Primary Cilia, Signaling Networks and Cell Migration

PhD Dissertation
IBEN RØNN VELAND
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Submitted to the PhD School of The Faculty of Science, University of Copenhagen, August 31st, 2013.
Abstract

Primary cilia are microtubule-based, sensory organelles that emerge from the centrosomal mother centriole to project from the surface of most quiescent cells in the human body. Ciliary entry is a tightly controlled process, involving diffusion barriers and gating complexes that maintain a unique composition of receptors and signal components in the cilium to regulate cellular processes such as transcriptional control or cytoskeletal reorganization. This dissertation focuses on selected signaling systems regulated by the primary cilium, including the PDGFRα, TGFβ and Wnt pathways, and how this controls directional cell migration as a physiological response.

The ciliary pocket is a membrane invagination with elevated activity of clathrin-dependent endocytosis (CDE). In paper I, we show that the primary cilium regulates TGF-β signaling and the ciliary pocket is a compartment for CDE-dependent regulation of signal transduction. Upon ligand-binding and activation in the cilium, TGFβ receptors accumulate and are internalized at the ciliary base together with Smad2/3 transcription factors that are phosphorylated here and translocated to the nucleus for target gene expression. These processes depend on formation of the primary cilium and CDE at the pocket region.

The ciliary protein Inversin functions as a molecular switch between canonical and non-canonical Wnt signaling. In paper II, we show that Inversin and the primary cilium control Wnt signaling and are required for polarization and cell migration. A number of central Wnt components localize to the fibroblast primary cilium, including the Wnt5a-receptor, Fzd3, and Dvl proteins. Inversin-deficient MEFs have an elevated expression of canonical Wnt-associated genes and proteins, in addition to dysregulation of components in non-canonical Wnt signaling and cytoskeletal organization. Further, cell migration and polarization in are impaired in Invs MEFs.

In two-dimensional cell migration, the centrosome is positioned between the nucleus and the leading edge with the primary cilium directed towards the direction of migration. PDGFRα is activated in the primary cilium upon stimulation with PDGF-AA, a chemotactic agent. In paper IV, we use methods described in paper III to show that the primary cilium controls directional cell migration in wound healing in PDGF-AA-mediated chemotaxis. In vitro and in vivo wound closure is impaired by defective cilia formation, which leads to uncontrolled cell movements.

Together, the results obtained from my PhD studies reflect the high level of complexity within signaling systems regulated by the primary cilium that control cellular processes during embryonic development and in tissue homeostasis. As such, this dissertation can contribute to expanding the current understanding of the genetic mechanisms underlying ciliopathies.

Cover images (from Paper II):

Fibroblast primary cilium (AcTub, red), with Frizzled-3 localization (green) and nucleus (DAPI, blue)
Migrating fibroblasts with polarized organization of F-actin (phalloidin, red), Ezrin (green) and nucleus (DAPI, blue)
Phosphorylated β-catenin (blue) at the primary cilium (GluTub, red) in an Inversin-mutated fibroblast transfected with InversinGFP (green).
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Acknowledgements

Initially, I would like to thank my supervisors, Søren Tvorup Christensen and Professor Karsten Kristiansen, for their support throughout my PhD studies. In particular, I wish to thank Søren for introducing me to the world of primary cilia and scientific thinking, writing, and networking - and especially for his persistent confidence in me.

I am extremely grateful for my colleagues in the Cilia Group and at the AKB fifth floor, who provided great discussions, new lab tricks and moral support. I would also like to thank Lotte B. Pedersen, Professor Stine F. Pedersen and Professor Peter Satir (AECOM) for inspiring, scientific discussions over the years.

Louise Lindbæk and Jacob Schrøder (SDU) are acknowledged for promising project proposals and late night drinks.

I also wish to thank Anni B. Nielsen and Søren L. Johansen for excellent technical assistance.

Lastly, I would like to thank the Faculty of Science for full financial support throughout my PhD studies, and the ASCB, Lundbeck and Oticon foundations for supporting my conference participations.
Preface

The overall aim of my PhD studies was to provide new insight into cell biological processes controlled by the primary cilium. My main objectives were: 1) To study aspects of ciliary signal transduction and 2) Relate this to a physiological response, i.e. cell migration.

This thesis is based on the following papers:

Paper I: **TGFβ Signaling is Associated with Endocytosis at the Pocket Region of the Primary Cilium.** Clement, CA; Ajbro, KD; Koefoed, K; Vestergaard, ML; Veland, IR; Henriques de Jesus, MPR; Pedersen, LB; Benmerah, A; Andersen, CY; Larsen, LA; Christensen, ST. (2013). Cell Reports 3:1-9

Paper II: **Inversin/Nephrocystin-2 is Required for Fibroblast Polarity and Directional Cell Migration.** Veland, IR; Montjean, R; Eley, L; Pedersen, LB; Schwab, A; Goodship, J; Kristiansen, K; Pedersen, SF; Saunier, S; Christensen, ST. (2013) PLoS ONE 8(4): e60193

Paper III: **Analysis of Primary Cilia in Directional Cell Migration in Fibroblasts.** Christensen, ST; Veland, IR; Schwab, A; Cammer, M; Satir, P. (2013). Methods in Enzymology 525: 45-58

Paper IV: **Directional Cell Migration and Chemotaxis in Wound Healing Response to PDGF-AA are Coordinated by the Primary Cilium in Fibroblasts.** Schneider, L; Cammer, M; Lehman, J; Nielsen, SK, Guerra, CF; Veland, IR; Stock, C; Hoffmann, EK; Yoder, BK; Schwab, A; Satir, P; Christensen, ST (2010) Cellular Physiology and Biochemistry 25(2-3): 279-392.

Chapter 1 contains an introduction to cilia biology, including assembly and functions of primary cilia and control of ciliary entry; in particular the nephrocystins, and especially Inversin/Nephrocystin-2, towards which my PhD studies have mainly been directed. Chapter 2 is dedicated to selected signaling systems coordinated by primary cilia, i.e. PDGFα, TGFβ and Wnt signaling, and chapter 3 concerns mechanisms in cell migration with focus on PDGF-AA mediated chemotaxis and Inversin-regulated polarization.

Two of the papers have been subdivided between chapters to match the overall thesis objectives, Ciliary Signaling and Cell Migration. As such, paper I is discussed in section 1.3. (The ciliary pocket) and section 3.2 (TGFβ signaling), and paper II is discussed in chapter 2.3 (Wnt signaling and Inversin) and 3.3 (Inversin in polarization and cell migration).

The experimental work was carried out mainly at The Department of Biology, University of Copenhagen, over a three-year period from September 1st, 2009 to August 31st, 2013, with additional experiments performed at the Institute of Vegetative Physiology, University of Münster, Germany.

This work was funded by a full PhD stipend granted by the Faculty of Science, University of Copenhagen.

Iben Rønn Veland

Copenhagen, August 31st, 2013
1. Introduction to primary cilia

1.1 Cilia and flagella

Cilia/flagella are microtubule (MT)-based and membrane-enclosed projections found on the surface of most eukaryotic cells. These organelles arose early in evolution as motile structures, yet have diversified to serve a wide range of motile and sensory functions. In mammals, primary cilia coordinate a series of signaling networks that play vital roles in developmental processes and in the etiology of syndromic disorders and diseases, collectively termed ciliopathies (Badano et al., 2006; Fliegauf et al., 2007; Hildebrandt et al., 2011; Waters and Beales, 2011).

The axoneme is the most prominent structural component of the cilium and may vary in composition in different cells types. Flagella and most motile cilia have a characteristic MT arrangement of nine outer doublets surrounding a central pair (9+2), and motility is driven by outer and inner arm dyneins that are connected to the outer doublets to control ciliary beat frequency and form, respectively (Satir and Christensen, 2008). In contrast, most primary cilia, which project as solitary organelles in vertebrate cells during growth arrest, lack the central MT pair (9+0) as well as dynein arms and are thus immotile (Christensen et al., 2007; Satir and Christensen, 2008). Exceptions to this classification include immotile 9+2 cilia that organize actin-based stereociliary bundles within the acustovestibular system (Dabdoub and Kelley, 2005; Jones et al., 2008) as well as immotile 9+2 cilia of olfactory sensory neurons (Lodovichi and Belluscio, 2012; Menco et al., 1992). Further, a motile variant of the 9+0 cilium possessing outer arm dynein is found on the embryonic node during early mammalian development, where it generates a leftward flow of the perinodal fluid that regulates left/right patterning in early embryonic development. The exact mechanisms of breaking bilateral symmetry are still speculative, but may involve signal transduction via non-motile, primary cilia at the surrounding nodal cells (Eley et al., 2004; McGrath et al., 2003; Norris, 2012; Okada et al., 2005; Watanabe et al., 2003). Consequently, mutations in many structural cilia genes are associated with randomized asymmetry/heterotaxy or even situs inversus (Norris, 2012).

The ciliary membrane forms as an extension of the cell membrane but is separated from the general surface of the cell by the ciliary necklace, or Y-links, at the transition zone between basal body and cilium proper (Gilula and Satir, 1972). The ciliary membrane differs in its composition of lipids and membrane-associated proteins due to impaired lateral diffusion at the ciliary necklace (reviewed in Kaneshiro, 1990; Christensen et al., 2007; Rohatgi and Snell, 2010), which may play a critical role as a diffusion barrier that organize the entry of proteins into the cilium (Nachury et al., 2010) as will be outlined in the below.

The assembly and maintenance of cilia relies on an ATP-driven, bidirectional motility process known as intraflagellar transport (IFT) (Kozminski et al., 1993; reviewed by (Bhogaraju et al., 2013; Pedersen et al., 2012; Pedersen and Rosenbaum, 2008). Large, multimeric IFT complexes are transported in anterograde direction along the ciliary microtubules by motor proteins of the kinesin-2 superfamily, such as Kif3a, whereas retrograde transport is executed by cytoplasmic dynein-2 (DynC2) (Pazour et al., 1998; Pedersen et al., 2012). By these means, IFT is part of the trafficking system that shuttles ciliary precursors from their site of synthesis in the cell body to their site of assembly at the ciliary tip or positioning along the axoneme (Bhogaraju et al., 2013; Pedersen et al., 2012; Pigino et al., 2009; Rosenbaum and Witman, 2002). As a consequence, the phenotypes resulting from disrupted synthesis of IFT particles, such as Ift88/Polaris (encoded by Tg737 in mice) or Ift172, or associated motor proteins,
e.g. the Kinesin-II subunit, Kif3a or the Dynch2 heavy chain, Dynch2h1, includes perturbed or prevented ciliary formation, such as in mammalian cells and in the unicellular algae, *Chlamydomonas reinhardtii* (Pazour et al., 1999; Pazour et al., 2000) (Huangfu et al., 2003; Takeda et al., 1999). The transport of ciliary membrane proteins were recently shown to be only sporadically active, and not strictly IFT-driven, indicating that integral membrane proteins within the cillum alternate between modes of IFT and diffusion (Ye et al., 2013).

1.2 Primary cilia

Primary cilia are the first type of cilia formed during embryogenesis (Sorokin, 1968b) and constitute solitary organelles, at least temporarily, on the surface of most non-dividing tissue cell types of the human body (Wheatley, 1995; www.bowserlab.org/primarycilia/ciliumpage2.htm). Whereas 9+2 cilia on multiciliated cells originate from numerous basal bodies preformed in a fibrogranular matrix (Dirksen, 1991), the basal body of the primary cillum is constituted by the mother centriole of the centrosome during growth-arrest (Sorokin, 1962; Tucker et al., 1979a; Tucker et al., 1979b). In a model proposed by Sorokin (1968), the cillum of an epithelial cell is derived from the basal body that directly attaches to the plasma membrane, while in non-epithelial cells, such as fibroblasts, primary cilia are formed within growing vesicles docked to the basal body, and emerging at the plasma membrane during or at the end of ciliogenesis (Sorokin, 1962; Sorokin, 1968a; Sorokin, 1968b). By the latter mechanism, the cilia-surrounding vesicle can develop into the ciliary pocket (see below) from which only a distal part of the fully-formed cillum is directly exposed to the extracellular space (Benmerah, 2013; Ghossoub et al., 2011; Pedersen et al., 2012; Sorokin, 1962; Sorokin, 1968a). In the early steps of ciliogenesis, the docking of a basal body to either plasma membrane or cytoplasmic vesicle requires the presence of the distal appendages. These structures anchor the basal body to the membrane at the transition zone and constitute the transition fibers, or alar sheets, which are positioned below the ciliary necklace (Anderson, 1972; Ishikawa et al., 2005; Pedersen et al., 2012; Pedersen and Rosenbaum, 2008). Hereby, the transition fibers form a propeller-like structure that serves as docking site for IFT particles (Anderson, 1972; Deane et al., 2001) and Golgi-derived vesicles with ciliary cargo to be exocytosed at the ciliary base (Follit et al., 2006; Pazour and Bloodgood, 2008; Rosenbaum and Witman, 2002). The process of polarized exocytosis depends on the exocyst complex, GTPase activity of Rab8 and -11, in addition to the BBSome, a octameric coating complex encoded by eight of the 16 genes responsible for the ciliopathy Bardet-Biedl syndrome (Jin et al., 2010; Loktev et al., 2008; Nachury, 2008; Nachury et al., 2010) (Das and Guo, 2011; Hsiao et al., 2012). Whereas the BBSome forms a coating complex that sorts vesicles with membrane proteins to primary cilia (Jin et al., 2010), Rab8/Sec4, Sec10, and -11 of the exocyst complex are required for recognition of the cilia-targeted proteins. In turn, these steps are also essential for generation of the ciliary membrane (Jin et al., 2010; Nachury, 2008) (Kaplan et al., 2010; Mazelova et al., 2009) (Fogelgren et al., 2011).

1.3 The ciliary pocket

The ciliary pocket is an invagination of the plasma membrane and a highly active site for clathrin-dependent endocysis (CDE) (Ghossoub et al., 2011; Molla-Herman et al., 2010; Poole et al., 1985; Rattner et al., 2010). As such, the formation of clathrin-coated pits (CCP) and -vesicles (CCV) in the ciliary pocket occurs at a three-time elevated rate compared to the plasma membrane (Molla-Herman et al.,
The exact functions of the ciliary pocket are only beginning to be explored (Benmerah, 2013; Ghossoub et al., 2011). The CDE at the pocket has been hypothesized to be involved in recycling of ciliary receptors via Rab GTPases (Christensen et al., 2012). Indeed, we recently suggested that Transforming Growth Factor beta (TGFβ) signaling is coordinated at the ciliary pocket through CDE-dependent internalization of activated receptors and phosphorylation of Smad2/3 transcription factors at the ciliary base followed by trafficking of activated Smad2/3 to the nucleus for target gene expression (see below; Clement et al., 2013a).

Interestingly, Tg737 orpk mouse embryonic fibroblasts (MEFs), which harbor a hypomorphic mutation in the Ift88 gene and form no or short primary cilia (Corbit et al., 2008; Schneider et al., 2005) also show defects in CDE at the base of stunted cilia, as judged by reduced formation of vesicles positive for clathrin assembly lymphoid myeloid leukemia protein (CALM), in addition to reduced internalization of fluorescent-labelled transferrin around the ciliary base (Clement et al., 2013a). These results indicate that Ift88 in addition to ciliary assembly also plays a role in organizing the ciliary pocket and/or formation of clathrin coated vesicles. Indeed, Ift88-mediated flagellum elongation in Trypanosomes is a prerequisite for proper development of the flagellar pocket, which is the only site for endocytosis and exocytosis in these protozoa, and displays striking similarity to the pocket of primary cilia (Ghossoub et al., 2011; Overath and Engstler, 2004). Consequently, diminished and disorganized pockets in ift88-mutated trypanosomes are not fully functional in terms of endocytosis (Absalon et al., 2008).

Whereas CDE is not a requirement for ciliogenesis in mammalian cells (Molla-Herman et al., 2010), this process does depend on polarized exocytosis of the ciliary components via the exocyst complex and BBSome, as mentioned above (Fogelgren et al., 2011; Jin et al., 2010; Nachury, 2008; Nachury et al., 2010). Ift88 is dispensable for docking of the mother centriole to the ciliary vesicle during early ciliogenesis, yet is required for ciliary elongation following this step (Joo et al., 2013) as evidenced by the occurrence of stunted cilia in Tg737 orpk MEFs (Corbit et al., 2008; Schneider et al., 2005). However, the exocyst component Sec10 was shown to interact directly with Ift88 in mammalian cells (Fogelgren et al., 2011) and is essential for cilia formation (Zuo et al., 2009). During early ciliogenesis, vesicles with ciliary cargo that fuse to the ciliary vesicle become part of this structure, ultimately emerging as the ciliary pocket (Benmerah, 2013). It is thus possible that Ift88 may be involved in receiving incoming vesicles during ciliogenesis via Sec10, thereby concomitantly enabling both ciliogenesis and vesicle expansion, resulting in pocket development. Supporting this view, Golgi-derived vesicles was observed to accumulate near the base of the stunted flagellum of the Tg737 orpk mouse spermatids (Kierszenbaum et al., 2011). The underlying mechanisms for the development and regulation of the ciliary pocket during ciliogenesis and cell signaling are only beginning to be elucidated. Electron microscopy studies of the ciliary pocket structure in ift mutants will aid in these aspects.

1.4 Control of ciliary entry

The control of ciliary entry is currently a major debated subject with the main controversy being the positioning and character of a diffusion and/or steric volume exclusion barrier (Breslow and Nachury, 2011; Hsiao et al., 2012; Nachury et al., 2010; Najafi and Calvert, 2012; Vieira et al., 2006). A lipid diffusion barrier has been detected in the peri-ciliary membrane, but remains to be further characterized (Nachury et al., 2010; Vieira et al., 2006).

Another hypothesis proposes a selective barrier with structural and functional resemblance to the nuclear pore complex, possibly involving the ciliary necklace (Gilula and Satir, 1972; Hsiao et al., 2012;
Rosenbaum and Witman, 2002). Favoring this model, components of the inner nucleoporin complex were reported to facilitate size exclusion at the ciliary base (Kee et al., 2012). Further, Importinβ-1, which co-operates with the Ran GTPase to control import and export via nucleoporins, localizes to centrosomes and primary cilium of MDCK cells, where it is required for ciliogenesis (Fan et al., 2007; Pedersden et al., 2008) and positioning of ciliary proteins (Dishinger et al., 2010; Hurd et al., 2011). In addition, several ciliary membrane proteins were shown to contain short amino acid motifs functioning as ciliary localization signals that mediate ciliary entry through the interaction with adaptors, scaffolding proteins and small GTPases for vesicular sorting and targeting of the proteins to the cilia (Berbari et al., 2008; Christensen et al., 2008; Folliet et al., 2010; Geng et al., 2006; Humbert et al., 2012; Pazour and Bloodgood, 2008; Satir and Christensen, 2014; Tao et al., 2009). Some of these motifs share homology to nuclear localization signals (Dishinger et al., 2010), and others putatively facilitate anchoring to lipid rafts (Nachury et al., 2010). Yet, recent work in Chlamydomonas suggests that ciliary entry is rather a diffusion-driven process that proceeds independently of IFT but relying more on the directed transport along cytoplasmic microtubules to the transition zone (Belzile et al., 2013).

Finally, recent studies have identified a ciliary gate at the transition zone of Caenorhabditis elegans sensory neurons, zebrafish and several mammalian cell types, containing at least four functional modules comprised by nephrocystins (Nphp, see below) and other proteins implicated in the NPHP-related ciliopathies, Joubert syndrome (JBTS), Meckel-Gruber syndrome (MKS), and BBS. The exact molecular character of this gating system is uncertain, but seems not to be involved in trafficking or sorting of cilia-transported vesicles in C. elegans, yet still restricts ciliary access of membrane-bound proteins (Williams et al., 2011). In mammalian cells, however, centrosomal nephrocystins do appear to be involved in polarized exocytosis (Sang et al., 2011). The overlapping ciliopathic disorders resulting from mutated versions of the underlying genes, of which only a few are linked to ciliogenesis per se, reflect the importance of the gating complexes for ciliary function (Garcia-Gonzalo et al., 2011; Huang et al., 2011; Sang et al., 2011; Szymanska and Johnson, 2012; Williams et al., 2011).

It should be noted that the proposed mechanisms on ciliary targeting, exclusion and protein sorting (Breslow and Nachury, 2011; Nachury et al., 2010; Satir and Christensen, 2014) are not mutually exclusive, and could in fact be combined in a multi-step process, depending on and adapting to the nature of the ciliary cargo.

### 1.5 The ciliary gate and the nephrocystins

The nephrocystins form a group of highly diverse proteins required for nephronal development and kidney homeostasis, and with mutations in their encoding genes being responsible for the autosomal recessive kidney disease, nephronophthisis (Benzing and Schermer, 2012; Hildebrandt et al., 2011; Hildebrandt and Zhou, 2007; Salomon et al., 2008). The most prominent trait of the disease spectrum is the formation of kidney cysts at the cortico-medullary border, which often manifests with extra-renal cysts, retinal degradation, cardiac abnormalities, laterality defects, and associated with the NPHP-related ciliopathies Senior-Løken syndrome (SLS), JBTS, MKS, and, to some extent, BBS (Halbritter et al., 2013; Hoff et al., 2013; Hurd et al., 2013; Schaefer et al., 2011; Tory et al., 2009; Wolf and Hildebrandt, 2011; Zhang et al., 2013). While more than half of the known causes of NPHP-related ciliopathies remain to be identified (Halbritter et al., 2013), the 14 or more nephrocystins that are currently acknowledged all localize to the ciliary/centrosomal axis (Arts et al., 2007; Attanasio et al., 2007; Chaki et al., 2011; Chang et al., 2006; Dawe et al., 2007; Delous et al., 2007; Efimenko et al., 2006; Fliegauf et al., 2006;
Hoff et al., 2013; Mahjoub et al., 2004; Mollet et al., 2005; Otto et al., 2003; Otto et al., 2005; Otto et al., 2010; Shiba et al., 2010; Tran et al., 2008; Vierkotten et al., 2007). Accordingly, a new classification of nephrocystins, based on the mammalian intraciliary localization, was recently suggested (Shiba & Yokoyama, 2012). Nphp class I proteins (Nphp1, 4-8, 11-13) are primarily found at the centrosome and/or transition zone of the cilium, whereas class II nephrocystins (2, 3, 9, 14) localize to the proximal cilium in a 2 µm subsection right above the transition zone (Hoff et al., 2013; Shiba et al., 2009; Shiba et al., 2010; Shiba and Yokoyama, 2012). Here, Inversin ties up Nphp3, Nphp9/Nek8, and Nphp14/Anks6 (Hoff et al., 2013; Shiba et al., 2010) in a region recognized as the Inversin compartment, which is defined by the extent of Inversin/Nphp2 accumulation (Shiba et al., 2009). The function of the Inversin compartment is still unclear, but its establishment in C. elegans, i.e., at the ciliary middle segment (Warburton-Pitt et al., 2012), partly relies on the yet uncharacterized Ankyrin-repeat and MYND domain protein, Ankmy2/DAF-25 (Wojtyniak et al., 2013).

The ciliary gate complex consists largely of class I nephrocystins and Mks and Jbts family proteins (Garcia-Gonzalo et al., 2011; Huang et al., 2011; Sang et al., 2011; Williams et al., 2011). Despite discrepancies between reported interactions in the different experimental setups, the existence of an Nphp1-4-8 module at the transition zone is supported in several studies, with Nphp8 regulating transition zone size and acting as a molecular anchor for Nphp1, 4 and other transition zone/ciliary gate proteins (Roepman et al., 2005; Szymanska and Johnson, 2012) (Delous et al., 2007; Huang et al., 2011; Sang et al., 2011; Williams et al., 2011). At the centrosome, Sang and colleagues (2011) showed that the previously characterized Nphp5-6 module (Schafer et al., 2008) is bridged to the 1-4-8 module by the class II protein, Nphp2/Inversin, and may direct interactions between Nphp5 and components of the exocyst complex (Sang et al., 2011). Interestingly, Nphp6/Cep290 is required for integrity of the ciliary necklace and gate function in Chlamydomonas (Craigie et al., 2010), and appears to be implicated in docking of cilia-targeted vesicles via the BBSome and Rab8 in mammalian cells (Kim et al., 2008; Zhang et al., 2013). Accordingly, Nphp5 and -6 were found essential for ciliogenesis in polarized epithelial cells (Sang et al., 2011). Based on these findings, it seems likely that the functional modules of the Nphp/Mks/Jbt gate complex play separate, yet cooperating roles in controlling ciliary access.

The reported modules, however, most likely represent a simplification of the dynamic and versatile interactions between the nephrocystins (Wolf and Hildebrandt, 2011). As such, another gate protein, B9d2 (Williams et al., 2008; Williams et al., 2011) were shown to be required for ciliary positioning of Nphp2/Inversin in zebrafish (Zhao and Malicki, 2011). The B9d proteins and Nphp8 restrain other transition zone proteins, such as Nphp11/Tmem67, to their proper position (Williams et al., 2011), and, in turn, Inversin was shown to cooperate with Nphp1 and -4 in regulating transition zone positioning in C. elegans (Warburton-Pitt et al., 2012). In this regard, Inversin was recently reported to be required for anchoring of cyclic nucleotide-gated cation channel subunits into the middle segment, i.e., Inversin compartment, in a subunit- and cell type-dependent manner (Wojtyniak et al., 2013). In mouse fibroblasts, a GFP-tagged Inversin (Inv-GFP) was detected at the mother centriole prior to cilia formation and extending into the cilium during elongation (Watanabe et al., 2003). This supports a role for ciliary Inversin in the very early steps of ciliogenesis, and is in agreement with the proposed functions of the ciliary gate during transition zone formation in during early ciliogenesis (Williams et al., 2011). It is therefore tempting to speculate that the Inversin compartment is involved in modulating and restricting the transition zone to a confined region in the forming cilium in cooperation with gate complex proteins.

In this context, however, it is important to note that the nephrocystins are not confined to their intraciliary modules, and several nephrocystins do display additional cellular localizations, such as cell-
contacts, as has been reported for Nphp1-5, and -8 (Nurnberger et al., 2002; Sang et al., 2011; Wolf and Hildebrandt, 2011), of which at least Nphp1-4 and -8 have been found to interact directly (Mahuzier et al., 2012; Mollet et al., 2005; Otto et al., 2003). Furthermore, some phenotypes, such as Nphp9/Nek8 defects, indicate extra-ciliary functions related to cell adhesion (Natoli et al., 2008), which may, however, in part be controlled via the cilium, as dealt with further below (Schneider et al., 2010; Veland et al., 2013). A more detailed description of Nphp2/Inversin on these matters will be discussed in the below.

1.6 Inversin

Inversin or Nphp2 is an enigmatic protein that has gained massive attention in recent years, due to its apparent multifunctional roles, especially in relations to the nphp complexes in the ciliary gate. Nphp2/Inversin (*inversion of embryo turning, Invs*; abbreviated *inv* in paper IV) is encoded by 16 exons and was first discovered as implicated in the establishment of left-right asymmetry, since *Invs* mice that have a homozygous deletion of exons 3-11, present with *situs inversus* (Mochizuki et al., 1998; Morgan et al., 1998; Yokoyama et al., 1993). Alternative splicing of the *Invs* gene in mice, as estimated from Northern blotting, RT-PCR and sequences analysis, suggests the presence of up to seven putative Inversin isoforms with predicted molecular weights ranging from 51.4 to 117.1 kDa (Morgan et al., 1998; Ward et al., 2004). In mammalian cells and tissues, four Inversin splice variants have been detected, with molecular weights of 90, 125, 140 and 165 kDa (Morgan et al., 2002a; Nurnberger et al., 2002). The full-length (165 kDa) isoform was shown to interact with β-tubulin (Nurnberger et al., 2004), and ciliary localization of Inversin has been determined in a range of cells and tissues (Morgan et al., 2002b; Nurnberger et al., 2004; Otto et al., 2003; Veland et al., 2013; Warburton-Pitt et al., 2012; Watanabe et al., 2003; Wojtyniak et al., 2013; Zhao and Malicki, 2011) with accumulation in the Inversin compartment above the transition zone (Shiba et al., 2009). Throughout the cell cycle, however, Inversin displays a highly dynamic localization pattern and has been detected at the centrosome during interphase and in spindle poles during mitosis in MDCK cells (Morgan et al., 2002a; Nurnberger et al., 2004; Werner et al., 2013). A slight effect of Inversin depletion can be detected in *C. elegans* (C. Williams and B. Yoder, personal communication), but Inversin is dispensable for ciliogenesis in mammalian tissues and cell cultures (Morgan et al., 1998; Shiba et al., 2005; Veland et al., 2013; Watanabe et al., 2003).

Inversin was also reported to localize to lateral surfaces of contacting cells (Nurnberger et al., 2002; Nurnberger et al., 2004; Simons et al., 2005), presumably at adherens junctional complexes, since the 125 kDa isoform of Inversin directly interacts with N-cadherin in addition to the α-, β- and γ-Catenins (Nurnberger et al., 2002). Inversin was previously suggested to form a complex with Nphp-1 and -4 (Hildebrandt and Zhou, 2007; Salomon et al., 2008) that are also found at cell-cell contacts of polarized epithelial cells together with Nphp8 (Sang et al., 2011). Supporting this, direct interactions between tagged versions of full length (FL) Inversin and Nphp1, -4, and -8 have been detected in HEK293T cells, which do not form primary cilia (Mahuzier et al., 2012; Otto et al., 2003).

Nuclear localization of at least the 90 and 140 kDa Inversin isoforms has been detected in MDCK-II and murine proximal tubule cells (Morgan et al., 2002a; Nurnberger et al., 2002), as was predicted from the presence of nuclear localization signals in these isoforms (Morgan et al., 1998). Albeit the 90 kDa isoform immobilized β-Catenin in immunoprecipitation analysis (Nurnberger et al., 2002), the putative roles of Inversin in e.g. regulation of gene transcription has not been subjected to further analysis.
Recently, an internal region spanning aa 615-832 in INVS was proposed to share some homology with the transcriptionally regulating IPC4 domain, but its potential functions in transcriptional processes remains to be validated (Lienkamp et al., 2012).

The amino acid sequence of FL Inversin covers 1062 aa in mice and 1065 in humans (Mochizuki et al., 1998; Morgan et al., 1998), and encodes 16 ankyrin (ank) repeats, two nuclear localization signals (a putative and a bipartite), two destruction boxes (D-box motifs), two calmodulin-binding IQ domains (Morgan et al., 2002b) and a C-terminal Ninein-homology domain, required for localization to the Inversin compartment (Shiba et al., 2009). Intriguingly, the ankyrin repeats were found sufficient for ciliary localization at the embryonic node but not in kidney cilia of mice (Watanabe et al., 2003). Likewise, the ciliary localization of the Inversin ank repeats were not restricted to the middle segment in *C. elegans* (Wojtyniak et al., 2013), but in zebrafish, the 553 N-terminal amino acids, which include the ank repeats, were both necessary and sufficient to bind B9d2, which cooperates with IFT proteins to establish ciliary Inversin localization (Zhao and Malicki, 2011). These findings demonstrate that integrity of the Inversin compartment is context-dependent rather than universal.

Inversin was reported to accumulate in inner medullary collecting duct (IMCD) cells exposed to fluid flow (Simons et al., 2005), and the FL protein interacts with Calmodulin (Morgan et al., 2002b; Yasuhiko et al., 2001), which also localizes to the cilium (Otto et al., 2005). Therefore, a role for Inversin in kidney development and homeostasis via flow sensing and Ca\(^{2+}\) signaling was proposed by several authors (Germino, 2005; Simons and Walz, 2006; Singla and Reiter, 2006). Nevertheless, in the absence of serum, fluid flow stimulation revealed no differences in intracellular Ca\(^{2+}\) increase between renal epithelial cells derived from *wt* and *Invs* mice, suggesting that a potential role for Inversin in flow sensing and Ca\(^{2+}\) signaling would lie down-stream of a flow detecting mechanisms (Shiba et al., 2005). Ciliary mechanosensing maintains nephronal tissue homeostasis (Praetorius and Leipziger, 2013; Praetorius and Spring, 2005), but flow detection may not be an absolute requirement for tubule formation (Praetorius and Spring, 2003) as spontaneous tubulogenesis can be induced in 3D-cultured immortalized urine bud cells by stimulation with fibroblast-conditioned medium (Sakurai and Nigam, 1997) or in MDCK cells over-expressing polycystin-1 (PC-1) (Boletta et al., 2000), a Ca\(^{2+}\) channel-regulator (Vandorpe et al., 2001). Polarized cell division and coordinated cell migration in a process known as convergence extension, however, is crucial in tubule development (Carroll and Yu, 2012; Germino, 2005; Simons and Walz, 2006), and in tissue repair upon renal injury, which is a process where functional primary cilia are essential (Davenport et al., 2007; Fischer et al., 2006; Piontek et al., 2007; Veland et al., 2009). Inversin depletion was recently shown to be associated with misoriented mitotic spindles and asymmetric cell division in mice kidney tubules and cell culture (Sugiyama et al., 2011; Werner et al., 2013). Further, distorted cell migration has been reported in the developing pronephros of Inversin-deficient zebrafish (Lienkamp et al., 2010) and mouse fibroblasts (Veland et al., 2013), confirming a role for Inversin in these processes.

Interestingly, expression of the N-terminus of Inversin was sufficient to impair the laterality defects but not prevent the formation of kidney cysts in *Invs* mice (Watanabe et al., 2003). However, Δ*Cinv* mice, expressing only the ank repeat region, displayed elevated cell proliferation and presumably increased G1/S transition (Sugiyama and Yokoyama, 2006), which is in agreement with the cystogenic kidney phenotype (Nadasdy et al., 1995; Simons and Walz, 2006). Within the Inversin compartment, the ank repeat domain of Nphp14/Anks6 binds Inversin, Nphp3 and the kinase domain of Nphp9/Nek8, hereby linking Nphp9/Nek8 to Inversin and Nphp3 (Hoff et al., 2013). While Nphp3 affects morphogenetic cell movement during gastrulation, suggesting that nphp3 is essential to regulate convergent extension (Zhou et al., 2010). Inversin, nphp9/Nek8 and nphp14/Anks6 have all been demonstrated essential for
zebrafish pronephros morphogenesis (Fukui et al., 2012; Hoff et al., 2013; Lienkamp et al., 2010), with Inversin acting upstream of nphp9/Nek8 (Fukui et al., 2012). Integrity of the Nphp2-3-9-14 module is facilitated by the binding and action of the Asparaginyl hydroxylase HIF1AN (Hoff et al., 2013), an oxygen sensor hydroxylating ankyrin-containing proteins (Wilkins et al., 2012), thereby linking defective or misregulated hypoxia signaling to cyst formation in nephronophthisis (Hoff et al., 2013).

The finding that the C-terminal part of Inversin (aa 554 – 1062) binds and targets cytoplasmic Dvl for proteasome-mediated degradation in vertebrates and mammalian cells was an important discovery in understanding the functions of Inversin in cellular signaling (Simons et al., 2005; Simons and Walz, 2006). By these means, Inversin may act at a decisive point between the two branches of Wingless-type mmtv integration site (Wnt) signaling (see below) that regulate diverse cellular processes, such as cell proliferation and cell polarization (Clark et al., 2012; Clevers and Nusse, 2012). This could explain the excessive level of cell proliferation in Invs and ΔCinv mice (Sugiyama et al., 2011; Sugiyama and Yokoyama, 2006) and is in accordance with disease expression (Bellavia et al., 2010) and recent findings in mouse fibroblasts (Veland et al., 2013). However, excessive proliferation in Invs mouse kidney tubules was not accompanied by elevated Wnt activity at E16.5 (Sugiyama et al., 2011), suggesting that Inversin exerts its effects before this timepoint during development (Lienkamp et al., 2012). Moreover, Nphp9/nek8 mRNA was shown to rescue phenotypes of Inversin-depleted zebrafish during pronephros morphogenesis (Fukui et al., 2012), illustrating that the mechanisms are far from understood.
2. Primary cilia and signal transduction

Probably, all types of cilia register changes in the extracellular environment through specific receptors and ion channels to control cellular processes during development and in tissue homeostasis (Bloodgood, 2010; Christensen et al., 2007; Koefoed et al., 2014). Immotile, primary cilia function strictly as sensory organelles, and are associated with the coordination of a growing number of signaling pathways governed by G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), transforming growth factor beta receptors (TGFβ-Rs), cell adhesion receptors, as well as TRP ion channels, such as PC-2 (Christensen et al., 2012; Clement et al., 2013a; Oh and Katsanis, 2013; Praetorius and Leipziger, 2013; Robbins et al., 2012; Yoder et al., 2002). The ciliopathies that result from defects in ciliary assembly or function thus cover a wide spectrum of developmental defects, syndromic diseases and pathologies (Badano et al., 2006; Fliegauf et al., 2007; Hildebrandt et al., 2011; Veland et al., 2009; Waters and Beales, 2011).

In this thesis report, I will focus on the role of the primary cilium in coordinating PDGFRα, TGFβ and Wnt signaling, the latter from the perspective of Inversin.

2.1 Platelet-derived growth factor receptor (PDGFR) α

PDGFRα is a single membrane-spanning receptor tyrosine kinase (RTK) (Claesson-Welsh et al., 1989; Heldin and Westermark, 1999) that is upregulated on mRNA and protein levels during growth arrest (Lih et al., 1996; Schneider et al., 2005) and localizes specifically to the primary cilium in many cell types (Christensen et al., 2012), including fibroblasts (Schneider et al., 2005). Interestingly, upregulation of receptor expression is dramatically reduced in cells with defects in formation of primary cilia, such as in Tg737/pork MEFS, indicating that the cilium is associated with regulation of transcriptional processes that controls PDGFRα expression during the process of ciliogenesis (Schneider et al., 2005). Receptor activation by dimerization in the cilium is induced by binding of PDGF-AA, the specific homodimeric agonist for PDGFRα, eventually leading to autophosphorylation of specific tyrosine residues in the cytoplasmic region that function as docking sites for adaptor proteins and effector molecules. These convey signal transduction through a series of downstream signaling modules, including the mitogenic activating protein kinase (MAPK) pathways that regulate changes in protein activities and/or gene expression, leading to proliferation, cell survival and migration during embryogenesis and in tissue homeostasis (Andrae et al., 2008; Christensen et al., 2012; Heldin and Westermark, 1999). Further, interactions between activated PDGFRα and phosphophatidyl inositol 3-OH kinase (PI3K) induces the activation of various other down-stream components, including the Rho GTPase family member, Rac1 (Hawkins et al., 1995; Hooshmand-Rad et al., 1997) and protein kinase C (PKC) for reorganization of the actin cytoskeleton, in addition to the Ras GTPase and protein kinase B (Akt) in stimulation of cell proliferation, cell cycle re-entrance and inhibition of apoptosis (Heldin and Westermark, 1999; Hooshmand-Rad et al., 2000; Schneider et al., 2005; Yeh et al., 2013).

The initial steps of PDGFRα signaling take place within the cilium, followed by activation of Akt and the Mek1/2-Erk1/2-Rsk pathway along the cilium or at the ciliary base (Clement et al., 2013b; Schneider et al., 2005; Schneider et al., 2009; Schneider et al., 2010). As will be outlined in the below, the ciliary pocket plays a critical function in regulation of TGFβ signaling and activation of Smad2/3 transcription factors in fibroblasts and in stem cells differentiating into cardiomyocytes (Clement et al., 2013a).
Although PDGFRα signaling and other receptor-mediated pathways are regulated through receptor internalization (Heldin and Westermark, 1999; Kapeller et al., 1993) it is currently unknown whether these pathways in a similar way are linked to CDE at the ciliary pocket.

2.2 Transforming growth factor (TGF) β - Paper I

TGFβ and and bone morphogenic protein (BMP) signaling controls various cellular process such as apoptosis, proliferation, differentiation, epithelial-mesenchymal transition (EMT) and migratory responses, and are thus useful for several aspects of embryogenesis and tissue homeostasis (Guo and Wang, 2009; Kubiczkova et al., 2012; Moustakas and Heldin, 2005; Moustakas and Heldin, 2012).

The binding of a TGFβ ligand to a type II TGFβ receptor (TGFβ-RII) induces formation of a heterotetrameric receptor complex of TGFβ-RI and TGFβ-RII, which conveys TGFβ-RII-mediated autophosphorylation of TGFβ-RI. This leads to docking and phosphorylation of the receptor (R)-Smads, Smad2 and -3, which then associate with the effector module, Smad4 and together translocate to the nucleus for targeted gene expression (Huang and Chen, 2012). Ligand-binding to the receptors induces endocytic internalization of the ligand-receptor complex by two distinct pathways; either by clathrin-dependent endocytosis (CDE) or through caveolae formation in lipid rafts (Balogh et al., 2013; Yao et al., 2002). CDE-generated CCVs and early endosomes (EE) enhances TGFβ signaling through the Smad anchor for receptor activation (Sara), that in turn is anchored to phosphatidylinositol-3 phosphate [PI(3)P] in EEs via its' FYVE-domain. Sara facilitates Smad2/3 binding to activated TGFβ-RI, while concomitantly impairing receptor degradation (Tsukazaki et al., 1998) Gillooly (Gillooly et al., 2001; Itoh et al., 2002; Tang et al., 2010). The inhibitory regulator Smad7 is transcriptionally upregulated by Smad2/4 and Smad3/4 complexes in response to TGFβ signaling, thus providing a negative feedback mechanism (von et al., 2000). Smad7 interferes with R-Smad/receptor binding (Nakao et al., 1997) and escorts the E3 ligases, Smad ubiquitylation regulatory factors (Smurf1/2) from the nucleus to the TGFβ receptor complex (Ebisawa et al., 2001; Itoh et al., 1998; Kavsak et al., 2000). Allegedly, this can either lead to proteosomal degradation of the complex or internalitization by the caveola-mediated endocytic pathway leading to lysosomal degradation (Balogh et al., 2013; Di Guglielmo et al., 2003; Kavsak et al., 2000; Mukhopadhyay and Riezman, 2007). Activation of the TGFβ-R complex also leads to phosphorylation and activation of other downstream effectors, including the MAP kinases Mek1/2-Erk1/2, which are especially important in EMT and mesenchymal cell migration; yet, the receptor-effector interactions in non-Smad TGFβ signaling are less well understood. Smads and MAP kinases can modulates each others’ activities, thereby generating a regulatory meshwork to fine tune downstream TGFβ-activated signaling outcomes (Huang and Chen, 2012; Kubiczkova et al., 2012; Moustakas and Heldin, 2005; Zuo and Chen, 2009).

Compartmentalization of the components involved in TGFβ signaling and their altered localization during pathway-progression is hypothesized to be of vast importance for regulation of the various outcomes, but has so far been less characterized than the biochemical aspects of TGFβ signaling (Balogh et al., 2013). The ciliary pocket is a cellular compartment with high CDE-activity (Molla-Herman et al., 2010), and could likely provide such a confined environment. In paper I, we investigated the possible connection between the primary cilium and its pocket region in regulating CDE-dependent TGFβ signaling in cultures of fibroblasts and stem cells differentiating into cardiomyocytes.

In fibroblasts (human foreskin fibroblasts and wt MEFs), we detected TGFβ-Rs I and II along the cilium and at the ciliary tip, but most predominantly in a region surrounding the ciliary base, where both
receptors accumulated upon stimulation with TGFβ1 ligand. Equally, we detected Smad2/3 as well as Smad4 at the ciliary base, and TGFβ1 stimulation induced a phosphorylation of Smad2/3 at the ciliary base followed by nuclear localization of Smads 2, 3, and 4 (Clement et al., 2013a). Immunofluorescence microscopy (IFM) analysis showed colocalization between TGFβ-Ri and CCVs, and inhibition of CDE at the ciliary pocket with Dynasore, which does not interfere with ciliogenesis (Kirchhausen et al., 2008; Molla-Herman et al., 2010), inhibited both Smad phosphorylations at the ciliary base and nuclear translocation of Smads 2, 3, and 4. Interestingly, we also observed ciliary base localization of the inhibitory Smad7 upon TGFβ1 stimulation (Clement et al., 2013a). These results support the conclusion that the ciliary pocket function as a unique site for regulated TGFβ signaling. Visualization of CCVs and EEs by internalization of labeled transferrin (Molla-Herman et al., 2010) and localization of CALM, Clathrin and TGFβ-Ri upon TGFβ1 stimulation revealed distinct differences in the extent and endocytic activity of the ciliary pocket in Tg737<sup>opk</sup> MEFs in comparison to the wt cells. Without affecting receptor expression, TGF-β signaling and nuclear translocation of Smad transcription factors were diminished in growth-arrested Ift88 MEFs with stunted primary cilia (Clement et al., 2013a). We hypothesize that TGFβ ligands bind to TGFβ-Rs in the primary cilium, followed by retrograde transport of the ligand-receptor complex to the ciliary pocket for internalization and activation through CDE. This should be further investigated using fluorescence-coupled ligands and receptors, similarly to the approach by which intraciliary transport related to Hedgehog signaling was determined (Rohatgi et al., 2007).

TGFβ signaling controls many developmental processes, including heart development (Arthur and Bamforth, 2011), and embryos of Tg737<sup>opk</sup> mice show cardiac anomalies (Clement et al., 2009) characteristic of aberrant TGFβ signaling (Arthur and Bamforth, 2011). Moreover, embryonic cardiac endothelial cells from Tg737<sup>opk</sup> mice display altered phosphorylation of Smad2 (Egorova et al., 2011). By employing Solexa-based transcriptional analysis and qPCR validation, we found an upregulation in TGFβ-related gene expression throughout the process of p19.cl6 cell differentiation into cardiomyocytes (Clement et al., 2013a); a process that relies on proper cilia formation (Clement et al., 2009). This is in agreement with previous studies showing that TGFβ signaling is involved in cardiomyogenesis of human embryonic stem cells (hESC) (Kitamura et al., 2007), and we found that this was equally associated with activation of Smad2/3 at the ciliary base in p19.CL6 cells and hESC during cardiomyogenesis (Clement et al., 2013a). The rate of cardiomyogenesis increased with TGFβ1 stimulation in a CDE-associated process at the ciliary base, as evidenced by western blotting (WB) and IFM analyses. Moreover, this region recruited FYVE-domain fused GFP (Clement et al., 2013a), a marker of PI(3)P containing EE (Gillooly et al., 2001). Cilia on the cardiac mesenchymal cells emerge from a ciliary pocket (Willaredt et al., 2012), in accordance with the model that cilia of non-epithelial cells are generated via the intracellular pathway and hence derive a pocket from the ciliary vesicle (Benmerah, 2013; Sorokin, 1968a). Together, these data support the conclusion that the cardiac anomalies of Ift88 mice can, in part, be explained by defective TGFβ signaling at the ciliary pocket.

The Mek1/2-Erk1/2 was also activated at the ciliary base by TGFβ1 stimulation, equally to Smad2/3; yet, only Smad2/3 phosphorylation depended on CDE in the ciliary pocket, as envisaged by the largely unaltered response in Erk1/2 phosphorylation when CDE was inhibited with Dynasore (Clement et al., 2013a). In line with this, TGFβ-mediated activation of MAP kinases to initiate EMT, but not Smad signaling, has been shown to depend on the formation of lipid rafts (Zuo and Chen, 2009), indicating that this branch of TGFβ signaling relies on caveolae-mediated endocytosis rather than CDE (Balogh et al., 2013). The ciliary membrane contains both the cholesterol-binding protein prominin-1 and raft-associated gangliosides, of which one, GM3, is highly concentrated in the cilium compared to the cell membrane (Janich and Corbeil, 2007; Vieira et al., 2006). Thus, although TGFβ-induced ciliary Mek1/2-
Erk1/2 signaling does not require CDE at the ciliary pocket, it cannot be ruled out that some receptor internalization occurs via lipid rafts/caveolae in the cilium or periciliary region. This is in agreement with the hypothesis that primary cilia on epithelial cells, were EMT occurs, are formed via the extracellular pathway, due to which they seldom have a pocket (Ghossoub et al., 2011; Molla-Herman et al., 2010; Sorokin, 1962; Sorokin, 1968a).

### 2.3 Wnt signaling

The Wnt signaling network consists of a highly complex signaling arrangement that traditionally is divided into canonical and non-canonical Wnt signaling (Kestler and Kuhl, 2008). Canonical signaling is effectuated by β-catenin whereas non-canonical signaling is β-catenin independent and mainly proceeds through Dishevelled (Dvl) (Gao and Chen, 2010; Rao and Kuhl, 2010; Veeman et al., 2003a; Vlada et al., 2009). In mammals, each response is initiated by the binding of one of 19 different Wnt ligands to a Frizzled (Fzd) receptor and context-dependent co-receptors such as Lrp5/6, Ryk or Ror2; the combination of which appear to be more important for the downstream response than the ligand type (Gordon and Nusse, 2006; Heisenberg and Tada, 2002; Rao and Kuhl, 2010). In addition to multiple ligands, the spectrum of Fzd receptors comprises 12 isoforms in mice and 10 in humans. The complexity of Wnt signaling is further increased by modulation from secreted antagonists, including Soluble Frizzled-related protein (Sfrp) and Dickkopf-related proteins (Dkk) or agonists such as R-spondin (Rspo) that sequester Wnt ligands or bind directly to Fzd or co-receptors (Cruciat and Niehrs, 2013; Niehrs, 2012; van, 2012). Nevertheless, in the absence of any receptor binding, β-catenin is constitutively being targeted for proteasomal degradation (Aberle et al., 1997) by a multimeric destruction complex consisting of Casein kinase 1 (Ck1), Axin, Adenomatous Poliposis Coli (Apc) and Glycogen Synthase Kinase 3 beta (Gsk3β) (Kimelman and Xu, 2006). In canonical Wnt signaling, Fzd-sequestered Dvl rescues β-catenin by destabilizing the degradation complex, thus permitting Jouberin (Ahi/Jbn)-facilitated translocation of β-catenin (and Dvl) to the nucleus (Gan et al., 2008; Itoh et al., 2005; Lancaster et al., 2011). Here, being stabilized by a c-Jun/Dvl interaction (Gan et al., 2008), β-catenin interacts with transcription factors of the T-cell/Lymphoid enhancer-binding factors (Tcf) family to activate Wnt target genes (Brantjes et al., 2002; Clevers and Nusse, 2012; Gan et al., 2008) implicated in proliferation, cell cycle progression, cell fate determination and differentiation (Clevers and Nusse, 2012; Rao and Kuhl, 2010).

Per definition, non-canonical Wnt signaling operates independently of β-catenin (Veeman et al., 2003a) and is primarily mediated by Dvl and a vast number of Dvl interaction partners and downstream effectors (reviewed by (Gao and Chen, 2010; Rao and Kuhl, 2010; Veeman et al., 2003a) and see chapter 3). Although Dvl itself is believed to process transcriptional activity, this has so far been conferred to canonical responses (Gan et al., 2008; Gao and Chen, 2010; Itoh et al., 2005). According to the prevailing hypothesis, Inversin promotes non-canonical Wnt signaling downstream of Fzd by recruiting or stabilizing membrane-associated Dvl, and is involved in the targeting of cytoplasmic Dvl for proteasomal degradation via the Anaphase Promoting Complex/Cyclosome, APC/C (Ganner et al., 2009; Lienkamp et al., 2010; Lienkamp et al., 2012; Simons et al., 2005; Veland et al., 2013). This allows Dvl at the membrane to elicit cytoskeletal rearrangements and polarization, including planar cell polarity (PCP), underlying morphogenesis and cell migration (Clark et al., 2012; Gao and Chen, 2010; Wallingford and Mitchell, 2011).
A link between the primary cilium and Wnt signaling was first hypothesized when Inversin was shown to suppress canonical Wnt signaling by regulating levels of Dvl in a proteasome-dependent manner (Simons et al., 2005). The question of whether the primary cilium regulates Wnt signaling has been intensely debated in recent years, yet remains inconclusive (Lienkamp et al., 2012; Oh and Katsanis, 2013; Wallingford and Mitchell, 2011). A growing list of Wnt and PCP components have been localized to the ciliary/centrosomal region in tissues and cultured cells (Awan et al., 2010; Kim et al., 2010; Veland et al., 2009; Veland et al., 2013; Zilber et al., 2013), including Dvl proteins (Awan et al., 2010; Veland et al., 2013; Zilber et al., 2013) and the specific Wnt5a receptor and core PCP component Fzd3 (Kawasaki et al., 2007; Luyten et al., 2010; Veland et al., 2013; Wang et al., 2006). Most notably, β-catenin phosphorylated by Gsk3β for subsequent proteasomal targeting (Aberle et al., 1997; Yost et al., 1998) accumulates at the ciliary base (Corbit et al., 2008). Supporting the view that the primary cilium restrains Wnt/β-catenin signaling and favors non-canonical pathways, Wnt/β-catenin hyper-responsiveness was reported in cultured fibroblasts with disrupted ciliary formation (Kif3a, Ift88 and Odf1 mutations, (Corbit et al., 2008)) or basal body integrity (Bbs1, -4 and -6, (Gerdes et al., 2007)). Further, Ift88 mice displayed classical PCP defects in the inner ear, including defects in the positioning of basal bodies (Jones et al., 2008). Yet, mice embryos with Kif3a, Ift88, Ift172, or Dynch2h1 mutations did not display apparent Wnt or PCP phenotypes at embryonic day 9.5, and fibroblasts derived from these embryos were reported to respond normally to Wnt ligands (Ocbina et al., 2009). Similarly, normal Wnt signaling was reported in ift88-deficient zebrafish (Huang and Schier, 2009); however, increased cytosolic localization of β-catenin as well as elevated Tcf/Lef expression was found in pancreatic cysts of the Tg73r^ocrk mouse (Cano et al., 2004). Further complicating the picture, several ciliary non-IFT proteins such as Jbn/Ahi1, Nphp3, -4, -8 and Fuzzy can affect both Wnt and/or PCP, and ciliogenesis, sometimes in a dosis-dependent manner (Bergmann et al., 2008; Burckle et al., 2011; Hsiao et al., 2009; Mahuzier et al., 2012; Vierkotten et al., 2007; Zilber et al., 2013), suggesting that cilia formation in some cases are downstream from Wnt and PCP signaling (Pedersen et al., 2008; Wallingford and Mitchell, 2011). A comprehensive list of cilia mutations and Wnt responses are given in (Oh and Katsanis, 2013). As outlined above, Inversin is a ciliary protein that affects both canonical Wnt and PCP (Lienkamp et al., 2010; Simons et al., 2005; Veland et al., 2013), yet has been demonstrated dispensable for cilia formation in mammalian tissues and cell culture (Morgan et al., 1998; Shiba et al., 2005; Veland et al., 2013; Watanabe et al., 2003).

### 2.4 Inversin in Wnt signaling - Paper II

In order to investigate the downstream effects of Inversin in terms of Wnt-related gene expression, we initially isolated and cultured MEFs from wt and Invs animals harboring a deletion of exon 4-12 of the Invs gene (Eley et al., 2004; Morgan et al., 1998). Both cultures were fully capable of entering growth arrest upon serum depletion and of generating primary cilia (Veland et al., 2013). We used these ciliated MEFs in Solexa-based transcription analysis, in addition to WB and IFM analysis, to further examine the expression and phosphorylation of central Wnt components, and their localization in relations to the primary cilium. The aspects of Inversin in relations to non-canonical signaling, polarization and cell migration will be dealt with further below (chapter 3.3).

In paper II, we confirmed the importance of Inversin in maintaining cellular Wnt balance, as Inversin’s absence led to a response similar to Wnt hyper-responsiveness in MEFs. The transcriptome analysis revealed major differences in mRNA expression of Wnt modulators, some of which are identified Wnt target genes (Christensen et al., 2013). Several Wnt/β-catenin co-activators were upregulated, including...
the gene encoding Pygopus, which facilitates nuclear accumulation of β-catenin (Kramps et al., 2002; Townsley et al., 2004) and Tcf4, which was shown to interact with Dvl and c-Jun in β-catenin-mediated gene expression (Gan et al., 2008). In addition to the deregulated expression of the Wnt antagonists Sfrp1 and -2, we also observed downregulation of the Wnt agonist R-spo and an significant upregulation of Sox9 (log2(Invs/wt) = 2.69, unpublished data), which has been demonstrated to function as a “destructor” of the Tcf/β-catenin complex (Akiyama et al., 2004). Dkk3, which was also upregulated, has been shown to impair canonical signaling by facilitating nuclear export of β-catenin simultaneously with activating non-canonical JNK signaling (Xiang et al., 2013). Together, these data strongly signify that Inversin is important to prevent excessive canonical Wnt signaling, and also point towards an activation of negative feedback mechanisms to counteract these effects in the Invs MEFs.

Curiously, both Apc mRNA and protein levels were down-regulated in Invs MEFs, as evidenced by WB. Similarly, we found decreased levels of Gsk3β protein in Invs MEFs, albeit no detectable difference in mRNA expression (Veland et al., 2013 and unpublished data). Apart from exerting a scaffold in the destruction complex that enables Gsk3β-mediated phosphorylation of β-catenin (Kimelman and Xu, 2006), Apc equally functions as a transcriptional repressor of Wnt target genes (Sierra et al., 2006). Thus, the decreased Apc and Gsk3β levels in the absence of Inversin could drive the cells even further towards excessive β-catenin signaling. Apc and Gsk3β have been detected in cilia/flagella; however, since Apc and Gsk3β are both essential regulators of MT dynamics, at least in cell migration (Etienne-Manneville and Hall, 2003; Schlessinger et al., 2009), it is possible that their roles in the primary cilium are related to maintaining axonemal integrity (Pedersen et al., 2008). As described in further detail below (chapter 3.2), several genes implicated in non-canonical Wnt signaling were also deregulated in the Invs MEFs (Veland et al., 2013). Together, these data support the findings of Simons et al. (2005) and show that Inversin is essential for regulating Wnt signaling and β-catenin activity.

Other nephrocystins than Inversin were reported to modulate Wnt signals by regulating the turnover of Dvl, including Nphp3, -4, and -8 (Bergmann et al., 2008; Burckle et al., 2011; Mahuzier et al., 2012) or β-catenin (Borgal et al., 2012). Nphp4 was recently shown to prevent canonical Wnt responses by interacting with Jade1 at the transition zone, and facilitate nuclear translocation of Jade1, which subsequently targets nuclear β-catenin for destruction (Borgal et al., 2012). Interestingly, Inversin can physically interact with Ahi/Jbn (Sang et al., 2011), which was recently shown to control the nuclear translocation of β-catenin (Lancaster et al., 2011). It is thus possible that Inversin, the Inversin compartment and the ciliary gate are implicated in restraining Ahi/Jbn in the cilium until a suitable signal for its release has been given, or is involved in regulation of the nuclear Dvl-β-catenin complex, in parallel with the mechanisms discovered for Nphp4 (Borgal et al., 2012).

While WB showed altered protein levels of all the Dvl isoforms in wt and Invs MEFs (Veland et al., 2013), our transcriptome analysis yielded no detectable differences in Dvl1-3 gene expression (unpublished results). This supports that the changes in Dvl proteins are regulated at the level of turn-over, which is controlled by Inversin (Fukui et al., 2012; Ganner et al., 2009; Simons et al., 2005). In this regard, we found an upregulation of the gene encoding Prickle, a core-PCP component that restricts β-catenin signaling in liver cancer cells by facilitating Dvl-3 degradation (Chan et al., 2006) and interacts with Inversin in vitro (Simons et al., 2005). However, both gain- and loss-of-function mutations impaired gastrulation movements in zebrafish (Veeman et al., 2003b) with the underlying mechanisms not being entirely understood. Interestingly, the functional differences between Dvl isoforms (Gao and Chen, 2010; Wynshaw-Boris, 2012) were also apparent in IFM analysis; we detected ciliary localization of endogenous Dvl1 and -3, but not Dvl2, in wt MEFs, and Dvl3 was absent from Invs MEF cilia (Veland et al., 2013). Recently, however, Cherry-tagged Dvl2 was reported to localize to cilia of wt MEFs, whereas
endogenous Dvl1 co-localized with γ-tubulin at the ciliary base (Zilber et al., 2013). When analyzing such contradictory results, the genetic background of the applied model system must be taken into account, as well as variations in experimental setup, including culturing methods and fixation techniques, and further analyses are clearly required to unravel the functional connection between cilia and Dvl. During fibroblast ciliogenesis, Dvl is recruited to the Rab8-positive vesicles by Fuzzy (Zilber et al., 2013) and regulates basal body positioning via mechanisms involving Inturned, Nphp8 and Sec2 in multiciliated tissue (Mahuzier et al., 2012; Park et al., 2008). However, the localization of both Dvl and several of its regulators in the transition zone and Inversin compartment indicates that ciliary Dvl localization and regulation is important for aspects of cilia functions past assembly. In this regard, we showed that the previously reported accumulation of phosphorylated β-catenin at the ciliary base (Corbit et al., 2008) was absent in Invs MEFs, but could be restored to wt-like levels with expression of FL InversinGFP, which localized to the primary cilium i.e. that re-expression of ciliary Inversin rescued the Wnt defects in Invs MEFs (Veland et al., 2013). It will be interesting to see how this is related to Dvl functions in the cilium.

Finally, it has been suggested that Inversin may itself possess transcriptional activity via a putative ICP4 homology domain (Lienkamp et al., 2012), but this remains to be investigated. Yet, the presence of nuclear localization signals (Morgan et al., 1998) and nuclear localization of the 90 and 140 kDa splice variants (Nurnberger et al., 2002) does point towards an additional role for Inversin in the nucleus, perhaps in transcriptional regulation besides from the indirect effect on β-catenin levels via Dvl (Simons et al., 2005; Veland et al., 2013). Indeed, the 90 and 125 kDa Inversin variants were reported to bind β-catenin directly (Nurnberger et al., 2002), but the β-catenin-Inversin interaction has, to our knowledge, not been characterized.
3. Primary cilia and cell migration

3.1 Migration

Directional cell migration is a fundamental process underlying many developmental and morphogenic processes, such as gastrulation, neurulation and organogenesis, and is crucial to wound healing and immune responses (Cordeiro and Jacinto, 2013; Solnica-Krezel and Sepich, 2012; Takahashi et al., 2013). Moreover, de-regulated cell migration is the leading cause of cancer mortality due to metastasis, where malignant cells invade the surrounding tissue (Wells et al., 2013; Yamaguchi and Condeelis, 2007). Cell migration is thus a highly coordinated process and involves a tight regulation yet continuous plasticity of the actin cytoskeleton (Insall and Machesky, 2009).

Cell motility is generated by cyclic processes of membrane protrusion and substrate adhesion in the direction of migration, i.e., at the leading edge of the cell, in concert with detachment and membrane retraction at the rear. Projections rich in filamentous actin (F-actin), such as filopodia and lamellipodia, attach to the substrate through focal adhesions (FAs). The tension resulting from the dynamic formation and turn-over of FA complexes is the main contributor to the forces driving cellular relocation (Beningo et al., 2001; Insall and Machesky, 2009; Stock et al., 2008), and the formation of filopodia and lamellipodia require polymerization and branching of F-actin. Nucleation of actin filaments is initiated by the Actin-related protein 2 and 3 complex (Arp2/3) (Welch et al., 1997), which is activated by the Wiscott-Aldrich Syndrome protein family member, WAVE (Machesky et al., 1999; Machesky and Insall, 1998). In turn, the WAVE regulatory complex entails cooperation between Rac1 and Arf1 of the Rho and Arf families of small GTPases, respectively (Koronakis et al., 2011). The Rho GTPases, RhoA, Rac1 and Cdc42 and a number of their associated guanine nucleotide exchange factors (GEFs) and activator proteins (GAPs) are essential regulators of many aspects of cell motility, and regulate both MT and actin cytoskeletal dynamics (Jaffe and Hall, 2005). For instance, Cdc42 is required for polarized generation of membrane protrusions through Rac1 and the Rac-GEF, βpix, which Cdc42 recruits to the leading edge (Cau and Hall, 2005).

The modulation of the actin cytoskeleton is also controlled by local, intracellular Ca^{2+} levels and pH, resulting in actin polymerization at the leading edge and de-polymerization at the stern. Cell migration thus critically depends on multiple ion transport proteins (Stock et al., 2013; Yamaguchi and Condeelis, 2007). One of these is the Na^+/H^+ Exchanger isoform NHE1 (Boedtkjer et al., 2012), which is transcriptionally up-regulated during quiescence in fibroblasts, and with its activity being dependent on ciliary PDGFβ receptor signaling (Clement et al., 2013b; Schneider et al., 2009). NHE1 is implicated in several aspects of cell motility, including the regulation of cell volume and pH in addition to the stabilization and anchorage of the actin cytoskeleton to the cell membrane. The latter occurs via the indirect interactions between NHE1 and the cytoskeletal linker proteins, Ezrin, Radixin and Moesin (ERM) (Arpin et al., 2011; Denker and Barber, 2002; Ivetic and Ridley, 2004; Stock et al., 2008). ERM proteins are structurally highly related and essential for lamellipodia formation, with their activation requiring both a yet unresolved interaction with phosphatidyl-inositol-4,5-biphosphate (PIP2) in the plasma membrane (Arpin et al., 2011) and subsequent phosphorylation of the actin-binding C-terminus, for instance by the Rho-kinase (Fievet et al., 2004; Matsui et al., 1998). Upon activation, ERM proteins can interact with a vast array of membrane proteins essential for cell motility, including NHE1, which associates with ERM proteins via its activator and adaptor molecule, NHE regulatory factor 1 (Nherf1) (Arpin et al., 2011; Cardone et al., 2007).
The establishment of an anterior-posterior axis i.e., a planar polarization of the cell is a prerequisite for coordination of the above mentioned mechanisms in directional cell migration. The polarization occurs in response to positional cues, such as chemo attractants and the disruption of contacts to neighbor cells, hereby guiding and adjusting the cytoskeletal reorganization throughout the migratory process (Etienne-Manneville, 2012; Gomes et al., 2005; Kupfer et al., 1982). One of the initial events in two-dimensional directed cell migration, such as a scratch assay, is a rearwards movement of the nucleus concomitantly with the re-positioning of the centrosome between the nucleus and the leading edge (Gomes et al., 2005). This process depends on the MT cytoskeleton and requires a Dvl/Cdc42/Par6/aPKC interaction (Cau and Hall, 2005; Etienne-Manneville and Hall, 2001; Nobes and Hall, 1999; Schlessinger et al., 2007).

In ciliated fibroblasts and vasculature smooth muscle cells (VSMCs), the primary cilium concomitantly reorients in parallel to the substratum, “pointing” in the direction of migration (Albrecht-Buehler, 1977; Lu et al., 2008; Schneider et al., 2010), indicating a role for the primary cilium in guiding and adjusting directional cell migration.

### 3.2 The primary cilium in directional cell migration and chemotaxis - Papers III&IV

In the presence of PDGF-AA, PDGFRα signaling promotes fibroblast chemotaxis and migration in cells cultured in the absence of serum (Hosang et al., 1989; Osornio-Vargas et al., 1996; Schneider et al., 2005) that induces growth arrest. Since growth arrest is associated with both formation of the primary cilium as well as transcriptional upregulation and ciliary targeting of PDGFRα (Clark et al., 1995; Lih et al., 1996; Schneider et al., 2005; Schneider et al., 2009), we investigated the role of the fibroblast primary cilium and associated PDGFRα signaling in directionally migrating mouse fibroblasts. The experimental procedures included live cell imaging and semi-automated single-cell tracking of *in vitro* wound healing (scratch) and micropipette assays in combination with IFM analysis, as described in paper III (Christensen et al., 2013). We also assessed *in vivo* wound healing by punch biopsies in wt and ORPK/Tg737Rpw mice that have impaired cilia formation, but are viable after birth, unlike Tg737oprk mice.

In scratch assays, primary cilia in wild type MEFs oriented in parallel to one another and perpendicularly to the wound. The tracking of individual cells showed that incubation with PDGF-AA increased the migration speed and the directional movement of the cells. In contrast, in Tg737oprk cells, which form stunted primary cilia, the migration speed was unaffected by PDGF-AA incubation, but the cellular directionality was decreased (Schneider et al., 2010). Using micropipettes to generate a PDGF-AA gradient (Christensen et al., 2013), ciliated wt MEFs responded immediately to PDGF-AA injection, and migrated uniformly towards the pipette, whereas Tg737oprk MEFs did not respond to PDGF-AA and moved around randomly (Schneider et al., 2010). We confirmed the necessity of the primary cilium for PDGFRα activation by immunoprecipitation of the endogenous receptor from PDGF-AA-stimulated wt and Tg737oprk MEFs, and subsequent WB with phospho-tyrosine specific antibodies and equal receptor amounts (Schneider et al., 2010). Together, these data show that the primary cilium coordinates PDGF-AA mediated chemotaxis in fibroblasts. Another study from our lab showed that PDGFRα-mediated migration in scratch assays depend on the activity of NHE1 (Schneider et al., 2009). NHE1 is upregulated during quiescence in fibroblasts, but unlike PDGFRα, this upregulation is independent of cilia formation (Schneider et al., 2009; Veland et al., 2013). Inhibition of NHE1 reduced the speed and directionality of PDGF-AA-mediated cell migration in wt MEFs, and this reduction was markedly decreased in Tg737oprk MEFs (Schneider et al., 2009). A more recent study showed that NHE1
translocates to the leading edge, possibly in vesicles along stable MTs, and is activated in response to PDGF-AA stimulation in an Akt and Mek1/2-dependent manner. As such, it was suggested that activation of Akt directs the polarized transport to the leading edge, whereas activation of the Mek1/2-Erk1/2-Rsk pathway modulates the position and incorporation of NHE1 in the lamellipodium (Clement et al., 2013b). By these means, PDGFRalpha signaling via the primary cilium probably continually adjusts the cell’s directionality in the chemotactic migratory response. These results support the conclusion that the primary cilium is part of the positioning machinery that coordinates directed migration in wound healing and developmental processes.

The reorientation of the ciliary/centromal axis can occur independently of ciliary signaling, as can lamellipodia formation in the presence of positional cues that do not involve ciliary signaling. Therefore, the mechanisms by which the primary cilium controls persistent directionality in cell migration are currently unknown, but have been hypothesized to involve trafficking along MTs extending from the centrosome, in which the ciliary basal body is anchored (Christensen et al., 2012). To increase our understanding of these processes, it would be helpful to apply live cell imaging of centrosome reorientation in Tg737opk MEFs together with monitoring of NHE1 translocation in response to PDGF-AA. Another option includes examination of migratory behavior concomitantly with manipulation of the primary cilium with optical tweezers. This, however, could simultaneously induce ciliary mechanosensation, and is therefore very complicated to investigate.

3.3 Inversin in polarization and cell migration - Paper II

The non-canonical Wnt responses are intimately linked and mainly coordinated by Dvl proteins with a range of interaction partners to regulate cell adhesion, polarization and migration responses (Clark et al., 2012; Gao and Chen, 2010; Veeman et al., 2003a), such as in PCP and wound repair (Caddy et al., 2010; Schlessinger et al., 2007; Wynshaw-Boris, 2012). Dvl can activate Ca\textsuperscript{2+} signaling via phospholipase C (Clark et al., 2012; Kuhl et al., 2001; Sheldahl et al., 2003), whereas in Ca\textsuperscript{2+}-independent non-canonical Wnt signaling, Dvl elicits remodeling of the MT and actin cytoskeletons via c-Jun N-terminal kinase JNK, the formin Daam1, and activation of RhoA, Rac1 and Cdc42 (Aspenstrom et al., 2006; Gao and Chen, 2010; Habas et al., 2001; Habas et al., 2003; Rosso et al., 2005; Schlessinger et al., 2009). It appears well-established that Inversin plays a role in establishment of PCP during convergence extension and patterning in vertebrates and mammals (Cirone et al., 2008; Lienkamp et al., 2010; Simons et al., 2005; Zhao and Malicki, 2011), but the cellular mechanism by which this is effectuated are currently unknown.

To investigate the role of Inversin in polarization and cell migration, we initially subjected ciliated wt and Invs MEFs to scratch assays and time lapse microscopy analysis, as described in Christensen et al. (2013). Single cell trajectories in scratch assays revealed that Invs MEFs have severely impaired motility and an even more inferior directionality than wt MEFs (Veland et al., 2013). This is in sharp contrast to the migratory behavior of Tg737opk MEFs, which displayed a higher rate of wound closure compared to wt MEFs. We conferred this to be an effect of excessive random migration due to the lost directionality in the absence of cilia (Schneider et al., 2010). The experimental setup in scratch assays does not allow for observation of the “running on spot” behavior (Christensen et al., 2013), but the applied trajectory analysis indicate a decreased directional persistence in Invs MEFs (Veland et al., 2013). Preliminary data in the Christensen lab show that Invs MEFs fail to transcriptionally upregulate and position PDGFR\alpha during growth arrest, but whether this is a secondary effect of the deregulated Wnt signaling in these cells is currently not clear, and will require further investigations of the PDGFR\alpha promoter. The
delineation of Inversin’s role in PDGFRα signaling could include PDGF-AA stimulation in scratch assays in addition to localization of endogenous and tagged PDGFRα in Invs MEFs. Moreover, the localization patterns of Inversin to stunted cilia of Tg737pdh MEFs have, to our knowledge, not been investigated.

Scratch assays combined with IFM analysis (Christensen et al., 2013) showed that Invs MEFs were severely compromised in their ability to properly position FAs and form lamellipodia (Veland et al., 2013). A similar redistribution of focal adhesion in Inversin-depleted HeLa cells accompanied by reduced migration in non-ciliated Invs dermal fibroblast where recently reported in a corresponding study (Werner et al., 2013). These authors also showed that filopodia persisted during mitosis in dividing Inversin-depleted HeLa cells (Werner et al., 2013), but we did not observe any effects on filopodia formation. Nevertheless, Invs MEFs displayed disordered F-actin bundles and aberrant expression, localization and activation of several motility-related proteins usually found at the leading edge, including the GTP-binding proteins RhoA, Rac1, and Cdc42, the ERM proteins, and NHE1. The polarized activation of ERM proteins were restored by expression of FL invsGFP, which also localized to the leading edge in addition to the primary cilium (Veland et al., 2013). In scratch assays, primary cilia of Invs MEFs were randomly orientated and not positioned in front of the nucleus and directed perpendicularly to the wound edge, as in wt cells. This could be a direct effect of the reduced protein levels of Dvl2 and -3 in Invs MEFs (Veland et al., 2013), since their simultaneous depletion with siRNA was previously shown to substantially decrease the rate of centrosome reorientation in fibroblasts (Schlessinger et al., 2007). Moreover, Dvl3 was absent from the base of Inv cilia, which could be explained by the decreased Dvl3 expression (Veland et al., 2013); however, restoration of Dvl3 levels with overexpression of GFP-tagged Dvl3 did not rescue any of the listed defects, nor did it restore localization of Dvl3 to the ciliary base (data not shown). Dvl can mediate rearrangements of the actin cytoskeleton via Daam1 and activation of the Rho GEF, Wgef (Habas et al., 2001), and the migration-deficient phenotype of Daam1 depleted COS cells includes defective polarization and centrosome re-orientation (Ang et al., 2010), similarly as Invs MEFs. The reported abnormal GTP binding of RhoGTPases in Invs MEFs may thus be a consequence of deregulated Dvl proteins (Veland et al., 2013). However, as the activity of RhoA, Rac1 and Cdc42 in cell migration relies both on the polarized localization of the GTP bound proteins, but also the availability of associated GEFs and GAPs (Etienne-Manneville and Hall, 2002; Nakamura, 2013). Therefore, another possibility is that RhoA, Rac1 and Cdc42 reside in the cytosplasm in their GTP-bound state, but cannot exert their effects in cytoskeletal modulation due to the absence of appropriate GAPs in their vicinity.

At present, we cannot determine whether the effects on Wnt signaling are related to Inversin in the cilium, at cell edges, or both, since the full-length InvGFP construct that rescued these defects, localized to both sites as well as in the cytoplasm and nucleus when overexpressed. However, at low expression levels, we usually observed InvGFP in the cilium. Interestingly, we and others have localized Fzd3, the specific receptor of Wnt5a (Kawasaki et al., 2007) to primary cilia, and this localization does not depend on Inversin (Luyten et al., 2010; Veland et al., 2013). Some aspects of non-canonical Wnt signaling have been shown to involve internalization of Fzd receptors via CDE, including Fzd3 in promotion of PCP (Yu et al., 2007). Since we recently discovered that ciliary TGFβ is associated with CDE at the ciliary pocket (Clement et al., 2013a), it is tempting to speculate that the pocket region could also be involved in internalization of Fzd3 in promotion of cell polarization. This could be investigated with a similar approach as applied in investigation of the TGFβ signaling (Clement et al., 2013a), and would include stimulation of fibroblasts with Wnt5a in combination with monitoring of receptor internalization and CDE.
Overexpression of Inversin has been shown to rescue endothelial cell migration defects induced with the chemical PCP suppressor, TNP-470 (methionin aminopeptidase 2 inhibitor) (Cirone et al., 2008), emphasizing that Inversin’s regulatory effect in cell migration is related to Wnt signaling. However, transcriptome analysis of ciliated wt and Invs MEFs showed downregulation of several genes involved in polarization and cell migration, including Dishevelled-associated activator of morphogenesis 2 (Daam2), Arpc1b of the Arp2/3 complex, its activator Wasf1/Wave1, and the tetraspanin-like Tm4sf1 (Veland et al., 2013), which controls endothelial cell migration (Zukauskas et al., 2011). Whether these genes are transcriptionally repressed by canonical Wnt signaling has, to our knowledge, not been determined, and may thus indicate other means by which Inversin can control migration processes.

The migratory deficiency of Invs MEFs was recently confirmed in the presence of serum (Werner et al., 2013), indicating that the effect on cell motility is unrelated to cilia formation. However, as high-confluent fibroblasts do form cilia even in the presence of serum, albeit in a less synchronized manner (Wheatley et al., 1994), a role for Inversin in regulating cell migration via the primary cilium cannot be excluded based on this experimental setup. The expression and localization of Inversin in cilia-defective cells, such as Tg737<sup>cre</sup> MEFS, will aid in this aspect.

As previously mentioned, the 90 and 125 kDa splice variants interact with catenins and N-cadherin (Nurnberger et al., 2002), all of which are essential components of fibroblasts adherens junctions (El Sayegh et al., 2007). Apart from mediating cell-cell contacts, adherens junctions enable actin polymerization to elicit membrane protrusions and regulate the activity of Rho GTPase (Etienne-Manneville, 2012). It may therefore be that Inversin participates in the detection mechanism that senses disruption of cell contacts, and is perhaps separated from the functions of FL Inversin in the cilium. Another possibility is that Inversin promotes cell polarization in connection with the sensing of local Ca<sup>2+</sup> changes, due to the reported interaction between Inversin and Calmodulin (Morgan et al., 2002a) and Inversin’s proposed role in Ca2+ responses in regulating Wnt signaling (Simons et al., 2005). This could be investigated by inducing and monitoring local changes Ca2+ and the migratory response (Ozkucur et al., 2011) in single Invs MEFs, in combination with expression of Invs constructs with mutated IQ-domain.

In conclusion, the apparent requirement of Inversin in morphogenic processes and tissue homeostasis can be explained by the vast array of polarization and motility defects in Invs MEFs, although the exact mechanisms remain to be determined. To detangle the various subcellular functions of Inversin, it will be necessary to express and monitor tagged versions of the different Inversin splice variants in systems deficient in Inversin and cilia formation, such as the Invs and Ift88 MEFs. Also, a more detailed outline of Inversin’s interaction partners could be obtained via stable isotope labeling in cell culture (SILAC) and mass spectrometry under conditions promoting and antagonizing cell migration.
4. Concluding remarks

The primary cilium was previously described as a cybernetic probe (Poole et al., 1985) or cellular GPS (Benzing and Walz, 2006) that coordinates the positioning of cells in connective and polarized tissues. As such, the primary cilium has proved to be essential for coordination of a variety of different signaling pathways critical in regulating cellular processes during development and tissue homeostasis. Despite recent advances, the genetics underlying the syndromic disorders and pathologies within the ciliopathy spectrum are still obscure, and, consequently, we know little as to how multiple signaling pathways are coordinated by the cilium to regulate cellular processes in tissues.

The overall aim of my thesis was to further our understanding of ciliary signaling systems and how this signaling takes part in regulation of cell migration. We have shown that prominent signaling systems important for polarization and migration of cells, such as PDGFRα, TGFβ and Wnt pathways, are associated with the primary cilium; in part through clathrin-dependent endocytosis at the ciliary pocket, which may function as a unique signaling hub for the coordinated interaction of multiple signaling networks during development and in tissue homeostasis. In terms of cell migration, our work on Inversin provides more pieces to the puzzle of understanding ciliary signaling and the resulting physiological responses in the context of cell polarization and motility. However, the results obtained during my PhD obviously raise more question than they answer. How are signaling networks coordinated at the primary cilium? What are the underlying mechanisms between cilia orientation and cytoskeletal reorganization? Clearly, more research within this area is required to untangle the mechanisms of ciliary signaling and crosstalk.


Summary

Primary cilia are microtubule-based sensory organelles that emerge from the centrosomal mother centriole to project from the surface of most quiescent cells in the human body. Ciliary entry is a tightly controlled process, involving ciliary diffusion barriers and gating complexes. By these means, the cilium maintains a unique composition of receptors and signal transduction molecules, and functions as a sensory microdomain that receives and relays extracellular cues to elicit cellular responses such as transcriptional control or cytoskeletal reorganization. As such, defects in assembly of function of primary cilia lead to a number of diseases and developmental defects, collectively termed ciliopathies. This dissertation focuses on selected signaling systems regulated by the primary cilium, including the PDGFRα, TGFβ and Wnt pathways, and how this controls directional cell migration as a physiological response.

In mesenchymal cell types, part of the cilium is embedded in the ciliary pocket, which is a membrane invagination with elevated activity of clathrin-dependent endocytosis (CDE). In paper I, it is shown that Transforming Growth Factor beta (TGFβ) signaling is activated in the cilium and transported to the ciliary base for CDE-mediated internalization and phosphorylation of Smad2/3 transcription factors. This is followed by translocation of activated Smad2/3 to the nucleus for target gene expression, and these processes depend on formation of the primary cilium. In conclusion, the primary cilium regulates TGF-β signaling in fibroblasts and differentiating cardiomyocytes, and the ciliary pocket is a compartment for CDE-dependent regulation of signal transduction.

The ciliary protein Inversin has been proposed to function as a molecular switch between canonical and non-canonical Wnt signaling. In paper II, it is shown that Inversin-deficient mouse embryonic fibroblasts (Invs MEFs) have an elevated expression of a range of genes and proteins implicated in canonical Wnt signaling, in addition to dysregulation of component associated with non-canonical Wnt signaling and cytoskeletal organization. These defects are physiologically evident from the impaired polarization and cell migration in Invs MEFs. A number of central Wnt components are localized to the primary cilium of wt MEFs. In conclusion, Inversin and the primary cilium control Wnt signaling and are required for polarization and cell migration.

In response to migration-promoting conditions in two dimensions, the centrosome and primary cilium orients between the leading edge and the nucleus and points in the direction of migration. PDGFRα was previously shown to become activated in the primary cilium upon stimulation with PDGF-AA. In paper IV, micropipette analysis and scratch assays (as described in paper III) are used to show that wound healing is impaired by defective cilia formation, which leads to uncontrolled cell movements. In conclusion, the primary cilium controls directional cell migration in wound healing.

Together, the results from this PhD study reflect the high level of complexity within signaling systems regulated by the primary cilium that control cellular processes during embryonic development and in tissue homeostasis. As such, this dissertation can contribute to increase the current understanding of the genetic mechanisms underlying ciliopathies.
Dansk sammenfatning


En tidlig polariseringsseffekt under migrationsfremmende forhold er en reorientering af centrosomet cellekernen, således at det primære cile peger i cellens migrationsretning. PDGFRa er tidligere vist at blive aktiveret i det primære cile. I artikel IV vises det, ved hjælp af mikropipetteanalyser og in vitro sår helingsanalyser, som beskrevet i artikel III, at et normalt, kemotaktisk repons til PDGF-AA i bindewævssceller afhænger af det primære cilie. Sårhelging, både in vivo og in vitro, hæmmes af defect ciledannelse, og fører til ukontrollerede cellebevægelser uden retnig. Det konkluderes at det primære cile regulerer retningsbestemt celmigration under sårheling.

De opnåede resultater diskuteres i forhold til den eksisterende litteratur om cilær signaling og retningsbestemt celmigration, og der perspektiveres til mulighederne for fremtidige klarlæggesmetoder. Resultaterne, der er fremkommet under dette PhD-studium, reflekterer tilsammen det høje kompleksitetsniveau indenfor cilært regulerede signaltransduktionssystemer, der kontrollerer cellulære processer under embryonal udvikling og vævshomeostase. Således kan denne afhandling bidrage til en øget forståelse af de genetiske mekanismer, der danner grundlag for forekomsten af ciliopatier.
Paper I
TGF-β Signaling Is Associated with Endocytosis at the Pocket Region of the Primary Cilium

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http://dx.doi.org/10.1016/j.celrep.2013.05.020

SUMMARY

Transforming growth factor β (TGF-β) signaling is regulated by clathrin-dependent endocytosis (CDE) for the control of cellular processes during development and in tissue homeostasis. The primary cilium coordinates several signaling pathways, and the pocket surrounding the base and proximal part of the cilium is a site for CDE. We report here that TGF-β receptors localize to the ciliary tip and endocytic vesicles at the ciliary base in fibroblasts and that TGF-β stimulation increases receptor localization and activation of SMAD2/3 and ERK1/2 at the ciliary base. Inhibition of CDE reduced TGF-β-mediated signaling at the cilium, and TGF-β signaling and CDE activity are reduced at stalled primary cilia in Tg737aprpc fibroblasts. Similarly, TGF-β signaling during cardiomyogenesis correlated with accumulation of TGF-β receptors and activation of SMAD2/3 at the ciliary base. Our results indicate that the primary cilium regulates TGF-β signaling and that the ciliary pocket is a compartment for CDE-dependent regulation of signal transduction.

INTRODUCTION

Transforming growth factor β (TGF-β)/bone morphogenetic protein (BMP) signaling plays critical roles in cell-cycle control, migration, differentiation, and other cellular processes throughout life (Guo and Wang, 2009). TGF-β signaling proceeds via formation of a heterotrimeric receptor complex of type I and II TGF-β receptors (TGF-βRI and TGF-βRII) whose activation leads to phosphorylation and activation of downstream effectors, including MEK1/2-ERK1/2 and receptor SMADs, SMAD2/3 (Huang and Chen, 2012). SMAD2/3 associates with SMAD4 in heterotrimeric complexes that translocate to the nucleus and regulate gene expression. Several feedback systems are activated upon TGF-β signaling, including SMAD7, which negatively regulates the pathway through receptor complex degradation. Internalization of TGF-β receptors (TGF-β-Rs) via clathrin-dependent endocytosis (CDE) enhances TGF-β signaling by formation of clathrin-coated vesicles (CCVs) and early endosomes (EEs) enriched in phosphatidylinositol-3 phosphate [PI(3)P]. PI(3)P anchors FYVE zinc finger domain-containing proteins, such as SMAD anchor for receptor activation (SARA) that conveys SMAD2/3 to the TGF-β-Rs and promotes TGF-βRI-mediated SMAD2/3 activation (Tang et al., 2010; Huang and Chen, 2012). Primary cilia coordinate multiple signaling pathways, such as receptor tyrosine kinase (RTK), Hedgehog (Hh), Wnt, Notch, and mTOR (Goetz and Anderson, 2010; Boehlke et al., 2010; Satir et al., 2010; Ezratty et al., 2011; Lienkamp et al., 2012; Christiansen et al., 2012). Thus, defective primary cilia lead to aberrant cell signaling, in turn causing a series of diseases known as ciliopathies (Hildebrandt et al., 2011; Waters and Beales, 2011). We previously reported that embryos of Tg737/litt88 null mice, which have defects in the formation of primary cilia, have cardiac anomalies (Clement et al., 2009a) characteristic of aberrant TGF-β signaling (Arthur and Bamforth, 2011). Further, in Tg737 mutant embryonic hearts, endothelial cells have altered SMAD2 phosphorylation and expression of α-smooth muscle actin (Egorova et al., 2011). Yet, a direct role for the primary cilium in coordinating TGF-β signaling is unknown.

In many cell types, the proximal part of the primary cilium resides in the cytoplasm within an invagination of the plasma membrane known as the ciliary pocket (CiPo), which is a unique domain for CDE (Poole et al., 1985; Rattner et al., 2010; Molla-Herman et al., 2010; Ghossoub et al., 2011), whose function is poorly understood. Here, we show that TGF-β signaling is associated with the CiPo region in fibroblasts and in stem cells during cardiomyocyte differentiation and identify the CiPo as a site of CDE-dependent activation of signal transduction.

RESULTS

TGF-β Induces Accumulation and Activation of TGF-β Signaling Components at the Primary Cilium in a CDE-Dependent Manner

The role of primary cilia in TGF-β signaling was first analyzed using cultures of growth-arrested fibroblasts. In human foreskin
Figure 1. TGF-β Signaling at the CiBa in Growth-Arrested hFF Cells

(A and B) WB of cells treated with TGF-β1 (2 ng/ml) for 0, 10, 30, and 180 min using antibodies as indicated.

(C) Quantification of p-SMAD2/3 levels in (A) relative to 0 min. Shown are means ± SD (n = 5). **p < 0.01; ***p < 0.001.

(D and E) DIC and IFM of cells with TGF-β1 for 0 and 30 min, (D) anti-anti-acetylated α-tubulin (Ac-tub), anti-TGF-β-RI(V-22), (E) anti-Ac-tub, and anti-TGF-β-RII(L-21). Bold arrows indicate primary cilia. Asterisks indicate CiBa. Open arrows indicate the ciliary tip.

(F) Quantification of TGF-β-RI and TGF-β-RII fluorescence levels at the CiBa region seen in (D) and (E). Shown are means ± SD (n = 24). **p < 0.01; ***p < 0.001.

(G) IFM of TGF-β-RI localization to the ciliary tip in cells with TGF-β1 for 180 min, anti-TGF-β-RI(V-22), and anti-Ac-tub. Bold arrows indicate primary cilia. Asterisks indicate CiBa.

(H) IFM of cells with TGF-β1 for 30 min, anti-Ac-tub and anti-SMAD4, anti-SMAD7, and DAPI. Bold arrows indicate primary cilia. Asterisks indicate CiBa.

(legend continued on next page)
fibroblasts (hFFs), TGF-β1 stimulation led to activation of SMAD2/3 and MEK1/2-ERK1/2 (Figures 1A–1C). Immunofluorescence microscopy (IFM) showed that TGF-β1-R1- and TGF-β1-R1R2 localized to the base of the primary cilium (CiBa) and sometimes to the ciliary tip in unstimulated cells. Upon 30 min of TGF-β1 stimulation, receptor localization increased at the CiBa (Figures 1D–1F) along with reduced ciliary tip localization. Occasionally, we observed receptor localization to the tip after 180 min of stimulation (Figure 1G), suggesting that activation of TGF-β signaling is associated with trafficking of receptors within the cilium and targeting to the CiBa. SMAD4 localized to the CiBa (Figure 1H) and TGF-β1 induced the phosphorylation of SMAD2/3 (p-SMAD2/3) and ERK1/2 (Figures 1I–1K and S1A) at CiBa, followed by nuclear translocation of SMAD4 and p-SMAD2/3 after 90 min of stimulation (Figures S1A and S1B). These results show that TGF-β signaling takes place at the cilium and that onset of signal transduction partly occurs at the CiBa followed by translocation of SMADs to the nucleus. We also detected SMAD7 at the CiBa (Figure 1H), indicating that deactivation of TGF-β signaling also occurs at the cilium.

To assess the role of CDE in TGF-β signaling at the cilium, hFFs were stimulated with TGF-β1 with and without Dynasore, which blocks the budding of clathrin-coated pits (CCPs) and CCP formation (Kirchhausen et al., 2008) and inhibits CDE at the CiPo region without reducing cilia formation (Molla-Herman et al., 2010). Dynasore inhibited β1-induced SMAD2/3 phosphorylation (Figures 1L and 1M) at the CiBa (Figure 1N and 1P) as well as nuclear localization of p-SMAD2/3 and SMAD4 after 90 min of TGF-β1 stimulation (Figures S1C and S1D). These results suggest that TGF-β signaling and activation of SMADs are associated with CDE at the CiBa. In contrast, activation of ERK1/2 was largely unaffected by Dynasore (Figures 1L, 1M, 1O, and 1P), indicating that this pathway is independent of CDE.

**Tg737orrpk Fibroblasts Have Reduced TGF-β Signaling and Decreased Activity of CDE at the Ciliary Base**

The importance of the primary cilium in TGF-β signaling via SMADs was tested using hypomorphic Tg737orrpk mutant mouse embryonic fibroblasts (MEFs) with stunted cilia (Schneider et al., 2005; Corbit et al., 2008). Compared to wild-type (WT) MEFs, p-SMAD2/3 levels were reduced in Tg737orrpk MEFs after TGF-β1 stimulation (Figures 2A and 2B). Tg737orrpk and WT MEFs expressed similar levels of TGF-β-R1 and TGF-β-R1R2 (Figure 2A), indicating that reduced TGF-β signaling in mutant MEFs is not due to altered receptor expression. However, upon 30 min of TGF-β1 stimulation, there was significantly less TGF-β-R1 (Figure 2C) and p-SMAD2/3 (Figure 2D) at the base of stunted cilia in Tg737orrpk MEFs compared to normal cilia in WT MEFs. This was associated with reduced nuclear translocation of p-SMAD2/3 and SMAD4 after 60 min of TGF-β1 stimulation (Figure S2A–S2D). Next, cells were cultured with Texas red transferrin (TR-Tf) for 10 min to visualize CCVs and EEs (Molla-Herman et al., 2010) (Figure S2E). In WT MEFs, TR-Tf localized as multiple puncta in the cytosol and around the primary cilium as reported for other cell types (Molla-Herman et al., 2010). In contrast, TR-Tf accumulation appeared less abundant at stunted cilia in Tg737orrpk MEFs, whereas the cytosolic labeling was similar to that of WT cells. Localization of clathrin assembly lymphoid myeloid leukemia (CALM)-positive puncta was also reduced around stunted cilia in Tg737orrpk MEFs (Figure 2E). IFM and isosurface three-dimensional (3D) visualization showed that CALM localized around the proximal part and base of WT primary cilia, and this localization was reduced in Tg737orrpk MEFs (Figure 2F). Multiple CALM-positive puncta at the CiPo of WT cilia colocalized with clathrin (Figure S2F) and with TGF-β-R1 (Figure 2G), indicating that TGF-β signaling is associated with CDE at the CiPo and that this is disrupted in Tg737orrpk MEFs.

**TGF-β Signaling Is Upregulated during Cardiomyocyte Differentiation**

TGF-β signaling plays a critical role in heart development (Arthur and Bamforth, 2011). To test whether it is associated with primary cilia during cardiomyogenesis, transcriptome analysis was performed on P19.CL6 cells during DMSO-induced cardiomyocyte differentiation (Habara-Ohkubo, 1996). Differentiation was evidenced by decreased nuclear localization of the stem cell marker SOX2 and increased nuclear localization of the cardiomyocyte transcription factor GATA4 as well as α-actinin localization to Z-lines of cardiac muscle cells at day 14 (Figure 3A). Primary cilia were formed at all stages of differentiation (Figure 3A) (Clement et al., 2009a). Transcription analysis revealed that several signaling pathways are affected during cardiomyogenesis, including TGFβ/BMP and Hh signaling (Figure 3B). Expression of cardiomyocyte markers and multiple TGF-β/BMP signaling components were upregulated during cardiomyogenesis (Figure 3C). Quantitative RT-PCR analysis (Figure 3D) confirmed these results. Expression of TGF-β-R1 (SMAD2, SMAD3, and SMAD4 all increased during differentiation in a bell-shaped fashion, peaking at day 7. TGF-β-R1 expression increased in a more linear fashion up to day 12. In contrast, SMAD7 was downregulated during the first few days, suggesting that SMAD7-dependent inhibition of TGF-β signaling is kept low during the initial stages of differentiation. At day 12, expression levels of TGF-β-R1, SMAD2, SMAD3, and SMAD4 decreased.

I and J) IFM of cells with TGF-β1 for 0 and 30 min, (l) anti-Ac-tub, anti-p-SMAD2/3, anti-TGF-β-R1R2(19), (J) anti-Ac-tub, anti-p-ERK1/2, and DAPI. Bold arrows indicate primary cilia. Asterisks indicate CiBa.

(K) Quantification of p-SMAD2/3 and p-ERK1/2 fluorescence levels at the CiBa region as seen in (l) and (J). Shown are means ± SD (n = 25). **p < 0.001.

(L) WB of cells treated with TGF-β1 for 0, 30, and 180 min with and without Dynasore (75 μM) using antibodies as indicated.

(M) Quantification of p-SMAD2/3 and p-ERK1/2 levels in Dynasore-treated cells compared to untreated cells with TGF-β1 for 30 min as seen in (L). Shown are means ± SD (n = 5). **p < 0.001. NS, not significant.

(N and O) DIC and IFM of cells with TGF-β1 for 30 min with and without Dynasore (75 μM). Bold arrows indicate primary cilia. Asterisks indicate CiBa.

(P) Quantification of p-SMAD2/3 and p-ERK1/2 fluorescence levels at the CiBa as seen in (N) and (O). Shown are means ± SD (n = 25). **p < 0.01. NS, not significant.
indicative of decreased TGF-β signaling at the end stage of differentiation. Western blot (WB) analysis confirmed that the TGF-β-Ri level peaks at day 7 of differentiation (Figures 3E and 3F).

**TGF-β Signaling Is Associated with CDE at the Ciliary Base during Cardiomyocyte Differentiation**

Next, the localization of TGF-β-Ri and p-SMAD2/3 during P19.CL6 differentiation at day 1 and 7 was analyzed. TGF-β-Ri accumulated around the CiBa during differentiation, along with increased SMAD2/3 activation (Figures 4A–4C). Specificity of the TGF-β-Ri antibody was confirmed by peptide competition assays (Figure 4D). Similar experiments were performed with hESC (Figure S3A), which as embryoid bodies (EBs) were induced to differentiate into cardiomyocytes expressing increased levels of GATA4 and NKX2-5 (Figures S3B and S3C). At day 20 of differentiation, TGF-β-Ri and p-SMAD2/3 accumulated at the hESC CiBa (Figures S3D and S3E). IFM and 3D isomeric reconstruction showed that ciliary TGF-β-Ri localization is largely confined to the area surrounding the CiBa and proximal part of the cilium (Figures 4E and 4F). This region comprises the CiPo, suggesting that TGF-β signaling takes places through CDE at this site during cardiomyocyte differentiation.
We analyzed the expression of cardiac marker proteins in P19.CL6 cells cultured in the presence of DMSO and various concentrations of TGF-β1. TGF-β1 stimulation increased the rate of cardiomyogenesis in a bell-shaped and concentration-dependent manner, with 0.2 ng/ml inducing the earliest onset of expression of the markers; at day 2, the level of GATA4 increased ~5-fold compared to cells with DMSO alone (Figures S4A and S4B). Similarly, nuclear GATA4 increased in the presence of TGF-β1 at 0.2 ng/ml, and this was abolished by the TGF-βRI antagonist SB431542 (Figure S4C). In accordance, TGF-β1 increased the expression of GATA4 and TGF-βRI, which was inhibited by SB431542 (Figure S4D). Next, cells at day 7 were serum depleted for 24 hr and stimulated with TGF-β1 with and without SB431542. TGF-β1 increased total cellular...
The increase in TGF-β signaling was associated with increased CiBa levels of p-SMAD2/3, which was abolished by SB431542 (Figures 4 G–4I). Thus, TGF-β signaling stimulates cardiomyogenesis and associates with the CiBa.

We transfected P19.CL6 cells with GFP-2xFYVE, a marker of PI(3)P-containing EEs (Gillooly et al., 2000), and confirmed that the CiPo region is enriched in EEs (Figures 4 J and 4K). The Golgi marker (GMAP210) localized around the periphery of the major accumulation of TGF-β-RIs at the CiPo, with partial TGF-β-RI colocalization to distinct puncta (Figures 4L and 4M). GMAP210 was shown to anchor IFT20 to the Golgi and regulate sorting or transport of ciliary proteins (Follit et al., 2008). Clathrin and CALM also localized in the CiPo region (Figure 4N) and colocalized with TGF-β-RIs (Figure 4O), suggesting that the CiPo of P19.CL6 cells is a site for CDE and internalization of TGF-β-RIs.

Please cite this article in press as: Clement et al., TGF-β Signaling Is Associated with Endocytosis at the Pocket Region of the Primary Cilium, Cell Reports (2013), http://dx.doi.org/10.1016/j.celrep.2013.05.020
DISCUSSION

Primary cilia coordinate many signaling pathways (e.g., Hh and RTK signaling) where ligands bind to their receptors in the ciliary membrane (Christensen et al., 2012; Goetz and Anderson, 2010) and initiate signal transduction within the ciliary compartment. We found that activation of TGF-β signaling occurs at the CIPo region in fibroblasts and in stem cells differentiating into cardiomyocytes. Here, activation was associated with accumulation of TGF-β-Rs after TGF-β stimulation and concomitant increased expression of TGF-β-Rs and downstream signaling components such as SMAD2/3/4 that activate target genes in TGF-β signaling. The CIPo in these cells is a site for CDE, as evidenced by markers for CCP, CCV, and EEs that colocalize with TGF-β-Ri. TGF-β signaling through SMAD2/3 at the CIPo was inhibited in hFFs treated with Dynasore and in Tg737opk MEFs with stunted primary cilia, which display reduced CDE. These results directly link TGF-β signaling to the primary cilium, where activation of the pathway may proceed through receptor internalization by CDE at the CIPo.

In fibroblasts, we occasionally observed localization of TGF-β-Rs at the ciliary tip, especially prior to and after prolonged TGF-β stimulation, indicative of intraciliary trafficking of receptors, which we hypothesize are transported after ligand binding to the CIPo for internalization and SMAD2/3 activation. TGF-β-Rs may also initiate signaling within the cilium similar to other receptor types, including PDGFRs, that activate MEK1/2-ERK1/2 in the cilium (Schneider et al., 2005). Alternatively, ligand binding to receptor populations in the plasma membrane in close proximity to the cilium/centrosome axis may be targeted to the CIPo. Targeting and transport of TGF-β-Rs to and within the cilium, as well as termination, recycling, and/or amplification of TGF-β signaling at the cilium, likely involve vesicular trafficking and exocytic and endocytic events that may be regulated by IFT88, Rab GTPases 8/11, and the exocyst complex, which dock at the CIBa and regulate ciliary protein and membrane transport (Das and Guo, 2011; Hsiao et al., 2012). Indeed, TGF-β-Rs were previously shown to be recycled through Rab11-dependent mechanisms (Mitchell et al., 2004), and Rab11 localizes to the CIBa (Hsiao et al., 2012).

Our analysis in MEFs confirms that CDE occurs elsewhere than at the CIPo, as evidenced by formation of CCVs and EEs at the cell periphery in both WT and Tg737opk cells (Figures 2E, 2F, and S2E). However, the CIPo comprises an endocytic compartment that is enriched in CCPs with a 3-fold increase in pits per surface unit at the CIPo membrane compared with the rest of the plasma membrane (Molla-Herman et al., 2010), indicating that a prominent part of CDE-mediated signaling takes place at the cilium. Indeed, the functional output of the TGF-β pathway relies on extensive crosstalk with other signaling pathways, including Hh, Wnt, Notch, mTor, and RTK (Guo and Wang, 2009; Lamouille et al., 2012), which previously were shown to be coordinated by the cilium/centrosome axis. Thus, our findings suggest that the CIBa region offers a unique site for the coordinated crosstalk between TGF-β signaling and other pathways during development and in tissue homeostasis.

Finally, our finding that CDE is disrupted at stunted primary cilia in Tg737opk MEFs may provide knowledge on how defective intraflagellar transport (IFT) leads to aberrant cell signaling coordinated by the primary cilium. Similar to TGF-β signaling, CDE controls the activity of RTK, Hh, and Wnt signaling through the internalization of ligand-bound receptors for either degradation or sustained signaling in endosomes or by recycling of the receptors back to the cell surface (Sorkin and van Zastrow, 2009; McMahon and Boucrot, 2011; Huang and Chen, 2012). In fact, the CIPo region was shown to be a site for both CDE and localization of tumor necrosis factor receptors in fibroblast-like type B synoviocytes (Rattner et al., 2010). Therefore, aberrant signaling observed in Tg737opk MEFs may in part be linked to aberrant CDE activity at the primary cilium, and it will be important to understand how IFT impinges on formation and/or function of the CIPo. Previous studies in trypanosomes showed that IFT mutant cells exhibit major disruptions in organization, orientation, and function of the flagellar pocket (Absalon et al., 2008), which displays remarkable similarity to the CIPo in mammalian cells (Overath and Engstler, 2004). Interestingly, IFT mutants showed major perturbations in vesicular trafficking and reduced endocytosis at the flagellar pocket (Absalon et al., 2008), supporting our finding that IFT88 is associated with regulation of CDE in the CIPo of primary cilia in fibroblasts. Taken together, we suggest that TGF-β signaling is linked to endocytosis at the CIPo and that crosstalk between TGF-β signaling and other signaling pathways is coordinated from this site.

EXPERIMENTAL PROCEDURES

Cell Cultures

Mouse embryonic carcinoma P19.CL6 cells were cultured as described previously (Clement et al., 2009b), and cardiomyocyte differentiation was induced with 1% DMSO (Merck). hFF cells and MEFs were cultured and serum deprived for ciliogenesis (Schneider et al., 2005; Schroder et al., 2011). hES/ES-L (RB005) were cultured (Laursen et al., 2007; Awan et al., 2010) and induced to differentiate into cardiomyocytes as EBs (Zhang et al., 2009).

Plasmids, Antibodies, and Reagents

GFP-2xFYVE plasmid was a gift from Harald Stenmark (Institute for Cancer Research, Oslo, Norway). Dynasore monohydrate (D7683-5MG) and SB-431542 (S4317) were from Sigma Aldrich, recombinant human TGF-β1 ligand (240-8) was from R&D Systems, DAPI (D1306) was from Molecular Probes, TR-TI (T2875) was from Life Technologies (T2875), and blocking peptide for TGF-β-Ri(V-22) (SC-398P) was from Santa Cruz. See Table S1 for a list of antibodies used.

RNA Isolation

Cells were grown in Petri dishes and were rapidly washed once in ice-cold sterile PBS prior to lysis (1% l-mercaptoethanol) and RNA purification with the NucleoSpin RNA II kit (Macherey-Nagel, 740955-50).

Transcriptome Analysis

Digital gene expression (DGE) tag profiling was performed using an Illumina Genome Analyzer II (’t Hoen et al., 2008). A total of 1 μg RNA per sample was analyzed using Illumina’s DGE Tag Profiling Kit according to the manufacturer’s protocol (version 2.1B). Tags were filtered and alignment against the mouse genome performed using SOAP (Li et al., 2008), and filtered tags were normalized to 1M tags and functional annotation was performed with DAVID Tools (http://david.abcc.ncifcrf.gov/summary.jsp). Gene set enrichment analysis was performed using the Broad Institute software (http://www.broadinstitute.org/gsea/index.jsp).
Quantitative RT-PCR was performed (Clement et al., 2009a) with the primers listed in Table S2. All samples were run in duplicates and normalized to the expression of four housekeeping genes: Gapdh, B2m, Hprt, and Psmd4. Data were analyzed using the comparative $C_\text{t}$ model (Livak and Schmittgen, 2001).

Nucleofection

P19.CL6 cells were transfected by nucleofection with the Nucleofector device II from Amxais Biosystems (Clement et al., 2009b).

SDS-PAGE and WB Analysis

The analysis was performed as described previously (Christensen et al., 2001). Immunoblots were scanned and processed for publication in Adobe Photoshop CS4 version 11.0. Band intensities were analyzed by densitometric scanning using UN-SCAN-IT 6.1 software (Silk Scientific). For statistical analysis, we used the Student’s t test for comparing the variation between two groups. When comparing three or more groups, we used the ANOVA test. All statistical calculations were performed on n = 3 or more. Significance levels were as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

Immunostaining, Microscopy, and Fluorescence Quantification

IFM was done as described previously (Christensen et al., 2001). Images were taken with a 3-phosphate in yeast and mammalian cells. EMBO J. 19, 4577–4588.

REFERENCES


Figure S1. Translocation of SMAD Transcription Factors to the Nucleus in Growth-Arrested hFF Cells Is Inhibited by Dynasore, Related to Figure 1

(A and B) DIC and IFM showing increased localization of phospho-SMAD2/3 and SMAD4 to the nucleus after 90 min of TGFβ-1 stimulation (2 ng/ml). Bold arrows: primary cilia; asterisks: CiBa; “n”: nucleus.

(C) DIC and IFM showing reduced nuclear localization of SMAD4 in cells treated with Dynasore (75 μM). Bold arrows: primary cilia.

(D) Quantification of SMAD4 fluorescence levels in the nucleus as seen in (C). Shown are means ± SD (n=20). ***p<0.001.
Figure S2. Translocation of SMAD Transcription Factors to the Nucleus and CDE at the Ciliary Base Region Is Reduced in Tg737orpk MEFs with Stunted Primary Cilia, Related to Figure 2

(A–D) IFM showing reduced nuclear localization of phospho-SMAD2/3 and SMAD4 in Tg737orpk MEFs as compared to wt MEFs after 60 min of TGFβ-1 stimulation (2ng/ml). Bold arrows: primary cilia; open arrows: stunted cilia; asterisks: CiBa. Dotted lines indicate circumferences of individual cells.

(E) IFM showing that CDE activity is reduced at stunted primary cilia in Tg737orpk MEFs as evidenced by reduced internalization of TR-Transferrin at this site. Bold arrows: primary cilia; open arrows: stunted cilia; asterisks: CiBa.

(F) IFM showing that CALM colocalizes to puncta of Clathrin at the ciliary base in wt MEFs. Localization of both Clathrin and CALM is reduced at the base of stunted primary cilia in Tg737orpk MEFs. Bold arrows: primary cilia; open arrows: stunted cilia; asterisks: CiBa.
Figure S3. hESC Cell Differentiation into Cardiomyocytes Is Associated with Activation of TGFβ Signaling at the Ciliary Base Similar to That Observed in P19.CL6 cells, Related to Figure 4

(A) DIC of cells forming clusters of cardiomyocytes at day 20 of differentiation.
(B) WB of GATA4 expression in cells differentiating after 1, 4, 9, 12 and 20 days of culturing.
(C) IFM showing that expression and nuclear localization of cardiomyocyte markers, GATA4 and NKX2-5, are increased at day 20 of differentiation.
(D and E) IFM showing increased localization of TGFβ-RI and phospho-SMAD2/3 at the base of primary cilia at day 20 of differentiation. Bold arrows: primary cilia; asterisks: CiBa.
Figure S4. TGFβ-1 Stimulates Differentiation of P19.CL6 Cells into Cardiomyocytes, and Cardiomyogenesis Is Inhibited by the TGFβ Receptor Antagonist, SB431542, Related to Figure 4

(A) WB of cells after 2, 4, 7, 10, 12 days of differentiation in the presence of DMSO and 0, 0.06, 0.2, 0.6, 2 and 6ng/ml TGFβ-1 using antibodies as indicated.

(B) Quantification of GATA4 levels at day 2, 7 and 12 at the different TGFβ-1 concentrations used in (A). Shown are means ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001.

(C and D) IFM (C) and WB (D) of the effects of SB431542 (2μM) on the expression and nuclear localization of GATA4 in the presence of DMSO and 0.2ng/ml TGFβ-1.
Inversin/Nephrocystin-2 Is Required for Fibroblast Polarity and Directional Cell Migration

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Abstract

Inversin is a cytoskeletal protein that critically regulates developmental processes and tissue homeostasis in vertebrates, partly through the degradation of Dishevelled (Dvl) proteins to coordinate Wnt signaling in planar cell polarity (PCP). Here, we investigated the role of Inversin in coordinating cell migration, which highly depends on polarity processes at the single-cell level, including the spatial and temporal organization of the cytoskeleton as well as expression and cellular localization of proteins in leading edge formation of migrating cells. Using cultures of mouse embryonic fibroblasts (MEFs) derived from inv−/− animals, we confirmed that both inv−/− and inv+/− MEFs form primary cilia, and that Inversin localizes to the primary cilium in inv+/− MEFs. In wound healing assays, inv−/− MEFs were severely compromised in their migratory ability and exhibited cytoskeletal rearrangements, including distorted lamellipodia formation and cilia orientation. Transcription analysis revealed dysregulation of Wnt signaling and of pathways regulating actin organization and focal adhesions in inv−/− MEFs as compared to inv+/− MEFs. Further, Dvl-1 and Dvl-3 localized to MEF primary cilia, and β-catenin/Wnt signaling was elevated in inv−/− MEFs, which moreover showed reduced ciliary localization of Dvl-3. Finally, inv−/− MEFs displayed dramatically altered activity and localization of RhoA, Rac1, and Cdc42 GTPases, and aberrant expression and targeting of the Na+/H+ exchanger NHE1 and ezrin/radixin/moesin (ERM) proteins to the edge of cells facing the wound. Phosphorylation of β-catenin at the ciliary base and formation of well-defined lamellipodia with localization and activation of ERM expressed in the leading edge of migrating cells were restored in inv−/− MEFs expressing Inv-GFP. Collectively, our findings point to the significance of Inversin in controlling cell migration processes, at least in part through transcriptional regulation of genes involved in Wnt signaling and pathways that control cytoskeletal organization and ion transport.

Introduction

Inversin (Inv or Nephrocystin-2) is encoded by the inversion of embryo turning (invs) gene [1–3] and was first discovered for its role during mammalian embryonic development in establishment of left-right asymmetry [4], which is reversed (situs inversus) in inv−/− mice with a homozygous deletion of exons 4–12, rendering only the first three exons transcribed [5]. Apart from laterality defects, the inv−/− mice exhibit cardiac, liver and kidney anomalies, including cyst formation in the extrahepatic bile ducts, pancreas and kidneys [1,3,4,6–9]. In humans, INVS was identified as the gene encoding Nephrocystin-2 (Nphp2) that is mutated in the recessive cystic kidney disease nephropathies type 2/infantile nephronophthisis [2], which, to a variable extent, is accompanied by situs inversus and other phenotypic traits of the inv−/− mouse and retinitis pigmentosa [10,11].

The four known mammalian Inversin splice variants of 90, 125, 140 and 165 kDa localize in a cell cycle-dependent manner to cell edges and primary cilia during growth arrest [12–14]. Primary cilia are microtubule-based, sensory organelles that emanate from the centrosomal mother centriole and coordinate a series of signaling pathways such as Hedgehog, Wnt and receptor tyrosine kinase (RTK) signaling during embryonic development and in tissue homeostasis [15–17]. Consequently, defects in the formation or function of primary cilia lead to a series of genetic disorders and diseases now commonly known as ciliopathies, including laterality defects, congenital heart disease, cystic kidney diseases and retinitis pigmentosa [18,19]. Endogenous Inversin was reported to localize to a confined region in the proximal segment of the primary cilium of mouse epithelial cells, referred to as the inv compartment [20]. Here, Inversin interacts with other Nphp proteins to form complexes with Meckel-Gruber syndrome (MKS) and Joubert
syndrome (JBS) proteins that control trafficking and signaling properties of the primary cilium [21–24].

Wnt signaling has been associated with the primary cilium due to the localization of several Wnt signaling components, including Inversin, at the ciliary/centrosomal axis in both hESC and differentiated cells [23–29]. Wnt signaling is initiated by the binding of a Wnt ligand to a Frizzled (Fzd) receptor and co-receptors, and has traditionally been divided into canonical and non-canonical Wnt pathways. Canonical Wnt signals recruits β-catenin from degradation by a complex comprising Gsk3β, Axin, Casein Kinase 1 (CK1) and adenomatos polyposis coli (APC), in turn leading to β-catenin-mediated gene transcription. In contrast, non-canonical Wnt signaling operates independently of β-catenin to control planar cell polarity (PCP) that refers to the organization of cells within the plane of a tissue [30–33].

PCP appears to be a prerequisite for correct cilia formation, yet Inversin may play a critical role in regulating Wnt signaling at the primary cilium to control PCP (discussed in [33,34]). In inv+/− mice, the hair patterning phenotype is reminiscent to that of PCP defects, and convergence extension movements are impaired by knockdown of Inversin in Xenopus laevis embryos [35]. Inversin was suggested to promote non-canonical Wnt signaling by recruiting or stabilizing Dishevelled (Dvl) at the plasma membrane in response to Fzd8 signal during Xenopus pronephros development [36]. Furthermore, Inversin was found to inhibit canonical signaling by degradation of Dvl-1 via the proteasome [35], which is hypothesized to localize at the ciliary base [37]. In support of these findings, elevated responsiveness to Wnt signals was reported in vertebrate cells with absent or incomplete primary cilia [24,27,31].

Mounting evidence links Wnt signaling, and in particular Dvl proteins, to control of the activity of Rho GTPases RhoA and Rac [38,39], but a role for Inversin in regulation of Rho GTPases has so far not been directly addressed. Cell motility and cytoskeletal organization are strongly influenced by the Rho GTPases [40–42], with Cdc42 and Rac1 involved predominantly in the formation of filopodia and lamellipodia, and RhoA in stress fiber formation [43]. In addition, an important reciprocal regulatory interaction exists between RhoA and the ezrin/radixin/moesin (ERM) family proteins, which play essential roles in single-cell polarization and cell migration [44]; yet, the possible link between Inversin and ERM proteins is unknown. Further, it is uncertain how Inversin regulates the reorganization of organelles and cytoskeleton during polarity processes and cell migration, and whether this relates to the function of Inversin in the primary cilium. Interestingly, primary cilia were found to orient in parallel to the migratory direction and towards the leading edge of fibroblasts and smooth muscle cells [45–47], suggesting that orientation of the cilium is part of the polarity system that transmits positional cues to migrating cells [48]. We recently showed that the primary cilium is required for directional cell migration in fibroblasts, in part by coordinating PDGFRβ-signaling [45] that regulates the spatial organization of translocation, incorporation and activation of the Na+/H+ exchanger 1 (NHE1) at the leading edge of the migrating cells [49,50]. NHE1 interacts directly with ERM proteins, is regulated by Rho GTPases, and in turn regulates leading edge Cdc42 activity, cytoskeletal organization, cell adhesion and migration [51]. Hence, the function and localization of NHE1 are potentially linked to Inversin signaling.

Here, we investigated the involvement of Inversin in control of directional cell migration by comparing wt mouse embryonic fibroblasts (MEFs) with MEFs derived from the inv−/− mouse. In wound healing assays, inv−/− MEFs exhibit reduced lamellipodia formation and cell migration compared to inv+/+ MEFs, in conjunction with disorientation of the primary cilium. We also show that Inversin, Dvl-1 and Dvl-3 localize to MEF primary cilia, and that canonical Wnt signaling is elevated in inv−/− MEFs, which furthermore show reduced ciliary localization of Dvl-3. This is associated with differential expression of genes involved in Wnt signaling and cytoskeletal organization, and altered regulation of RhoA, Rac1, Cdc42, NHE1, and ERM proteins. Collectively, our findings point to the significance of Inversin in controlling the migratory behavior of fibroblasts, at least in part through transcriptional regulation of genes involved in Wnt/PCP signaling and pathways that control cytoskeletal organization and ion transport.

Materials and Methods

Isolation of inv+/+ and inv−/− MEFs

Mouse embryonic fibroblasts (MEFs) were established from inv−/− and inv+/+ E13.5 embryos. Animals were held according to Home office guidelines to maintain health and ensure welfare. Animals were housed in IVC cages and supplied with food and water ad libitum and provided with environmental enrichment to enhance their standard of living and avoid stereotypical behaviors (such enrichment included sunflower seeds - encourages foraging behavior, torn paper bedding - encourages nest building and feeling of security, mouse houses - again encourages security and wellbeing, bacon pellets - foraging and also variation in diet). Methods of sacrifice were as per the Home Office Schedule 1 regulations and are detailed as follows; pregnant females were exposed to carbon dioxide in a rising concentration and death ensured by neck dislocation. The embryos were isolated and placed in ice cold PBS and subsequent decapitation carried out. Newcastle Ethics Review Committee approved this study.

Viscera, liver and heart were discarded from the embryos, and the remaining embryo was cut into fine pieces in the presence of Trypsin-EDTA (Invitrogen). Further Trypsin-EDTA was added and the digested tissue was incubated in a Petri dish at 37°C, 95% humidity and 5% CO2 until individual cells were visible under microscope. In a 50 ml tube, the digested tissue was mixed with 37°C Dulbecco’s Modified Eagle’s Medium (DMEM, high glucose without sodium pyruvate), 10% heat-inactivated foetal bovine serum (FBS), non essential amino acids, and 50 U/ml penicillin/streptomycin (all from Gibco) and allowed to settle. The top layer was separated from the bottom layer and cultured in a T25 tissue culture flask (Cellstar) for future experiments. The bottom layer was placed in a separate tissue flask and kept as a backup.

Cell Culture

inv+/+ and inv−/− MEFs were grown in 45% DMEM, 45% F-12+4.3-L-Glutamine with 10% FBS and 50 U/ml penicillin/streptomycin (all from Gibco) in T75 flasks (Cellstar) at 37°C, 95% humidity and 5% CO2. The cells were passaged every 3-4 days by trypsinization (Trypsin-EDTA, Gibco) at a confluence of ~80% and were never used beyond passage 15 to minimize clonal selection. Growth arrest and cilia formation in experimental cells were induced as described previously [52] by growing cells to near confluence and replacing the growth medium with a serum-free equivalent for 24–48 h after 2×wash in PBS.

Characterization of inv+/+ and inv−/− MEFs by qPCR

Analysis

RNA from inv+/+ versus inv−/− MEFs was purified with a Qagen Extraction Kit, treated with DNase I, and one µg of total RNA was reverse-transcribed using Superscript II (Invitrogen). Relative expression levels of the Nphp2 mRNA were determined by
quantitative RT-PCR using Absolute SYBR Green ROX Mix (ABgene) and a set of primers specific for the Nphp2 gene (forward primer: 5'-ACTTGTGACCAGCATATGCTG-3', reverse primer: 5'-AGGAGAAAACATTGAACCTTGTCIT-3'). Nphp2 expression was normalized to Gapdh mRNA expression (forward primer: 5'-TGGACCAACTGGTTAAG-3', reverse primer: 5'-GGATGCAGGGATGATGGTC-3'). Nphp2 expression analysis was performed with the 2^-ΔΔCt method and values are expressed as the average of triplicates. Levels of Nphp2 expression in the different MEFs were normalized to those of wt MEF cells and confirmed the essential absence of Inversin in inv^-/- MEFs (Figure S1).

Transcriptome Analysis
Cycling or growth arrested cells were lysed in lysis buffer with 1% β-mercaptoethanol, and RNA was purified using the NucleoSpin RNA II kit (MACHEREY-NAGEL, cat. no. 740955-50), as recommended by the manufacturer. Digital Gene Expression tag profiling (DGE) was performed essentially as previously described [53] using Illumina's Digital Gene Expression Tag Profiling Kit according to the manufacturer's protocol (version 2.1B). One μg of RNA was used for preparation of cDNA on magnetic Oligo(dT) beads using Superscript II (Invitrogen). The cDNA was digested with NlaIII, ligated to GEX MlaIII Adapter 1, digested with MmeI, ligated to GEX Adapter 2 and amplified by PCR. Amplicons were gel purified. Cluster formation was performed according to Illumina's instructions using a DNA concentration of 1–4 pM and amplification for 35 cycles. Sequencing was conducted on the Illumina Genome Analyzer II (GAIIL). Tags were filtered and alignment against the mouse genome was performed using SOAP [54] and normalized to 1M tags. Functional annotation was performed using DAVID Tools (www.david.abcc.ncifcrf.gov/summary.jsp). Gene Set Enrichment Analysis (GSEA) was performed using Broad Institute software (http://www.broadinstitute.org/gsea/index.jsp).

Transfections
Cells were transfected with a BosEx vector containing full length mouse Inversin fused to green fluorescent protein (Inv-GFP) [55] kindly provided by Dr. Hiroshi Hamada, Osaka University, Japan. Transfections were carried out as previously described, either by nucleofection with the Nucleofector device II from Amaxa Biosystems [56] or using DharmaFECT transfection reagent (Thermo Scientific) [57]. For nucleofection, 2×10^6 suspended cells were nucleofected with 2 μg plasmid DNA in MEF2 buffer. For DharmaFECT transfections, cells were grown to 50% confluence on coverslips in six well trays and incubated four hours with 1.5 μg plasmid DNA premixed with 5 μl DharmaFECT under serum free conditions. Subsequently, the cells were cultured and treated as described below.

Antibodies and Staining Reagents
Primary antibodies: mouse anti-acetylated α-tubulin, Sigma (T7451); mouse anti-β-actin, Sigma (A5441); mouse anti-Adenomatous polyposis coli, Santa Cruz (SC-53165); rabbit anti-β-catenin, Epitomics (1247-S); rabbit anti-phospho-β-catenin, Cell Signaling (9561S); rabbit anti-Cdc-42, Cell Signaling (24628); goat anti-centrin2, Santa Cruz (SC-8025); rabbit anti-detyrosinated α-tubulin (Glu-tubulin), AbCam (AB48389); mouse anti-Dishevelled-1, Santa Cruz (SC-8025); rabbit anti-Dishevelled-2, Cell Signaling (3224); rabbit anti-Dishevelled-3, Cell Signaling (3218); mouse anti-ezrin, Cell Signaling (E8987); rabbit anti-ezrin/radixin/moesin, Cell Signaling (3142); rabbit anti-phospho-εzrin/radixin/moesin, Cell Signaling (3142); chicken anti-GFP, AbCam (ab13970); mouse anti-GFP, AbCam (ab1218); rabbit anti-GFP, Santa Cruz (sc-8334); mouse anti-GSK3β, BD Transduction, (61021); goat anti-Inversin, Santa Cruz (SC-47195); rabbit anti-moesin, Epitomics (2036-1); mouse anti-NHE1, Santa Cruz (sc-136239); rabbit anti-radixin, Epitomics (2042-1); rabbit anti- phospho- Retinoblastoma protein, Cell Signaling (9308S); mouse anti-vinculin, Sigma (V9131); mouse anti-Rac1, Transduction Laboratories (610651); mouse anti-RhoA, Cytoskeleton (ARH03). The rabbit anti-NHE1 antibody was a kind gift from Dr. Josette Noel at University de Montreal, Canada.

Secondary antibodies and staining reagents for immunofluorescence (all from Molecular Probes): Alexa Flour® 350 Donkey IgG, anti-mouse (A10055), Alexa 350 Fluor® Donkey IgG, anti-rabbit (A10059); Alexa Fluor® 488 Donkey IgG, anti-goat (A1053); Alexa Fluor® 568 Donkey IgG, anti-mouse (A10037); Alexa Fluor® 488 Donkey IgG, anti-mouse (A21202); Alexa Fluor® 488 Donkey IgG, anti-rabbit (A21206); 4', 6-diamino-2-phenylindole dichloride (D1306); Alexa Fluor® 350 Phalloidin (A22201); Rhodamine-phalloidin (R415). Secondary antibodies for western blot analysis from Sigma: goat anti-mouse F(ab')2 specific alkaline phosphatase conjugated (A1293); Goat anti-rabbit F(ab')2 specific alkaline phosphatase conjugated (A3937); Rabbit anti-goat whole molecule alkaline phosphatase conjugated (A1187).

Immunofluorescence and DIC Microscopy Analysis
Cells were grown on HCl-rinsed coverslips in six-well trays (TTP) and serum deprived to induce cilia formation, then fixed for differential interference contrast (DIC) and immunofluorescence microscopy analysis (IFM) as previously described [52]. Images were obtained with a cooled CCD Optronics camera on a Nikon-Japan, Eclipse E600 epifluorescence microscope or with a cooled CCD Olympus DP72 camera on an Olympus BX63 epifluorescence microscope. Images were digitally processed for overlays with Adobe Photoshop CS4.

SDS-PAGE and Western Blotting
Cells were grown in Petri dishes, washed in ice-cold PBS and lysed in hot lysis buffer (0.5% SDS, 1 mM Tris-HCl, 1 mM sodium ortho-vanadate and Complete protease inhibitor cocktail (Santa Cruz)). Cell lysates were homogenized by sonication (PowerMED), and SDS-PAGE and Western blotting was performed as previously described [58]. Band intensities were estimated from densitometric values using Un-SCAN-IT gel (Silk Software).

Migration Assays
inv^-/- and inv^-/- MEFs were grown to 100% confluence (monolayer) in T2 flasks (Falcon) followed by serum deprivation to induce cilia formation, and directional cell migration was analyzed by wound healing assays [59]. Micrographs were obtained in 10 min intervals over a time course of 6 h. The circumferences of cells at the wound edge were marked at each time step throughout the entire image stack using Amira 4.1 (TGS, http://amiravis.com). Net translocation, velocity and movement of the cells into the wound (“x-direction” of a virtual coordinate system) were calculated as in [49]. In this experimental setup, translocation into the y-direction (parallel to the wound) was close to zero for inv^-/- as well as inv^-/- MEFs (data not shown) in accordance with [49].
GTPase Activity Assays

Cells were maintained in culture in the presence of serum until 100% confluence, then serum-starved for 24 h, scratched (40 times per 150 mm petri dish) and allowed to migrate for 30 min or 4 h as indicated. The relative levels of GTP-bound RhoA, Rac1, or Cdc42 were determined by an effector pulldown assay as described by [60]. Lysis of MEFs and pull down assay were done according to the manufacturer’s instructions (Cytoskeleton). In brief, cells were lysed in ice cold buffer and cell lysates were immediately clarified by centrifugation. An aliquot of cell extract was incubated with the GST-PAK fusion protein (Rac1 and Cdc42 assay) or with the GST-rotekin fusion protein (RhoA assay). The bead pellet was washed and re-suspended in 2×Laemmli sample buffer. Proteins bound to the beads were separated on a 15% SDS polyacrylamide gel, transferred onto a nitrocellulose membrane, and membranes were blotted with RhoA, Rac1 or Cdc42 antibodies. The band density was quantified by Bio1D software (Vilber Lourmat), and the relative densities of pulled down RhoA, Rac1 or Cdc42 were normalized to the total RhoA, Rac1 or Cdc42 (input) in the same sample.

Statistics

Statistical significance of the obtained data was tested with two-tailed Students’ t-test for ciliation frequencies and WB analysis, one-way analysis of variance (ANOVA) for migration assays, and Mann-Whitney U test for GTPase activity assays. Significance levels are indicated with asterisks (*: p<0.05, **: p<0.001, ***: p<0.0001).

Results

Directional Cell Migration is Inhibited in inv−/− MEFs

The two-dimensional migratory behavior of growth arrested inv+/+ and inv−/− MEFs was studied using in vitro wound healing assays and time-lapse video microscopy. Six h after wound induction, inv+/+ MEFs had migrated more than halfway into the wound, while the inv−/− MEFs had barely moved (Figure 1A). Figure 1B shows the migratory tracks of inv+/+ and inv−/− MEFs over 6 h. All trajectories were normalized to a common starting point, with the radius of each red circle representing the average cell displacement after 6 h. As shown in the left hand insert in Figure 1C, the overall velocity of inv−/− MEFs was about 25% of that in inv+/+ cells, whereas the distance traveled was less than 15% (right hand insert of Figure 1C). The average velocity of directionally migrating cells was obtained as the slope of translocation into the wound (x-direction, see Figure 1A) plotted against time (Figure 1C), and for inv−/− MEFs, this was approximately 10% of that of the wt (Figure 1C). We next evaluated the ability of the cells to form leading edge lamellipodia, filopodia, and focal adhesions. In contrast to inv+/+ MEFs, inv−/− MEFs failed to form well-defined lamellipodia whereas filopodia formation appeared largely unaffected. Inv−/− MEFs also displayed a more diffuse distribution of actin bundles and focal adhesions as detected with anti-vinculin compared to inv+/+ cells (Figure 1D), although total vinculin expression was not altered (Figure 1E,F). Thus, inv−/− MEFs are severely compromised in their ability to migrate and this is associated with defective formation of leading edge lamellipodia.

Characterization of Primary Cilia in inv+/+ and inv−/− MEFs

Immunofluorescence microscopy (IFM) analysis with acetylated α-tubulin (α-tub) and drezosynized α-tubulin (Glut-tub) antibodies showed that both wt and inv−/− MEFs form primary cilia of approximately 5 µm in length with a well-defined ciliary basal region, which was marked with Centrin antibody (Figure 2A,B). Cilia were formed at a frequency of about 90% after both 24 and 48 h of serum deprivation and in inv+/+ and inv−/− MEFs (Figure 2C). Both inv+/+ and inv−/− MEFs entered growth arrest upon serum deprivation as evidenced by a clear reduction in S807/S811 phosphorylation of Retinoblastoma protein (Rb) (Figure 2D), and ciliary formation occurred only in cells negative for nuclear localization of the proliferation-marker, Ki67 (Figure 1E). These results show that Inversin is not required for cells to enter growth arrest and form primary cilia.

In accordance with previous reports [20], IFM analysis showed localization of endogenous Inversin in wt MEFs to the lower part of the cilium, which signifies the Inversin compartment [20] (Figure 2F). No corresponding immunoreactivity was observed in inv−/− MEFs, confirming the Inversin specificity of the antibody. As a further control, cells were transfected with Inv-GFP that localized to primary cilium in both inv+/+ and inv−/− MEFs (Figure 2G). In some cases, Inv-GFP localized to the entire length of the cilium as well as to the cilial base region (data not shown), which may indicate the dynamic trafficking of the protein to and from the Inversin compartment in cells expressing high levels of the construct. We next evaluated the ability of the primary cilia to reorient in the direction of migration, as previously shown for migrating fibroblasts [43,46]. In inv+/+ MEFs, the primary cilia were positioned in front of the nucleus and oriented towards the leading edge. In marked contrast, primary cilia of inv−/− MEFs appeared randomly positioned and lacked an axis of orientation towards the edge of the wound (Figure 2H). These results indicate that Inversin critically regulates the orientation and correct positioning of the cilium, which is part of the polarity process required for cell migration.

Aberrant Wnt/β-catenin Signaling in inv−/− MEFs

In order to delineate the mechanisms through which Inversin exerts its effects on directional cell migration, we initially performed transcriptome analysis on total RNA isolated from quiescent inv+/+ and inv−/− MEFs. As listed in Table 1, the analysis showed a significant change in the expression of Wnt signaling related genes in inv−/− MEFs, as evidenced by a major change in the expression profile of genes encoding Wnt ligands, ligand scavengers and secreted proteins that impact on Wnt signaling (Rspo1, Sfrp1, Sfrp2, Dkk3), Wnt receptors and co-receptors (Fzd2, Fzd5, Ros2), various isoforms of casein kinases (Csk2a1, Csk2b, Csk1g2), cyclins (Cnd1, Cnd2), and protein phosphatases (Ppp2r1a, Ppp2r5d, Ppp3r1, Ppp4r1), as well as transcriptional regulators (Chd8, Gsc, Fox1l, Tcf4, Tcf7l1). Interestingly, we found an upregulation of several β-catenin target genes, such as Sfrp2 [61], Tcf4 [62], Tcf7l1, Wisp2 [63], Cnd1 [64,65], Cnd2 [66] and Gsc [67] in inv−/− MEFs, consistent with increased canonical Wnt/β-catenin signaling in these cells. These findings are in agreement with previous studies showing that Inversin acts as a molecular switch between Wnt signaling pathways, by inhibiting Wnt/β-catenin signaling via degradation of cytoplasmic Dvl-1 [33]. The transcriptome analysis further revealed a major change in the expression profile of those Wnt signaling-related genes that regulate pathways associated with actin cytoskeleton reorganization and targeting of receptors and regulatory proteins to the leading edge of migrating cells. For instance, Neurf1, encoding Na+/H+ exchange regulatory factor-1, which inhibits β-catenin activity through the tethering of Fzd to the actin cytoskeleton [68] and functions as an adapter protein for ezrin/radixin/moesin (ERM) proteins and activator of Na+/H+...
exchanger 1 (NHE1) to control directional cell migration [69–71], is strongly downregulated in inv<sup>−/−</sup> MEFs.

In order to verify whether inv<sup>−/−</sup> MEFs exhibit increased canonical Wnt/β-catenin signaling, WB and IFM analyses were carried out to study the expression, localization and phosphorylation of vinculin.

**Figure 1. Wound healing assays on cell migration and localization of focal adhesions in growth-arrested inv<sup>+/+</sup> and inv<sup>−/−</sup> MEFs.**

- **A** Light microscopy of inv<sup>+/+</sup> and inv<sup>−/−</sup> MEFs in wound healing assay at t = 0 h (left) and t = 6 h (right).
- **B** Trajectories of growth-arrested inv<sup>+/+</sup> (N = 27) and inv<sup>−/−</sup> (N = 28) MEFs in wound healing assay. Each line represents the migration of one cell within a 6 h period. The red circles illustrate the mean translocation of the cells.
- **C** Mean velocity and translocation of cells into the wound. (D) IFM analysis of localization of focal adhesions by anti-vinculin (red) in cells in wound healing assays and the actin cytoskeleton was stained with phalloidin (F-Actin, blue). Open arrows mark direction of migration into the wound and green dotted lines mark the front of cells facing the wound. (E,F) SDS-PAGE and WB analysis of expression of vinculin in growth-arrested inv<sup>+/+</sup> and inv<sup>−/−</sup> MEFs.

**doi:** 10.1371/journal.pone.0060193.g001
Figure 2. Formation and orientation of primary cilia and localization of Inversin in growth-arrested inv+/+ and inv−/− MEFs. (A) IFM analysis of primary cilia (closed arrows) with anti-acetylated alpha tubulin (Ac-tub, red) and their basal body region with anti-Centrin-2 that marks the two centrioles (asterisks) of the centrosome (Ctn-2, green). (B) IFM analysis of primary cilia (closed arrows) co-labelled with Ac-tub (red) and anti-glutamylated alpha-tubulin (Glu-tub, green). (C) Ciliation frequencies of inv+/+ and inv−/− MEFs upon 24 and 48 h of serum-free incubation, represented as mean ± S.E.M. (n = 3). (D) WB analysis of inv+/+ and inv−/− MEFs in the presence (+) and absence (−) of serum with anti-phospho-Retinoblastoma protein, which is downregulated in growth arrested cells. (E) IFM analysis of primary cilia (Ac-tub, red, and closed arrows) formation in growth arrested cells. Nuclei (DAPI, blue) of cycling cells shows localization of anti-Ki67 (green). (F) IFM analysis of Inversin (green) localization to primary cilia (Glu-tub, red, closed arrows) of inv+/+ and inv−/− MEFs. Asterisks (*) indicate ciliary base, nuclei are stained with DAPI (blue). (G) IFM analysis of Inv-GFP (green) and Inversin (red) localization to primary cilia (Ac-tub, blue, closed arrows) in mock and Inv::GFP transfected cells. The base of the cilium is identified by Differential interference constrast microscopy, DIC (asterisks). (H) IFM analysis of inv+/+ and inv−/− in wound healing assays after 30 min (top panel) and 4 h (lower panel) migration. Open arrows indicate direction of migration. Primary cilia are stained with Ac-tub (upper panel, red, closed arrows) or Glu-Tub (lower panel, green) and nuclei are stained with DAPI (blue). In lower panel, the actin cytoskeleton is stained with phalloidin (F-actin, red). Green dotted lines mark the edge of cells facing the wound. doi:10.1371/journal.pone.0060193.g002
Table 1. Transcriptomic analysis of up- and down-regulated genes in growth arrested inv−/− relative to inv+/+ MEFs with listed p values (n = 3) and common protein names.

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doi:10.1371/journal.pone.0060193.t001

Inversin Controls Cell Migration

lation of β-catenin and Dvl-1-3, as well as APC and GSK3β. WB analysis showed that inv−/− MEFs displayed an increased protein level of Dvl-1, whereas protein levels of Dvl-2, Dvl-3, APC and GSK3β were significantly reduced, compared to inv+/+ MEFs (Figure 3A,B). Further, inv−/− MEFs showed a reduction in the GSK3β-mediated phosphorylation of β-catenin and, accordingly, an increase in the β-catenin protein level (Figure 3A,B). These results support the conclusion that canonical Wnt/β-catenin signaling is upregulated in inv−/− MEFs.

Given the reported interaction between Inversin and Dvl proteins [35], we next examined the localization of Dvl-1, -2, and -3 in inv+/+ and inv−/− MEFs. We detected Dvl-1 and Dvl-3, but not Dvl-2, in the primary cilia (Figure 3C–E). Similarly to Inversin, Dvl-1 localized to the lower part of primary cilia of wt and mutant cells (Figure 3C). In contrast, Dvl-3 was present only at the basal region of the cilia and was, notably, absent in the primary cilia of inv−/− MEFs (Figure 3E). All three Dvl isoforms were also detected in the cytosol and at the plasma membrane (data not shown). In accordance with [72] there was a distinct accumulation of phosphorylated β-catenin (p-β-cat) at the basal region of the cilia in inv−/− MEFs. p-β-cat staining was reduced and appeared more dispersed at the ciliary base in inv−/− MEFs (Figure 3F). We further observed localization of Fzd-3 to primary cilia of both inv+/+ and inv−/− MEFs (Figure 3G), indicating that...
Wnt signaling may be regulated directly by activation of receptors in the cilium, and that ciliary targeting of Fzd-3 is independent of Inversin. Finally, expression of Inv::GFP in inv^{-/-} MEFs restored the distinct accumulation of p-\beta-catenin at the basal region of the cilium (Figure 3H,I), confirming a specific role of Inversin in regulating \beta-catenin stability at the primary cilium. Taken together, these results demonstrate that inv^{-/-} MEFs exhibit increased canonical Wnt signaling and changes in the localization of Wnt signaling components at the primary cilium.
Aberrant Activation and Localization of Rho GTPases in \textit{inv^{+/−}} MEFS

Rho GTPases are essential regulators of cell motility, in part via the downstream effectors Arp2/3 and WAVE/WASP [43]. To test the hypothesis that Inversin plays a role in regulation of Rho GTPase activity, effector domain-binding assays were employed to estimate the fractions of active Cdc42, Rac1 and RhoA in \textit{inv^{+/−}} and \textit{inv^{−/−}} MEFS in wound healing assays. 30 min after onset of migration (induced by introducing multiple scratches in the cell culture), a 5-fold increase of Rac1 activity and a small, yet significant, increase in Cdc42 activity were detected in \textit{inv^{−/−}} MEFS compared to wt, whereas RhoA activity did not differ significantly between \textit{inv^{+/−}} and \textit{inv^{−/−}} cells (Figure 4A). Interestingly, upon 4 h of migration, a 4-fold increase in Cdc42 activity and an 11-fold increase in Rac1 activity were observed in \textit{inv^{−/−}} MEFS compared to wt, whereas RhoA activity was only slightly, albeit significantly, increased compared to wt (Figure 4B). Notably, the expression levels of Cdc42 and RhoA were markedly decreased in \textit{inv^{−/−}} MEFS compared to wt, whereas Rac1 expression was reduced at 30 min yet not at 4 h, after introduction of the wound (Figure 4A–B).

Correct targeting of Rac1 and RhoA at the leading edge is essential for proper directional migration [73–75], and we therefore next examined the localization of these proteins by IFM analysis. In \textit{inv^{+/−}} MEFS, both GTPases localized diffusely to the cytosol as well as to distinct punctae in cytoplasmic protrusions of migrating cells (Figure 5A,B, left panels). In comparison, \textit{inv^{−/−}} MEFS showed a markedly reduced Rac1 and RhoA localization at cell surfaces facing the wound (Figure 5A,B, right panels). Quantitative image analysis confirmed that localization of both Rho GTPases at the leading edge was significantly reduced in \textit{inv^{−/−}} MEFS (Figure 5C,D). These results show that while Rho GTPase activity was increased in \textit{inv^{−/−}} MEFS, the defective targeting of Rac1 and RhoA in these cells could compromise the organization of the cytoskeleton required for cell migration. Indeed, transcriptome analysis revealed a large extend of deregulated gene expression within pathways regulating focal adhesions, the actin cytoskeleton and adherens junctions in \textit{inv^{−/−}} MEFS (Figure 5E). In this regard, expression of \textit{Wasf1} (encoding Wave1) and \textit{Arp2/3}, both of which are regulated by Rac1 and Cdc42 and essential for actin reorganization during lamellipodia formation [43], were significantly downregulated in \textit{inv^{−/−}} MEFS (Figure 5F). These findings demonstrate that Inversin regulates the activation and localization of Rho GTPases and the expression of a large number of key proteins in cell migration.

![Figure 4. Inversin affects the activity of the Rho GTPases.](image-url)

Activity of Rac-1, RhoA and Cdc42 was determined by pull-down in migrating \textit{inv^{+/−}} and \textit{inv^{−/−}} MEFS 30 min (A) or 4 h (B) after scratch. Histograms represent the mean ± S.E.M. of three independent experiments.
doi:10.1371/journal.pone.0060193.g004
Expression and Leading Edge Localization of ERM Proteins Are Altered in inv−/− MEFs

In light of the known roles of RhoA, Rac, and Cdc42 in regulating ERM proteins [44], and the known involvement of ERM proteins in the formation of leading edge lamellipodia [76], we next asked whether inv−/− MEFs exhibited altered expression and activity of ERM proteins. WB analysis using an antibody recognizing total ezrin, radixin and moesin (ERM) showed an about 10-fold decrease in the proteins level(s) of ezrin and/or radixin (both 80 kDa) in inv−/− MEFs, whereas the level of moesin (75 kDa) was unaltered. Separate examination of the individual ERM proteins confirmed this observation and showed that the 80 kDa ERM protein with reduced expression in inv−/− MEFs corresponds to ezrin. Interestingly, ERM protein activation (represented by their phosphorylation at T567/564/558) was also markedly reduced in inv−/− cells (Figure 6B,E).

To investigate whether lack of Inversin also alters ERM trafficking to the leading edge of migrating cells, IFM analysis was carried out in wound healing assays. Typical lamellipodia in the direction of the wound, with prominent cortical F-actin and clear leading edge ezrin localization, were formed in inv+/+ MEFs (Figure 6C, left panel), but in inv−/− MEFs, ezrin was largely absent at the plasma membrane, in congruence with the low expression of ezrin in these cells (Figure 6C, right panel). Notably, localization of radixin and moesin to wound-approaching cell edges was also absent in inv−/− MEFs, despite the fact that the mutant cells expressed these proteins at a level comparable to that of wt cells (Figure 6D). Further, phosphorylated ERM proteins were strongly expressed at the leading edge in inv+/+ MEFs, but were absent from the cell edge facing the wound in inv−/− MEFs (Figure 6E). Finally, expression of Inv::GFP in inv−/− MEFs restored formation of well-defined lamellipodia in the direction of the wound with localization of both ERM and phospho-ERM to the leading edge of migrating cells (Figure 6F,G). Collectively, these results support the conclusion that Inversin plays a major role in regulation of the expression, regulation, and leading edge localization of ERM proteins.

Expression and Leading Edge Localization of NHE1 are Altered in inv−/− MEFs

ERM proteins physically link the actin cytoskeleton to the Na+/H+ Exchanger NHE1, which we and others have shown to localize to the leading edge and play an essential role in directional cell migration [49,77,78] in a partially primary cilium-dependent manner [49,79]. We therefore hypothesized that NHE1 localization would be disrupted in the inv−/− cells. IFM analysis of quiescent cells in wound healing assays confirmed targeting and colocalization of NHE1 with ezrin at the leading edge of wt MEFs, (Figure 7A, upper panel). In contrast, NHE1 was absent from cell edges facing the wound of inv−/− MEFs (Figure 7A, lower panel). We also performed IFM analysis on cells cultured at low confluence, allowing them to move freely with no contact to neighboring cells. In these experiments, inv−/− MEFs formed lamellipodia with clear leading edge localization of NHE1, which colocalized with ezrin (Figure S2A,B, left panels), whereas inv−/− MEFs showed no detectable localization of either protein at the cell surface (Figure S2A,B, right panels). In accordance with our previous findings [49], NHE1 was upregulated during growth

Figure 5. Inversin affects the localization of the Rho GTPases and gene expression in migration-related pathways. (A,B) IFM analysis of Rac-1 (A, green) or RhoA (B, green) localization after 4 h migration of inv+/+ and inv−/− MEFs. Arrows indicate Rac1/RhoA localization at the leading edge. The actin cytoskeleton is stained with phalloidin (F-actin, red) and nuclei are stained with DAPI (blue). (C,D) Quantification of leading edge staining of Rac1 (C) and RhoA (D) in inv+/+ and inv−/− MEFs represented as mean ± S.E.M. (n = 3). (E,F) Transcriptomics of growth arrested MEFs with listed p values (n = 3). Migration-related pathways with number and percentage of differentially expressed genes (DEGs) in inv−/− compared to inv+/+ MEFs (E). Downregulation of specific genes controlling actin polymerization in inv−/− relative to inv+/+ MEFs (F). doi:10.1371/journal.pone.0060193.g005
Inversin regulates both the cell-cycle-dependent expression and the lamellipodial targeting of ERM proteins and NHE1, as well the phosphorylation of ERM proteins.

Figure 6. Inversin affects expression, regulation and localization of ERM proteins. (A) WB analysis of growth arrested inv+/+ and inv−/− MEFs with antibodies against total ezrin/radixin/moesin (ERM), phosphorylated ERM (T567 of ezrin, T564 of Radixin, T558 of moesin, p-ERM), ezrin, radixin and moesin, and α-tubulin as control, with indications of the 80 (1) and 75 (2) kDa bands. (B) Quantification of WB from (A); Histograms represent mean ± S.E.M. (n=3). (C,E) IFM analysis of growth arrested inv+/+ and inv−/− MEFs in wound healing assays, with phalloidin staining of the actin cytoskeleton (F-actin, red) and nuclei with DAPI (blue). Open arrows indicate direction of migration, and arrowhead indicate leading edge staining of ezrin (C, green), Radixin (D; upper panel, green), moesin (D; lower panel, green), and p-ERM (E, green). (F,G) DIC and IFM analysis on lamellipodium formation and localization of ERM (F, red) and p-ERM (G, red) to the leading edge of migrating cells in Inv-GFP (green) transfected inv−/− MEFs. The actin cytoskeleton is stained with phalloidin (F-actin, blue). Open arrows indicate direction of migration. doi:10.1371/journal.pone.0060193.g006

arrest in inv+/+ MEFs, to more than twice the level in cycling cells. Notably, this upregulation was abolished in inv−/− MEFs (Figure 7B,C). Similarly, we observed a significant, growth arrest-associated upregulation of ezrin in inv+/+ MEFs, but not in inv−/− MEFs (Figure 7B,C). Collectively, the data in Figure 6 and 7 show that Inversin regulates both the cell-cycle-dependent expression and the lamellipodial targeting of ERM proteins and NHE1, as well the phosphorylation of ERM proteins.
Discussion

We show here that Inversin plays a critical role in regulation of cellular pathways associated with polarity control in cell migration. Inv
2
+/2 MEFs exhibit marked defects in cell migration, in conjunction with defective orientation of primary cilia towards the leading edge, altered Wnt signaling, and marked changes in motility-related regulation of Rho GTPases, ERM proteins, and NHE1. In addition, Inversin deficiency elicited marked changes in the RNA expression profiles of genes involved in the regulation of cytoskeletal organization, including the NHE-regulatory factor, Nherf1, as well as Ark2/3, Wac1, and clusters of genes regulating formation and function of adherence junctions and focal adhesion. These findings are the first to characterize, at the single-cell level, the roles of Inversin in regulating transcriptional processes and polarity pathways during cell migration.

Inv
2
+/2 MEFs exhibited reduced migration speed and defects in lamellipodia formation in wound healing assays. As previously reported for fibroblasts [45,46], primary cilia in inv
+/+ MEFs were positioned in front of the nucleus, pointing towards the leading edge and in parallel to the path of migration. This was in sharp contrast to inv
−/− MEFs, in which primary cilia were randomly positioned and lacked an axis of orientation towards the edge of the wound. On sensory cells in the vertebrate inner ear, PCP signaling controls the position of the kinocilium, which then directs the organization of the stereocilia [34,80]. In line with previous findings [45,49], we suggest that ciliary orientation and positioning are critically linked to the polarity of migrating cells and that Inversin plays a significant role in regulating these processes.

Since Inversin acts as a molecular switch between the β-catenin and PCP pathways [35,36], we hypothesized that ciliary orientation might be regulated by Inversin through the facilitation of non-canonical Wnt/PCP signaling. In order to investigate this in further detail, we carried out gene and protein expression analysis on Wnt signaling components in inv
+/+ and inv
−/− MEFs. Our transcriptome analysis revealed a dramatic change in expression of several genes in both canonical and non-canonical Wnt signaling. Most importantly, there was a major up-regulation of multiple β-catenin target genes, confirming that Inversin deficiency leads to up-regulation of canonical Wnt signaling. As an example, Gsc, a canonical Wnt-responsive gene encoding Goosecoid [81] that controls cell migration in Xenopus embryos [82], was upregulated more than 1,500-fold in inv
−/− compared to inv
+/+ MEFs. In concurrence with these data, our protein expression analysis confirmed up-regulation of canonical Wnt signaling in inv
−/− MEFs as judged by increased protein levels of Dvl-1 and β-catenin, consistent with a reduction in S33/37,T41-phosphorylation of β-catenin at the primary cilium. Further, the protein levels of
Dvl-2 and Dvl-3 were significantly reduced in inv-/- MEFs, and this was associated with a prominent reduction in ciliary accumulation of Dvl-3 at the ciliary base. These results suggest that Inversin partly acts through Dvl-3 at the cilium to control PCP signaling. Indeed, Dvl proteins critically regulate the accumulation of Dvl-3 at the ciliary base. These results suggest this was associated with a prominent reduction in ciliary wound healing [87]. It can thus be speculated that in increased activation of Cdc42 in MEFs attenuated centrosome reorientation of the centrosome to face the leading edge [74,85,86]. In accordance with our findings, the Par6/aPKC complex, in reorientation of the centrosome to face the leading edge [74,85,86]. In accordance with our findings, increased activation of Cdc42 in MEFs attenuated centrosome reorientation toward the leading edge and migration during wound healing [87]. It can thus be speculated that in inv-/- MEFs, the reduced Dvl-2 level could lead to insufficient aPKC/ Dvl-2 interaction. In response, the Cdc42/Par6/aPKC signal transduction may compensate, leading to an over-activation of Cdc42, which in turn may induce the inhibition of centrosome reorientation and migration observed in inv-/- cells.

Wnt/Fzd/Dvl signalling can directly stimulate the activity of Rac, Cdc42 and RhoA [38,39], and Cdc42 can secondarily activate Rac via the recruitment of the Rac-GEF [PPIX] [74]. Hence, we hypothesize that the Rho GTase dysregulation in inv-/- MEFs is at least in part downstream from the observed changes in Wnt signaling. Consistent with the role of Rac in control of cellular directionality and velocity [73,89], and the reported link between elevated Rac activity and loss of fibroblast motility [90], we detected strongly increased Rac1 activity in inv-/- MEFs. Interestingly, a similar decrease in Rac1 expression accompanied by an increased activity was observed in primary vascular smooth muscle cells treated with siRNA against Nherf1 [71], which we found downregulated in inv-/- MEFs. The high level of activation of Cdc42 and Rac1 in the inv-/- MEFs could in turn suppress RhoA activity [87,90], which may explain why the activity of this GTase was only slightly increased in inv-/- cells. Despite the modest effect on RhoA activity, inv-/- MEFs exhibited extensive formation of actin bundles and altered focal adhesion patterns compared to wt cells. The dramatic loss of Wasp1 and Actp2/3 RNA in inv-/- MEFs may result directly from the absence of Inversin and dysregulated β-catenin signaling, or may be a negative feedback effect of the elevated Rac1 activity in these cells. Whatever the precise mechanism, it seems likely that the loss of these two essential regulators of lamellipodium formation plays a major role in the impaired formation of lamellipodia in inv-/- MEFs.

Rho GTases and ERM proteins reciprocally regulate each other [44,91], hence, the defective ERM protein activation in inv-/- MEFs could contribute to the abnormal Rho GTase activity; a relation which would be novel in the context of Inversin-Wnt signaling. ERM proteins, in turn, play essential roles in regulation of cell polarity and motility, and directly link the actin cytoskeleton to NHE1. We and others have shown NHE1 to play essential roles in directional fibroblast motility, at least in part through its effects on intra- and extracellular pH in the leading edge region [92-94] and through signaling in the primary cilium [49,50]. In this regard, PDGFRβ signaling in the cilium was shown to regulate targeting of NHE1 to the leading edge partly through the AKT pathway that initiates NHE1 translocation to the edge and partly through the MEK1/2-ERK1/2-p90RSK pathway that controls the spatial organization of NHE1 translocation and incorporation and therefore specifies the direction in which the leading edge forms [50]. Indeed, inhibition of the AKT pathway leads to defective cell migration [50] similarly to what is observed for inv-/- MEFs, suggesting a potential cross-talk between ciliary PDGFRβ and Inversin-mediated Wnt signaling in coordination of NHE1 translocation and directional cell migration. Further, the parallel dysregulation of NHE1 and ezrin expression and localization in inv-/- MEFs, in conjunction with their known physical interaction and the strong migration defective phenotype observed in these cells, are in accordance with the existence of a tight functional link between NHE1 and ezrin that is important in regulation of cell motility. Studies in Drosophila melanogaster have shown that dysregulation of Na+/H+ exchange activity leads to PCP defects due to defective recruitment of Dvl to Fzd in the plasma membrane [95]. In this context, it is noteworthy that the absence of Inversin is accompanied by a decreased expression of Nherf1, which binds F-actin to regulate ezrin and RhoA activity [70,71] and to suppress Wnt/β-catenin signaling [68]. Furthermore, Nherf1 was reported to localize to pseudopodial tips along with NHE1 and stimulate NHE1 activity, leading to increased invasiveness of breast cancer cells [96], and loss of Nherf1 enhances Wnt/β-catenin signaling, which is associated with hyperproliferation in breast cancer [68].

In conclusion, loss of Inversin abolishes directional migration of MEFs in a manner correlated with dysregulation of Wnt signaling, Rho GTases and ezrin activity, F-actin organization and with loss of localization of Rho GTases, ERM proteins and the Na+/H+ exchanger NHE1 to the leading edge. In conjunction with the known roles of these proteins in regulation of cell motility, our findings suggest the existence of a signaling axis consisting of Inversin, Wnt, Rho GTases, ERM proteins, and NHE1 that may contribute importantly to the regulation of fibroblast polarization and motility, in a manner partially associated with the primary cilium.

Supporting Information

Figure S1 qPCR analysis of Inversin (Inv) mRNA expression in inv-/- relative to inv+/- MEFs. (A) Data are presented as mean ± S.E.M. (n = 3). (TIF)

Figure S2 DIC and IFM analysis of low-confluent, serum starved inv+/- and inv-/- MEFs. (A,B) The actin cytoskeleton is stained with phalloidin (F-actin, blue), and arrowheads indicate cortical localization of NHE1 (A, B, green) and ezrin (B, red). In (A), microtubules are detected with anti-α-tubulin (α-tub, red). (TIF)

Acknowledgments

We are grateful to Dr. Hiroshi Hamada (Osaka University, Japan) for the inv-GFP construct. The authors would like to thank Anni Bech Nielsen and Sabine Mally for excellent technical assistance.
Author Contributions
Conceived and designed the experiments: IRV STC AS KK SFP SS. Performed the experiments: IRV LEY STC AS TJ KK SFP SS. Analyzed the data: IRV LEY STC AS TJ KK SFP SS. Contributed reagents/materials/analysis tools: JG KK SFP SS. Wrote the paper: IRV SFP.

References


68. Inversin Controls Cell Migration


Suppl. Figure 1: Veland et al
Paper III
CHAPTER THREE

Analysis of Primary Cilia in Directional Cell Migration in Fibroblasts

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Abstract

Early studies of migrating fibroblasts showed that primary cilia orient in front of the nucleus and point toward the leading edge. Recent work has shown that primary cilia coordinate a series of signaling pathways critical to fibroblast cell migration during development and in wound healing. In particular, platelet-derived growth factor receptor alpha (PDGFRα) is compartmentalized to the primary cilium to activate signaling pathways that regulate reorganization of the cytoskeleton required for lamellipodium formation and directional migration in the presence of a specific ligand gradient. We summarize selected methods in analyzing ciliary function in directional cell migration, including immunofluorescence microscopy, scratch assay, and chemotaxis assay by
micropipette addition of PDGFRα ligands to cultures of fibroblasts. These methods should be useful not only in studying cell migration but also more generally in delineating response pathways in cells with primary cilia.

1. INTRODUCTION

The primary cilium is a microtubule-based, solitary organelle that emanates from the centrosomal mother centriole during growth arrest of most cell types of the human body to coordinate signaling pathways that critically regulate cellular processes during development and in tissue homeostasis (Christensen, Clement, Satir, & Pedersen, 2012; Satir & Christensen, 2007). Consequently, defects in ciliary formation or compartmentalization of ciliary receptors and downstream components in signal transduction lead to a series of pathologies, now known as ciliopathies (Hildebrandt, Benzing, & Katsanis, 2011; Waters & Beales, 2011). Here, we describe methods in analyzing ciliary function in directional cell migration in fibroblasts, which when defective causes developmental disorders and is implicated in diseases such as fibrosis, tumorigenesis, and cancer cell invasion.

Recognition of the relationship of primary cilia to fibroblast migration antedates the modern era of quantitative study. The fibroblast primary cilium was described by Sorokin in the early 1960s (Sorokin, 1962), and Tucker and coworkers studied its formation during growth arrest and relationship to the cell cycle (Tucker, Pardee, & Fujiwara, 1979). Several investigators, notably Albrecht-Buehler, noted that the primary cilium points in the direction of cell migration (Albrecht-Buehler, 1977). However, analysis of the ciliary signaling pathways in cell migration has only been achieved in the past decade (Christensen, Pedersen, Satir, Veland, & Schneider, 2008; Jones et al., 2012; Lu et al., 2008), particularly with the realization that growth arrest-specific proteins, especially platelet-derived growth factor receptor alpha (PDGFRα), were associated with the pathway in lamellipodia formation and directional cell migration (Schneider et al., 2010, 2005, 2009). As outlined in this chapter, major advances in immunomicroscopy, scratch assay, and micropipette analysis have contributed to our understanding of primary cilia in cell migration.

The role of primary ciliary in PDGFRα signaling and cell migration began with cultures of NIH3T3 fibroblasts, but an important tool for analysis are mouse embryonic fibroblasts (MEFs) derived from either Tg737opk mice or their wild-type (wt) littermates. Serum deprivation in NIH3T3 or wt
MEFs leads to the formation of primary cilia and upregulation of PDGFRα, while Tg737opk mutant MEFs function as controls where neither event occurs (Schneider et al., 2005). PDGF-AA is a specific ligand for PDGFRα. A combination of Western blot and immunolocalization experiments using this ligand shows that PDGFRα is transported to and imported into the growing cillum where it dimerizes, becomes phosphorylated, and signals via the AKT and MEK1/2–ERK1/2 pathways to control directional cell migration by influencing the transport and positioning of an Na+/H+ exchange protein to the lamellipodium (Clement et al., 2012; Schneider et al., 2010, 2005, 2009). While the use of mutants to define the signal transduction pathways is extremely useful, the system can also be probed using ciliary knockout procedures, RNAi or inhibitors. Since little is actually known about the way primary cilia control cellular events or cytoskeletal organization, it is probable that variations of these techniques will prove useful in not only following cellular changes in other primary cilia signaling systems, certainly where cell migration is involved, but also more generally in delineating pathways from the cilium itself into the cytoplasm and nucleus. In this chapter, with the fibroblast system as a model, we provide protocols for techniques to localize signaling proteins and to measure directional cell migration that is dependent upon primary cillum signaling.

2. LOCALIZATION OF PRIMARY CILIA BY IMMUNOFLUORESCENCE MICROSCOPY

2.1. Fibroblast cultures and immunofluorescence microscopy analysis

NIH3T3 cells and MEFs form primary cilia at a frequency of 70–90% in cell cultures that are deprived of serum to induce growth arrest. Maximum ciliation usually occurs by 24–48 h of serum starvation. The cilia are detected by immunofluorescence microscopy (IFM) with antibodies against acetylated (Ac-tub) or glutamylated α-tubulin (Glu-tub), which are post-translational modification enriched in primary cilia (Pedersen, Schroder, Satir, & Christensen, 2012). The basal body of primary cilia is monitored by antibodies recognizing components of the centrosome, for example, centrin, pericentrin, and γ-tubulin. The nucleus is stained with 4′,6-diamodino-2-phenylinodole (DAPI). Double staining with antibodies to signaling or cytoskeletal proteins is employed for colocalization (Fig. 3.1A). A new method employs SEM together with IFM to localize proteins along the cillum without immunogold labeling (Fig. 3.1B).
2.1.1 Cell cultures

NIH3T3 fibroblasts and MEFs are grown in T75 tissue culture flasks at 37 °C, 95% humidity, and 5% CO₂ in growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM, NIH3T3 cells) or DMEM and F12-Ham in a 1:1 relationship (MEFs) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Under these conditions, the fibroblast doubling time is 22–24 h. The cells are maintained at maximum confluence of 80% and passaged every 3–4 days by trypsination. Growth arrest of experimental cells and cilia formation are induced by serum starvation where the growth medium is replaced with a serum-free

Figure 3.1 Visualization of primary cilia in cultures of growth-arrested fibroblasts. (A) Differential interference contrast (DIC) and immunofluorescence microscopy of primary cilia (arrows) using various combinations of antibodies directed against α-tubulin (tub), acetylated α-tubulin (Ac-tub), and glutamylated α-tubulin (Glu-tub) as well as pericentrin (Pctn) that localizes to the centrosome, that is, the ciliary base. F-actin was stained with phalloidin and nuclei were stained with DAPI. The lower left and the upper right panels show shifted overlays. (B) Correlative light and electron microscopy (CLEM) of a primary cilium, combining immunofluorescence images of Ac-tub localization (red) and nuclear staining with DAPI (blue) from the light microscope with scanning electron micrographs (SEM) with nanometer precision. Courtesy of Johan Kolstrup.

2.1.1 Cell cultures

NIH3T3 fibroblasts and MEFs are grown in T75 tissue culture flasks at 37 °C, 95% humidity, and 5% CO₂ in growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM, NIH3T3 cells) or DMEM and F12-Ham in a 1:1 relationship (MEFs) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Under these conditions, the fibroblast doubling time is 22–24 h. The cells are maintained at maximum confluence of 80% and passaged every 3–4 days by trypsination. Growth arrest of experimental cells and cilia formation are induced by serum starvation where the growth medium is replaced with a serum-free
equivalent after two washes with 37 °C phosphate-buffered saline (PBS) (Schneider et al., 2005).
1. Remove growth medium and carefully wash cells in 5 ml 37 °C PBS.
2. Remove PBS and add 1 ml trypsin–EDTA. Incubate until cells have rounded up and begun to detach.
3. Add 9 ml growth medium and gently pipet up and down a few times to ensure that all cells are in suspension.
4. Transfer a volume of cell suspension, adjusted to growth area, desired confluence and experimental day, to culture flasks or dishes with fresh growth medium.

2.1.2 Immunofluorescence microscopy
Unless otherwise stated, this procedure is carried out at room temperature. To ensure that cell structures are kept intact, it is important not to let the cells dry out at any time.
1. Remove starvation medium and wash the cells by adding 1.4 ml ice-cold PBS to each dish/well.
2. Remove PBS and fix the cells with the same volume 4% paraformaldehyde in PBS for 15 min.
3. Wash fixed cells twice in PBS and then permeabilize cell membranes with 0.2% Triton-X and 1% bovine serum albumin (BSA) in PBS for 12 min.
4. Quench cells with 2% BSA in PBS (blocking buffer) for 30 min and then transfer coverslips to humidity chambers consisting of H2O-soaked Whatman paper overlaid with parafilm in a Petri dish.
5. Incubate coverslips with 100 μl of primary antibodies diluted in blocking buffer for 90 min or overnight at 4 °C.
6. Wash cells 3 × 5 min in blocking buffer and then incubate with 100 μl fluorochrome-conjugated secondary antibodies in blocking buffer for 45 min. For visualization of the actin cytoskeleton, F-actin can be stained with fluorochrome-conjugated phalloidin concomitantly with secondary antibody incubation.
7. Wash again 3 × 5 min in blocking buffer and incubate cells for a few seconds with DAPI to stain nuclei.
8. Wash 3 × 5 min in PBS and mount the coverslips cell side down on ethanol-cleansed microscope slides in a small drop of mounting medium (glycerol with 10% of 10 × PBS and 2% N-propyl gallate). Press gently on coverslips to remove air bubbles and excess mounting medium, and seal the edges with nail polish. Store slides in the dark at maximum 4 °C.
9. Visualize antibody localization and cell structures with an epifluorescence or confocal microscope (Fig. 3.1A).
3. CELL MIGRATION AND ORIENTATION OF PRIMARY CILIA IN SCRATCH ASSAYS

3.1. Setting up the scratch assay

The scratch assay is a simple method and useful tool for analysis of cell migration in two dimensions (Nobes & Hall, 1999). Although fibroblasts in vivo rarely migrate in a plane, this assay benefits from being relatively easy to monitor and allows for investigation of cell behavior and ligand application under tightly controlled conditions. Differences between migration assays in one, two, or three dimensions have been discussed in Baker and Chen (2012) and Cukierman, Pankov, Stevens, and Yamada (2001).

1. Grow the cells to a monolayer on acid-cleansed coverslips in small Petri dishes/six-well trays (for IF analysis) or in T2 culture flasks (for live-cell imaging), and serum starve for 24 h (NIH3T3 cells) or 48 h (MEFs) to induce growth arrest and primary cilia formation.

2. Induce a scratch in the monolayer with a sterile 10-μl pipette tip and change the starvation medium to remove cell debris.

3. Allow the cells to recover in the incubator for 1 h before adding ligands and/or initiate monitoring, to enable pCO₂, pH, temperature, and humidity equilibration.

3.2. Live-cell imaging and analysis

In scratch assays, the migratory behavior of fibroblasts can be characterized with three parameters: translocation, speed of migration, and directionality. Translocation is a measure of sustained migration and reflects the net distance covered by a cell during the course of the experiment, that is, it is the distance between its starting and final position at the beginning and end of the experiment, respectively (Fig. 3.2A and B). It summarizes stochastic movements of the cells and contributions from directed migration induced by the wound-healing condition (Dieterich, Klages, Preuss, & Schwab, 2008; Dreval, Dieterich, Stock, & Schwab, 2005; Schwab et al., 2006). The smaller the time interval between two consecutive images is, the larger is the contribution of lamellipodial dynamics—that is, of actin dynamics—to the speed of migration. Since migration parallel to the wound is usually close to zero, the velocity of cells migrating into the wound is a measure of directionality in this experimental setup (Schneider et al., 2009).
1. Set up the scratch assay (Section 3.1) in T2 flasks.

2. After step 2 in Section 3.1, optionally add ligands and/or pharmacological modulators (50 ng/ml PDGF-AA, 10 μM 5-(N-ethyl-N-isopropyl)amiloride, various signal pathway inhibitors, or 10% FBS). Place the flask in a 37 °C heating chamber on the stage of an inverted phase contrast microscope equipped with 10× or 20× objective.

3. Immediately initiate monitoring with a video camera controlled by HiPic software (Hamamatsu) and capture images at 5-min intervals over 5 h.
5–24 h depending on the speed of wound closure. For data analysis, use Amira software (http://www.tgs.com) to mark the circumference of individual cells close to the wound edge at each time step throughout the entire image stack to obtain time-dependent cell outlines for further calculations (Dieterich et al., 2008; Dreval et al., 2005). Such segmentation also allows the investigator to evaluate the cells’ morphology (e.g., projected cell area, structural index; see Fig. 3.2A and B). Alternatively, the visually determined cell centers could be marked using ImageJ. It is our experience that automatic tracking of individual fibroblasts is not reliable in this experimental setup.

4. Determine $x$- and $y$-coordinates (in μm) and transfer these to Excel (Microsoft). Initially, quantify migration as movement of single cell centers by perceiving coordinates as geometric means of equally weighed pixel positions within cell outlines. Thus, the time sequence of the cell center $(x(t), y(t))$ represents the trajectory of the movement of a single cell in one experiment. Such trajectories can be normalized to a common starting point in order to depict the behavior of the respective cell population (Fig. 3.2C).

5. Calculate translocation as the mean distance between the position of each cell center at the beginning and at the end of the experiment for the observed group of cells.

6. Dissolve time-dependent cell center positions into numerical values of $x$- and $y$-coordinates and fit the means to functions of time $(x(t) = v_x \times t$ and $y(t) = v_y \times t)$. Estimate the velocities $v_x$ and $v_y$ (in μm/min) and their uncertainties with the least-square fit routine of gnuplot (http://www.gnuplot.info/).

### 3.3. Orientation of the primary cilium during cell migration

This method combines the scratch assay with IFM.

1. Set up the scratch assay (Section 3.1) on coverslips in small Petri dishes or six-well trays.

2. After step 2 in Section 3.1, allow the cells to migrate in the incubator and proceed with DIC microscopy and the IF protocol (Section 2.1.2) using antibodies against, for example, Ac-Tub or Glu-Tub. At the time of fixation, the cells should still be in the linear phase of migration (e.g., scratch closure of $\sim 50\%$), showing localization of the primary cilium in front of the nucleus and orienting toward the leading edge and parallel to the path of migration (Fig. 3.2D). Alternatively, prepare the dish with the scratch assay for SEM using a critical point dry procedure.
4. CELL MIGRATION AND CILIARY SIGNALING IN MICROPIPETTE ASSAYS

4.1. Setting up the micropipette assay

The goal of the micropipette assay is to provide a localized source of diffusible molecules, to image cells’ reaction to the chemical, and to measure or diagram responses. This method has been characterized for use with yeast orienting toward a source of mating factor (Segall, 1993), *Dictyostelium* streaming toward cyclic AMP (Segall & Gerisch, 1989), macrophage crawling to CSF, metastatic carcinomas (Bailly, Yan, Whitesides, Condeelis, & Segall, 1998), and with MEFs with primary cilia orienting toward a PDGF-AA gradient (Schneider et al., 2010) in addition to other systems.

The Eppendorf FemtoJet is an instrument of choice because of the ease of operating the XYZ micromanipulation integrated with the regulated pressure supply. A less expensive alternative would be a manual XYZ micromanipulator and a syringe pump. Eppendorf Femtotips II capillaries or similar ones pulled with a Sutter P97 micropipette puller can be used.

For imaging, phase contrast microscopy provides superior imaging of adherent cells, but may be substituted with other light microscopy techniques such as Nomarski or bright field.

Environmental control is important for proper cell physiology. Options range from a fully enclosed environmental chamber with humidity and gas control to simple heated stage. In the work reported in Schneider et al. (2010), a heated stage and buffered culture media (HEPES or L15) proved sufficient.

Other standard concerns of live-cell microscopy apply. For instance, some cell types may be light sensitive. Light sensitivity may be avoided by having an electronic shutter only allow light to the sample during camera exposures, keeping the intensity low and using longer or high-gain camera exposures, and by putting heat filters in the light path or using narrow wavelength LEDs that do not emit UV or heat. For fibroblasts, a simple green interference filter with halogen illumination should be sufficient.

1. In advance, plate cells in dishes. Cells behave differently depending on the substrate. The bottom of the dish, therefore, needs to be both physiologically relevant and thin enough to use with the microscope optics.

2. To make fibroblasts or other cultured cells grow primary cilia, cells are usually serum starved or media changed to buffer as appropriate and
placed in a non-CO$_2$ incubator. This assay works best when cells are used before confluency so that individual cell response can easily be followed. Check microscopically.

3. Choose a suitable ligand to load into the microneedles for the experiments. Examples that could be instructive for primary cilia signaling include PDGF-AA, Sonic Hedgehog, somatostatin, serotonin, leptin, etc. and are dependent on the cells in culture. Previous study should suggest an optimum concentration of response. For gradient production, approximately 10 $\times$ concentration should be loaded. A small fluorescent dextran (for instance, FITC–dextran at 100 $\mu$g/ml) may be included to visualize diffusion from the needle tip by epifluorescence microscopy.

4. Solution should be filtered (0.2 $\mu$m) or spun to remove particles that can clog the micropipette tip.

5. Fill a micropipette with the ligand–dye solution. There are flexible plastic microloader tips made by Eppendorf which fit on a standard PipetMan to deliver 1–5 $\mu$l to the glass capillary. Carefully insert the microloader tip into the micropipette (Eppendorf Femtotip needle) opening from the large open side. Insert the microloader all the way down to the tip of needle and then gently release the ligand–dye solution as you move the tip of the microloader up and away from the bottom of needle. Avoid creating bubbles which could block the flow of solution.

6. Attach the needle to a tube apparatus that will connect to the FemtoJet pump or similar.

7. If at any time you think that maybe the pulled end of the needle might have touched something, then it probably did, is broken, and you should prepare a new one.

8. Set the flow, making sure that there is pressure inside the needle. Check that the needle is not clogged and that the solution is completely filling the needle (Fig. 3.3A).

9. Set the microscope objective to 10 $\times$ or other magnification dependent on the size of cells, the field size required, and the spatial resolution needed.

10. The dish of cultured cells with/without primary cilia is placed on microscope and transmitted illumination Kohler aligned.

11. Find a field of cells to image.

12. Begin the time lapse. This initial imaging will provide a baseline of cell motility. Intervals and total length are experiment dependent.
Figure 3.3 Chemotaxis of growth-arrested fibroblasts in the micropipette assay. (A) Testing of the micropipette needle with a dye solution to ensure that the tip is not clogged (upper panel) or that the bore is not too large (lower panel). (B) PDGF-AA-mediated chemotaxis of cells toward a gradient of PDGF-AA coming from the tip (circle) of the micropipette needle monitored by time-lapse video microscopy (upper and middle panels). In the lower panel, green dots represent cells moving toward the tip of the micropipette and red dots illustrate cells not moving toward the tip during the 4-h time period of the experiment. (C) The upper panel shows a simulated color “temperature” map for all cells moving uniformly toward the tip of the micropipette, for example, the source of the PDGF-AA. The middle panel shows an actual color “temperature” map for cells migrating toward the gradient of PDGF-AA after the 4-h time period of the experiment. The lower panel shows the map for cells in a setup where PDGF-AA was replaced with buffer in the micropipette. Panels (B) and (C) were reproduced from Schneider et al. (2010) with permission from S. Karger AG Basel.
Figure 3.3B shows selected frames of a time-lapse image showing the position of cells at the beginning of the experiment (upper panel) and after 4 h of migration toward the needle (middle panel).

13. Bring the tip of the needle to the center of the transmitted light.
14. Carefully lower the needle into the dish. The needle must be under pressure before dropping into the culture media.
15. Steer needle into field and lower to the bottom of the dish without crashing into the substrate.
16. Fluid coming out of needle should be visible by transmitted light or if a dye has been added, by fluorescence.
17. Cell culture grade mineral oil may be poured over the top of the media to prevent evaporation.
18. It may be necessary to slightly misalign the condenser to adjust for the meniscus formed at the top of the media where the needle is inserted.
19. Image for the time needed. Intervals and total length are experiment dependent.

4.2. Live-cell imaging and computational analysis

In the pipette assay, the migratory behavior of fibroblasts or other cells can be characterized with multiple parameters but the most relevant here are translocation and persistent migration. This is essentially the same as in the scratch assay, but here the parameter is motion converging on a single point or small region.

For a quick and simple report of movement, ImageJ has a stack projection method that calculates the standard deviation of pixel values through time. In the resultant image, stationary features mostly disappear and features that change are highly contrasted.

Color “temperature” maps (Schneider et al., 2010) showing the average movement of populations of cells can be generated based on the initial and final position of each cell. Mark starting and ending positions of all cells that remain in the field for the entire duration of the recording and are not stationary (defined as remaining within a 30-pixel radius). This produces a list of trajectories with movement toward or away from the pipette indicated (Fig. 3.3B, lower panel). Based on the trajectory of each cell, a color “temperature” map can be generated to express the average probability of cells traveling to all locations in the field and this can be overlaid on the actual location of the pipette (Fig. 3.3C). Control maps where each cell is moving randomly can also be generated similarly. These functions are coded in ImageJ macros.
Whereas these temperature maps provide excellent visualization, Imarus, Volocity, Amira, MatLab, various plugins for ImageJ and Fiji, or other software can also be used to quantify movement. If cells are not touching, these software systems may automatically compute paths. Also, these software systems are able to calculate more granular data on persistence, periodic motion, and morphology. In a comparison test, Imarus, Volocity, and ImageJ report equivalent results. The analysis at the end of Section 3.3 may be applied here. Each software has its own step-by-step protocol.

5. SUMMARY

The methods described above for (1) immunofluorescence localization of primary cilia in fibroblast cultures; (2) their orientation with respect to the cell axis during directional migration; (3) setting up and utilizing scratch assays to measure directional cell migration in wound healing; (4) studying cell signaling and response with micropipette assays; (5) live-cell imaging; and (6) analysis of individual cell and population migration parameters, have all been successfully tested. Figures 3.1–3.3 provide selected results. The methods are readily reproducible and can easily be adapted to other instrumentation and computer analysis programs, some of which have been indicated, as well as to other cell culture systems to study the generation and persistence of primary cilia and their role in directional cell movement and in signaling.

ACKNOWLEDGMENTS

This work was supported by The Lundbeck Foundation, including a visiting Professorship to P. S., The Danish National Science Research Council, and the Novo Foundation (S. T. C.).

REFERENCES


Directional Cell Migration and Chemotaxis in Wound Healing Response to PDGF-AA are Coordinated by the Primary Cilium in Fibroblasts

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Key Words
Fibroblasts • Cell migration • Primary cilia • Wound healing • PDGFRα • PDGF-AA

Abstract
Cell motility and migration play pivotal roles in numerous physiological and pathophysiological processes including development and tissue repair. Cell migration is regulated through external stimuli such as platelet-derived growth factor-AA (PDGF-AA), a key regulator in directional cell migration during embryonic development and a chemoattractant during postnatal migratory responses including wound healing. We previously showed that PDGFRα signaling is coordinated by the primary cilium in quiescent cells. However, little is known about the function of the primary cilium in cell migration. Here we used micropipette analysis to show that a normal chemosensory response to PDGF-AA in fibroblasts requires the primary cilium. In vitro and in vivo wound healing assays revealed that in ORPK mouse (IFT88Tg737Rpw) fibroblasts, where ciliary assembly is defective, chemotaxis towards PDGF-AA is absent, leading to unregulated high speed and uncontrolled directional cell displacement during wound closure, with subsequent defects in wound healing. These data suggest that in coordination with cytoskeletal reorganization, the fibroblast primary cilium functions via ciliary PDGFRα signaling to monitor directional movement during wound healing.

Introduction
Directional cell migration plays a critical role in embryonic development and in maintenance of tissue homeostasis. Migration relies heavily on the concerted action of chemosensory stimuli; the dynamic
reorganization of the cytoskeleton, particularly the actin cytoskeleton [1-6]; the formation and release of cell-matrix contacts and the local ion homeostasis across the plasma membrane [7, 8]. Migration is directed when a chemotactic gradient is imposed onto the cells. Platelet-derived growth factors (PDGFs) are important chemosensory regulators of the migratory response during development and in wound healing [9, 10]. PDGFs are essential chemotacticants that promote distribution of oligodendrocyte progenitors throughout the developing CNS in vivo [11, 12], while PDGF receptors (PDGFRs) are strongly expressed in the neural crest mesenchyme and are mandatory for spreading/migration of various populations of cells [11-13]. Additionally, PDGFs are significant chemotactants during postnatal migratory responses such as in wound healing [14]. As part of the mechanisms that control speed and directionality of migrating cells, the Na+/H+-exchanger, NHE1, which is

In NIH3T3 fibroblasts and mouse embryonic fibroblasts (MEFs), PDGFRβ is localized at the cell membrane [20]. In fibroblasts, PDGFRβ signaling operates in part via the Nck family of Src homology (SH) 2/SH3 domain adaptors to regulate downstream modulators of actin dynamics [2, 4]. Nck adaptors are required for cytoskeletal reorganization and chemotaxis stimulated by PDGF-BB. Nck-deficient cells fail to display cytoskeletal rearrangements, including the formation of membrane ruffles and the disassembly of F-actin, typically shown by their wild type (wt) counterparts in response to PDGF-BB. Other proteins, such as Akt and Rac-1, controlling actin polymerization, bundling and disassembly, are also influenced by PDGFRβ signals [3, 5, 6].

PDGFRα signaling, along with PDGFRβ signaling, regulates cell migration [10]. PDGF-AA acts exclusively through the PDGFRα homodimer, PDGFRαα. We previously showed that during growth arrest (in the Go/G1 phase) in fibroblasts, PDGFRα expression is up-regulated and the receptor is targeted to the primary cilium where ligand-dependent activation of the receptor and the Mek1/2-Erk1/2 pathway occurs, indicating that PDGFRα-mediated signaling via the PDGFRα homodimer in cell cycle entry is coordinated by the primary cilium [20].

The primary cilium is a microtubule-based organelle that emanates from the mother centriole into the extracellular environment as an antenna-like structure. In most cultured cells, primary cilia emerge during growth arrest, i.e., at Go/G1, following centrosomal docking to the plasma membrane. In most cases, the cilium is disassembled in late G2, so that the engaged centrioles are available for mitotic spindle formation [21-23]. In the present work, we have used NIH 3T3 cells and primary cultures of MEFs, which we previously showed form primary cilia only during growth arrest after serum starvation for 24-48 h, and cilia are absent in subconfluent cells grown in the presence of serum [20]. As a control we used fibroblasts from the ORPK (IFT88Tg737Rpw) mouse, which we will refer to as Tg737 MEFs. Tg737 encodes the protein polaris/IFT88, which is part of the intraflagellar transport (IFT) protein complex responsible for assembly and maintenance of the primary cilium [24]. Consequently, Tg737 MEFs form no or very short cilia [20]. A single primary cilium contains many different signal transduction systems in order to carry out diverse signaling processes during development and in tissue homeostasis [25], and it is likely that the composition of signal systems closely reflects the functionality of the cell type in different tissues, i.e., that some ciliary signal systems are tissue specific. Some signaling interactions can be unique features of primary cilia on fibroblasts or mesenchymal cells, versus cilia that protrude from the apical surface into a lumen as seen on epithelial and endothelial cells. Emerging evidence indicates that cell migration is directly or indirectly related to primary cilium assembly and/or ciliary signaling [26]. Originally, Albrecht Buehler discovered that primary cilia in migrating 3T3 fibroblasts were oriented predominantly in parallel to the substrate and to the current movement direction [27]. In various cell systems previous work has shown that the centrosome and Golgi apparatus come to lie in front of the nucleus and towards the direction of eventual cell migration [28-32]. In a more detailed study, Katsumoto et al. demonstrated that reorientation of primary cilium together with the centrosome and stable cytoplasmic microtubules in 3Y1 rat cells occurs prior to initiation of migration [33]. They proposed that the direction of migration is determined by the orientation of the centrioles, which is controlled by the primary cilium. More recently, in vitro wound healing assays provided other examples of orientation of primary cilia towards the leading edge in cultures of smooth muscle cells [34, 35].

Here we show that in response to PDGF-AA, migration of quiescent fibroblasts in culture is regulated by their primary cilium, such that cells from wt mice with
normal primary cilia show chemotaxis towards a PDGF-AA gradient with an increase in migration speed and directional cell movement in wound closure in vitro. During migration the primary cilium is oriented parallelly to the direction of migration, often pointing towards the leading edge of the migrating cell. In contrast, Tg737 MEFs do not show chemotaxis, nor do they respond to PDGF-AA by regulating speed or direction during wound closure. Further, Akt is phosphorylated at the base of the primary cilium in wt MEFs in the presence of PDGF-AA, but this is blocked in mutant MEFs. The ORPK mice show a reduced rate of wound repair in vivo, but this is not blocked in ORPK MEFs. PDGF-AA, but this is blocked in mutant MEFs. The ORPK mice show a reduced rate of wound repair in vivo. These results indicate that signaling through the primary cilium, probably continuing via the PI3 kinase-Akt pathway, activates directional migration of tissue fibroblasts. In coordination with actin and microtubule cytoskeletal reorganization mediated through PDGF or other cell membrane-based signaling pathways, signaling through the primary cilium is necessary for the sensing of a PDGF-AA-mediated chemotactic gradient in wound healing.

Materials and Methods

Cell culturing
NIH3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FBS) and 100 U/ml penicillin-streptomycin at 37°C, 5% CO₂, 95% humidity. Primary cell cultures of Mouse Embryonic Fibroblasts (MEFs) from wt and Tg737 mice were grown in 45% DMEM and 45% F12-ham supplemented with 10% FBS and 10ml 1-1 penicillin-streptomycin. Cells were serum starved for 48h to induce growth arrest. About 90% of the cells were ciliated after serum starvation. In some experiments cells were stimulated with 50ng/ml PDGF-AA (R&D Systems, 221-AA).

Immunofluorescence (IF) Microscopy
Cells were grown on glass cover slips to 100% confluence, serum starved for 48h and fixed in 4% formaldehyde or methanol [36], permeabilized in 0.2% triton X100, quenched in PBS with 2% BSA, and incubated with primary antibodies at room temperature for 2h. Cells were washed in PBS and incubated with secondary antibodies for 1h. Fluorescence was visualized on Microphox-FXA and Eclipse E600 microscopes (Nikon, Tokyo, Japan). Primary antibodies: Primary cilia were detected with mouse acetylated alpha-tubulin antibody (1:5000 Sigma, T6793) or anti-detyrosinated-tubulin (glu-tub, 1:100, Abcam, AB3201); phospho-Akt (1:5000, Cell Signaling, 587F11). Secondary antibodies: (1:600, GARα + β [338]; A11070, GAMα + β [348]; A11019 Molecular Probes). Nuclei were stained with DAPI (Molecular Probes, D1306).

SDS PAGE, Immunoprecipitation and Western Blotting Analysis
Cells were grown in petri dishes and washed in PBS, 150 µl 0.1% SDS lysis buffer was added and cells were scraped off and transferred 10 times through a 27 gauge needle, followed by centrifugation at 16.000xg. The protein concentrations were calculated using a BCA protein kit (Pierce, 23209). Rabbit anti-PDGFRα was added to lysates for IP in RIPA buffer with no SDS and incubated overnight at 4°C. Protein A- and G-conjugated sepharose (1:1) equilibrated in RIPA was added and incubated for 2 h at room temperature. The beads were washed with RIPA and the precipitate was dissolved in sample buffer. Proteins from whole cell lysates and immunoprecipitates were separated by SDS PAGE on 10% NuPAGE Bis-Tris gels using NuPAGE MOPS SDS running buffer (NP0002), Fermentas protein standards and Novex XCell (E19001) system, and electrophoretically transferred to nitrocellulose membranes using XCell II blot module (Novex). Membranes were blocked for 2h at room temperature prior to incubation with primary antibodies in blocking buffer over night at 4°C: anti-PDGFRα (1:600, Santa Cruz, sc-338); anti-phospho-PDGFRα Y754 (1:200, Santa Cruz, sc-12911-R), anti-Akt (1:500, Cell Signaling, 9272); anti-phospho-Akt (1:200, Cell signaling 587F11) and anti-Phospho-Tyr (1:200, Santa Cruz, sc-12911-R), anti-β-actin (1:10,000, SigmaAldrich, A5441). Primary antibodies were detected using alkaline phosphatase-coupled secondary antibodies in blocking buffer for 1h (GAR & GAM 1:1200, A3937 & A12293) and visualized with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution, BCIP/NBT (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The developed blots were scanned and band intensity was estimated from arbitrary densitometric values obtained using UN-SCAN-IT software. The data were tested for significance using analysis of variance (ANOVA) or Kruskal Wallis Test (nonparametric ANOVA). The level of significance was set at p< 0.05; (***): p<0.001; (**): p<0.01.
Wound healing assays

Cells were grown to confluence in growth media and serum starved for 48 h. A wound was made using a pipette tip and the culture medium was changed to fresh serum-free medium, and the cells were allowed to recover for 1 h in the incubator after the scratch was made. Subsequently, the cells were incubated with and without 50ng/ml PDGF-AA or 10% FBS and placed in a heating chamber (37°C) on the stage of an inverted microscope (Axiovert 25 or Axiovert 40C; 10x or 20x; Zeiss, Oberkochen, Germany). Migration was monitored for ~4 h with a video camera (Hamamatsu, Hersching, Germany) controlled by HiPic software (Hamamatsu). Images were taken at 5 min intervals and stored as stacks of Tiff-files. The circumferences of individual cells were marked at each time point through the entire image stacks with Amira software (TGS, France; http://www.amiravis.com/) as described [37, 38]. These segmentation data were used for further analysis. Migration was quantified as the movement of the cell centre per time unit. All experiments were repeated at least three times and data in graphs are presented as the mean values +/- S.E.M. The data were tested for significance using analysis of variance (ANOVA) or Kruskal Wallis Test (nonparametric ANOVA). The level of significance was set at p< 0.05; (**): p<0.01; (***): p<0.001. Symbols: (O) marks the tip of the micropipette.

Micropipette Analysis

Cells were grown to 30-40% confluence and serum starved for 48 h. Chemotaxis experiments were performed by continuously ejecting small amounts of PDGF-AA from a micropipette (0.5 µg/ml in the pipette) to create a gradient in the vicinity of growth-arrested wt and Tg737orpk MEFs. Cell movement in the presence of a PDGF-AA gradient was monitored with time lapse video microscopy, taking images at 5 min intervals. For system specifications please see: http://www.aecom.yu.edu/aif/instructions/ccd4/index.htm. A Femtojet Micromanipulator 5171 (Eppendorf-Brinkman Instruments) and a pump (model Femtojet; Eppendorf) were used to control the position of the micropipette and the pressure required for the chemoattractant flow. Femtotip II micropipettes (defined opening with 0.5 µm inner diameter and 0.7 µm outer diameter ±0.1 µm) as per http://www.eppendorfna.com/products/ECET_tips_de.asp) were positioned within 1 µm of the cover slip and pressure set at 30 to 45 hPa [39]. The data were tested for significance using ANOVA or Kruskal Wallis Test: (**): p<0.01; (***): p<0.001. Symbols: (O) marks the tip of the micropipette.

In vivo Wound Healing of Wt and ORPK (IFT88αI737Rpw) mutant mice

P15 ORPK mutant (mt) mice and corresponding wild type (wt) littermates were wounded via 4 mm full thickness punch biopsy and assessed for rate of wound closure by secondary intention. Wound closure was monitored by digital imaging or by using digital calipers (Fisher). Wound edges and areas were analyzed using Image J (Find Edges) algorithm and Image J scale and measurement tools or by calculating the wound ellipse area from caliper measurements. Significance was tested using a two-tailed student’s t-test. Wound closure was defined as no scab or a scab < 2mm². All animals in this study were maintained in AALAC certified mouse facilities at UAB and in accordance with IACUC regulations and protocols at the University of Alabama at Birmingham. The data were tested for significance using analysis of variance (ANOVA) or Kruskal Wallis Test (nonparametric ANOVA). The level of significance was set at p< 0.05; (**): p<0.001; (***): p<0.001.

Results

Orientation of primary cilia during in vitro wound healing

To investigate the role of the primary cilium in cell migration, we initially studied the orientation of the cilium during wound healing in cell cultures. Scratch assays were performed followed by immunofluorescence analysis on cultures of confluent, growth arrested NIH3T3 fibroblasts (Figure 1A). We found that in serum-free medium primary cilia in the first row of cells facing the wound often oriented towards the wound within 30-60 min after the scratch was made (Figure 1B). The primary cilium emanates from the centrosome that was found predominantly in front of the nucleus (Figure 1B, inset). Stable cytoplasmic microtubules marked with Glu-tub were also detected towards the leading edge (Figure 1B, inset). Occasionally, cilia were observed pointing towards the trailing edge, albeit still oriented parallelly to the direction of migration. Similar observations were reported in 3T3 cells [27], in 3Y1 cells [33], and in cultures of smooth muscle cells (SMC) [34, 35]. In contrast, primary cilia on non-migrating cells in subsequent rows were oriented in all directions and not specifically towards the wound (Figure 1B). IA analysis was performed to show GFP-tagged PDGFRα localization to the primary cilium in cultures of growth arrested MEFs (Figure 1C). This subcellular distribution is similar to that of the endogenous PDGFRα detected with antibodies [20].

Primary cilia coordinate PDGF-AA-mediated migration speed and directionality

Next we determined the migratory speed and displacement of growth-arrested cells in scratch assays in the presence and in the absence of PDGF-AA, the specific ligand for dimerization of PDGFRα and activation of the homodimeric receptor [20, 40]. In these experiments we used primary cultures of mouse embryonic fibroblasts (MEF); wt MEF and Tg737orpk MEF. In the absence of ligand, Tg737orpk MEFs had a significantly higher migratory speed than wt MEFs.
Fig. 1. Primary cilia orient in the direction of cell movement during wound healing. NIH 3T3 cells were grown to 100% confluency on glass cover slips and serum starved for 24-48h. Wounds were made in confluent layers of growth-arrested cells using a fine pipette tip (A). Immunofluorescence (IF) was used to detect orientation of the primary cilia pointing in the direction of cell migration (asterisks) during wound healing in the first row of cells facing the wound. Primary cilia were detected using mouse acetylated alpha-tubulin primary antibody (B). Insets in figure 1B show the orientation of the primary cilium detected with rabbit detyrosinated tubulin antibody (Glu-tub (green), asterisk) emerging from the centrosome detected with goat pericentrin antibody (Pctn, red) pointing towards the leading edge of a migrating cell (stippled line). The nucleus was stained with DAPI (blue) (B, insets). The shorter arrows in inset B show stable microtubule structures that point in the direction of the leading edge. Ciliary localization of GFP-tagged PDGFRα in cells serum starved to induce growth arrest and formation of primary cilia (C) [20]. The inset shows a high resolution image of GFP-PDGFRα in the cilium (shifted overlay). Primary cilia were detected with mouse acetylated alpha-tubulin antibody (tb (red), arrows) using IF microscopy.

Fig. 2. Primary cilia regulate PDGF-AA-mediated migratory speed and directional cell movement. Scratch assays were performed on confluent layers of serum starved wt and Tg737orpk MEFs serum with and without PDGF-AA or 10% FBS. Migration was monitored for ~4 h for calculation of migration speed (µm/min) and cellular displacement (µm) with and without PDGF-AA (A & B). The displacement is the distance between the cells’ positions at the beginning and at the end of the experiment (B). Cell migration for growth-arrested wt and Tg737orpk MEFs was also illustrated by trajectories (C & D).

The trajectories of 20-30 cells for each condition were normalized to common starting points. Each track represents the movement of one cell during the 4h period described in A & B. The speed (E) and displacement (F) of wt and Tg737orpk MEFs was also monitored in the presence of either PDGF-AA or serum relative to control cells. (p<0.01); however, the mutant cells were unresponsive to PDGF-AA (2A). In contrast, the migratory speed of wt MEFs, initially lower than that of Tg737orpk MEFs, increased by about 40% (p<0.001) to a level higher than...
that of Tg737orpk MEFs (p<0.01) upon PDGF-AA addition (Figure 2A). Figures 2C & 2D show trajectories of growth arrested MEFs in the presence and absence of PDGF-AA. All trajectories are normalized to a common starting point and the radii of the red circles represent the mean distances covered within appr. 4 h. These data are summarized in Figure 2A. The mean displacement of wt MEFs increased three-fold upon incubation with the ligand (p<0.01), whereas mutant cells were unaffected by addition of PDGF-AA. In non-arrested cells, there were no differences in migratory speed or displacement between mutant and wt cells.

To investigate whether migration in serum starved and growth-arrested wt and Tg737orpk MEFs can be...
**Fig. 4.** PDGF-AA signals through the primary cilium and activates Akt at the ciliary basal body in the centrosome. Western blotting analysis of phospho-PDGFRα (phosphorylated at tyrosine in position 754) and phospho-Akt upon PDGF-AA stimulation in wt and Tg737ortpk MEFs (A). Quantification of Akt phosphorylation in wt and Tg737ortpk MEFs before and after stimulation with PDGF-AA (B), shows that the level of Akt phosphorylation increases approximately 7-fold in wt MEFs (p<0.01). Figure 4C shows PDGFRα activation in wt and Tg737ortpk MEFs. Immunoprecipitated PDGFRα was stimulated for 10 min with 50 ng/ml PDGF-AA, and the level of phospho-receptor was detected with anti-phosphotyrosine antibody (p-Tyr). (D) IF analysis on the localization of phospho-Akt (second panel, red) before and after three minutes stimulation of growth-arrested wt and Tg737ortpk MEFs with 50 ng/ml PDGF-AA. The primary cilium was stained with anti-detyrosinated-tubulin (glu-tub (first panel, green), arrows), which marks both the primary cilium and the two centrioles [24] (asterisks). The third panel of inserts shows the merged images.

**Fig. 5.** Wound Healing Defects in ORPK (IFT88Tg737Rpw) mutant mice. P15 ORPK mutant (mt) mice (A) and corresponding wild type (wt) littermates (B) were wounded and assessed for rate of wound closure by secondary intention. All wt animals achieved complete wound closure by day 7 post wounding (B, right panel). In contrast, most ORPK mutants did not have wound closure by this time period (A, right panel). The effect was further quantified by measuring the rate of closure measured at 3 and 7 days post wounding. (C) shows average wound closure rates (mm²/hour) during seven days of wound healing of five wild type and four mutant mice. The average wound closure rate was found to be significantly reduced in mutant cells (p<0.05). (D) shows average percentage of wound area remaining three and seven days after wounding relative to day of wounding (day 0). The percentage of wound area remaining was found to be significantly reduced in only wild type cells (p<0.01).

stimulated by signals other than PDGF-AA, we performed scratch assays re-adding serum as a chemoattractant (Figures 2E and 2F). While wt cells responded equally well to PDGF-AA and serum, only serum increased the speed and displacement of mutant cells. The relative levels of response of mutant cells is lower than that of wt
cells, since mutant cells have a higher initial migratory speed and displacement than wt without stimulus (Figure 2A & 2B). The migratory speed of wt and mutant cells in the presence of serum was identical (0.21±0.01 and 0.21±0.02 μm/min, respectively), and the displacement in the presence of serum was not significantly different (49.49± 3.59 and 51.84± 5.66 μm).

**PDGF-AA mediated chemotaxis is controlled by the primary cilium**

In order to directly investigate the role of fibroblast primary cilia in coordinated cell movement directly, we analysed the significance of the cilium for chemotaxis towards gradients of PDGF-AA. The gradient was established by continuously ejecting the ligand from a micropipette placed in the vicinity of the cells. We used growth-arrested cells at a confluence of ca. 50%, so that the cells could move in any direction along the surface of the culture dish. We observed that wt MEFs, which had primary cilia under these conditions (Figure 3A), were strongly affected by the gradient of PDGF-AA and moved directly towards the pipette tip. In sharp contrast, Tg737orpk MEFs, which have significantly shortened non-functional primary cilia (Figure 3A), did not respond to the gradient, and continued to move randomly (Figure 3B & 3C). Figure 3C summarizes the movement of individual wt or Tg737orpk cells monitored during a 4 h period. Starting and end-point positions of each cell are indicated by the white line and the green/red dots, respectively. Green dots represent the end-point of cells that moved directly towards the gradient; red dots label cells not moving towards the pipette. Before addition of PDGF-AA, Tg737orpk MEFs moved with a high frequency of “running on spot” with a high turnover of ruffling and formation of lamellipodia in different directions, but little cell displacement. A similar pattern of movement was observed for wt cells albeit at a lower frequency.

To visualize the overall patterns of migration in a systemic way, we developed a colorimetric analysis (Figure 3D). The coordinates of cell movement are converted into a color scale that shows the level of coordinated migration in the presence of a stimulus gradient. For comparison, we simulated the color scale for all cells moving uniformly towards the source of a chemoattractant (Figure 3E). This color scale was used as reference for our experimental analysis on migration of wt and Tg737orpk MEFs. In the presence of a PDGF-AA gradient, cells in cultures of wt MEFs produced a color scale similar to that of the reference (Figure 3F, upper left panel), whereas cells in the absence of the PDGF-AA gradient produced a scale visibly different from the reference, with no uniform migration towards the tip of the pipette (Figure 3F, lower left panel). Tg737orpk MEFs, both in the presence and absence of the PDGF-AA gradient, produced a color scale almost identical to that of wt MEFs with no gradient of PDGF-AA (Figure 3F, right panels). In sharp contrast to cells in cultures of Tg737orpk MEFs, where essentially no cells moved towards the gradient of PDGF-AA (p<0.001), quantitatively, about 50% of the cells in cultures of wt MEFs (p<0.01) moved uniformly and directly towards the gradient of PDGF-AA (Figure 3G).

**PDGF-AA signals through Akt at the ciliary base**

To explore the mechanism by which ciliary signaling via PDGFRα affects the speed and direction of migration, we investigated the activation of the receptor and the Akt-pathway upon stimulation with PDGF-AA in wt and Tg737orpk MEFs. Western blot analysis showed that PDGFRα and Akt are activated, with the level of Akt phosphorylation increasing about 7-fold in wt MEFs (Figure 4A & 4B). This increase was absent in Tg737orpk MEFs. PDGFRα is a growth arrest specific protein in fibroblasts [41], and we previously demonstrated that up-regulation of PDGFRα in quiescent cells is inhibited in Tg737orpk MEFs [20]. In order to investigate whether lack of activation of the receptor and Akt in Tg737orpk MEFs is due to lower levels of PDGFRα, equal amounts of PDGFRα were analyzed after immunoprecipitation from PDGF-AA-stimulated wt and Tg737orpk MEFs. Activation of the receptor occurred only in wt cells (Figure 4C). Then we used immunofluorescence microscopy to examine the localization of increased Akt phosphorylation in wt MEFs after PDGF-AA stimulation. We show that the level of phospho-Akt increases at the base of the cilium, i.e. at the mother centriole (Figure 4D). There is little or no increase in background staining of phospho-Akt in the cytoplasm.

**Wound Healing defects in ORPK (IFT88Tg737Rpw) mutant mice**

The in vitro data suggest that cilia have important in vivo roles in regulating wound healing. To assess this possibility, we conducted small punch biopsy wound healing assays in wild type and IFT88Tg737Rpw (ORPK) mutants from which the MEF cells described above were derived (Figure 5). The wounds formed fibrin clots at approximately the same time in both mutants and

Schneider/Cammer/Lehman/Nielsen/Guerra/Veland/Stock/Hoffmann/Yoder/Schwab/Sati/Christensen
wild types, suggesting that there was no defect in platelet clotting or subsequent PDGF release. However, Tg737orpk animals analyzed had defects in wound closure, with wounded ORPK mutant animals being unable to achieve a significant wound closure by seven days post wounding (Figure 5A & 5B). This effect was quantified by measuring the rate of closure, where the average wound closure rate in mm² per hour during seven days after wounding was significantly reduced in mutant cells ($p<0.05$) (Figure 5C). Further, the average percentage of wound area remaining was found to be significantly reduced only in wild type cells ($p<0.01$) (Figure 5D).

**Discussion**

*Primary cilia orient in the direction of wound healing*

We have investigated the orientation of primary cilia during wound healing *in vitro* and the role of primary cilia in PDGF-AA mediated cell migration. We show that the primary cilium during migration orients predominantly in parallel to the direction of migration, and often towards the leading edge when the centrosome lies in front of the nucleus. Similar observations were made in a variety of other cell types [27, 33-35], indicating that ciliary reorientation is a general phenomenon of the migratory response in growth arrested cells, in which reorientation of the centrosome towards the leading edge seem to occur upon wounding before the cells move [34, 42]. In fibroblasts the primary cilium may therefore move with its basal body, projecting forward as the nucleus moves rearward and the basal body tilts [35, 42]. Whether the primary cilium is the follower of the centrosome or vice-versa, and the exact mechanisms underlying these processes remain to be elucidated. However, these observations support the model that orientation of the primary cilium is linked to cellular mechanisms that control directional cell migration. Presumably, this orientation is controlled in concert with not only centrosomes but also the dynamic reorganization of the cytoskeleton as demonstrated by Katsumoto et al. in 3Y1 rat cells [33]. As will be discussed in the following, formation of a primary cilium from the centrosome in growth-arrested cells may act as a unique sensory site from which changes in environmental cues are relayed to the centrosome to control the migratory response.

*Primary cilia control PDGF-AA mediated migration speed, directionality and chemotaxis in mouse fibroblasts*

We further investigated the migratory speed and directionality of growth-arrested wt and Tg737orpk MEFs in the presence and absence of PDGF-AA in scratch assays. We showed that PDGF-AA significantly increases both migration speed and displacement of growth arrested wt MEFs. In contrast, Tg737orpk MEFs were unaffected by PDGF-AA, suggesting an essential role of primary cilia in PDGF-AA-mediated regulation of the migratory response. Without the ligand, however, growth arrested mutant cells generally have a higher speed and subsequently higher displacement than their wt counterparts. These results imply that loss of the cilium, and potentially the lack of a cilium orienting parallelly to the direction of migration in quiescent fibroblasts, leads to a lack of regulatory control of the migratory speed and displacement of cells; this is a characteristic of fibrosis and tumor cell invasion. However, in the absence of PDGF-AA, PDGFRA-signaling is minimal in both wt and Tg737orpk MEFs [20], indicating that the observed increased migration speed of mutant cells is not related to aberrant PDGFRA-signaling. In non-arrested cells, there were no differences in migratory speed or displacement between mutant and wt cells, suggesting that PDGF-AA signaling is a key regulator of cell migration specifically during cellular growth arrest. We further demonstrated that both quiescent wt and Tg737orpk MEFs can be stimulated equally by serum. These data show that quiescent cells lacking the primary cilium are able to respond to factors other than PDGF-AA, but that signaling through the cilium plays a major role in PDGF-AA-mediated migration. This is an important observation since PDGF-AA is an essential mediator during development and in the early processes of wound repair *in vivo* [10, 14, 43-45].

In order to measure the function of the primary cilium in the chemotactic response to PDGF-AA, we performed micropipette analysis in serum-starved cultures of MEFs at a low confluency. This allows the cells to move in any direction in two dimensions in the culture dish. Wt cells moved directly towards the gradient of PDGF-AA, whereas mutant cells moved randomly and unaffected by PDGF-AA. These data illustrate an essential role of the primary cilium in PDGFRA-mediated cell migration and directional cell movement in quiescent fibroblasts. The overall displacement...
of mutant MEFs in the presence of PDGF-AA was reduced relative to wt MEFs but was characterized by a high frequency of "running on spot", as evidenced by a high turnover of ruffling and formation of lamellipodia in different directions, exactly as if PDGF-AA had not been added. Thus, the observed increase in both migration speed and displacement of confluent mutant MEFs in scratch assays relative to wt MEFs in the absence of PDGF-AA could be a result of passive migration promoted by expanding cells in subsequent rows of cells, pushing on the first row of cells directly facing towards the wound, coupled with a high rate of lamellipod formation. Evidently, this physical interaction between cells that results in actual migration was absent at low confluence of cells in the micropipette analysis.

Activation of Akt at the ciliary base is part of the PDGF-AA-mediated response

We observed that in the presence of PDGF-AA, Akt is phosphorylated at the base of the primary cilium in wt MEFs. This corresponds an increase in the level of phospho-Akt in western blot analysis of PDGF-AA stimulated wt MEFs that is blocked in mutant MEFs. Lack of Akt phosphorylation in PDGF-AA stimulated mutant cells is not caused by reduced levels of PDGFRα, which suggests that activation of PDGFRα occurs in wt cells, because the receptor needs to localize to the primary cilium in order to become activated. One possibility is that PDGFRα only dimerizes to PDGFRαα after it reaches the cilium. This suggests that ciliary PDGF-AA-mediated signaling leading to directional migration in wt MEFs involves the activation of Akt, initially at the ciliary base. Increased levels of phospho-Akt are blocked in mutant MEFs. Additionally, we show that activation of PDGFRα only occurs in wt cells, supporting the conclusion that lack of Akt phosphorylation in mutant cells may not be caused by reduced levels of PDGFRα, but a mistranslocation of PDGFRα.

Akt is known to affect cell migration and cell motility by a variety of molecular mechanisms including transcriptional regulation of motility genes, actin cytoskeletal reorganization and dynamics, and control of cellular interactions with the extracellular matrix [46]. The Akt/PI3 kinase pathway also promotes stabilization of the microtubule cytoskeleton at the leading edge in migrating fibroblasts after PDGF addition. Onishi et al. [47] and Vidali et al. [5] showed that an Akt/PI3 kinase pathway remains functional upon PDGF stimulation (presumably including PDGFRαα stimulation) in Rac-1 null MEFs, which do not organize their actin into lamellipodia, but migrate nonetheless. Taken together, these results support the conclusion that the primary cilium is critical for PDGFRαα-mediated signaling via Akt and other pathways to control microtubule stabilization at the leading edge in cell migration, producing directional cell movement. Further, in the absence of PDGFRα, activation the overall level of Akt phosphorylation is kept at a low and comparable level in both wt and Tg737^orp^ MEFs, indicating that increased directional migration speed in mutant cells is not caused by aberrant Akt signaling, but by changes in the activity of regulatory components downstream or independent of Akt.

Defective wound healing in ORPK (IFT88Tg737Rpw) mutant mice

By investigating the possible role of primary cilia in regulating wound healing in vivo, we found that Tg737^orp^ animals had significant defects in wound closure compared to wt animals. These data suggest that primary cilia play a role in physiological wound repair, and that PDGFRα signaling in primary cilium in fibroblasts is part of this repair process, since impaired wound healing is associated with defects in PDGF-AA signaling in diabetic mouse models [48]. It is important to note that the analysis of wound repair in vivo is complex and multiple signaling pathways, in addition to PDGFRα, may be impaired by loss of cilia function. The ORPK mutants on the FVB/N inbred background have multiple health problems including growth retardation, hydrocephalus, polycystic kidney, liver and pancreatic diseases and hyperkeratosis in the skin that may contribute to an impaired wound healing response [49]. To fully address this issue in the absence of other potentially confounding phenotypes will require the use of conditional mutants that will specifically disrupt ciliary function in the skin fibroblasts. However, our in vitro model is consistent with the observed defects in in vivo wound healing, with PDGFRαα contributing to directional migration of fibroblasts. The mutants likely achieved partial closure via fibroblast migration in response to additional signals, much as the in vitro fibroblasts migrate in response to serum stimulus. At in vivo wound healing conditions, the establishment of gradients of chemotactic mediators for directional cell migration, including PDGF-AA, is coordinated by platelets, whereas serum released to the wound serves to form the fibrin/fibrinogen network in clotting that provides the provisional ECM, which supports migration of fibroblasts and immune cells into the wound [14]. Consequently, fibroblasts that lack the

Schneider/Cammer/Lehman/Nielsen/Guerra/Veland/Stock/Hoffmann/Yoder/Schwab/Sati/Christensen

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primary cilium are blind to PDGF-AA and move more randomly.

Conclusions and perspectives

In tissue fibroblasts, the primary cilium may be part of the positioning machinery that coordinates cell polarity, which is essential for directed migration in wound healing and developmental processes [50]. In the absence of chemosensory stimuli, signals from the cilium may restrain excessive cell migration to prevent the uncontrolled and/or incorrect displacement of cells that is seen in Tg737 orpk MEFs in scratch assays. During the establishment of a gradient of chemosensory stimuli and initiation of migration, the primary cilium may function as a cellular GPS [51] to monitor the directional movement and organize the coordinated actions of chemosensory stimuli and the dynamic reorganization of the cytoskeleton. It seems probable that signaling molecules generated within the primary cilium leave the cilium and impinge on the centrosome, surrounding the ciliary basal body [52], where proteins such as activated Akt could modulate actin, microtubule and cell junction reorganization and generalized cell motility. A second PDGFRα-mediated signaling pathway that could affect the migratory response is the Mek1/2-Erk1/2 pathway, which is specifically activated in the primary cilium upon PDGF-AA stimulation in fibroblasts [20].

A possible link between PDGFRα signaling and leading edge events was recently suggested to involve the activation of the Na+/H+ exchanger 1 (NHE1) [53], which is located mainly in the leading edge of the cell where it works as a central player in the regulation of cell migration [15, 54]. Both the PI3K-Akt and the Mek1/2-Erk1/2 pathways activate NHE1 [53-55]; the Mek1/2-Erk1/2 pathway acting via p90Ribosomal S6 kinase (p90Rsk) to phosphoaryl the C-terminal part of NHE1 [56, 57]. Recently PDGF-BB was shown to mediate activation of NHE1 and actin cytoskeleton remodeling via Akt in fibroblasts [58]. One hypothesis is that PDGF-AA-mediated activation of Akt at the centrosome affects both NHE1, microtubule cytoskeleton stabilization and actin reorganization towards the leading edge, which in turn affects the generation of leading edge lamellipodia [52, 26]. In this scenario the cilium/centrosome axis could coordinate the reorientation and remodeling of stable microtubules, which is known to regulate turnover of focal adhesions and trafficking of membrane proteins to the leading edge [59]. In smooth muscle cells, primary cilia containing EGFR, integrins and polycystins 1 and 2 orient towards the leading edge during migration [34, 35], supporting the model in which the primary cilium coordinate a whole series of different signal transduction pathways that are critical in reorientation and remodeling of stable microtubules towards the leading edge in directional cell migration.

In terms of tumor cell invasion, signaling systems that may be coordinated by the primary cilium besides PDGFRα signaling include the Hedgehog (Hh) and Wingless/Int (Wnt) pathways, which are important regulators of the migratory response in a variety of cell types and which, when mutated, cause cancer [60-63]. Indeed, several types of cancer cells are recognized by a significantly lower frequency of primary cilia [64, 65], and changes in primary cilium signaling has been linked to increased progression of various types of cancers [64, 66-68]. As an example, Wong et al. [67] and Han et al. [68] showed that the primary cilium has a dual role as a unique signaling organelle that can either mediate or suppress tumorigenesis depending on the nature of the oncocigenic initiating event. It would be interesting to know if primary cilia dysfunction causes a change in the migratory behavior in cancer cells similar to that of Tg737 mutant MEFs, which migrate with disrupted directionality and increased speed compared with the wt MEFs.

Even though many signaling pathways may be involved in the responses to PDGF and other serum factors, our results emphasize that in fibroblasts, PDGFRα signaling originating in the primary cilium is a component of the response, essential for efficient directional migration in the presence of PDGF-AA, and important for chemotaxis in wound healing. Probably, directional migration of fibroblasts is similarly regulated through interactions between the cilium and ECM; potentially in concert with chemoattractants in embryonic patterning and adult tissue reorganization. This may be a unique feature of primary cilia on cells deep within tissues such as in fibroblasts versus cilia that protrude from the apical surface into a lumen as seen on epithelial and endothelial cells. In fibroblasts, defects in building the primary cilium or in the targeting of PDGFRα to it have crucial consequences on cellular and physiological levels and may be responsible for human diseases and migration-related disorders involving PDGF pathway mutations [69, 70].
Abbreviations

GPS (Global positioning system); MEF (Mouse embryonic fibroblast); ORPK (Oak Ridge polycystic kidney); PDGF-AA (Platelet-derived growth factor AA); PDGFR (Platelet-derived growth factor receptor).

Acknowledgements

This work was supported by grants to S.T.C. and L.S. (Lundbeck Foundation grant number R9-A969, Fonden af 1870, funds from the University of Copenhagen), S.K.N. (Novo Nordic foundation), E.K.H. and S.T.C (The Danish Natural Science Research Council, 21-04-0535 and The Danish Cancer Society, DP05072), A.S. (Deutsche Forschungsgemeinschaft Schw 407/9-3, and 10-1 and IZKF Münster), C.S. (“Innovative Medical Research” Fund of the University of Münster Medical School, ST 2 1 06 01), and by a Pilot and Feasibility award to B.K.Y. from the UAB Skin Diseases Research Center (SDRC) supported by an NIH P30 (AR050948-03, C. Elmets). We thank Anni Bech Nielsen, Sabine Mally and Dr. Jacco Van Rheezen for excellent technical assistance.

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Cell Physiol Biochem 2010;25:279-292


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