primary cilia of both human pancreatic progenitor cells of the initial ductal epithelium and in cultures of human pancreatic adenocarcinoma duct cell lines, and that expression of ciliary Hh components may be linked to pancreatic organogenesis and pancreatic cancer.

Hh Signaling and Primary Cilia in the Developing Human Pancreas

Previous studies have shown that the graded response to activation of the Hh signaling pathway is critical in pancreatic organogenesis in the mouse, where Hh signaling is activated only at late developmental stages (Kawahira et al., 2005; Lau et al., 2006; Cano et al., 2007; van den Brink, 2007). It is likely that organogenesis of the human pancreas also depends on the graded response to Hh signaling, because the transcriptional network that controls pancreatic development is highly conserved in mammals (Wilson et al., 2003), although the time point of onset of development and maturation of the endocrine system differs in humans and rodents (Ostrer et al., 2006). Recently, the primary cilia was shown to act as a sophisticated switch by which cells turn Hh signaling on and off by the regulated movement of Smo into the cilium and Ptc out of the cilium upon stimulation of the Hh pathway in fibroblasts (Rohatgi et al., 2007) and hESC (Kiprilov et al., 2008), where Smo probably functions to activate Gli transcription factors (Christensen and Ott, 2007). To analyze the significance of the primary cilia in Hh signaling during organogenesis of the human pancreas, we studied sections of the developing human pancreas at different developmental stages. These stages included embryonic stage 7.5 weeks, which is before development of the endocrine system, and stages 14 and 18 weeks, where endocrine progenitors bud from the ductal epithelium to form the endocrine compartment (Fig. 1). Based on the recent data from Rohatgi et al. (2007), our findings that Smo and Gli2 are absent from pancreatic primary cilia at embryonic stage 7.5, but highly concentrated in cilia in 14- and 18-week-old fetuses (Fig. 2) suggest that the Hh pathway has become activated during developmental stages. This further supports the conclusion that development of the human pancreas is regulated by a graded Hh signaling response, and that this could be coordinated by the primary cilium. Indeed, the loss of Gli3 staining in the ductal epithelium after week 7.5, that is, before formation of the endocrine system, is consistent with the interpretation that Gli3 staining at week 7.5 may represent the up-regulated level of the repressor form of Gli3 (Fig. 5F), consistent with low level of Hh signaling at this developmental stage. Therefore, disruption of pancreatic developmental in mice with defects in primary ciliary assembly (Can et al., 2004, 2006; Zhang et al., 2005), may partly be due to loss of coordinated Hh signaling during genesis of the pancreas.

Another interesting observation is the presence of long cilia-like structures that seem to bridge with corresponding structures emerging from adjacent or opposite cells of the lumen surface (Fig. 1). This is particularly prominent in the developing ducts at week 7.5. Bridging of primary cilia was also observed in collecting ducts of the mouse developing kidney (Liu et al., 2005b), but the function of physical contact between individual primary cilia is unknown. Although highly speculative at this point, the intertwining of cilia could be part of the sensory signaling machinery that controls the initial development of ducts and perhaps endocrine progenitors. In the developing kidney, the formation of tubular systems is controlled by invarin, which acts as a
molecular switch between the canonical and noncanonical Wnt pathways (Simons et al., 2005), and inversin potentially signals from the primary cilium to suppress β-catenin expression in the canonical pathway and to up-regulate the noncanonical pathway to organize planar cell polarity and correct tubular formation (Christensen et al., 2007). Because loss of the primary cilium in the developing mouse pancreas is associated with increased β-catenin expression (Cano et al., 2004; Zhang et al., 2005), it is possible that cilia in the developing pancreas regulate both Wnt and Hh signaling, although it is presently unknown whether Hh or Wnt signaling is affected by physical contact between primary cilia in the ductal epithelium. Further experiments are required to explore this.

**Hh Signaling and Primary Cilia in Human Pancreatic Duct Cell Lines**

Hh is implicated as an important mediator of human pancreatic carcinoma, as evidenced by, for example, the increased expression of positive Hh regulators and decreased expression of negative Hh regulators in CFPAC-1 or PANC-1 cells (Thayer et al., 2003; Berman et al., 2003). Here, we show that essential components of the Hh pathway, including Smo, Ptc, and Gli2, are present in primary cilia of human pancreatic ductal adenocarcinoma cell lines, CFPAC-1 and PANC-1, consistent with the idea that the primary cilium continues to coordinate Hh signaling in cells derived from the mature pancreas. We also suggest that aberrant Hh signaling in these cancer cell lines may be associated with the autonomous activation of the signaling pathway in the cilium as judged by high levels of Smo and low levels of Ptc in the cilium, which is accompanied by the formation of Gli2(FL) forms in the cilium and low levels of Gli3(R) in the nucleus.

In support of the hypothesis that the primary cilium may comprise the site for aberrant Hh signaling in human pancreatic cancer tumorigenesis, we show that in contrast to normal cells, which require Hh pathway stimulation in the cultures for Smo to enter the cilium and activate Hh target genes (Rohatgi et al., 2007; Kiprilov et al., 2008), Smo is strongly localized to the primary cilium in both CFPAC-1 and PANC-1 cells, even in the absence of external stimulation of the Hh pathway by SAG. Further and in contrast to primary cultures of mouse embryonic fibroblasts, Ptc is expressed at a higher level than that of MEFs, which require the primary cilium and stimulation of the Hh pathway to increase their expression of Ptc. The activator form of Gli2, Gli2(FL), localizes to the tip of primary cilium, which may comprise the site for activation of Gli transcription factors in Hh signaling. We then examined the expression and localization of Gli3 before and after SAG stimulation by comparing the localization of endogenous Gli3 by IF assessed using Gli3-H-280, which recognizes the processed form of Gli3 with localization of GFP-Gli3(FL) and GFP-Gli3(R) expressed in transfected growth-arrested NIH3T3 fibroblasts. As shown in Figure 5J, only GFP-Gli3(R) localized to the nucleus and to cytoplasmic puncta. These data are consistent with the conclusion that the repressor form is absent from the primary cilium and is repressing Hh target genes in the absence of Hh signaling. We then performed IF analysis in NIH3T3 cells in the absence and in the presence of SAG and compared the staining with Gli3-H-280 to localization of the protein expressed from the GFP constructs. As shown in Figure 5I (left panels) anti-Gli3 stains non-stimulated cells similar to that of the GFP-Gli3(R) construct. Upon activation of the Hh pathway nuclear Gli3 staining is strongly reduced, supporting the conclusion that the antibody in IF analysis is detecting the repressor form of Gli3. Finally, we examined the localization of Gli3 in CFPAC-1 cells, which showed very weak cytosolic and nuclear staining of Gli3 in both stimulated and nonstimulated cells. This result would be expected if Hh signaling activity is elevated in these cells because Hh signaling represses Gli3 expression (Marigo et al., 1996; Büscher and Rüther, 1998; Schweitzer et al., 2000), and is further evidenced by the accumulation of Smo in the cilium of these cells.

**CONCLUSIONS**

Our data indicate a functional role of the primary cilium in coordinating Hh signaling during development of the human pancreas and potentially in tumorigenesis of the adult human pancreas. During development the primary cilium may sense and relay the graded response to activation of the Hh signaling pathway that controls human pancreatic organogenesis at the onset of fetal development. In the adult, the autonomous activation of the Hh pathway in pancreatic tumorigenesis may be linked to aberrant Hh signaling in the primary cilium of the pancreatic cells. Further analysis will be required to understand the potential significance of Gli processing in the cilium and how the switch between activator and repressor forms of Gli2 and Gli3 is regulated by the cilium during development and tumorigenesis of the pancreas and potentially of other human tissues and organs. Furthermore, it will be important to identify the molecular mechanisms that coordinate translocation of Smo and Ptc in and out of the cilium, which ultimately may impinge on the activation and deactivation of the Hh pathway, which is of relevance in human health and development.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**

The human pancreatic exocrine duct cell lines PANC-1 (ATCC, #CRL-1469) and CFPAC-1 (ATCC, #CRL-1918; passages 8–36) were from the American Type Culture Collection and were cultured in Dulbecco’s modified Eagles medium with glutamax (DMEM; Invitrogen) and Iscove’s modified Dulbecco’s medium with glutamax (IMDM; Invitrogen), respectively. Both growth media were supplemented with 10% heat inactivated fetal calf serum (FCS; Invitrogen) and 1% penicillin-streptomycin (Penicillin G sodium: 10,000 U/ml and streptomycin G sodium: 10,000 µg/ml) (PS; Invitrogen) and kept in a humidified air chamber at 37°C and 5% CO₂. Passing of cells was performed by trypsinization. NIH3T3 Swiss mouse embryonic fibroblasts and wt, Ptc−/− and Tg737neo+ mouse embryonic fibroblasts (MEFs) were cultured as de-
Tissues

Human embryos and fetuses were obtained from extra uterine pregnancies, Caesarean sections, or postglandinduced legal abortions donated to the Developmental Biology Unit, ICMM, at the Panum Institute, University of Copenhagen, Denmark. The embryos and fetuses ranged from 8 to 180 mm crown-rump length (CRL), corresponding to 6th–19th ovulation weeks. Informed consent was obtained according to the guidelines of the Helsinki Declaration II. Additional samples from legal first trimester abortions from the Laboratory of Reproductive Biology, Rigshospitalet, and Frederiksborg Hospital (both University of Copenhagen) were also included in this study. Informed consent was obtained according to the Helsinki declaration II and approved by the ethical committee of Copenhagen and Frederiksborg Communities (KF 258206). Calculation of embryonic and fetal age was based on information about the last menstrual period, and measurements of CRL and foot lengths.

Antibodies, Blocking Peptides, and Staining Reagents

Primary antibodies (dilutions in parenthesis for IF analysis): mouse monoclonal antibodies from SigmaAldrich: anti-acetylated α-tubulin (acet. tb. cat. no. T6795; 1:5,000), anti-β-actin (cat. no. A5441; 1:5,000), and α-tubulin (cat. no. T5168; 1:2,000). Rabbit polyclonal anti-detyrosinated α-tubulin (glu-tub; ab48389; 1:500) was from Abcam. Polyclonal antibodies from Santa Cruz Biotechnology: rabbit anti-Smo (cat. no. LS-A2668; 1:100) from MBL, and (2) Smo (cat. no. 38686) from Abcam. Secondary antibodies for immunofluorescence microscopy analysis (all from Molecular Probes and diluted at 1:600): Alexa Fluor 568-conjugated goat anti-mouse IgG (cat. no. A11019), Alexa Fluor 568-conjugated rabbit anti-mouse IgG (cat. no. A11061), Alexa Fluor 488-conjugated goat anti-rabbit IgG (cat. no. A11008), and Alexa Fluor 488-conjugated donkey anti-goat IgG (cat. no. A1055). Secondary antibodies for Western blot analysis (all from Jackson Immunoresearch and diluted 1:5,000): alkaline phosphatase conjugated goat anti-rabbit, rabbit anti-goat, and goat anti-mouse. Nuclei were stained with 4’,6-diamidine-2-phenylindole-dihydrochloride (DAPI, 1:600) from Molecular Probes, and F-actin was stained with rhodamine phalloidin (1:100; Molecular Probes, Invitrogen). Blocking peptides sc-6155P, sc20290P, sc-6149P, and sc-6149P were from Santa Cruz Biotechnology.

Immunohistochemistry of the Developing Human Pancreas

Tissue specimens were dissected into appropriate tissue blocks and fixed for 12–24 hr at 4°C in one of the following fixatives: 10% buffered formalin, 4% Formol-Calcium, Lillie’s AAF, or Bouin’s fixatives. The specimens were dehydrated with graded alcohols, cleared in xylene, and embedded in paraffin wax (Merck, melting point 52°C). Serial sections, 3–5 μm thick, were cut in transverse, sagittal, or horizontal planes and placed on siliconized slides. Representative sections of each series were stained with hematoxylin and eosin, or with toluidine blue. For immunohistochemistry (IHC) sections were dewaxed, rehydrated and washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.6 mM KCl, 6.5 mM Na2HPO4, and 1.5 mM KH2PO4) as previously described (Teilmann and Christensen, 2005), followed by rinsing with blocking buffer (5% bovine serum albumin in PBS) for 15 min before incubation with primary antibodies for at least 1.5 hr at room temperature or overnight at 4°C. The sections were then washed three times in blocking buffer, incubated for 45 min in dark with fluorochrome-conjugated secondary antibodies and DAPI in blocking buffer. After washing, sections were mounted in PBS with 70% glycerol and 2% N-propylgallate and sealed with nail polish. Samples were observed on an Eclipse E600 microscopes (Nikon, Tokyo, Japan) with EPI-FL3 filters and MagnaFire cooled CCD camera (Optronics, Goleta, CA), and digital images were processed using Adobe Photoshop 6.0.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Cell lysates were prepared by using 1% sodium dodecyl sulfate (SDS) lysis buffer. Lysates were sonicated and centrifuged to separate off cell debris. Protein concentrations were measured using a DC Protein Assay from Bio-Rad. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Christensen et al. (2001). Briefly, proteins were separated under reducing conditions with NuPAGE 10% Bis-Tris precast gels (Invitrogen), fol-
lowed by electrophoretic transfer to nitrocellulose membranes (Invitrogen). Before incubation with antibodies for 2 hr/over night the membranes were blocked with 5% milk/TBST (10 mM Tris/HCl [pH 7.5], 120 mM NaCl, 0.1% Tween 20) and 0.5% Na-azide. Antibodies were diluted in 5% milk/TBST as indicated: anti-Ptc (1:200), anti-Gli2 (N-20) and (G-20) (1:200), anti-Gli3 (H-280) and (N-19) (1:100), anti-β-actin (1.5,000), and anti-α-tubulin (1.2,000). Membranes were washed several times in TBST followed by incubation with alkaline phosphatase-conjugated secondary antibodies. Blots were developed with BCIP/TNBT from KPL. The developed membranes were blocked with 5% milk/TBST (10 mM Tris/HCl [pH 7.5], 120 mM NaCl, 0.1% Tween 20) and 0.5% Na-azide.

**SAG Stimulation**

Cultures of PANC-1, CP-PAC1 cells and NIH3T3 fibroblasts (80% confluency) were serum starved for 48–72 hr and incubated in the presence and the absence of 1 μM SAG (Alexis Biochemicals, San Diego, CA) for 0 and 24 hr, followed by IF analysis with anti-Smo and anti-Gli3 and by WB analysis with anti-Ptc and anti-Gli2. Primary cilia were visualized with anti-acet. tb and nuclei with DAPI. All images were taken with equivalent time exposures.

**Transfection of Cells With Fluorescent-Tagged Plasmids**

CFPAC-1 cells were cultured on coverslips placed in six-well test plates (NUNC A/S, Denmark) and grown to 70% confluency. The medium was changed to DMEM/IMDM for 1 hr, followed by incubation with 1 μg/ml plasmid (YFP-Smo, provided by P. Beachy) and 5 μl of lipofectamine 2000 (Invitrogen) per well. After 4 hr of transfection the medium was changed to DMEM/IMDM with 10% FCS, and after the cells had reached 90% confluency they were serum-starved for 48 hr to induce growth arrest. Cells were then fixed and permeabilized and subjected to immunocytochemistry analysis as described above. NIH3T3 cells were cultured and transfected under the same conditions as above, except that serum-free DMEM was used during the 4-hr transfection period. The following plasmids were used: full length Gli2::GFP, GFP-Gli2/FL, full length Gli3::GFP, GFP-Gli3/FL, Gli3repressor::GFP, GFP-Gli(R) (Buttitta et al., 2003). All the vectors were constructed in the pShuttle backbone (Clontech).

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Human embryonic stem cells in culture possess primary cilia with hedgehog signaling machinery

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Introduction

Driving human embryonic stem cells (hESCs) along specific differentiation pathways remains a significant challenge for translational medicine and the development of hESC therapies. During early embryology, signaling pathways, such as hedgehog (Hh) and Wnt, are critical for human development (Corbit et al., 2005; Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005) and, recently, have been shown to be mediated by the primary cilium (for reviews see Michaud and Yoder, 2006; Singla and Reiter, 2006; Christensen et al., 2007; Satir and Christensen, 2007). Therefore, in the search for mechanisms regulating hESC differentiation, it is vital to first establish the existence of primary cilia and the localization of signaling components in undifferentiated hESCs.

Primary cilia are single, generally nonmotile, cilia with a 9 + 0 axoneme, differing from the 9 + 2 arrangement of motile cilia. Primary cilia are implicated as key cellular sensory structures involved in signal transduction and coordination of intra- and intercellular signaling pathways (for reviews see Michaud and Yoder, 2006; Singla and Reiter, 2006; Christensen et al., 2007; Satir and Christensen, 2007). Stimulation of the pathway results in the concerted movement of Ptc1 out of, and smoothened into, the primary cilium as well as up-regulation of GLI1 and PTC1. These findings show that hESCs contain primary cilia associated with working Hh machinery.

Supplemental Material can be found at: http://jcb.rupress.org/cgi/content/full/jcb.200706028/DC1
hESCs. The presence of this organelle is not limited to specific culture conditions. HESCs from H1 (male) and H9 (female) lines (approved by the National Institutes of Health; Olivier et al., 2006) were grown on matrigel without feeder cells (described in Yao et al. [2006]) with serum replacement for 6 d. Primary cilia were first identified by immunofluorescence (IF) markers of acetylated tubulin (AcTb) in both H1 and H9 hESCs after 6 d of culture in DME:F12 with serum replacement (Figs. 1A and 2A). Primary cilia became more prominent after starvation of hESCs by placement in DME:F12 without serum replacement (i.e., starved) for 24 h (Fig. 1B). Another hESC line, LRB003 (female; studied in the Denmark laboratory and supported by funds independent of the National Institutes of Health; Laursen et al., 2007), was cultured in monolayers on 0.1% gelatin with conditioned medium from cultured human foreskin fibroblasts (hFF), and primary cilia were observed after 4 d as the cells entered growth arrest in confluent colonies in the culture dish (Fig. 1, D and E).

Confinement that the hESCs remained undifferentiated was made by IF using the transcription factor OCT-4 (Fig. 1, A, D, and E) and stage-specific embryonic antigen 4 (not depicted). Both markers were used to assure undifferentiated hESCs. Anti-AcTb identified potential primary cilia (Fig. 1, B and C) and antibodies against tumor rejection antigen 1-85 (Tra-1-85) marked human cells (see Fig. 3D). After 5 d in culture, short (~2–3 μm) AcTb extensions characteristic of primary cilia were seen on ~33% of H1 hESCs (25 cilia/75 cells counted from five left–right asymmetry axis, limb and heart development, and neurogenesis (Corbit et al., 2005; Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). In the adult, Hh signaling is involved in stem cell maintenance and tissue homeostasis.

We hypothesized that primary cilia might be found in hESCs, wherein they could play a critical role in hESC differentiation parallel to that in normal early embryogenesis. In this study, we demonstrate that primary cilia are a general feature of hESC lines and that essential signaling components of the Hh pathway are present and functional in primary cilia of undifferentiated hESCs. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images provide definitive evidence and reveal novel features of hESCs and their primary cilia. To date, this is the first study conclusively showing the presence of these unique organelles in hESCs by definitive confocal and electron micrographs of hESC primary cilia and by dynamic colocalization of key signaling molecules essential for early development and known to be functional in the Hh signaling pathway, as was recently demonstrated in primary cilia of cultured mouse fibroblasts (Rohatgi et al., 2007).

Results and discussion

In this study, we demonstrate that the primary cilium is a dynamic ultrastructural feature in three different lines of undifferentiated hESCs. The presence of this organelle is not limited to specific culture conditions. HESCs from H1 (male) and H9 (female) lines (approved by the National Institutes of Health; Olivier et al., 2006) were grown on matrigel without feeder cells (described in Yao et al. [2006]) with serum replacement for 6 d. Primary cilia were first identified by immunofluorescence (IF) markers of acetylated tubulin (AcTb) in both H1 and H9 hESCs after 6 d of culture in DME:F12 with serum replacement (Figs. 1A and 2A). Primary cilia became more prominent after starvation of hESCs by placement in DME:F12 without serum replacement for 24 h (Fig. 1B). Another hESC line, LRB003 (female; studied in the Denmark laboratory and supported by funds independent of the National Institutes of Health; Laursen et al., 2007), was cultured in monolayers on 0.1% gelatin with conditioned medium from cultured human foreskin fibroblasts (hFF), and primary cilia were observed after 4 d as the cells entered growth arrest in confluent colonies in the culture dish (Fig. 1, D and E).
surfaces of the cells, which is in contrast to the many microvilli that are shorter and have a smaller diameter (Fig. 2B). SEM also demonstrated paddle tips at the ends of some primary cilia (Fig. 2, C and D). Confocal images (Fig. 2A) show the outward orientation of primary cilium from growth-arrested cells in a monolayer, whereas mitotic cells lack a primary cilium (Pan and Snell, 2007). To show definitively that the structures are primary cilia, we fixed hESC cultures in situ and processed them for TEM. Some colonies were cut parallel to and just above their free surfaces to give cross-sectional views of projecting structures, and other sections were oriented through the cell bodies perpendicular to this direction to show longitudinal views of the cilia and their basal bodies. Cross sections near the apical surfaces of the cells showed axonemes, which are enclosed by a unit membrane (Fig. 2E). The 9 + 0 pattern can be clearly observed in cross sections close to the basal body, as are a disarray of nine doublets, including 8 + 1, 6 + 1, and other patterns (Fig. 2E), either from the same cell but at different sections along the length of the cilium approaching the tip or in different cells at varying stages of ciliary growth. One centriole pair can also be observed close to the cell surface with a primary cilium growing from one of the centrioles (Fig. 2F), which has become the ciliary basal body. Primary cilia often emerge from a concavity in the cell,
Gli transcription factors that enter the nucleus to control differential processes during early and late embryogenesis. Smo was previously reported to be a constituent of nodal cilia, Madin Darby canine kidney cell cilia, and other primary cilia (Corbit et al., 2005; May et al., 2005), and Gli2 was found at the tip of mesenchymal primary cilia during limb formation (Haycraft et al., 2005).

Time-dependent studies in mammalian differentiated cells support a model in which SHh triggers the removal of Ptc from the primary cilium, permitting Smo to enter the cilium and initiating signaling (Rohatgi et al., 2007). We therefore tested whether Smo, Ptc, and Gli2 are present in hESC primary cilia, and we followed the movement of Smo and Ptc in and out of the cilium upon stimulation by Smo agonist (SAG). The use of SAG to induce activation of SHh signaling has been established by Chen et al. (2002). In transfected LRB003 hESCs, YFP:Smo strongly and almost exclusively localizes to the primary cilium (Fig. 3A). The ciliary staining of YFP:Smo was remarkably higher than that of anti-Smo (Fig. 4A) because of overexpression of Smo from the construct. Furthermore, with a Gli2-specific antibody, which may be interpreted as a small depression in the cell's apical surface as shown in the SEM (Fig. 2D) and TEM (Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200706028/DC1) images. Rarely (in <1% of observed cells), two primary cilia originate within one cell (unpublished data). A rich array of polyribosomes and cytoplasmic microtubules, running parallel to the apical surface, are seen near the basal body (Fig. S1A). Immediately below this level, a ciliary rootlet emerging from the basal body and microfilament bands of the adherens junctions of the confluent hESCs can be found (Fig. S2). In addition, lamellar-type vesicles are observed both intracellularly and extracellularly, adherent to the hESC surface (Fig. 2, F and G; and Fig. S1B).

Next, we examined whether components of the Hh signaling system were present and functional in the hESC primary cilium. It has been reported previously that the sonic Hh (SHh) receptors patched (Ptc) and smoothened (Smo) and their downstream effectors Gli1, 2, and 3 are expressed in hESCs (Rho et al., 2006). In various cells, upon binding of SHh to its receptor Ptc, Smo is activated, which is followed by the processing and activation of Gli transcription factors that enter the nucleus to control differential processes during early and late embryogenesis. Smo was previously reported to be a constituent of nodal cilia, Madin Darby canine kidney cell cilia, and other primary cilia (Corbit et al., 2005; May et al., 2005), and Gli2 was found at the tip of mesenchymal primary cilia during limb formation (Haycraft et al., 2005). Time-dependent studies in mammalian differentiated cells support a model in which SHh triggers the removal of Ptc from the primary cilium, permitting Smo to enter the cilium and initiating signaling (Rohatgi et al., 2007). We therefore tested whether Smo, Ptc, and Gli2 are present in hESC primary cilium, and we followed the movement of Smo and Ptc in and out of the cilium upon stimulation by SAG. The use of SAG to induce activation of SHh signaling has been established by Chen et al. (2002). In transfected LRB003 hESCs, YFP:Smo strongly and almost exclusively localizes to the primary cilium (Fig. 3A). The ciliary staining of YFP:Smo was remarkably higher than that of anti-Smo (Fig. 4A) because of overexpression of Smo from the construct. Furthermore, with a Gli2-specific antibody,
we show that Gli2 strongly localizes in a punctuate pattern along the entire length of primary cilia but is absent in the nucleus of these cells (Fig. 3 B). Also, in H1 and 9 hESCs, anti-Gli2 localizes to the primary cilia (Fig. 3, B and D), whereas Smo localizes to the base of ~2/3 of the cells with primary cilia (Fig. 3 F). In addition, by fluorescence immunolocalization, small amounts of SHh can be localized near the base of the cilia, which is clearly located to the side of the primary cilium (Fig. 3 C, z series) in ~3/4 of the ciliated H1 cells (Fig. 3, C and E). In LRB003 cells, upon stimulation with SAG, the ciliary level of Smo starts to increase beginning at 1 h (Fig. 4 B) as compared with 0 h (Fig. 4 A). This is followed by a major accumulation of Smo along the length of the cilium at 4 h of SAG treatment (Fig. 4 C). This infers that translocation of Smo along the cilium is initiated by the docking of Smo at the base of the cilium. The opposite pattern of translocation can be seen for Ptc, which leaves the cilium upon SAG stimulation (Fig. 4, D–F). This movement of Hh components into and out of the cilium (Fig. 4), along with the z series showing SHh located to the side of the primary cilium (Fig. 3 C), eliminates the possibility of nonspecific antibody binding to the centrosome in light of the fact that centrosomes never migrate up the cilium. Experiments similar to that described by Orozco et al. (1999) are planned for the direct viewing of intracellular and ciliary transport of intraflagellar transport motor proteins and their Hh cargoes in hESCs that would establish mechanisms of trafficking. Knockdown experiments, for example, using siRNA of KIF3A, would be informative and are presently underway.

The addition of 5 μM Shh or 10 μg/ml SAG to H1 hESCs for 18 h up-regulated GLI1 (approximately twofold) and PTC1 (approximately fivefold) mRNA levels compared with baseline levels of these components without exogenous ligand stimulation, as determined by real-time PCR with GAPDH as an internal control (Fig. 5). As expected, GLI2 mRNA was essentially nonresponsive. Cyclopamine, a Smo inhibitor (Lipinski et al., 2006), modestly inhibited the up-regulation in the presence of inducers under the conditions used (Fig. 5). GLI2 mRNA was not affected. These data are consistent with the dynamics of the Hh signaling machinery, as described by Rohatgi et al. (2007), in differentiated cells and, together with the localization studies of Hh signaling pathways, support the conclusion that Hh signaling proceeds through hESC primary cilia. Whether or not the SHh ligand is produced by the hESC and whether the function of the signal is to maintain the cells undifferentiated or act as a precursor to differentiation remains to be determined.

The presence of the extracellular lamellar bodies in undifferentiated hESCs may also be related to Hh or other signaling pathways. Similar vesicles have been reported to be involved in
signaling in association with cells with nodal or primary cilia in several embryonic tissues. It would be interesting if the lamellar vesicles seen here are indeed akin to nodal vesicular parcels containing Shh signals, as described in early embryonic nodal cells (Tanaka et al., 2005; Hirokawa et al., 2006), or to prominin-1-containing particles of dividing neuroepithelial cells of the developing mammalian central nervous system (Dubreuil et al., 2007). The content of the H1 lamellar vesicles remains to be investigated further.

In summary, definitive confocal and transmission electron micrographs, coupled with SEM and IF microscopy, conclusively demonstrate the presence of primary cilia with many known features in hESCs. For a detailed review of primary cilia ultrastructure in differentiated cells, see Sari and Christensen (2007). Because Hh signaling pathways of embryological development and patterning operate via primary cilia, it is perhaps not surprising to find Ptc, Smo, and Gli2 localized and potentially functional within the hESC primary cilia. Whether Shh is released in cultures before or after differentiation of HESCs is unclear, and whether important receptors of other signaling pathways, such as Wnt (Gerdes et al., 2007; Pan and Thomson, 2007), are localized in hESC primary cilia remains to be determined.

Collectively, our results suggest that primary cilia may be involved in the regulation and coordination of the first steps of hESC differentiation and/or the maintenance of the undifferentiated state/self-renewal. Because hESCs hold promise for the treatment of many diseases and provide an excellent system for studying mechanisms involved in early human development, these findings provide the groundwork to determine specific aspects of early differentiation controlled by the machinery of primary cilia. This knowledge may ultimately reveal pathways for manipulation of hESC differentiation into specific cell and tissue lineages.

Materials and methods

Cell cultures (Albert Einstein College of Medicine)

hESCs from H1 and 9 lines (National Institutes of Health approved) were maintained in a humidified incubator at 37°C with an atmosphere consisting of 6% CO₂, 7% O₂, and 87% N₂ and were grown on matrigel (BD Biosciences) without feeder cells in DME nutrient mixture F12 (Ham; Invitrogen) with L-glutamine and 15 mM Hepes, supplemented with the serum replacements N2 (chemically defined supplement containing 1000 mg/liter human transferrin, 50 mg/liter insulin recombinant full chain, 0.6 mg/liter progesterone, 161 mg/liter putrescine, and 173 mg/liter selenite; Invitrogen) at 100× concentrate of Bottenstein’s N2 formulation (Bottenstein, 1985) and B27 (50× serum supplement designed for the long-term viability of hippocampal and other neurons of the central nervous system; Invitrogen), in addition to 20 ng/ml of basic FGF (R&D Systems), BSA fraction V, 1% nonessential amino acids, 50 U/ml penicillin, 50 ng/ml streptomycin, 1 mM L-glutamine, and 1-thioglycerol added for 6 d (as described in detail in Yao et al. [2006]) and observed by phase microscopy using an inverted light microscope (CK40; Olympus). To passage the cells, differentiated cells were scraped in PBS under a binocular magnifier with a Pasteur pipette scraper (elongated and twisted using heat), treated with prewarmed collagenase type IV for 5 min to detach the hESC colonies, aspirated, concentrated using a macrocentrifuge (Eppendorf), and either plated on 6-well tissue culture plates (Thermo Fisher Scientific) coated with 1:4 matrigel for propagation, on gamma-irradiated flasks (Eppendorf), and either plated on 6-well tissue culture plates (Thermo Fisher Scientific) coated with 1:4 matrigel for propagation, on gamma-irradiated flasks (Eppendorf), and either plated on 6-well tissue culture plates (Thermo Fisher Scientific) coated with 1:4 matrigel for propagation, or on carbon-coated glass coverslips on the bottom of each well of a 6-well plate covered with 1:4 matrigel for TEM or SEM. The cultures were monitored microscopically and at day 6 were either maintained for an additional day in the same supplemented medium or starved in plain DME: F12 for 24 h. The cells were then prepared for IF microscopy using the protocol described in IF Microscopy (Albert Einstein College of Medicine).

The LRB003 cell line (not approved by the National Institutes of Health) was studied, as described in the next section, in the Copenhagen laboratory and solely supported by Danish funding agencies (see Acknowledgments).

Cell cultures (Copenhagen)

The hESC line R8003 (Laursen et al., 2007) was initially cultured on 35-mm dishes (Thermo Fisher Scientific) coated with 0.1% gelatin (Sigma-Aldrich) on a confluent layer of mitotically inactivated hFF (Line #CCD-1112Sk; American Type Culture Collection). The hESC culture medium consisted of the following: knockout DMEM, 1.5% knockout serum replacement, 2 mM Glutamax, nonessential amino acids, 50 U/ml penicillin, 50 ng/ml streptomycin, and 0.1 mM β-mercaptoethanol (Invitrogen); and 4 ng/ml basic FGF (R&D Systems). Cells were maintained in a humidified incubator at 37°C with an atmosphere consisting of 6% CO₂, 7% O₂, and 87% N₂. After 5–7 d of incubation, hESCs were passaged using trypsin (Invitrogen) for experimental culturing conditions in a feeder-free environment. The cells were plated on 16-well glass slides (Thermo Fisher Scientific) coated with 0.1% gelatin (BD Biosciences) in the absence of hFF. The conditioned media used consisted of hFF supernatant and hESC culture media (1:1).

IF microscopy (Albert Einstein College of Medicine)

hESCs from H1 and 9 cell lines were cultured in Dulbecco’s PBS without calcium and magnesium (Mediatech, Inc.) at RT, and then fixed in 3.7% paraformaldehyde in PBS for 15 min. They were then rinsed three times with PBS, incubated in 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min, and blocked with 2% BSA in PBS for 1 h at RT or overnight at 4°C, and primary antibodies (monoclonal anti-AcTb mouse anti-human IgG2b [Sigma-Aldrich];
purified polyclonal anti-α-tubulin rabbit anti-human IgG (Biologend); purified polyclonal anti-zinc finger protein GLI2 rabbit anti-human IgG (Aviva Systems Biology); monoclonal anti-Tra-1-85 mouse anti-human IgG1 [Millipore]; and PE-conjugated monoclonal anti-stage-specific embryonic antigen 4 mouse anti-human IgG3 [R&D Systems]) were added in a 1:300 dilution in blocking buffer for 1 h at RT or overnight at 4°C. Rabbit anti-human Oct3/4 polyclonal IgG (Santa Cruz Biotechnology, Inc.); rabbit anti-human Smo polyclonal IgG (Santa Cruz Biotechnology, Inc.); and rabbit anti-human SHh antibody polyclonal IgG (Cell Signaling Technology) were used at dilutions of 1:100 in blocking buffer and incubated overnight at 4°C. The cells were then washed three times in PBS with 5-min incubations between washes. The secondary antibodies Cy3-conjugated AffiniPure goat anti-mouse IgG (H + L) and Cy5-conjugated AffiniPure goat anti-rabbit IgG (H + L) (Jackson Immunoresearch Laboratories) were added at 1:400 dilution in blocking buffer and incubated for 1 h at RT in the dark. All appropriate controls were done for the IF experiments described. Negative controls consisted of cells incubated with secondary antibody only. The cells were then washed again three times in PBS with 5-min incubations between washes and taken for IF imaging or stored at 4°C. IF imaging was performed on an inverted (BX70; Olympus) and a confocal microscope (described in detail in Confocal microscopy; TCS SP2 AOBS; Leica) and viewed at a final magnification of 600 using CY3 (red) and 5 (far red) fluorescence filters. A cooled charge-coupled device camera (Sensicam QE; Sony) and IP Laboratory software (BD Biosciences) were used to capture the images, whereas ImageJ (National Institutes of Health) and Photoshop CS2 version 9.0.2 (Adobe) were used to view and analyze the data.

IF microscopy (Copenhagen)

After 1 wk of incubation, hESCs on 16-well glass slides were washed once with PBS (136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.7 mM KH₂HPO₄) and then fixed with 4% paraformaldehyde for 20 min. After three 5-min washes with PBS, the wells were permeabilized with 0.1% Triton X-100 for 20 min. After three 5-min washes with PBS, the wells were blocked with 4% PBS for 45 min. Wells were incubated overnight at 4°C in the following primary antibodies: monoclonal mouse anti-AcTb at 1:1,000; polyclonal goat anti-pericentrum, polyclonal goat anti-centrin, polyclonal rabbit anti-GH2, polyclonal rabbit anti-OC1-4, polyclonal rabbit anti-Pc (Santa Cruz Biotechnology, Inc.) at 1:200; and polyclonal rabbit anti-Smo (MBL International) at 1:200. The next day, cells were washed five times with PBS and allowed to stand 5 min, followed by three more quick washes with PBS. The cells were incubated 1 h with the following secondary antibodies: Alexa Fluor488-conjugated goat anti-rabbit IgG, Alexa Fluor555-conjugated donkey anti–mouse IgG, and Alexa Fluor647-conjugated donkey anti–mouse IgG (Jackson Immunoresearch Laboratories). Secondary antibody incubation was occasionally followed by DAPI incubation. Cells were visualized on a microscope (Eclipse E600; Nikon) with EPI-FL3 filters and a cooled charge-coupled device camera (Magnafire; Optronics), and digital images were processed using Photoshop.

Confocal microscopy (Albert Einstein College of Medicine)

Images were collected with a confocal microscope (TCS SP2 AOBS) with 60x oil immersion optics. Laser lines at 488, 543, and 633 nm for excitation of DAPI, Cy3, and Cy5, respectively, were provided by an Ar laser and a HeNe laser. Detection ranges were set to eliminate crosstalk between fluorophores.

SAG stimulation (Copenhagen)

Confluent cultures of U87003 cells were incubated in the presence of 1 μM SAG (Qiagenene) for 0, 1, and 4 h, followed by IF microscopy analysis with rabbit anti-Smo and anti-Pc. Primary cilia were visualized with anti-AcTb and nuclei with DAPI. All images were taken with equivalent time exposures.

Exposure of H1 hESCs to SHhN, SAG, and cyclopamine (Albert Einstein College of Medicine)

Recombinant human SHh (C24II), amino terminal peptide (SHhN; R&D Systems), and SAG were dissolved in PBS containing 0.1% BSA. Cyclopamine (Toronto Research Chemicals) was dissolved in 95% ethanol. SHhN, SAG, and cyclopamine, in medium containing 0.5% serum, were applied to H1 hESCs in culture (in triplicate) at concentrations of 5 μM, 10 μg/ml, and 1 μM, respectively, for 18 h. The exposure time and concentrations used were derived from Lipinski et al. (2006).


The primary cilium coordinates early cardiogenesis and hedgehog signaling in cardiomyocyte differentiation

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Summary

Defects in the assembly or function of primary cilia, which are sensory organelles, are tightly coupled to developmental defects and diseases in mammals. Here, we investigated the function of the primary cilium in regulating hedgehog signaling and early cardiogenesis. We report that the pluripotent P19.CL6 mouse stem cell line, which can differentiate into beating cardiomyocytes, forms primary cilia that contain essential components of the hedgehog pathway, including Smoothened, Patched-1 and Gli2. Knockdown of the primary cilium by Ift88 and Ift20 siRNA or treatment with cyclopamine, an inhibitor of Smoothened, blocks hedgehog signaling in P19.CL6 cells, as well as differentiation of the cells into beating cardiomyocytes.

Introduction

Heart development in vertebrates is initiated in embryos shortly after gastrulation by aggregation of cardiomyocyte progenitor cells that become allocated from the mesodermal population (Sucov, 1998). The mouse embryonal carcinoma (EC) P19 cell line is a common cell model system to study early heart differentiation in vitro because the P19 EC cells can differentiate into beating cardiomyocytes when stimulated with dimethyl sulfoxide (DMSO) (Skerjanc, 1999; Paquin et al., 2002). The heart transcription factors Gata4 and Nkx2-5 are markers for early cardiomyocyte differentiation (Grépin et al., 1997; Lints et al., 1993). Gata4 is a tissue-restricted transcription factor that is found in the heart but not in skeletal muscle (Grépin et al., 1994) and is necessary for proper heart tube development at the ventral midline (Kuo et al., 1997). The Gata4 and Nkx2-5 genes are, together with MEF2C, desmin and cardiac actin, expressed in the cardiomyocyte population before fusion of the linear heart tube (Lynes, 1994). Mice lacking Gata4 and Nkx2-5 die because of severe defects in heart formation (Sucov, 1998).

A series of signal transduction systems have been implicated as essential coordinators of early cardiogenesis, including hedgehog (Hh), Wnt, bone morphogenetic protein (BMP) and platelet-derived growth factor receptor (PDGFR) signaling (Washington Smoak et al., 2005; Kwon et al., 2008; Hirata et al., 2007; van Wijk et al., 2007). In mammals, Hh signaling is induced by three different ligands, including Sonic hedgehog (Shh), which controls left-right asymmetry, digit patterning in the limbs and development of the lung and heart (Tsukui et al., 1999; Johnson et al., 1994; Bellusci et al., 1997; Washington Smoak et al., 2005). In the adult mouse heart, Hh signaling is required for proangiogenic gene expression and maintenance of the adult coronary vasculature, and it specifically controls the survival of small coronary arteries and capillaries (Lavine et al., 2008). In vertebrates, the secreted Hh proteins bind to the transmembrane patched protein-1 (Ptc1) hereby abolishing the inhibitory effect of Ptc1 on the seven-transmembrane receptor Smoothened (Smo). This allows Smo to transduce a signal via Gli transcription factors to the nucleus for expression of Hh target genes. There are three Gli transcription factors, Gli1-Gli3. Gli1 functions as a constitutive activator (Hynes et al., 1997; Ruiz-I Altaba, 1999), whereas Gli2 and Gli3 have an N-terminal transcriptional repressor domain and a C-terminal transcriptional activator domain. Smo might be the controlling molecule in the Hh signaling pathway that mediates the proteolytic events between the activating and repressing form of Gli2 and Gli3 in an Hh-dependent manner (Huangfu and Anderson, 2006). P19 EC cells normally require aggregation to form embryoid bodies in suspension induced by DMSO before differentiation analysis (Skerjanc, 1999). However, overexpression of Shh has been observed to induce the expression of cardiac muscle factors Gata4 and Nkx2-5 via Gli1and Gli2, which results in differentiation of the cells into cardiomyocytes in the absence of DMSO (Gianakopoulos and Skerjanc, 2005). This supports the conclusion that Hh signaling is critical during early development.

E11.5 embryos of the Ift88tm1Rpw (Ift88-null) mice, which form no cilia, have ventricular dilation, decreased myocardial trabeculation and abnormal outflow tract development. These data support the conclusion that cardiac primary cilia are crucial in early heart development, where they partly coordinate hedgehog signaling.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/17/3070/DC1

Key words: Primary cilia, P19.CL6 cells, Cardiac development, Mouse, Heart, Hedgehog signaling, siRNA, Ift88, Ift20, Cyclopamine
cardiogenesis. Therefore, P19 EC cells offer a unique model cell system to investigate the mechanisms by which Hh signaling is coordinated by the cells during differentiation in early cardiogenesis.

Recent reports have indicated that primary cilia have an important role in a number of vertebrate developmental processes. Primary cilia are microtubule-based organelles, organized in a 9+0 axonemal ultrastructure, which are assembled and maintained via a process termed intraflagellar transport (IFT) in most mammalian cells during growth arrest (Rosenbaum and Witman, 2002; Pedersen et al., 2008). Primary cilia are thought to function as mechano- and chemosensory organelles that specifically coordinate a series of cellular signal transduction pathways during development and in tissue homeostasis, including Hedgehog (Hh), PDGF and Wnt signaling (reviewed by Christensen et al., 2007; Christensen et al., 2008; Wong and Reiter, 2008; Gerdes and Katsanis, 2008). Consequently, defects in assembly of the primary cilium or mutations in ciliary signaling components lead to severe developmental disorders and diseases, now referred to as ciliopathies (reviewed by Pan, 2008; Lehmann et al., 2008; Davenport and Yoder, 2005). One of the first diseases to be related to dysfunctional primary cilium, was polycystic kidney disease (PKD), which was originally identified in mice mutated in the gene encoding Ift88/Polaris in the Oak Ridge Polycystic Kidney mouse (ORPK mouse, Ift88^Rpf or Ift88^F370R^Rpu) (Moyer et al., 1994). Ift88 is a subunit of the IFT particle complex B required for functional IFT and assembly of the primary cilium (Pazour et al., 2000; Murcia et al., 2000; Haycraft et al., 2001; Taulman et al., 2001; Yoder et al., 2002; Luckner et al., 2005). No other function of Ift88 is known, and genes encoding IFT are found only in organisms that possess cilia.

One may of the essential Hh signaling components, such as Gli2, Gli3, Smo and Ptc, localize to primary cilium in a number of cell types, including fibroblasts (Haycraft et al., 2005; Rohatgi et al., 2007), epithelial cells in renal tubules (Harris and Torres, 2009) and the exocrine duct of the pancreas (Nielson et al., 2008), as well as in human embryonic stem cells (Kiprilov et al., 2008; Breunig et al., 2008). It was suggested that the concerted movement of Smo and Ptc into and out of the cilium creates a switch by which cells can turn Hh signaling on and off during development and tissue homeostasis (Corbit et al., 2005; Rohatgi et al., 2007; Christensen and Ott, 2007). In this scenario, binding of ligands to Ptc in the cilium might activate the Hh pathway by removal of Ptc from the cilium in a process that is associated with ciliary enrichment of Smo (Rohatgi et al., 2007). In vitro activation of Smo in cells exposed to Shh is blocked in mouse embryonic fibroblasts (MEFs) lacking Ift172 or the dynein retrograde motor, Dyn2C2h1 (Obcina and Anderson, 2008). The heterotrimeric kinesin complex comprising the motor subunits Kif3a and Kif3b and the nonmotor protein KAP is responsible for microtubule-based anterograde translocation destined for membranous organelles, as well as for ciliogenesis (Yamazaki et al., 1995; Haraguchi et al., 2006).

Furthermore, in mice lacking Kif3a and Smo there is a failure in the maturation of radial astrocytes that would normally develop into the dentate gyrus, which is responsible for maintenance of adult neurogenesis (Han et al., 2008). Disruption of Kif3a results in severe developmental abnormalities in the neural tube, cardiovascular insufficiencies and randomized left-right development (Takeda et al., 1999; Nonaka et al., 1998). Consequently, mutations in IFT proteins and other proteins associated with the cilium and the centrosome might result in dysfunctional Hh signaling and/or Gli processing with severe developmental disorders in mammals, including skeletogenesis (Gouttenoire et al., 2007), limb development (Haycraft et al., 2007), neural tube formation (Gorivodsky et al., 2008), cerebellar development (Chizhikov et al., 2007; Spassky et al., 2008), mammary gland development and defects in ovarian function (Johnson et al., 2008). Recently, Brueckner and co-workers (Slough et al., 2008) showed that the embryo heart at embryonic day 9.5 (E9.5) in Kif3a^-/^- mice has abnormal development of endocardial cushions (ECCs) and reduced trabeculation, indicating that primary cilium could coordinate processes in cardiac morphogenesis.

Since Kif3 family proteins regulate cellular processes in mammalian cells that are not necessarily related to the primary cilium (Teng et al., 2005; Haraguchi et al., 2006; Corbit et al., 2008), there may be a need for a more thorough investigation on the role of the primary cilium in early cardiogenesis and Hh signaling, which is crucial for cardiomyocyte differentiation. In this study, we investigated the role of the primary cilium in Hh signaling and early cardiogenesis by: (1) characterization of primary cilium and their role in Hh signaling and differentiation of the pluripotent P19.CL6 cell line, an isolated subclone from the P19 cell line, into cardiomyocytes and (2) microscopy analysis of defects in heart development in E11.5 embryos from wild-type (WT) and Ift88-null (Ift88^Gtm1^Bpu) mice. P19.CL6 cells have no requirement for being cultured in suspension and form embryoid bodies before differentiation (Uchida et al., 2007). This allowed us to follow the function of the primary cilium in the initial phases of differentiation from day 1 over a 2 week period, until beating cardiomyocytes formed. By analyzing the protein and mRNA levels of Gata4, Nkx2-5 and α-actinin, we can determine the effect on cardiogenesis when shutting down Hh signaling with cyclopamine treatment, a Smo-specific antagonist (Chen et al., 2002), or knocking down primary cilia by Ift88 and If20 siRNA. If20 is associated with the Golgi complex, and knockdown of this IFT particle reduces ciliary assembly without affecting Golgi structure (Follit et al., 2006). We show here that P19.CL6 cells form primary cilia and that Hh signaling components such as Ptc1, Smo and Gli2 localize to the cilium in these cells. Furthermore, cyclopamine halts Hh signaling, preventing P19.CL6 cells from forming beating clusters of cardiomyocytes. Ift88 and If20 siRNA knockdown strongly reduced the assembly of primary cilium, mRNA and protein levels of Gata4, Nkx2-5 and α-actinin, expression of Ptc1 and Gli1, and nuclear localization of Gli1. Furthermore, the induced loss of primary cilium with Ift88 and If20 siRNA reduced and delayed the number of beating clusters of cardiomyocytes. In E11.5 embryos of Ift88-null (Ift88^Gtm1^Bpu) mice, which lack primary cilium, we saw ventricular dilation, abnormal outflow tract development and abnormal myocardial trabecular morphology compared with the wild type. These data support the conclusion that primary cilium are crucial for differentiation of P19.CL6 cells into cardiomyocytes and in early heart development in the mouse, partly via coordination of Hh signaling.

Results
P19.CL6 cell morphology and the effect of cyclopamine on cardiomyocyte differentiation
To investigate the cellular events during early heart development, we studied the morphology and changes in expression of cardiac transcription factors in P19.CL6 cell cultures during their differentiation into cardiomyocytes over a 2 week period. We first analyzed the effect of cyclopamine on Hh signaling and cardiomyogenesis (Fig. 1). Addition of 1% DMSO to the growth medium of P19.CL6 cells led to the formation of 15-20 beating
clusters of cardiomyocytes at day 12 on average in a 9.6 cm² culture dish (Fig. 1A,L). At this time point, the individual clusters had a diameter of 0.2-0.6 mm and the clusters could be observed in small networks beating synchronously at a frequency of about 60 rhythmic contractions per minute. Comparable structures of cardiogenic cell clusters were observed in differentiating P19.SI cells, another
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subclone of P19 cells, which contracted synchronically by about 2 weeks (Angello et al., 2007). The network between clusters in differentiated P19.CL6 cells later develop into a thick unified layer of cardiac muscle epithelium (data not shown). Analysis of Gata4 and Nkx2-5 by immunofluorescence microscopy (Fig. 1B-C), showed that Gata4-positive cells were present from day 2 onwards during cardiomyocyte differentiation. Nkx2-5-positive cells appeared at a later stage (~day 9) during cardiomyogenesis. To verify that the P19.CL6 cells form cardiomyocytes, we stained with an antibody against the cardiomyocyte marker α-actinin, which localizes in the Z-line on α-cardiac muscle stress fibers (Fig. 1D-F). The structural organization of P19.CL6 cardiomyocyte muscle fibers takes place at approximately day 12. Primary cilia, localized with anti-detyrosinated tubulin (Glu-tub), label cells that have entered growth arrest (Satir and Christensen, 2007) and were present at all stages of heart development (Fig. 1D-F). This was particularly prominent in cells that had formed contact with other cells, either as confluent monolayers before the beginning of differentiation or as multilayered cell clusters during the subsequent phases of differentiation and formation of the beating cardiomyocyte. mRNA levels of Gata4, Nkx2-5 and α-actinin were analyzed by quantitative RT-PCR (Q-PCR) (Fig. 1G) and showed an increase over the 2 week growth period that matched the appearance of positive cells observed by immunofluorescence microscopy. As a control, the protein levels of Gata4, Nkx2-5 and α-actinin in western blot (WB) analysis (Fig. 1H), followed the increase in mRNA levels and localization intensities of the proteins upon immunofluorescence analysis; Gata4 expression was upregulated after day 2 and Nkx2-5 and α-actinin at around day 9. The effect of cyclopamine on P19.CL6 morphology indicated that a concentration of 0.3 μM of this Smo inhibitor was too low to suppress cardiomyocyte development (Fig. 1I). However, at 10 μM or more, the cyclopamine became toxic and affected cell viability. Furthermore, cyclopamine concentrations equal to or greater than 1 μM altered the cell morphology in the culture and the cells aggregated in disorganized clusters that adhered poorly to the culture dish. The addition of 3 μM cyclopamine to the culture medium had a significant negative effect on the mRNA levels of Gata4, Nkx2-5 and α-actinin at day 9, compared with levels in control cells (Fig. 1J). A similar reduction in mRNA expression of the cardiomyocyte markers was observed at day 12 (data not shown).

Stem cell markers during P19.CL6 differentiation

To verify that DMSO promotes the differentiation of P19.CL6 cells into cardiomyocytes and not other cell lineages, we followed the shift in cellular expression of stem cell markers into Gata4-positive cells. Confirmation that all P19.CL6 cells remained undifferentiated at day 1 was made by immunofluorescence analysis using the transcription factors Sox2 and Oct4, both markers for undifferentiated cells (Pesce and Scholer, 2001; Masui et al., 2007). Both markers colocalized to the nuclei of all cells at day 1 (Fig. 2A), at which time no cells were positive for Gata4 (Fig. 1B; Fig. 2B, left panels). During differentiation at day 5 after DMSO
treatment, we observed a shift in the cellular expression of Sox2 to Gata4 such that cells were either Sox2 or Gata4 positive (Fig. 2B, middle panels). About 40% of the cells were Gata4 positive at this time point of differentiation. At day 12, the number of Gata4-positive cells increased to about 80%, whereas the remaining cells expressed Sox2 (Fig. 2B, right panels). As a control, all cells left in growth medium without DMSO for 12 days remained undifferentiated (Fig. 2C). These results show that DMSO primarily promotes the differentiation of P19.CL6 cells into cardiomyocytes and not other cell lineages.

Cyclopamine inhibits the Hedgehog signaling pathway and heart development in P19.CL6 cells

The results of cyclopamine treatment in P19.CL6 cells presented in Fig. 1 indicate that the Hh signaling pathway is turned off (Fig. 3) and that normal cardiogenesis requires the Hh signaling pathway to become activated, as previously suggested for P19 EC cells (Gianakopoulos and Skerjanc, 2005). Elevated mRNA levels of Gli1 and Ptc1 during P19.CL6 differentiation at day 9 were no longer apparent upon 3 μM cyclopamine treatment (Fig. 3A). The Hh transcription factor Gli2 exists in a full-length activator form (Gli2-A) and in repressor forms (Gli2-R), which are formed after C-terminal degradation of the protein (Pan et al., 2006). We here show that 3 μM cyclopamine at day 5 reduces the level of the full-length form of Gli2 (~160 kDa), as judged by WB analysis with an antibody directed against the internal region of Gli2 (Gli2-G20) that recognizes only the full-length form of Gli2 (Nielsen et al., 2008) (Fig. 3B). WB analysis was also performed at day 9 using an antibody directed against the N-terminal region of Gli2 (Gli2-N20), which recognizes processed forms of Gli2 (Nielsen et al., 2008).

In this analysis Gli2-N20 detected a protein band at ~60 kDa, which increased in intensity in the presence of cyclopamine (Fig. 3C). Both protein bands were eliminated by addition of blocking peptide to the antibodies. These results might indicate that inhibition of Hh signaling by cyclopamine treatment is associated with processing of Gli2 into repressor forms, although further analysis will be required to identify their function in Hh signaling.

The effect of cyclopamine on Gli1 expression was also investigated by immunofluorescence analysis at day 2, 5 and 9 of differentiation. The level of Gli1 expression greatly increased in the nucleus at day 5 and 9 (Fig. 3D-F), and this increase was largely abolished in the presence of 3 μM cyclopamine. Confirmation that Gli1 increased in cells that differentiate into cardiomyocytes was made by immunofluorescence analysis, which showed colocalization of Gli1 and Nkx2-5 in cells at day 9 (Fig. 3G). Similarly, Gli2 and Gli3 expression levels at day 5 in the absence and in the presence of 3 μM cyclopamine was observed (Fig. 3H-I). We used Gli2 (G-20) and Gli3 (C-20) antibodies, which recognize the full-length forms of each transcription factor. In both cases, cyclopamine strongly reduced the nuclear localization of Gli2 and Gli3. These results support the conclusion that cyclopamine prevents P19.CL6 cells from differentiating into cardiomyocytes by shutting down Hh signaling in the early phases of differentiation.

Hedgehog signaling components localize to primary cilia in P19.CL6 cells

Hh signaling was previously suggested to be coordinated by primary cilia (Liu et al., 2005). To confirm formation of primary cilia in cultures of P19.CL6 cells, we initially performed immunofluorescence analysis using anti-pericentrin (Pctn), which
labels the centrosome, and anti-acetylated α-tubulin, which labels primary cilia in growth-arrested cells (Schneider et al., 2005). As shown in Fig. 4A, primary cilia were clearly observed in cells either in confluent monolayers or in multilayered cell clusters, as also indicated with Glu-tub in Fig. 1D-F. Co-localization studies with α-tubulin and antibodies directed against Ptc1, Smo and Gli2 showed that all three Hh components localize to the primary cilium as indicated in Fig. 4B-D. Immunofluorescence analysis showed the ciliary localization of Ptc-1 and Gli2 in cells expressing Gata4 and Nkx2-5, respectively (Fig. 4E-F), which confirms that Hh components localize to cilia in cells differentiating into cardiomyocytes. We also observed that the intensity of ciliary Ptc1 and Smo fluctuated in the cell cultures during cardiogenesis. This was particularly evident around the forming cell clusters, which tended to have more Smo and less Ptc1 in the cilium (data not shown). At the end stage of differentiation, however, we also observed that ciliary Ptc1 often increased in intensity in the cilium. These observations could indicate that the primary cilium in P19.CL6 cells is part of the signaling machinery that coordinates activation of the Hh pathway during differentiation, and that Ptc1 participates in a negative regulatory feedback inhibition in the developing cardiomyocytes. In this scenario, either newly expressed and/or pre-existing Ptc-1 might translocate to the primary cilium in cells differentiating into cardiomyocytes. We also observed that the intensity of ciliary Ptc1 and Smo fluctuated in the cell cultures during cardiogenesis. This was particularly evident around the forming cell clusters, which tended to have more Smo and less Ptc1 in the cilium (data not shown). At the end stage of differentiation, however, we also observed that ciliary Ptc1 often increased in intensity in the cilium. These observations could indicate that the primary cilium in P19.CL6 cells is part of the signaling machinery that coordinates activation of the Hh pathway during differentiation, and that Ptc1 participates in a negative regulatory feedback inhibition in the developed cardiomyocytes. In this scenario, either newly expressed and/or pre-existing Ptc-1 might translocate to the primary cilium at the end stage of differentiation, although further analysis is required to examine the importance of changes in ciliary localizations of Hh components during differentiation of P19.CL6 cells into beating cardiomyocytes.

Knocking down the primary cilium with Ift88 and Ift20 siRNA prevents cardiogenesis

To investigate more directly the importance of primary cilia in early cardiogenesis, we investigated the effects of knockdown of Ift88/Polaris and Ift20 on differentiation of P19.CL6 cells into cardiomyocytes. Using antibodies against Ift88 and Ift20 (Fig. 5A) we initially observed that Ift88 predominantly localizes to the ciliary base and tip, whereas Ift20 had a centrosome and Golgi localization at the primary cilium in P19.CL6 cells. This localization is identical to that observed in other cell types (Pazour et al., 2000; Taullman et al., 2001; Folliot et al., 2006; Haycraft et al., 2005). Confirmation that Ift88 and Ift20 localized to the cilia, centrosome and Golgi in cells that differentiate into cardiomyocytes was made by immunofluorescence analysis, which showed localization of both IFT proteins in cells that express Nkx2-5 at day 9 (Fig. 5B).

For knockdown studies, we nucleofected cells either with a siRNA construct targeting Ift88 alone or in combination with a siRNA construct targeting Ift20 (Ift88+Ift20); the Ift88 construct expresses GFP. Then, we followed the effect of the knockdown constructs on expression rates of Ift88 and Ift20, assembly rates of primary cilium, expression rates of Gata4, Nkx2-5 and α-actinin, and rates of beating cardiomyocytes. Using Q-PCR analysis, we found that Ift88 and Ift88+Ift20 knockdown reduced the level of Ift88 mRNA to about 50% compared with mock-transfected cells under both conditions 3 days after nucleofection (Fig. 5C). Ift20 mRNA was reduced to about 25% when transfected with Ift88+Ift20 siRNA but it was not affected by Ift88 nucleofection alone. These results indicate that Ift88 siRNA does not affect the expression of Ift20 and vice versa. Furthermore, western blot analysis showed that the protein levels of Ift88 (~95 kDa) and Ift20 (~16 kDa) are significantly reduced by siRNA nucleofection (Fig. 5D). As a control for the Ift88 antibody, we performed western blot analysis on NIH3T3 cells, wt MEFs and Ift88Tg737Nrpkn MEFS (Tg73prkkn MEF) showing that the Ift88 protein band at 95 kDa is absent in mutant fibroblasts (Fig. 5E). This has also been reported in prlxcre.Ift88tm5 conditional mutants (Haycraft et al., 2007). Immunofluorescence analysis was performed to show that cells nucleofected with the Ift88 siRNA construct expressing GFP grew no or very short cilia (~1 μm) compared with mock-transfected cells, which formed cilia of ~4 μm in length (Fig. 5F). Furthermore, in mock-transfected cultures, about 70% of the cells formed primary cilia, which was reduced to about 30% in Ift88-transfected cells (Fig. 5G). A combination of both Ift88 and Ift20 siRNA reduced the frequency of ciliated cells further to about 20%.

Q-PCR analysis demonstrated that knockdown of the primary cilium was associated with a reduction of mRNA expression rate of Gata4 to about 40% and 25% relative to mock-transfected cells with Ift88 and Ift88+Ift20 siRNA, respectively (Fig. 6A). Immunofluorescence analysis confirmed that cells nucleofected with the Ift88 siRNA construct expressing GFP were Gata4 negative (Fig. 6B) and Sox2 positive (Fig. 6C), indicating that knockdown of the primary cilium maintains cells in their undifferentiated state. In

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**Fig. 4.** Ciliary localization of Hh-signaling components in P19.CL6 cells. (A) Immunofluorescence microscopy (IF) analysis of acetylated tubulin (tb) on primary cilia after 24 hours (tb, red; pericentrin, green; DAPI, blue). Asterisk indicates centrosome region and arrows show primary cilium. (B) IF analysis of Ptc1 localization to the primary cilium. (C) IF analysis of Smo localization to the primary cilium. (D) IF analysis of Gli2 localization to the primary cilium. (E) IF analysis of Ptc1 and Gata4 localization at day 12. (F) IF analysis of Gli2(H-300) and Nkx2-5 localization at day 12. Arrows in B-F indicate the primary cilium.
addition, Ift88+Ift20 siRNA effectively blocked expression and nuclear localization of Gata4 (Fig. 6D). Similarly, Q-PCR and western blot analysis showed that Ift88+Ift20 siRNA reduced the mRNA and/or protein levels of Nkx2-5 and α-actinin at day 9 (Fig. 6E,F). Finally, we analyzed the number of clusters of beating cardiomyocytes after addition of siRNA (Fig. 6G). The cultures were scanned daily for beating clusters; day \([X]\) marking the day of the first observed beating cluster in a 9.6 cm² culture dish. The cells were then recounted for beating clusters the day after \([X+1]\). The time point for formation of beating clusters could vary a few days from one experiment to another, although all experiments showed beating clusters, on average, at day 12. In a screen of more than eight independent experiments, we observed no major differences in the time point for appearance of beating clusters in mock-treated versus non-treated cells. As shown in Fig. 6G, both Ift88 and Ift88+Ift20 siRNA reduced the number of beating cardiomyocytes from about 12 beating clusters in mock-transfected cells to about 3 and 1.5 beating clusters on average in Ift88 and Ift88+Ift20 siRNA nucleofected cells, respectively, at day X+1. The size of the beating clusters in siRNA nucleofected cells was reduced to about 20% of the size of clusters in mock-transfected cells, and no networks were observed between individual clusters. These results support the conclusion that primary cilia are crucial regulators of P19.CL6 cardiogenesis.

Knockdown of Ift88 and Ift20 blocks Hedgehog signaling

To further investigate the significance of primary cilia in coordination of Hh signaling in P19.CL6 cardiogenesis, we initially analyzed the effect of Ift88+Ift20 siRNA on the expression levels of Ptc1 mRNA (Fig. 7A) and Gli1 mRNA (Fig. 7B) at day 5 of differentiation by Q-PCR analysis. In both cases, knockdown of the cilium largely reduced the expression levels of both Hh signaling markers to about 12% and 4%, respectively, relative to mock-transfected cells. Immunofluorescence analysis demonstrated that Ift88+Ift20 siRNA blocked the expression and nuclear localization of Gli1 at day 5 (Fig. 7C), which was comparable with that observed in the presence of cyclopamine (Fig. 3E). Therefore, the primary cilium might control P19.CL6 cardiogenesis partly by coordinating the Hh signaling machinery.

Heart defects in E11.5 Ift88-null mice

The Ift88-null (Ift88<sup>em1Rpw</sup>, Ift88<sup>−/−</sup>) mouse has multiple developmental phenotypes including random left-right axis specification, neural tube closure and patterning abnormalities, hepatic and pancreatic ductal defects, polydactyly, cerebellar hypoplasia and retinal degeneration because of malfunctioning or loss of primary cilia (Lehman et al., 2008). Since knockdown of Ift88 severely inhibits cardiomyogenesis in P19.CL6 cells (Fig. 6), we hypothesized that the loss of Ift88 in Ift88<sup>em1Rpw</sup> (Ift88<sup>−/−</sup>) mice would cause heart defects. This hypothesis was tested by investigating cardiac tissue sections from WT and Ift88<sup>−/−</sup> embryos at day E11.5 (Fig. 8). In the embryonic hearts of homozygous Ift88<sup>−/−</sup> mice (Fig. 8A,B), we observed malformations of the cardiac outflow tract (OFT) and the ventricles. The length of the distal truncus in Ift88<sup>−/−</sup> mice was significantly shorter compared with
that of WT mice, and the OFT cushions seemed to be malformed, with a thinner appearance (Fig. 8A). The WT mice had expanded cardiac cushion tissue and a distinct transformation of endocardial cells into mesenchyme, whereas the epithelial-mesenchymal transformation (EMT) was virtually absent in the Ift88–/– mice. The ventricles of Ift88–/– mice appeared dilated and empty because of greatly reduced ventricular trabeculation compared with that in WT mice (Fig. 8B). Furthermore, there was an increased volume of the pericardial space in the Ift88–/– embryo compared with that of the WT embryo (arrowheads in Fig. 7B). We verified that the mouse embryos were of the correct genotype (Fig. 8C) and immunohistochemical analysis showed that the Ift88-null mice had no or very short primary cilia, as expected (Fig. 8D).

Immunohistochemical analysis also showed that Gli2 localizes to primary cilia in the developing heart of WT embryos, such as in the ventricular body wall (Fig. 8E, top panel). This localization is absent in Ift88+/– embryos (Fig. 8E, bottom panel). These in vivo findings support the conclusion that primary cilia have an important role in cardiogenesis by coordinating Hh signaling.

**Discussion**

Primary cilia have a critical role in the coordination of a number of developmental processes in mammals, such as embryonic left/right determination, skeletal patterning, limb formation and neurogenesis (Nonaka et al., 1998; Gouttenoire et al., 2007; Haycraft et al., 2007; Breunig et al., 2008). Brueckner and co-workers (Slough et al., 2008) demonstrated that E9.5 Kif3a−/− mouse embryos have abnormal development of ECCs and reduced trabeculation, indicating that primary cilia in the heart could regulate processes in cardiac morphogenesis, because Kif3a is required for ciliary assembly amongst other cellular functions (Slough et al., 2008). Here, we studied the function of the primary cilium in early cardiogenesis by examining differentiated clusters of cardiomyocytes, and by analysis of defects in heart development in Ift88-null E11.5 mouse embryos, which form no or very short primary cilia.

Primary cilia in P19.CL6 stem cell differentiation

P19.CL6 stem cells spontaneously differentiate into clusters of beating cardiomyocytes in the presence of DMSO (Habara-Ohkubo, 1996). We show that P19.CL6 stem cell form primary cilia and that essential components of the Hh pathway, Ptc1, Smo and Gli2, localize to P19.CL6 cilia. This is the first discovery of primary cilia in this cell line. Before cardiomyocyte differentiation, the cells are kept in their pluripotent state, as evidenced by expression of the stem cell markers Sox2 and Oct4. Inhibition of the Hh pathway by cyclopamine blocks DMSO-induced differentiation of P19.CL6 cells into clusters of beating cardiomyocytes by obstructing the expression and nuclear localization of the heart transcription factors Gata4 and Nkx2-5, which mark early cardiomyocyte differentiation (Grepin et al., 1997; Lints et al., 1993). Further, cyclopamine altered the cell morphology; cells aggregated in disorganized clusters associated with a decreased expression of α-actinin and a lack of α-actinin organized into the Z-line on α-cardiac muscle stress fibers, which indicate fully differentiated cardiomyocytes. The inhibitory effect of cyclopamine on Hh signaling in P19.CL6 cells was
confirmed by inhibition of DMSO-induced upregulation of Gli1 and Ptc1 mRNA expression and nuclear localization of Gli1, Gli2 and Gli3. In support of the conclusion that cyclopamine inhibits Hh signaling by processing of Gli2, we used western blot analysis to show that the level of the full-length form of Gli2, which might function as an activator form of Gli2, is reduced in cyclopamine-treated cells. Concomitantly, in the presence of cyclopamine, we observed an increase in a processed form of Gli2, which might represent an inhibitory form of Gli2 (Pan et al., 2006). These results confirm that Hh signaling is required for differentiation of P19.CL6 cells into cardiomyocytes, and that Hh signaling may be associated with primary cilia in P19.CL6 cells.

To determine the function of the primary cilium in regulation of Hh signaling and in cardiomyogenesis, we carried out experiments in which the primary cilium was knocked down by Ift88 and Ift20 siRNA. Initially, we showed that Ift88 uniquely localizes to primary cilia and that Ift20 localizes to the centrosome and Golgi region in P19.CL6 cells, as previously shown in differentiated cells (Pazour et al., 2000; Follit et al., 2006; Haycraft et al., 2005). Ift88 is a subunit of the IFT particle complex B and is required for functional IFT and ciliary assembly (Pazour et al., 2000; Lucker et al., 2005). Ift20 functions in the delivery of ciliary membrane proteins from the Golgi complex to the cilium and strong knockdown of this IFT particle blocks ciliary assembly without affecting Golgi structure (Follit et al., 2006). Ift20 is anchored to the Golgi complex by the golgin protein GMAP210, and mice lacking GMAP210 die at birth with a pleiotropic phenotype that includes ventricular septal defects of the heart, although cells have normal Golgi structure (Follit et al., 2008). Knockdown of Ift88 in P19.CL6 cells reduced the frequency of ciliated cells by more than 50% and inhibited the expression levels of both Gata4 and Nkx2-5 to about 40% at day 5 of transfection, and this was associated with a decrease in nuclear localization of Gata4 followed by a reduced number of beating cardiomyocytes at around day 12. We also observed that cells transfected with Ift88 siRNA were Sox2 positive, indicating that knockdown of the cilium maintains cells in their undifferentiated state and the cells then do not undergo apoptosis or differentiate into other cell lineages. We next performed double knockout of Ift88 and Ift20 to show that an augmented reduction of primary cilia is associated with an additional decrease in mRNA expression levels of Gata4 and Nkx2-5 and numbers of clusters of beating cardiomyocytes in accordance with a strong reduction in protein levels of Gata4, Nkx2-5 and α-actinin. Since knockdown of Ift88 and Ift20 produces an inhibitory response on Hh signaling that is similar to that of cyclopamine in P19.CL6 cells at day 5 of differentiation (i.e. strongly reduces the expression levels of Ptc1 and Gli1 as well as the nuclear localization of Gli1), we conclude that differentiation of P19.CL6 cells into cardiomyocytes is governed by the primary cilium, partly by regulation of Hh signaling.

A number of observations have indicated a crucial function of primary cilia in differentiation processes in mammalian stem cells. Human embryonic stem cells (hESCs) possess primary cilia (Kiprilov et al., 2008) that contain a series of signal transduction components, including PDGFRα (Awan et al., 2009) and members of the Hh and Wnt signaling systems (Awan et al., 2009; Kiprilov et al., 2008), which are important in stem cell maintenance, differentiation and proliferation. It was further shown that primary cilia are crucial for the development of neural stem cells needed for proper development of the hippocampal region (Han et al., 2008) and in development of the neocortex and cerebellum (Spassky et al., 2008). These results imply that stem cell primary cilia per se might coordinate cellular processes in early development, including cardiogenesis. The mechanism by which Ptc1, Smo and Gli2 coordinate Hh signaling in the cilium of P19.CL6 is presently not understood. Our preliminary data suggest that Ptc1 and Smo become differentially localized to the cilium during the differentiation stages towards formation of beating cardiomyocytes, as previously described for cilia after stimulation of the Hh pathway in hESCs, fibroblasts, MDCK cells and epithelial cells from the exocrine ducts of the human pancreas (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Rohatgi et al., 2007; Nielsen et al., 2008). The regulated movement of Ptc1 out of the cilium and Smo into the cilium might create a switch by which cells can regulate Gli processing and turn Hh signaling on (reviewed by Christensen and Ott, 2007). Further experiments are required to investigate this in detail to understand how the primary cilium might function as a specialized organelle that integrates positive and negative inputs on Hh signaling in P19.CL6 cell differentiation.

Primary cilia are important for cardiogenesis in vivo

The cardiac phenotype of E11.5 Ift88−/− embryos, where ciliogenesis is inhibited, resembles in part, the cardiac phenotypes of Pkd2−/− and Kif3α−/− mice (Slough et al., 2008), which have malformed endocardial cushions, decreased trabeculation and increased pericardial space. Slough et al. (Slough et al., 2008) showed, by comparison to lrd−/− embryos, that decreased trabeculation and increased pericardial space are not related to abnormal development of the left-right asymmetry, which is initiated at the node of the

Fig. 7. Effect of knockdown of Ift88 and Ift20 on Hh signaling in P19.CL6 cells. (A,B) Quantitative RT-PCR analysis of Ptc1 (A) and Gli1 (B) relative mRNA levels after Ift88+Ift20 siRNA nucleofection vs mock control on day 5. (C) Immunofluorescence microscopy analysis of Gli1 expression in Ift88-Ift20 nucleofected P19.CL6 cells compared with mock control on day 5 (Gata4, green; DAPI, blue). **P<0.01.
The primary cilium in cardiogenesis

mouse at E7.75 and coordinated by nodal cilia. Therefore, our results on the Ift88<sup>−/−</sup> mouse support these findings (Slough et al., 2008) and suggest that these defects in cardiac morphogenesis are not caused by defects in nodal cilia, but in cardiac primary cilia in the developing heart. We also show that that Ift88<sup>−/−</sup> E11.5 embryos have malformations of the cardiac outflow tract (OFT), indicating that primary cilia also have an essential role in formation of the OFT. Brueckner and colleagues (Slough et al., 2008) did not investigate OFT malformations.

A series of signal transduction pathways have been implicated in the morphogenesis of the embryonic heart after establishment of the left-right asymmetry, some of which could be coordinated by the cardiac primary cilium. Our in vitro data on P19.CL6 cells show that Hh signaling is one of the ciliary pathways necessary for cardiomyocyte differentiation, and we surmise that ciliary Hh signaling in a similar manner coordinates in vivo morphogenesis of the heart. As an example, Gli2 localizes to primary cilia in both P19.CL6 cells and in the developing heart of WT embryos, and this localization is disrupted in Ift88<sup>−/−</sup> embryos. Interestingly, the OFT phenotype of Ift88<sup>−/−</sup> embryos resembles that of Shh<sup>−/−</sup> mice (Washington Smoak et al., 2005; Goddeeris et al., 2007), suggesting that aberrant Hh signaling due to defects in assembly of primary cilia is involved in shortening of the OFT and potentially in other cardiac structures in Ift88<sup>−/−</sup> embryos. Indeed, cardiac expression of Nkx2-5 during heart development is blocked in the Smo<sup>−/−</sup> mouse embryo at the 2- to 3-somite stage (which corresponds to E9), suggesting that Nkx2-5 is a specific cardiac target of Hh signaling and when blocked, underlies the defective heart morphogenesis observed in Smo mutants (Zhang et al., 2001). Similarly, our results show that knockdown of the primary cilium in P19.CL6 cells inhibits Hh signaling and blocks the expression of Nkx2-5, supporting the conclusion that primary cilia have an important role in heart development, in part by coordinating Hh signaling. Whether the primary cilium regulates cardiogenesis by coordinating other signaling pathways, such as Wnt, BMP and PDGF signaling, is presently unknown. Wnt and PDGF signaling are regulated by the cilium in a series of other cell types involved in growth control, migration and differentiation (reviewed by Christensen et al., 2008; Gerdes and Katsanis, 2008). BMP promotes the induction of cardiomyocytes from the mouse stem cell line P19.SI (Angello et al., 2007) and human embryonic stem cells (Takei et al., 2009). Based on their findings on Pkd2<sup>−/−</sup> mouse embryos, Slough and colleagues (Slough et al., 2008) also hypothesized that primary cilia in the heart and/or vasculature can function as mechanosensors to...
Materials and Methods

Análisis y preparación de tejido

materias y métodos

Primary antibodies

Antibodies from Santa Cruz: rabbit anti-Gli3 (H-280) (Cat. no. SC-20688), goat anti-Gli2 (G-20) (SC-20291), goat anti-Gli3 (N-19) (SC-6155), goat anti-Gli2 (N-20) (SC-20290), goat anti-Gli3 (C-20) (SC-6154), rabbit anti-Gli2 (H-300) (SC-28674), goat anti-patched (G-19) (SC-6144), rabbit anti-Gata4 (H-112) (SC-9053), goat anti-Nkx2.5 (N-25) (SC-1897), anti-Goat Oct3/4 (C-20) (SC-8629), rabbit anti-Gli1 (Abcam AB14149); rabbit anti-Smo (MBL International LS2666); mouse anti-α-actinin (Sigma-Aldrich, A2547); mouse anti-acetylated tubulin, Sigma (T7451); rabbit anti-detyrosinated α-tubulin (Glus-tubulin, Chemicon AB3201); goat anti-Sox2 (R&D systems) (AF2018), mouse anti-sox2 (R&D systems) (MAB208). Secondary antibodies for immunofluorescence from Molecular Probes: Alexa Fluor® 488, Alexa Fluor® 568, and Alexa Fluor® 647, respectively (Molecular Probes, 35738). Blocking peptides (BPs) (Santa Cruz): BP for goat anti-Gli2 (G-20) (SC-20291-P); BP for goat anti-Gli2 (N-20) (SC-20290-P).

siRNA constructs and transfection

The cells were transfected with the plasmid pGIP76.13 and pJAF135.45 encoding siRNA targeted at mouse Ift88 and Ift20, respectively, by nucleofection with the Nucleofector device II from Amaxis Biosystems. We followed the recommended protocol for P19 cell transfection and used a Nucleofector Kit V. The recommended amount of cells for transfection was 2×10^6 and with 2 μg plasmid. Complementary oligonucleotides corresponding to the coding region of mouse Ift20 and mouse Ift88 were annealed and cloned into pGIP76.13 digested with BglII and HindIII to produce pGIP768.12 and pJAF135.45, respectively (Follit et al., 2006).

Immunofluorescence

The cells were grown on coverslips in six-well trays (TPP) and subjected to immunofluorescence microscopy analysis as described (Schneider et al., 2005). Pictures were captured with a cooled CCD Optronics camera on a Nikon-Japan, Eclipse E600 epifluorescence microscope and the digital images processed with Photoshop 6.0.

Histochemistry

Representative sections of WT and Ift88<sup>−/−</sup> mice were stained with hematoxylin and eosin (H&E). In preparation for immunohistochemistry, tissue sections were heated for 1 hour at 60°C. Then, sections were deparaffinized for 10 minutes in xylene, 2 minutes in 99% ethanol, twice for 15 minutes in 99% ethanol, 15 minutes in 96% ethanol, 15 minutes in 70% ethanol and a final 15 minutes in H2O. The sections were then placed in a tub with running tap water for 10 minutes and in PBS for 10 minutes. Sections were circled with a PAP pen and incubated in blocking buffer (PBS with 5% BSA) for 20 minutes. Immunohistochemistry was performed as described (Nielsen et al., 2008).

SDS-PAGE and western blot analysis

Cells were grown in Petri dishes and were rapidly washed once in PBS and spun down at 500 g for 5 minutes, after which 350 μl lysis buffer with 1% β-mercaptoethanol was added. Proteins were purified using NucleoSpin kit protocol (Macherey-Nagel) for RNA or protein analysis. SDS-PAGE and western blotting were performed as described (Schneider et al., 2005).

Quantitative RT-PCR

RNA was purified using a NucleoSpin kit (Macherey-Nagel). For cDNA synthesis we used DNase I Amplification grade (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). The Q-RT-PCR reactions were performed on a 7500 fast Realtime PCR-system from Applied Biosystems with Lightcycler Fast Start DNA Master plus SYBR Green I (Roche). Primers used are listed in supplementary material Table S1.

Statistics

We used one-way analysis of variance (ANOVA) test on n=3 or more. Significance levels were divided into three categories (*P<0.05, **P<0.01, highly significant; ***P<0.001, extremely significant).

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Ocbina, P. J. and Anderson, K. V.


CHAPTER 9

Using Nucleofection of siRNA Constructs for Knockdown of Primary Cilia in P19.CL6 Cancer Stem Cell Differentiation into Cardiomyocytes

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Abstract

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VI. Summary

Acknowledgments

References

Abstract

Primary cilia assemble as solitary organelles in most mammalian cells during growth arrest and are thought to coordinate a series of signal transduction pathways required for cell cycle control, cell migration, and cell differentiation during development and in tissue homeostasis. Recently, primary cilia were suggested to control pluripotency, proliferation, and/or differentiation of stem cells, which may comprise an important source in regenerative biology. We here provide a method using a P19.CL6 embryonic carcinoma (EC) stem cell line to study the function of the primary cilium in early cardiogenesis. By knocking down the formation of the primary cilium by nucleofection of plasmid DNA with siRNA sequences against genes essential in ciliogenesis (IFT88 and IFT20) we block hedgehog (Hh) signaling in P19.CL6 cells as well as the differentiation of the cells into beating cardiomyocytes (Clement et al., 2009). Immunofluorescence microscopy, western blotting, and quantitative PCR analysis were employed to delineate the molecular and cellular events in cilia-dependent cardiogenesis. We optimized the nucleofection procedure to generate strong reduction in the frequency of ciliated cells in the P19.CL6 culture.

I. Introduction

Primary cilia are organelles that emanate from the surface of most growth-arrested mammalian cells. They consist of a microtubule (MT)-based axoneme organized in a 9 + 0 axonemal ultrastructure ensheathed by a bilayer lipid membrane continuous with the plasma membrane, but which contains a distinct subset of receptors and other proteins engaged in signaling pathways in developmental processes and tissue homeostasis. Primary cilia are formed via a process termed intraflagellar transport (IFT), which is essential for the assembly and maintenance of almost all eukaryotic cilia and flagella (Cole and Snell, 2009; Pedersen et al., 2008). Separating the two membrane compartments at the ciliary base is a region known as the “ciliary necklace” (Gilula and Satir, 1972), which is connected by fibers to the transition zone of the basal body, which may function as a pore where ciliary precursors and IFT proteins accumulate prior to entering the ciliary compartment via IFT, a process essential for assembly of virtually all cilia and flagella. IFT is a bidirectional transport system that tracks along the polarized MTs of the ciliary axoneme. IFT is composed of large protein complexes, known as IFT particles, and the motor proteins heterotrimeric kinesin-2 (kinesin-2) for
anterograde (base to tip) transport of ciliary building blocks, and cytoplasmic dynein 2 for retrograde (tip to base) transport of ciliary turnover products (Pedersen et al., 2008).

The signaling pathways being coordinated by the developed primary cilium include Hh, Wingless (Wnt), platelet-derived growth factor receptor (PDGFR)α, Ca²⁺, neuronal and purinergic receptor signaling, and communication with the ECM (Satir et al., 2009). Accordingly, defects in assembly or function of the primary cilium are a major cause of human diseases and developmental abnormalities and disorders now commonly referred to as ciliopathies (reviewed in Lehman et al., 2008).

Recent observations indicate that primary cilia in stem cells coordinate signaling pathways, including Hh signaling, in cell differentiation during embryonic development and potentially in regulation of stem cell maintenance and/or pluripotency (reviewed in Veland et al., 2009). Stem cells hold great promises for their possible therapeutical abilities since they can give rise to all three germinal layers and differentiate to form specific cell types dependent on the environment and specific factors present. Stem cells may also be important targets against cancer. Hh regulates cell proliferation and differentiation in numerous embryonic tissues and Hh ligands are expressed in the notochord, the floorplate of the neural tube, the brain, the limb bud zone of polarizing activity, and the gut (Odent et al., 1999). Hh signaling is further required in homeostasis of mature tissues and is implicated in human cancers (Beachy et al., 2004) and neurodegenerative disorders (Bak et al., 2003). A screen for embryonic patterning mutations characteristic of defective Hh signaling first indicated a link between IFT proteins, Hh signaling, and nervous system development (Huangfu et al., 2003). Subsequent studies confirmed that Hh signaling is coordinated by the primary cilium to control targets of the Hh pathway by Gli transcription factors (Corbit et al., 2005; Liu et al., 2005; reviewed in Wong and Reiter, 2008). Functioning Hh components, including Ptc-1, Smo, and Gli transcription factors are localized in primary cilia of human embryonic stem cells (Kiprilov et al., 2008) and neuronal development proceeds ciliary Hh signaling in adult neural stem cell formation, specification of neural cell fate, hippocampal neurogenesis and development of cerebellum and neocortex (Breunig et al., 2008; Han et al., 2008; Komada et al., 2008; Spassky et al., 2008). Similarly, primary cilia are involved in the coordination of Hh signaling, for example, in limb bud formation (Haycraft et al., 2007), skeletogenesis (Gouttenoire et al., 2007), mammary gland development and ovarian function (Johnson et al., 2008), molar tooth number (Ohazama et al., 2009), and development of the pancreas (Nielsen et al., 2008).

Primary cilia and Hh signaling are also implicated in early cardiogenesis as evidenced by defective heart development in knockout mice with defects in ciliary assembly, including decreased trabeculation, increased pericardial space, and malformations of the cardiac outflow tract (Clement et al., 2009). Further, knock down of the primary cilium in the pluripotent P19.CL6 EC stem cell line blocked Hh signaling and differentiation of cells into beating cardiomyocytes in vitro (Clement et al., 2009). The P19.CL6 cell line is a subclone from the P19 cell line that spontaneously differentiate into clusters of beating cardiomyocytes in the presence of dimethyl sulfoxide (DMSO) (Habara-Ohkubo, 1996). Further, P19.CL6 cells have no requirement for being
cultured in suspension and form embryoid bodies before carrying out the analysis on cardiac differentiation (Uchida et al., 2007). This allows the investigator to follow the function of the primary cilium in the initial phases of differentiation from day 1 through a 2-week period until the formation of beating cardiomyocytes.

II. Rationale

Here we provide a detailed and optimized method for nucleofecting P19.CL6 EC cells with IFT88 and IFT20 siRNA plasmid DNA to produce a high transfection percentage to knockdown primary cilia in cultures of P19.CL6 cells during their differentiation into cardiomyocytes. IFT88 is a subunit of the IFT particle complex B required for functional IFT and assembly of the primary cilium (Pedersen and Rosenbaum, 2008). IFT20 is associated with the Golgi apparatus, and knockdown of this IFT particle reduces ciliary assembly without affecting Golgi structure (Follit et al., 2006). Following knockdown of the cilium cell differentiation can be assessed by light microscopy (LM), immunofluorescence microscopy (IFM), SDS-PAGE, western blotting (WB), and quantitative PCR (qPCR) analysis in order to follow changes in DMSO-induced formation of beating cardiomyocytes, expression and localization of stem cell and cardiomyocyte markers, and activation of Hh signaling.

III. Materials

A. Cell Line and Cell Culture Reagents

The P19.CL6 cell line is of mouse origin isolated from embryonal carcinoma tissue. The originator is Habara, Akemi and registered with Murofushi, Kimiko, Japan (ref nr. 2406 3467).

MEM Alpha medium (Gibco, Cat#22561-021)
Penicillin/streptomycin (Gibco, pen/strep, Cat#15140-148)
Phosphate-Buffered Saline (PBS)
Fetal bovine serum (FBS, Gibco, Cat#10 160-177)
Trypsin (Trypsin-EDTA, Gibco, Cat#15 400-054)
T75 cell culture flasks (Cell star, Cat#658 170)
T25 cell culture flasks (Cell star, Cat#690 175)
6-well trays (TTP, Cat# 92006)
Petri dishes (Cell Star, 60 x 15 mm, Cat# 628 160)
DMSO (MERCK, Cat#1.02952.1000)

B. Reagents and Solutions for Nucleofection

Nucleofector device II (Amaxa Biosystems)
Nucleofector Kit V (Amaxa Biosystems)
2 µg of IFT88 or IFT20 siRNA plasmid DNA (high grade, high concentration)
MEM Alpha medium (Gibco, Cat#22561-021)
C. Reagents and Solutions for IFM Analysis

- Microscope slides and glass coverslips (12 mm diameter)
- Concentrated HCl
- Humidity chamber
- Ethanol (96% v/v and 70% v/v)
- Paraformaldehyde (PFA), 4% w/v solution
- Blocking buffer: 2% w/v Bovine Serum Albumin (BSA) in PBS
- Permeabilization buffer: 0.2% v/v Triton X-100, 1% w/v BSA in PBS
- DAPI (4',6-diamidino-2-phenylindole, dihydrochloride)
- Mounting medium (PBS, 2% w/v N-propylgallate, 85% v/v glycerol in PBS)
- Nail polish
- Antibodies and fluorescent reagents (Table I)

D. Western Blot

- NOVEX system (XcellSure Lock)
- Precast NuPAGE 10% and 12% BIS-TRIS 12-well gels
- Nucleospin kit (Macherey-Nagel, Cat#740 933.50) for RNA/protein isolation
- BSA protein standard (Pierce Biotechnology, Rockford, IL, USA)
- Protein assay (BioRAD, DC based on Lowry’s method)
- Running buffer (Invitrogen, Cat#NP0001)
- Transferbuffer (Invitrogen, Cat#NP0006-1)
- Non-fat dry milk blocking buffer
- NuPAGE Antioxidant (Invitrogen, Cat#NP0005)
- Ethanol (96% v/v)
- TBST
- Antibodies (Table I)

Table I

<table>
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<th>Used Antibodies and Fluorescent Reagents</th>
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<tr>
<td><strong>Primary Antibodies</strong></td>
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<tr>
<td>Mouse anti-α-actinin (Sarcomeric), (Sigma Aldrich, Cat#A-7811)</td>
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<tr>
<td>Goat anti-Nx2 (N-19), (Santa Cruz, Cat#SC-8697)</td>
</tr>
<tr>
<td>Mouse anti-β-actin (Sigma Aldrich, Cat#A-5441)</td>
</tr>
<tr>
<td>Mouse antiacetylated Tubulin, (Sigma Aldrich, Cat#T7451)</td>
</tr>
<tr>
<td>Rabbit antidetyrosinated α-Tubulin (Glub-Tubulin, Chemicon, Cat#AB3201)</td>
</tr>
<tr>
<td>Rabbit anti-IFT20 (see Follit et al. 2006)</td>
</tr>
<tr>
<td><strong>Secondary Antibodies and Fluorescent Agents</strong></td>
</tr>
<tr>
<td>Donkey anti-goat (DAG), Alexa Flour® 488 (Molecular Probes, Eugene, OR, USA, Cat#A11055)</td>
</tr>
<tr>
<td>Donkey anti-mouse (DAM), Alexa Flour® 568 (Molecular Probes, Cat#A10037)</td>
</tr>
<tr>
<td>Goat anti-rabbit (GAR), F(ab')2-specific Alkaline phosphatase-conjugated (Sigma Aldrich, Cat#A3937)</td>
</tr>
<tr>
<td>Goat anti-mouse (GAM), F(ab')2-specific Alkaline phosphatase-conjugated (Sigma Aldrich, Cat#A1293)</td>
</tr>
<tr>
<td>DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes, Cat#D1306)</td>
</tr>
</tbody>
</table>
E. Quantitative RT-PCR

Nucleospin kit (Macherey-Nagel, Cat#740 933.50) for RNA/protein isolation
SuperScript™ II reverse transcriptase (Invitrogen, Cat#18064-014)
DNase I Amplification grade (Invitrogen, Cat#18068-015)
PCR-grade nuclease-free water
dNTP mix, 10 mM of each dNTP
RNAse inhibitor (rRNasin from Promega)
qPCR plate (Applied Biosystems, Foster City, CA, USA Cat#4346906)
Lightcycler® FastStart DNA MasterPLUS SYBR Green I (Roche, Cat#030515885001)
RNA purifying kit (Macherey-Nagel, Cat#740 933.50) for RNA/protein Ammonium
Buffer with 15 mM MgCl₂ (Ampliqon III, Cat#AMP300305)
TAQ-polymerase (5 units/µl, Ampliqon III)
Agarose, ethidium bromide, TAE buffer (4.84 g Tris base, 1.14 ml glacial acetic acid, 2 ml
0.5 M Na₂ EDTA pH 8.0, ddH₂O up to 1 l), nucleic acid molecular weight marker
PCR primers (Table II)
Mouse universal reference total RNA (Stratagene, Cat#740100)
E.N.Z.A. gel extraction kit (Omega Bio-Tek Inc., Norcross, GA, USA, Cat#D2500-00)

IV. Methods

A. Introductory Remarks and Experimental Outline

Growth arrest and formation of primary cilia in cultures of mammalian cells can be
induced either by depletion of serum and/or by growing cells to confluency. In
cultures of P19.CL6 EC stem cells primary cilia are formed in the presence of
serum as the cells leave their pluripotent stage and enter the differential steps
induced by DMSO to form clusters of beating cardiomyocytes, most probably because physical contact between individual cells promotes the entrance into G0 (Clement et al., 2009). This allows the investigation of the function of primary cilia in stem cell differentiation and cardiomyogenesis in vitro by knocking down the cilia. Cultures of P19.CL6 cells in 9.6 cm² Petri dishes may form up to about 20 clusters of cardiomyocytes with a diameter of 0.2–0.6 mm that beat synchronously at a frequency of about 60 rhythmic contractions per minute around day 12 in the presence of DMSO (Clement et al., 2009). Prior to differentiation cells are positive for stem cell markers Sox2 and Oct4, which are replaced by Gata4 positive cells at day 2, Nkx2–5 positive cells around day 8, and α-actinin positive cells marking the Z-line on α-cardiac muscle stress fibers around day 12 concomitantly with the onset of contractions of the mini hearts. During these steps of cell proliferation and differentiation, the expression of the transcriptional target genes for Hh signaling, Gli1 and Ptc-1 are highly upregulated: both cardiomyocytes differentiation and Hh signaling being blocked in the presence of the Smo antagonist and Hh-signaling inhibitor, cyclopamine (Clement et al., 2009).

In this protocol we performed siRNA knockdown of the primary cilium in P19.CL6 cells by nucleofection, which is a transfection method that enables efficient and reproducible transfer of nucleic acids such as DNA, RNA, and siRNA into cells. Nucleofection is also known as Nucleofector Technology, which was invented by Amaxa (http://www.amaxa.com). Nucleofection uses a combination of optimized parameters generated by a device termed Nucleofector with cell-type specific reagents and buffers. The substrate is transferred directly into the cell nucleus and cytosol. Here we nucleofected P19.CL6 cells with IFT88 and IFT20 siRNA plasmid DNAs in order to knock down the cillum and follow its consequences in stem cell maintenance, cell differentiation, and Hh signaling. The IFT88 plasmid DNA is expressing GFP, which enables quantification of nucleofection rates and direct visualization of its consequences on ciliary formation and cell differentiation. Here we present a detailed protocol on the nucleofection procedure, followed by qualitative and quantitative analysis on cell differentiation and Hh signaling by IFM, SDS-PAGE, WB, and qPCR analysis. The protocol for qPCR analysis is presented comprehensively, while IFM, SDS-PAGE, and WB analyses are portrayed in less detail.

### B. Cell Culturing and Passaging

The cells were grown for passaging in T25 cell culture flasks at 37°C, 5% CO₂, and 95% humidity. The cells were passaged every 2–3 days by trypsination, and grown in MEM Alpha cell culture media, containing 1% penicillin/streptomycin and 10% FBS. Prior to trypsination the cells were washed once in 37°C PBS and reseeded at 10–15% confluency (avoid growing cells into 100% confluency). To induce cardiomyocyte differentiation, the medium was supplemented with 1% DMSO and grown under normal incubator conditions. Experimental cells were seeded at a confluency of about 30% in T75 cell culture flasks, 6-well trays, or Petri dishes.
C. Nucleofection of P19.CL6 Cells with IFT80 and IFT20 siRNA (Plasmid DNA)

Sufficient cells were cultivated in T75 flasks, enough to provide each sample with 2 x 10^6 cells. Preferably, the cells should be passaged the day before nucleofection but if the experiments require the cells to enter a stage of differentiation (e.g., DMSO-induced differentiation that needs siRNA knockdown at day 2–3), it is still possible to carry out the nucleofection with a high transfection rate. This will disrupt cell culture morphology since the cells will need to be resuspended in nucleofection solution and reseeded.

Before the actual nucleofection step, prepare the following:

- 6-well trays/dishes with the appropriate working volume of differentiation media are put inside the incubator (37°C/5% CO2) and prewarmed 20 min before nucleofection.
- Prewarm the supplemental Cell Line Nucleofector Solution V to room temperature.
- Thaw up your highly purified IFT88 and IFT20 siRNA plasmids. Amount of µg is determined by optimization (e.g., 2 µg DNA per sample, see Section IV.D).

The medium was removed from the T75 culture flasks and washed once in 37°C PBS. The cells were then trypsinized in 1 × Trypsin-EDTA for 5 min in 37°C/5% CO2 and resuspended in an appropriate volume of 37°C growth medium (preferably freshly made). The volume was adjusted so that the cell density was high enough to collect 2 x 10^6 cells in a 1.5 ml Eppendorf tube. The cells were counted in a hemocytometer to determine the actual number of cells. The cells were then spun down at 90 x g for 10 min at room temperature. The cell pellet was resuspended in 100-µl Cell Line Nucleofector Solution V per 2 x 10^6 cells per sample. Do not keep the cells in the Nucleofector Solution V for more than 15 min.

Each sample is nucleofected in the following steps:

- Add 2 µg of siRNA plasmid DNA into the 100-µl solution containing the cell pellet.
- Gently mix the DNA, cells, and Cell Line Nucleofector Solution V three times in a 1000-µl pipette and transfer the sample to an Amaxa-certified cuvette (make sure the sample covers the cuvette bottom with no air bubbles).
- Close the cuvette with the lid and insert into the Nucleofector device II and run program C-020.
- Transfer 500 µl media from the prewarmed 6-well tray/dish using the supplied plastic pipettes into the cuvette and transfer the whole sample back into the 6-well tray/dish. Avoid transferring white foam “popcorn” to the 6-well tray/dish since this contains only cell debris

The 6-well trays/dishes are put into the incubator as soon as possible in 37°C/5% CO2 to provide as little stress as possible. Analysis of the samples and the effect of IFT88 and IFT20 siRNA knockdown can be performed 24 h after nucleofection. At this time point the maximal effect of the IFT88 and IFT20 knockdown can be found. The optimal time for siRNA plasmid expression can be determined by selecting different time points after nucleofection and verifying the level of mRNA knockdown with IF analysis of a GFP-reporter inserted in the siRNA plasmid.
D. Methods for Optimization with IFT88 siRNA Plasmid for High Transfection Rate

To find the optimal transfection rate, we used the pmaxGFP\textsuperscript{TM} positive control vector supplied by Amaxa biosystems. An experimental setup with variable amounts of pmaxGFP\textsuperscript{TM} (1 µg, 1.5 µg, 2 µg, 2.5 µg, 3 µg, and 4 µg) was nucleofected into 2 × 10^6 cells. The cells were grown on coverslips in 6-well trays at 37°C/5% CO\textsubscript{2} for 24 h. The cells were fixed in 4% paraformaldehyde for 15 min at RT, followed by two washes in PBS, and then permeabilized in 0.2% Triton X-100, 1% BSA for 12 min. Cells were then stained with DAPI to visualize total cell numbers on the coverslips and the total-cell/transfected-cell ratio could hereby be determined. Also variable time points after nucleofection (12, 24, 36, 48, and 72 h) were tested to determine at which time point the transfection rate was the highest. The recommended DNA/cell number ratio supplied by Amaxa biosystems was 2 \times 10^6 cells with 2-µg DNA plasmid followed by analysis after 24 h. The optimal transfection rate for our experiments was found under the recommended conditions and was close to 80%. Cell viability was good—estimated to ~70%. Furthermore, it is highly recommendable to perform both positive and negative nucleofection controls (cells + solution + DNA – program) (cells + solution – DNA + program) to assess influences of nucleofection or purity of DNA on cell viability.

E. Nucleofection for Light and Immunofluorescence Microscopy Analysis

After nucleofection the cells were transferred to 6-well trays containing coverslips and grown in 37°C/5% CO\textsubscript{2} for at least 24 h to visualize the optimal effect of the transfection. After 1, 5, 8, and 12 days of culturing in differentiation medium the cells were subjected to IFM in order to analyze the frequency of ciliated cells. The protocol used for IFM and detection of primary cilia is identical to that described in Chapter 3 by Thorsteinsson et al. (this volume). For detection of primary cilia we used primary antibodies against acetylated α-tubulin (1:1500) and detyrosinated α-tubulin (1:600). Similarly, one can use antibodies directed against specific markers of cardiomyogenesis in order to follow the time course for differentiation, and how nucleofection with IFT88 and IFT20 siRNA constructs impinge on stem cell maintenance and cardiomyocyte differentiation with antibodies directed against Oct4 and Sox2 (stem cell markers) and Gata4, Nkx2–5, and α-actinin (cardiomyocytes markers) (Clement et al., 2009). Further, expression and localization of Hh signaling components such as Gli transcription factors are assessed by IFM and the formation of beating clusters of cardiomyocytes is easily detected by light microscopy.

F. Nucleofection for Western Blot Analysis

After nucleofection the cells were transferred to Petri dishes and grown in 37°C/5% CO\textsubscript{2} for at least 24 hours. Hereafter the cells were cultured for 1–12 days in differentiation medium. For WB analysis cells were washed in PBS, spun down at 500 × g for 5 min, and then added with 350-µl lysis buffer with 1% β-mercaptoethanol. Proteins were purified using Nucleospin kit protocol (Macherey-Nagel, Cat# 740 933.50) for
RNA/protein. After protein precipitation the cells were added with 2% SDS, 1% Glycerol lysis buffer and sonicated, centrifuged at 20,000 × g to precipitate nonsoluble material. The protein concentration was compared with a BCA protein standard (Pierce Biotechnology) and measured with a Protein assay so that an equal amount of protein could be loaded in each lane. For SDS-PAGE a NOVEX system was used with precast NuPAGE 10% and 12% BIS-TRIS 12-well gels (Schneider et al., 2005).

G. Nucleofection for Quantitative Real-Time RT-PCR (qPCR) Analysis

After nucleofection the cells were treated as in the above for WB analysis in order to isolate total RNA accordingly to the Nucleospin kit protocol for RNA/protein and to assess transcriptional processes in Hh signaling and cell differentiation. RNA was treated with DNase I Amplification grade and cDNA was produced from 1µg total RNA using SuperScript™ II reverse transcriptase. PCR primers for amplification of housekeeping genes (Gapdh, B2m, Hprt, Psmd4, Rp13a, Alas1, Pbgd2), cardiomyocyte markers (Mef2C, Myh6, Myh7) and Hh signaling genes (Smo, Ptc-1) were designed using Oligo version 6.23 (Table II). PCR annealing temperature was optimized in a QuatroCycler temperature gradient thermocycler (VWR, West Chester, PA, USA) using universal mouse cDNA as template. For each primer pair PCR fragments were excised from an agarose gel and extracted using an E.N.Z.A. gel extraction kit. Standard curves were generated by 10-fold serial dilutions of the PCR fragments. The quantitative real-time RT-PCR (qPCR) reactions were performed in a 7500 fast Real-time PCR system (Applied Biosystems,) using a Lightcycler Fast Start DNA Master plus SYBR Green 1 kit (Roche, Copenhagen, Denmark).

For each gene the cycle threshold (Ct) value was converted to a relative expression (E) value using the standard curve. Due to the high sensitivity of qPCR, differences in the efficiency of the cDNA synthesis and differences in PCR reaction kinetics between samples may lead to incorrect quantitation of the expression. To correct for intersample variation, samples are normalized by dividing the E value of the gene of interest with the E value of an endogenous control. The ideal endogenous control for qPCR analysis is a gene that displays similar reaction kinetics and expression profiles in all samples (VanGuilder et al., 2008). Usually, housekeeping genes are used as endogenous controls. However, not all housekeeping genes are resistant to experimental conditions; thus to ensure a robust expression profile of the endogenous control in all samples, we normalized using the average E value of at least three housekeeping genes with similar expression profiles across the samples. Only normalized E values were compared in the experiments.

V. Results and Discussion

A. Timetable and Markers of Differentiation in P19.CL6 Cells

In order to delineate the onset for DMSO-induced P19.CL6 stem cell differentiation we initially used light microscopy analysis to evaluate the time point for formation of beating clusters of cardiomyocytes. As depicted in Fig. 1A most cell cultures form
beating clusters around day 12, clusters appearing in small networks that beat at a frequency of about 60 rhythmic contractions per minute. These results are consistent with the findings that clusters of cells at this time point are positive for \(\alpha\)-actinin that marks the Z-line on \(\alpha\)-cardiac muscle stress fibers (Fig. 1C, upper panels) (Clement et al., 2009). To follow differentiation of P19.CL6 cells in more detail we then measured the transcription of the cardiomyocyte markers \(Gata4\), \(Nkx2-5\), \(Actc2\),

Fig. 1 Morphology and expression profile of cardiomyocyte markers in P19.CL6 cells during cardiomyocyte differentiation induced by 1% DMSO. (A) Light microscope images of P19.CL6 cell morphology at day 1 prior to differentiation and at day 12 where cells have formed beating clusters of cardiomyocytes (open arrow). (B) Quantitative RT-PCR analysis on Myh6, Myh7, and Mef2c mRNA levels relative to expression of housekeeping genes during days 1–10 of P19.CL6 differentiation. (C) Immunofluorescence microscopy analysis at days 1 and 12 of localization of \(\alpha\)-actinin and primary cilia (lower panels) and \(\alpha\)-actinin and \(Nkx2-5\) (upper panels). Upper panels: \(Nkx2-5\): green; \(\alpha\)-actinin: red; DAPI: blue. Lower panels: primary cilia (arrows, detyrosinated \(\alpha\)-tubulin, glu-tb): green; \(\alpha\)-actinin: red, DAPI: blue. (See Plate no. xx in the Color Plate Section.)
Mef2C, Myh6, and Myh7 using qPCR. As exemplified in Fig. 1B cardiomyocyte marker genes are transcriptionally upregulated around day 4 after DMSO addition, their expression levels peaking around day 10. These results are in agreement with previous data, showing that cardiomyocyte markers are upregulated at around day 2 and 6 in P19.CL6 cells stimulated with DMSO (Clement et al., 2009). Finally, we performed IFM analysis to show that clusters of cells either at day 1 in undifferentiated cells or day 12 in differentiated cells (positive for Nkx2–5 and α-actinin; Fig. 1C, upper panels) formed primary cilia (Fig. 1C, lower panels) as evidenced by staining with antidetyrosinated α-tubulin (Glu-tb) that is highly enriched in primary cilia of vertebrate cells (Gundersen and Bulinski, 1986). This allows the investigator to examine the role of the primary cilium by RNAi methods in differentiation of P19. CL6 cells using qPCR analysis on the expression of cardiomyocyte genes, Myh6, Myh7, and Mef2c.

B. Nucleofection with IFT88 and IFT20 siRNA and Its Consequences on Ciliary Formation in P19.CL6

Nucleofection was performed with IFT88 and IFT20 siRNA plasmid constructs in order to inhibit the formation of primary cilia in cultures of P19.CL6 cells and then subsequently analyze the effect of ciliary knockdown on cardiomyocyte differentiation. In this regard there are a number of analyses to perform to ensure optimal knockdown efficiency and minimize unwanted effects on cell behavior, such as cell viability caused by the transfection method. As described in Section IV.D, transfection rates can initially be determined with a pmaxGFP™ positive control vector, in which the rate of transfection can be monitored and estimated by green fluorescence in nucleofected cells. In this analysis we found that the optimal transfection rate of 80% was achieved by nucleofection of 2 × 10⁶ P19.CL6 cells with 2-µg plasmid DNA 2 days after the addition of DMSO to the cell cultures, followed by analysis after 24 h. In this setup cell viability was estimated to about 70%. We then used these parameters to analyze cell viability and transfection rate after nucleofection with IFT88 siRNA GFP plasmid. The cells were brought into suspension and nucleofected with the plasmid, and after 24 h of subsequent cell culture the cells were fixed, stained with DAPI, and the transfection rate was calculated to be about 70–80% as judged by IFM (Fig. 2A). In total, the cells had 3 days of differentiation with 1% DMSO in the culture media, hence the day 3 in Fig. 2A.

In order to examine the effect of IFT88 knockdown on formation of primary cilia, we performed IFM analysis with antibodies directed against either detyrosinated α-tubulin or acetylated α-tubulin, the latter also marking primary cilia and other stable cellular MTs (Piperno and Fuller, 1985). As indicated in Fig. 2B, the number of primary cilia at day 6 was markedly reduced in cells nucleofected with IFT88 siRNA plasmid as compared to mock transfected cells. Further, primary cilia emerging from cells in IFT88 siRNA-nucleofected cells were often shorter than in the control cells, supporting the conclusion that IFT88 is required for ciliary assembly. The percentage of ciliated cells at day 3 was further enumerated by IFM analysis as
9. Knockdown of Primary Cilia in P19.CL6 Cells

shown in Fig. 2C. In controls, the percentage of ciliated cells was calculated to be about 75%, whereas this number was reduced to about 30% in IFT88 siRNA-nucleofected cells. As a further control, we found no differences in cell viability between mock and IFT88 siRNA-nucleofected cells, which was estimated to be about 70% in both cases (Clement et al., 2009). We then went on to perform a double knockdown of the primary cilium in P19.CL6 cells using both IFT88 and IFT20 siRNA plasmid DNA. As indicated in Fig. 2C, the number of ciliated cells was further reduced to about 20%, showing that a reduction in both IFT88 and IFT20 has a stronger inhibitory effect on ciliary formation than IFT88 alone.

The level of IFT20 knockdown by nucleofection can also be verified on protein levels with WB analysis. We show here (Fig. 2D) an example of such an analysis of IFT20 protein levels in cells subjected to IFT20 siRNA knockdown compared to mock transfected cells on day 3, 6, and 10. Initially protein levels were greatly reduced on day 3 and 6 compared to control cells. At day 10, however, reduction in the protein level was less pronounced, indicating a loss in siRNA of IFT20. A similar phenomenon was observed with IFT88 siRNA (not shown). A plausible explanation for this is that a portion of the transfected cells may lose their siRNA plasmid over time as a consequence of continuous cell divisions such that the number of plasmid-containing cells in the

Fig. 2 Nucleofection of IFT88 and IFT20 plasmid siRNA in P19.CL6 cells. (A) Immunofluorescence microscopy analysis of cells nucleofected with GFP-expressing IFT88 siRNA plasmid at day 3. Transfection rate efficiency: ~70–80% (IFT88 siRNA GFP: green; DAPI: blue). (B) Immunofluorescence microscopy analysis of primary cilia at day 6 of differentiation after nucleofection with mock and IFT88 siRNA (primary cilia were localized with antiacetylated α-tubulin, tb: red; DAPI: blue). (C) Bar graph showing the percentage of ciliated cells in mock, IFT88, and IFT88 + IFT20 siRNA-nucleofected P19.CL6 cells at day 3. Reproduced with permission from Clement et al. (2009). (D) Western blot analysis of IFT20 (~20 kDa) and β-actin (~43 kDa) protein levels in P19.CL6 cells after 3, 6, and 10 days of IFT20 siRNA nucleofection versus mock transfected cells. (See Plate no. xx in the Color Plate Section.)
culture is reduced. This also means that the percentage of ciliated cells in nucleofected cultures may increase over time around day 10. Since nucleofection requires that cells are kept in suspension, it was not an option to perform a second round of nucleofection of P19.CL6 cells at day 10 without disrupting cell morphology and clusters of cardiomyocytes, which begin to form at this time point after DMSO stimulation.

C. Nucleofection with IFT88 and IFT20 siRNA and Its Consequences on Differentiation and Hh Signaling in P19.CL6 Cells

To analyze the effects of IFT88 and IFT20 knockdown on cardiomyocyte differentiation and Hh signaling we initially performed qPCR analysis on the mRNA levels of myocyte enhancer factor 2c (Mef2c) and myosin heavy chain 7 (Myh7). As shown in Fig. 3A the

![Fig. 3](image-url)

**Fig. 3** Knockdown of IFT88 and IFT20 by siRNA nucleofection inhibits cardiomyocyte differentiation and Hh signaling in P19.CL6 cells. (A) Bar graph showing quantitative RT-PCR analysis on Mef2c and Myh7 mRNA levels relative to expression of housekeeping genes after siRNA nucleofection versus mock at day 5. (B) Number of beating cardiomyocyte clusters in mock, IFT88, and IFT88+IFT20 siRNA-nucleofected P19.CL6 cells at day 12. (C) Bar graph showing quantitative RT-PCR analysis on Ptc1 and Gli1 mRNA levels relative to expression of housekeeping genes siRNA nucleofection versus mock at day 5. Reproduced with permission from Clement et al. (2009).
mRNA levels of both markers of cardiomyocytes differentiation were largely reduced by about 75 and 60%, respectively, compared to mock transfected cells. Similarly, it was shown that IFT88 and IFT20 knockdown reduced the mRNA and/or protein levels of Gata4, Nkx2–5, and α-actinin as judged by qPCR, WB, and IFM analysis (Clement et al., 2009), supporting the conclusion that the primary cilium is critical in the regulation of P19.CL6 cell differentiation into cardiomyocytes. This was further sustained by the observation that knock down of IFT proteins reduced the number of clusters of beating cardiomyocytes at day 12. Figure 3B presents data from a single and representative experiment in which 15 beating clusters in mock transfected cells were reduced to 3 and 1 clusters in IFT88 and IFT88 + 20 siRNA-nucleofected cells, respectively. Moreover, the clusters of cardiomyocytes in IFT siRNA-nucleofected cells were abnormally small with no or very little networks between the individual clusters. It is likely that these irregular clusters of cardiomyocytes are formed as a consequence of a partial loss of plasmid DNA in IFT88 and IFT88 + 20 siRNA-nucleofected cells.

Early cardiogenesis is regulated by a number of different signal transduction pathways, including Hh, Wnt, bone morphogenetic protein (BMP), and PDGFR signaling (Hirata et al., 2007; Kwon et al., 2008; van Wijk et al., 2007; Washington Smoak et al., 2005). In conjunction with defects in cardiomyogenesis qPCR analysis showed that IFT88 + 20 siRNA inhibited Hh signaling in P19.CL6 cells. Under normal cardiomyocyte formation the key elements in Hh signaling, Gli1 and Ptc-1, are upregulated throughout differentiation from day 1 to 9 (Clement et al., 2009). As shown in Fig. 3C, the relative mRNA levels of Gli1 and Ptc-1 at day 5 were reduced to about 5 and 10%, respectively, of the level in mock transfected cells (Clement et al., 2009). These results indicate that Hh signaling in P19.CL6 cell differentiation is coordinated by the primary cilium. Additional experiments will be required to investigate whether IFT88 + 20 siRNA and ciliary knockdown in P19.CL6 cells also affects other signaling pathways, including Wnt, PDGFR, and BMP signaling, which may provide further insight into the function of the primary cilium in cardiomyogenesis and heart development.

VI. Summary

We have described a detailed siRNA-based nucleofection protocol for examining the role of the primary cilium in differentiation of P19.CL6 cancer stem cells into cardiomyocytes. Knockdown of IFT88 and IFT20 with their corresponding siRNA plasmid DNA inhibits ciliary formation, Hh signaling, and differentiation of cells into cardiomyocytes as judged by qPCR, IFM, SDS-PAGE, and WB analysis. In addition to identifying cilia-related signaling pathways, the nucleofection method can be extended to identify other genes that are involved in P19.CL6 stem cell maintenance, proliferation, and differentiation.

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References


9. Knockdown of Primary Cilia in P19.CL6 Cells


Clement et al. Figure 1

A  
Day 1  
Day 12

![Image of cellular growth comparison between Day 1 and Day 12]

B  
Relative mRNA levels

<table>
<thead>
<tr>
<th>Protein</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myh6</td>
<td>1</td>
</tr>
<tr>
<td>Myh7</td>
<td>0.1</td>
</tr>
<tr>
<td>Mef2c</td>
<td>0.1</td>
</tr>
</tbody>
</table>

C  
Day 1  Day 12

![Images of cellular marker changes between Day 1 and Day 12]
Clement et al. Figure 2

A Day 3

B mock: Day 6 Ift88 siRNA: Day 6

C Day 3

D

Percentages of ciliated cells

Ift20

β-actin

Day 3 Day 6 Day 10