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Gene Expression in mESC Deficient in FGFR Signalling and the Identification of the Novel Gene Ened

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Gene Expression in mESC Deficient in FGFR signalling and the Identification of the Novel Gene Ened

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Doctoral dissertation

Front cover: Mouse embryos at day 9.5, 10.5 and 11.5 after fertilization. In situ hybridized embryos expressing the novel gene Ened are shown. Ened expression was observed from day 9.5 in the heart and the future gastrointestinal tract. On day 10.5 the expression extended to the developing peripheral nervous system. On day 11.5 Ened expression appeared in the lens epithelium.

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To Virginia and Veronika

Abstract

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of blastocyst stage embryos, and upon differentiation into embryoid bodies (EBs) they recapitulate the genetic, cellular and morphological events occurring in early embryogenesis. Among several signalling pathways and inductive factors, the fibroblast growth factors (FGFs) and their receptors (FGFRs) have been shown to be important during early embryogenesis. Previously it has been shown that ESCs expressing a mutant form of FGFR failed to form the two characteristic cell layers of EBs: the endoderm and the ectoderm. In addition, due to the impaired FGF/FGFR signalling, the mutant EBs failed to synthesize laminin-111 and collagen type IV, the main building blocks of the basement membrane (BM) protein network. The aim of this thesis was to elucidate the molecular and biological changes occurring due to impaired FGF/FGFR signalling in differentiating EBs. A large scale microarray study was performed that provided insights into the global gene expression changes occurring in both wild-type (normal) and FGF/FGFR signalling deficient EBs, which resulted in an extensive catalogues of significantly expressed genes in both cell lines. Results showed that during wild-type EB differentiation a rapid down-regulation of pluripotency related genes and up-regulation of genes related to morphogenesis and development occurred. Analysis of FGF/FGFR deficient EBs showed a significant decrease of genes encoding endodermal and BM related proteins. In addition, an increase of mesodermal and pluripotency related gene transcripts was observed. Such transcripts are normally activated later during EB development. The assumption was that the most significantly decreased genes could be involved in FGF/FGFR signalling. To further test this theory, we chose to elucidate the expression pattern of the hitherto uncharacterized gene *1110032E23Rik* whose expression was significantly decreased in FGF/FGFR mutant EBs. By in situ hybridization analysis, the expression of *1110032E23Rik* was examined in both mouse and *Xenopus laevis* (frog) embryonic development. Results showed that the expression of *1110032E23Rik* in mouse embryos was restricted to epithelia such as the lining of the gastrointestinal tract, urogenital tract, heart, skin and lens, and in addition to the peripheral nervous tissues. Expression analysis of the mouse *1110032E23Rik* orthologue in frog embryos showed that mice and frogs share several expression domains such as the eye, heart, kidney and epidermis. The expression was observed at locations where active FGF/FGFR signalling takes place and BMs are present. Based on the expression pattern observed in mice, we named this uncharacterized gene *Ened* (Expressed in Nerve and Epithelium during Development).

List of articles

This thesis is based on the following papers referred to in the text by their Roman numerals I-III.

I. **Meszaros, R.,** Åkerlund, M., Hjalt, T., Durbeej, M., Ekblom, P. (2007). Gene expression profiling of differentiating embryonic stem cells expressing dominant negative fibroblast growth factor receptor 2. Matrix Biol. 26, 197-205. *

II. **Meszaros, R.,** Strate, I., Pera, E.M., Durbeej, M. (2008). Expression of *Ened* during Mouse and *Xenopus* Embryonic Development. *Conditionally accepted*.

III. **Meszaros, R.,**[#] Åkerlund, M.[#], Durbeej, M. (2008). Global gene expression analysis during early mouse embryonic stem cell differentiation. *Manuscript in preparation.*

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[#] Shared authorship.

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Abbreviations

ANOVA	analysis of variance
BM	basement membrane
dnFGFR2	dominant negative fibroblast growth factor receptor 2
EB	embryoid body
ECM	extracellular matrix
EMI	epithelial-mesenchymal interaction
EMT	epithelial-mesenchymal transition
Ened	expressed in nerve and epithelium during development
ESC	embryonic stem cell
EST	expressed sequence tag
EmVE	embryonic visceral endoderm
ExVE	extraembryonic visceral endoderm
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GO	gene ontology
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
ICM	inner cell mass
LIF	leukemia inhibiting factor
NCC	neural crest cell
ParE	parietal endoderm
PrE	primitive endoderm
RTK	receptor tyrosine kinase
TE	trophectoderm
VE	visceral endoderm
WT	wild-type

1. Introduction

1.1. Fibroblast Growth Factors, their receptors and signalling

The beginning of the fibroblast growth factor (FGF) signalling field can be traced back to year 1939, when bovine brain extracts were shown to promote proliferation of fibroblast cell lines in vitro (Torwell, 1938). In the early 1970s Gospodarowicz and colleagues confirmed and followed up the studies by Torwell et al, by purifying and characterizing the first FGFs: FGF1 and FGF2 from bovine brain extracts (Gospodarowicz, 1974; Gospodarowicz et al., 1978). These studies subsequently led to the identification of a large family of proteins; the FGFs and their receptors (FGFRs), by now recognized as one of the key regulators during embryonic development and in the adult organism.

1.1.1. FGFs and their receptors

Signalling by FGFs controls a diversity of biological processes, including early embryonic development, organogenesis, cell proliferation, differentiation, migration and apoptosis.

The FGF gene family is composed of 22 structurally related members that are readily secreted into the extracellular matrix (ECM) where they bind with varying affinity to their receptors (Ornitz and Itoh, 2001). Exceptions are FGF1, FGF2, FGF9, FGF16 and FGF20 that lack a signal sequence for secretion; however they can be found both on the cell surface and in the ECM. In addition, FGF1 and FGF2 can remain intracellulary and localize to the nucleus and stimulate mitogenesis (Powers et al., 2000). The members of the FGF11 subfamily (FGF11 to 14) also lack a signal sequence for secretion and are thought to act as intracellular factors with no known activity against FGFRs (Zhang et al., 2006).

FGFRs are members of the receptor tyrosine kinase (RTK) family. The extracellular ligand-binding region of FGFRs contains three immunoglobin (Ig-like) domains (IgI, IgII and IgIII), an acidic box between IgI and IgII, a heparin binding domain, a single transmembrane domain and a split intracellular tyrosine kinase domain (Powers et al., 2000) (Figure 1A).

There are 4 FGFR genes (FGFR1 to FGFR4), which through alternative mRNA splicing produce up to 15 different FGFR isoforms (Johnson and Williams, 1993). Also, a novel member of the FGFR family, termed FGFRL1, has been recently

identified (Wiedemann and Trueb, 2000) and is believed to modulate or inhibit the FGF/FGFR signalling cascade since it lacks the tyrosine kinase domain (Trueb et al., 2003).

The gene structures, nucleotide and amino acid sequences are highly conserved within the different FGFs and FGFRs, and this conservation of the FGF/FGFR signalling system extends throughout most of animal evolution (Itoh and Ornitz, 2008).

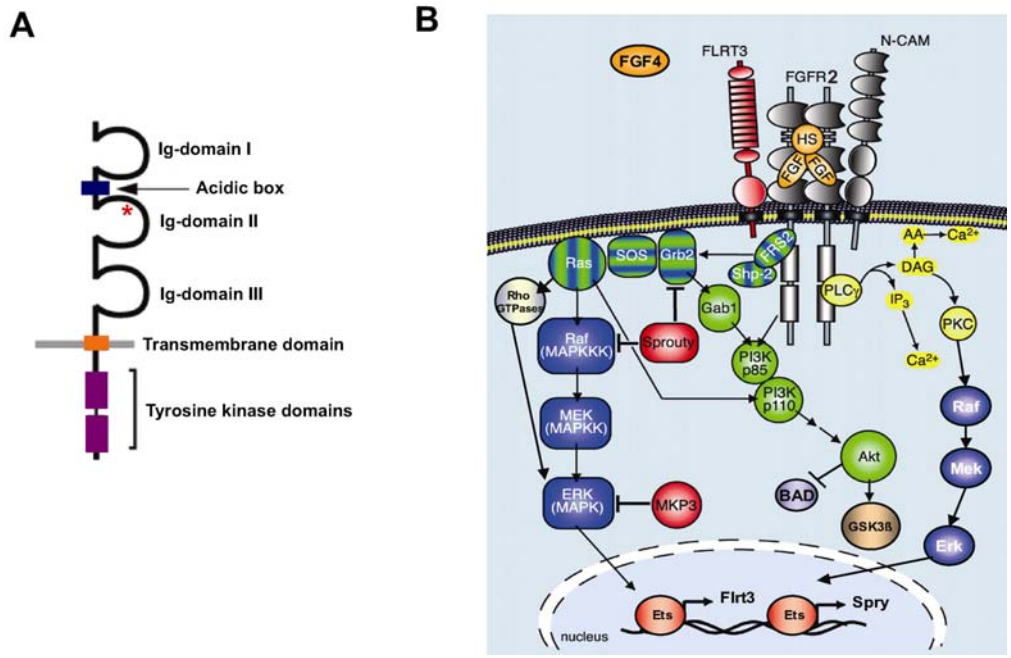

Figure 1. FGFR structure and FGF/FGFR signalling cascade. (A) Schematic structure of FGFR with its different domains indicated (se text for details). The heparin binding site on Ig-domain II is marked with an asterix. (Modified from Dickson et al, 2000). (B) The three main pathways induced by an FGFR ligand (example FGF4). Signals are relayed through the MAPK, PI3K/AKT and PLCγ pathways that lead to a wide variety of responses depending on the cell type. Raf can be activated through PKC in a Ras-independent manner (se text for details). (Modified from Böttcher and Niehrs, 2005).

1.1.2. FGF/FGFR signalling

Ligand-induced receptor dimerization is a requirement for RTK activation. The interaction of heparan sulfate proteoglycans (HSPGs) with the extracellular part of FGFR is important for the proper activation of FGF/FGFR signalling (Thisse and Thisse, 2005). Receptor dimerization brings the cytoplasmic domains of the receptors in close vicinity to each other providing the opportunity for receptor trans-

autophosphorylation, subsequent tyrosine kinase activation and initiation of downstream signalling pathways.

FGFRs exist in monomeric form until activated by their FGF ligand in a sequential manner: (i) initial binding of one FGF to its FGFR; (ii) binding may be stabilized by heparin or HSPGs that facilitates the transient receptor dimerization with another FGFR monomer; (iii) binding of HSPG molecule and subsequent binding of a second FGF, dimerization of the monomeric receptors and formation of a stable FGF/FGFR complex (Ornitz, 2000). Interestingly, both homodimeric and heterodimeric receptor-pairs can form between the different FGFR isoforms (Ueno et al., 1992). Dimerization leads to autophosphorylation on critical tyrosine residues in the cytoplasmic domain of the receptor, and initiate the recruitment and activation of downstream signalling partners (Eswarakumar et al., 2005).

1.1.3. FGF/FGFR signalling pathways

The main pathways activated by FGFs are the Ras-MAP kinase (MAPK) pathway, the PI3 kinase/AKT pathway, and the PLCγ pathway (Schlessinger, 2000) (Figure 1B).

The key component of FGFR activated MAPK pathway is the activation of FRS2α docking protein that functions as a platform for recruitment of the adaptor proteins GRB2 and SOS, and the docking protein GAB1 to form a complex which stimulates the activation of the oncogenic Ras (that belong to the Ras GTPase family) from an inactive GDP-bound to an active GTP-bound state. The activated Ras recruits Raf to the cell membrane. This can activate the phosphorylation of the dual-specificity kinases MEK1 and 2, which in turn can phosphorylate ERK1 and 2 that translocate into the nucleus where they phosphorylate or activate trascription factors such as Ets. This in turn activates expression of specific FGF target genes such as *Spry* and *Flrt3* that act as a negative or a positive regulator on FGFR signalling, respectively (Thisse and Thisse, 2005). This activation leads to among else cell differentiation and proliferation. Ras can also activate small Rho GTPases (Rac, Rho and CDC42) that may converge with ERK and lead to cytoskeletal reorganization.

The PI3K/AKT pathway is activated by either the p85 PI3K regulatory subunit that is recruited to the activated tyrosine kinase residue on the FGFR receptor, or by the p110 subunit binding to activated Ras. In addition, PI3K can be activated by the GRB2/SOS/GAB1 complex that results in the phosphorylation of PIP2, which then generates PIP3 and subsequently induce AKT. This results in the translocation of

AKT to the plasma membrane where it is phosphorylated and activated by PDK. In turn, AKT may phosphorylate a variety of target substrates such as GSK3β that result in the down-regulation of GSK3β activity. Other target molecules are pro-apoptotic factors such as BAD whose activity is inhibited. In this manner, the PI3K/AKT pathway promotes cell survival (Schlessinger, 2000).

The PLCγ pathway is activated when PLCγ binds with its SH2 domain to the tyrosine kinase domain of the autophosphorylated FGFR that leads to hydrolysis of PIP2 into IP3 and DAG. IP3 induces Ca^{2+} release from intracellular stores, which then binds to calmodulin and activates calmodulin dependent protein kinases. DAG, on the other hand, activates members of the PKC family of protein kinases. In turn, the activated PKCs are able to phosphorylate and stimulate Raf, which leads to the activation of the MAPK pathway in a Ras-independent manner (Schlessinger, 2000).

1.2. Basement membranes, early embryonic development and embryonic stem cells with emphasis on FGF/FGFR signalling

1.2.1. Basement membranes

Basement membranes (BMs) are thin, sheet-like structures of specialized extracellular matrix (ECM) underlying epithelial and endothelial cells and surrounding muscle, peripheral nerve and fat cells (Figure 2A). The BM function is to provide structural support, divide tissues into compartments and regulate cell behavior such as adhesion, migration, differentiation, cell survival and apoptosis through interaction with specific receptors. The main network forming components of the BM are various isoforms of laminins, collagen type IV, and this network is stabilized by interactions with the heparin sulfate proteoglycan perlecan, nidogens, agrin, BM 40 (SPARC/osteonectin), fibulins and collagens type XV and XVIII (Ekblom et al., 2003).

Laminins are a large family of cross-shaped heterotrimeric glycoproteins (Figure 2B). To date, 16 isoforms of laminin have been found, derived from five α, three β and three γ subunits (Aumailley et al., 2005). Laminin α1 is one of the earliest expressed laminin α-chain during embryonic epithelial morphogenesis, although in adult tissues the expression is more restricted to epithelial BMs such as the lens epithelium, proximal tubules in the kidney, ovary, testis and parenchymal BM in the brain blood vessels (Falk et al., 1999). Laminin α2-chain is mainly found in muscle,

α3 in skin and intestinal epithelia, α4 in mesenchyme or mesenchyme-derived cells (bone-marrow, blood vessels) and α5 is ubiquitously expressed in adult tissues (Colognato and Yurchenco, 2000; Ekblom et al., 2003). A major cell-binding domain is located in the C-terminal G domain of the laminin α chains. The laminin G domain consists of five laminin type G domain (LG) modules. The LG modules interact with cell surface receptors like integrins, dystroglycan and syndecans, but also with heparin and ECM molecules like perlecan and fibulins. The α-chain's N-terminal domains also have integrin binding sites, but their main role (along with the β- and γ-chain N-terminal modules) is the formation of large laminin polymers (Tunggal et al., 2000).

The nonfibrillar collagen type IV family is composed of six distinct α chain monomers: α1-α6 chains, and αl(IV) and α2(IV) chains appear to be ubiquitously distributed in tissues (Hudson et al., 1993). The monomers self-associate into a triple helical polymer, which in turn assemble with other collagen polymers to form a complex branching network that serves as a scaffold for the other BM molecules. It stabilizes the structure of BM through interactions with other BM components such as BM40, nidogens and laminin. Collagen IV binds to integrins on the cell surface and to the ECM through fibronectin (Hudson et al., 1993).

The binding of BM components to the cell surface through receptors such as integrins, dystroglycan, heparin and cell surface sulfates/sulfatides (Figure 2B) is crucial for maintaining cell-to-matrix contact.

Integrins are heterodimeric transmembrane receptors composed of α- and β-subunits that mediate cell adhesion to the BM and ECM, and also serve as linkers to the cytoskeleton and transmembrane growth factor receptors. They transmit survival signals through the PI3K/AKT pathway, and signals for migration, cell division and differentiation through the MAPK pathway (Bouvard et al., 2001), and thus can be linked to the FGF/FGFR signalling pathway.

Dystroglycan is a part of the dystrophin glycoprotein complex and is post-translationally cleaved to an α- and a β-subunit. It binds to the laminin α1-chain LG module 4, and it has been shown to have a role in branching morphogenesis in kidney, lung and salivary gland during epithelial development (Durbeej and Ekblom, 1997). Deletion of dystroglycan leads to early embryonic lethality (Williamson et al., 1997).

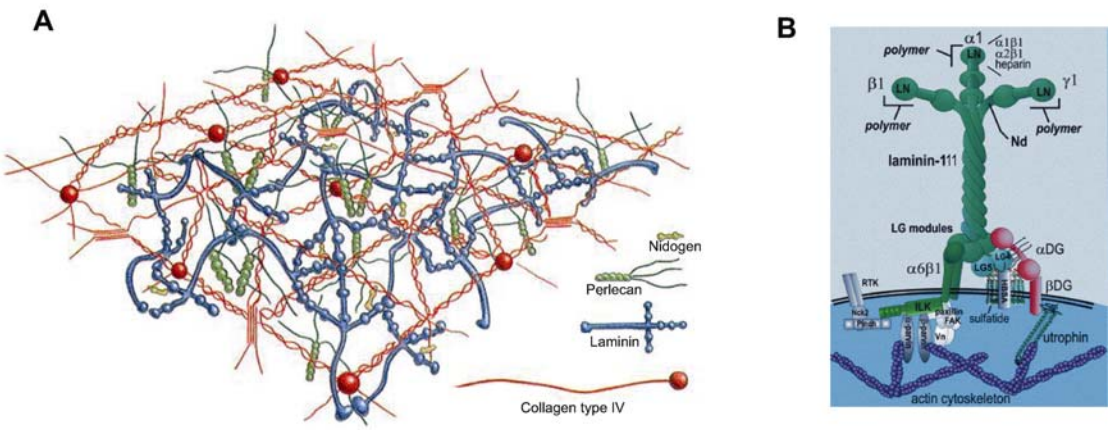

Figure 2. Laminin-111 and basement membrane structure. (A) Molecular structures of the BM showing laminins and collagen type IV forming the two main networks of the BM. This network is stabilized among else by perlecan and nidogen. (Modified from Alberts et al, Molecular Biology of the Cell, Fourth edition, 2002). (B) Laminins, in this figure laminin-111, are composed of α-, β- and γ-chains that form a cross-shaped heterotrimeric glycoprotein. The N-terminal LN modules provide polymerisation with other laminins, or binding to integrins (example α1β1 and α2β1 integrins), nidogen (Nd) and heparin. Laminin LG modules link with their α-chain to the cell surface and cytoskeleton via cell-adhesion receptors such as dystroglycan (DG), integrins (example α6β1 integrin), cell surface HSPGs and sulfatides. Through integrins and integrin-linked kinase (ILK), laminins may interact with growth factor receptors (RTKs) such as FGFRs. (Modified from Li et al, 2003).

Heparin and heparane sulfates (HS) linked to core proteins form heparan sulfate proteoglycans (HSPGs). Growth factors may be stored and concentrated by HSPGs that are found on cell surfaces (such as syndecans and glypicans), or in the BM. Binding studies in cells lacking HS showed that FGFR expressed in these mutant cells were incapable of FGF binding (Yayon et al., 1991).

In mammals, the first laminins to be expressed during embryogenesis are laminin α1β1γ1 (laminin-111) and laminin α5β1γ1 (laminin-511) that have partially overlapping expression patterns and functions in the embryo. However, only laminin-111 appears to be essential for early embryonic development. LAMC1 -/- (laminin γ1-chain deficient) embryos die at embryonic day 4.5 (E4.5) during the implantation stage (Smyth et al., 1999), and LAMB1 -/- (laminin β1-chain) and LAMA1 -/- (laminin α1-chain) embryos die after implantation around E5.5 and E6.5, respectively (Miner et al., 2004). The slightly later phenotype of LAMA1-/- embryos is most likely due the functional compensation of laminin α5-chain (Miner et al., 2004). In contrast, LAMA5 -/- (laminin α5-chain deficient) embryos die at late embryogenesis, around E16.5, although deformities in organs were apparent from E9 (Miner et al., 1998). The late phenotype of LAMA5 -/- embryos could be due the compensation of laminin

α1-chain in the developing organs. Deletion of the collagen IV isoform $\alpha1(IV)_2\alpha2(IV)$ in mouse embryos cause embryonic lethality around E11 (Poschl et al., 2004). In these mutants, the BM was assembled, although the structural integrity could not be maintained. These data point out the important role of laminin-111 and collagen IV in BM assembly and for normal embryonic development.

1.2.2. Early mouse embryonic development

FGF/FGFR signalling is essential for proper embryonic development. In the pre-implantation mouse embryo, FGFR2 is one of the first signal transduction molecules to be expressed in the compacted morula around E2 (Haffner-Krausz et al., 1999). In addition, mRNAs of the FGF ligands FGF1, FGF5 and FGF8 are expressed from the oocyte stage (unfertilized egg), and FGF4 and FGF8 can be detected from the 2-cell and 4-cell stage, respectively, at high levels in the embryo (Zhong et al, 2006). This suggests a maternal contribution of FGF ligands in the developing embryo, however the zygotic genome is activated at 2-cell stage after fertilization when the maternal mRNA starts to degrade (Hamatani et al., 2004). In addition, mRNAs for all three chains of laminin-111 can be detected between the cells at this stage (Ekblom et al., 2003).

At around E3, the first cell-linage decision takes place, forming the early blastocyst composed of two distinct pluripotent cell populations: the interior cells called the inner cell mass (ICM) and the outer layer forming the trophectoderm (TE). The ICM expresses FGF4, the ligand for FGFR2 that is now expressed in the adjacent TE (Rappolee et al., 1994). Also, expression of FGFR3 in the TE and FGFR4 in both TE and ICM can be detected at E3.5 (Rappolee et al., 1998). TE cells acquire the characteristics of epithelial cells in being flattened and joined together by tight junctional complexes, where the connected E-cadherin/WNT/β-catenin pathway plays a key role (De Vries et al., 2004), thus E-cadherin -/- embryos fail to form the TE due to impaired compaction (Larue et al., 1994). At this stage, TE cells express in addition to *Fgfr2* the genes *Eomes* and *Cdx2*, while genes located in the ICM in addition to *Fgf4* are *Gata6, Lefty1, Nanog, Nodal, Oct4, Sox2* and *Tdgf1* (Chazaud et al., 2006; Pfister et al., 2007), which indicates that the ICM manifests heterogeneity already at E3.5 and up-regulates genes related to primitive endoderm (PrE) and epiblast (that will form the embryo proper). FGF/FGFR signalling through the MAPK

pathway is required for the ICM to exit self-renewal by directing the action of *Bmp4* and repressing *Nanog* expression (Chazaud et al., 2006; Kunath et al., 2007). GRB2 is an adaptor protein in the MAPK pathway, and in GRB2 -/- embryos it was shown that *Gata6* expression was lost and PrE development was abrogated (Chazaud et al., 2006). As the blastocyst develops, the trophectodermal cells begin to pump fluid into intracellular spaces and later into extracellular spaces, forming the blastocoelic cavity.

At about E4, ICM cells in contact with the blastocoele begin to form the PrE, and the inner approximately 20 cells remain pluripotent as the nonpolarized ICM (epiblast), that later will contribute to three cell layers of the future embryo, *i.e.* the embryonic endoderm, mesoderm and ectoderm. At this stage, two BM structures emerge: one is deposited by the TE and will form Reichert´s membrane; the other is deposited between the newly formed PrE and undifferentiatied epiblast by the PrE, and will form the embryonic BM (Yurchenco et al., 2004). Here, the importance of FGF/FGFR signalling could be pointed out. To form the first BM structures, the epithelial layers of TE and PrE need to induce their transcriptional machinery to produce BM components. It has been shown that FGF4 -/- mouse embryos die due the impaired proliferation of ICM before implantation (Feldman et al., 1995), while the targeted disruption of the binding and anchoring site for FGFR2 resulted in peri-implantational lethality due to trophectodermal defects in the embryos (Arman et al., 1998). Also, a dominant negative truncated form of FGFR4 cDNA expressed in mouse embryos resulted in lethality in the early blastocyst stage embryos (Chai et al., 1998). Collectively these data suggest the importance of FGF/FGFR signalling in pre-implantational embryogenesis, when the epithelial layers produce the first BM components.

At implantation around E5, the blastocyst express among else *Amn, Cer1, Dab2, Gata4, Gata6, Hnf4* and *Lefty1* in the newly formed PrE (Pfister et al., 2007). At this stage, a portion of the PrE differentiates into visceral endoderm (VE), which remains in contact with the epiblast. The TE undergoes rapid proliferation and intrudes into the blastocoelic cavity and forms a conical structure (so called egg-cylinder). The other portion of the PrE in contact with the TE migrates laterally to line the blastocoele, forming the parietal endoderm (ParE). The ParE cells migrate along the TE toward the distal end of the embryo with the help of the TE secreted Reichert´s membrane, ParE themselves secrete more BM components that incorporate into the Reichert´s membrane, resulting in a thick sheet of BM (Murray and Edgar, 2001). At

this stage, the TE express *Fgfr2, Cdx2* and *Eomes*; the epiblast starts to express *Oct4, Nanog* and *Tdgf1*, while the VE expresses *Dab2, Amn, Foxa2* and *Otx2*. The Reichert´s membrane was not formed in LAMA1, LAMB1 and LAMC1 -/- embryo (Miner et al., 2004; Smyth et al., 1999) that prevented the migration of PrE cells, which indicates important roles of the BM for normal development. In addition, GATA6 -/- embryos fail to express *Dab2* and die around E5.5 of gestation due defects in PrE differentiation (Koutsourakis et al., 1999; Morrisey et al., 2000), which is a further indicative of the complex morphological interludes that might be governed by FGF/FGFR signalling.

At around E6 in the pre-streak embryo, the VE will divide into the extramebryonic VE (ExVE) and embryonic VE (EmVE), resulting in distinct gene expression profiles. The ExVE expresses among else *Gata4, Hnf4α, Sox7* and *Sox17*, while the EmVE expresses *Afp, Bmp2, Cer1, Fgf5, Fgf8, Lefty1* and *Nodal* (Pfister et al., 2007). Also, epiblast cells in contact with the embryonic BM will polarize and form the columnar embryonic ectoderm (the first embryonic epithelium). At this point, cavitation is also induced that will result in the formation of the proamniotic cavity of the embryo.

This induction of the epiblast will result in the formation of the definitive germ layers of the embryo: mesoderm, endoderm, and the ectoderm, which marks the start of gastrulation at E6.5. This is accomplished by the polarization of the undifferentiated epiblast cells in contact with the embryonic BM. Without this contact, the embryonic ectoderm cannot form and the embryos cease to gastrulate. This has been demonstrated in the LAMB1 -/- and LAMC1 -/- mutant mouse embryos (Miner et al., 2004) and embryos that lacked the carboxyterminal LG4-5 domain of laminin α1-chain (Scheele et al., 2005).

Gastrulation involves major morphological changes in the embryo accompanied by distinct gene expression patterns for the regionalization, epithelial-to-mesenchymal transition (EMT) and anterior-posterior patterning of the primitive streak embryo. The VE is replaced by the definitive endoderm and expresses *Cer1, Dab2, Dkk1, Frz8* and *Sox17*, while the mesoderm express *Lefty2, Ifitm1, Mixl1, Mesp1, Smad1* and *Wnt3*. At the posterior part the primitive streak express genes such as *Fgf8, Mixl1* and *Wnt3a*, while in the anterior primitive streak express *Fgf4, Foxa2, Gsc, Nodal* and *Nog*. Generally, genes encoding signalling ligands, co-receptors and intracellular transducers are expressed in the patterning epiblast, while genes coding

for receptors and antagonists are expressed in the VE, which is consistent with the graded activity of WNT, Nodal and BMP signalling (Pfister et al., 2007). However, FGF/FGFR signalling cannot be excluded at this stage. FGFR1 -/- embryos die during gastrulation due to accumulation of cells at the posterior streak and mesodermal defects (Deng et al., 1994), and FGF8 -/- embryos die later, around E9, when they display failure of cell movement away from the primitive streak (Sun et al., 1999). Cells that undergo EMT need to leave the primitive streak but instead accumulate and are unable to migrate away from the streak region in these mutants, which could indicate a role of FGF/FGFR signalling in the primitive streak presumably by regulating the production of proteins necessary for cell migration.

1.2.3. Embryonic stem cells and their differentiation into embryoid bodies

Early embryonic development is difficult to study due to the small size of the embryo. Hence, *in vitro* techniques have been developed to elucidate early genetic and morphological events taking place during the early stages of embryonic development.

The ICM contains about 20 cells in the blastocyst stage embryo around E3.5. These cells, called embryonic stem cells (ESCs) can be isolated and cultured *in vitro*, and maintained and expanded in an undifferentiated state for extended periods of time while they retain their pluripotency after extensive passaging (Gardner and Brook, 1997). On a Petri dish in suspension culture and upon withdrawal of leukaemia inhibiting factor (LIF), ESCs aggregate into embryoid bodies (EBs) that differentiate into round spherical structures. The development of EBs resembles early embryonic development. During their differentiation, EBs forms the derivatives of the three embryonic germ layers of endoderm, ectoderm and mesoderm, where a BM (equivalent to the embryonic BM) separates the endoderm from the ectoderm (Coucouvanis and Martin, 1995).

Generally, undifferentiated ESCs express the same set of genes that is detected in the ICM, where the so called core-transcription factors *Nanog, Oct4* and *Sox2* keep ESCs in a pluripotent state. LIF is required for maintenance of pluripotency in mouse ESCs, and exerts its effects through the JAK/STAT pathway. Although it is still not clear exactly how these factors are regulated, the WNT, BMP and FGF singnalling pathways have been implicated in maintaining pluripotency (Boiani and Scholer, 2005; Kunath et al., 2007). Open withdrawal of LIF and plating the undifferentiated ESCs on a Petri dish that does not support adhesion of the cells, ESCs aggregate and

by day 1 of EB culture, they start to form the first epithelial germ layer of the EBs, *i.e.* the endoderm. This stage corresponds to the first emergence of the PrE in E4 embryos. Between day 2 and 3 of EB culture the endoderm secretes the underlying BM, which resembles embryonic development around E4 to E4.5 when the PrE deposits the embryonic BM. Around day 4 to 5 of EB culture, the BM induces the polarization of the underlying undifferentiated ESCs in the center of the EB structure. This will result in the formation of the second epithelial germ layer, *i.e.* the ectoderm, which resembles the embryonic stage E6. In addition, a cavity starts to form in the center of the EB that corresponds to the proamniotic cavity in the gastrulating embryos (Weitzer, 2006).

The importance of FGF/FGFR signalling during these early stages of EB development has been pointed out by different studies, where mutations of FGFs or FGFRs have been used to elucidate the effects of this signalling pathway.

Transcripts for *Fgfr1* and *Fgfr2* are expressed in undifferentiated ESCs, while the expression of *Fgfr3* and *Fgfr4* is absent (Esner et al., 2002). However, all four FGFRs are expressed in the developing EBs, where *Fgfr1* and *Fgfr2* transcripts are detected in the ectoderm, while *Fgfr3* and *Fgfr4* transcripts are restricted to the endoderm (Chen et al., 2000). To circumvent the possibility of FGFR heterodimer formation between different FGFR isoforms, a dominant negative FGFR2 cDNA that lack a part of the tyrosine kinase domain of the receptor was introduced into ESCs. This mutation inhibited the activity of all FGFR isoforms in the dnFGFR2 mutant EBs and disrupted the first step during EB differentiation: the formation of endoderm. Hence, the subsequent secretion of the BM components laminin-111 and collagen IV, and the formation of ectoderm were also abrogated (Chen et al., 2000). Targeted disruption of FGFR1 in ESCs showed a similar phenotype as the dnFGFR2 mutation, where EBs did not form endoderm (Esner et al., 2002). Also, differentiating FGF4 -/- EBs could not form endoderm without the addition of FGF4 to the cell cultures (Wilder et al., 1997), and these EBs retained *Oct4* expression (Kunath et al., 2007), which implies that FGF4 expression is required for the down-regulation of pluripotency markers and the commitment of ESCs to differentiation. Moreover, removing the expression of the FGFR signalling downstream target ERK (MAPK3) by generating ERK -/- ESCs showed that EB derivates could not differentiate and retained their pluripotency markers (Kunath et al., 2007). These data emphasize the

significance of a correctly functioning FGF/FGFR signalling in exiting the ESC pluripotent state to could initiate the epithelial morphogenesis.

Further experiments examining the effects of mutant FGF/FGFR signalling showed that the PI3K/AKT pathway, rather than the MAPK pathway, was involved in the epithelial morphogenesis during EB differentiation (Chen et al., 2000). Furthermore, FGFR signalling was mediated through GATA4 and GATA6 during endoderm development (Li et al., 2004), where the expression of *Gata4* was induced by *Gata6* (Morrisey et al., 1996). GATA4 -/- and GATA6 -/- EBs could not form endodermal structures either (Capo-Chichi et al., 2005; Soudais et al., 1995). Additional elucidation of endoderm induction showed that GATA4 and GATA6 induced *lamb1* and *lamc1* expression through the transcription factors COUP-TFs I and II (Fujikura et al., 2002; Murray and Edgar, 2001), which coupled the FGF/FGFR signalling pathway to the induction of GATA4 and GATA6 and the subsequent induction of the BM component laminin-111. Additional players for laminin induction are SOX7 and SOX17 that are expressed by the ExVE in developing embryos. Culturing of mouse F9 embryonal carcinoma cells (that can be induced to form EB structures upon addition of retinoic acid) showed that SOX7 and SOX17 induced the expression of *Lama1* (laminin α1-chain) by binding to its enhancer site (Niimi et al., 2004).

DnFGFR2 EBs expresses other BM components such as nidogen and perlecan, although these components were not localized to the BM due the lack of the network forming components laminin-111 and collagen IV. Interestingly, co-cultivation of dnFGFR2 EBs with normal EBs rescued the mutant EB phenotype, and the same rescue was observed when adding Matrigel (a gel-like composition of BM components) or laminin-111 to the dnFGFR2 EBs (Li et al., 2001a). This indicates that dnFGFR2 EBs, although they cannot differentiate, retain molecular properties to recognize external inductive signals, such as the BM component laminin-111. The rescued dnFGFR2 EBs did not show any signs of endodermal differentiation, however they formed a polarized ectoderm beneath the BM that accumulated on the outer aspect of the EBs (Li et al., 2001a). In contrast, LAMC1 -/- EBs formed the outer endodermal epithelia but failed to produce a functioning BM. This phenotype could also be rescued by the addition of laminin-111, which induced the formation of a BM network and the subsequent polarization of the ectoderm and cavity formation (Li et al., 2002). Moreover, EBs with a targeted deletion of the globular domain of laminin

α1-chain LG4-5 could form endoderm (although endodermal differentiation may be incomplete, Åkerlund et al., unpublished data) and a BM. However, ectoderm polarization failed (Scheele et al., 2005). Thus, these data pinpointed the domain of laminin-111 required for the induction of polarization of the ectodermal epithelia. The small Rho GTPase deficient CDC42 -/- EBs also formed endoderm and BM, although they lacked a polarized ectoderm (Wu et al., 2007), similar to LAMC1 -/- and laminin α1-chain LG4-5 domain -/- EBs. The inhibition of the Rho-kinase ROCK in EBs showed a similar phenotype (Li et al., 2004).

Cavity formation during embryonic and EB development involves the formation of multiple foci of cell death which merge to a single, centrally located cavity surrounded by the polarized ectoderm. Cavitation has been ascribed to the exerted effects of BMP4 (Coucouvanis and Martin, 1999). BPM2 is expressed in the endoderm and BMP4 in the ectoderm and the reciprocal signalling between these factors from endoderm to ectoderm promotes endoderm differentiation and cavitation. However, BMP4 is only expressed for a short period of time in the ectoderm, and the fact that ectoderm can be polarized without an existing endoderm upon addition of laminin-111 to the cell cultures, challenged this view. In addition, EBs deficient in the autophagy genes *Atg5* and *Beclin1* could not form a cavity (Qu et al., 2007). Autophagy has been linked to apoptosis, where the newly characterized gene *Dram* was shown to be a direct target of p53 mediated apoptosis (Crighton et al., 2007). It is therefore possible that the developing ectoderm itself produces and secretes factors necessary for apoptosis; however gene expression studies in developing embryos and EBs showed that transcripts are present for apoptotic factors at early stages of development before ectoderm development (Hamatani et al., 2006).

Taking together, these observations suggest that endoderm differentiation requires FGF/FGFR signalling, while ectoderm polarization and differentiation require the presence of laminin-111. The cascade of the signalling and molecular interactions could be depicted as: FGF4 => FGFR2 => PI3K/AKT pathway => GATA6 and GATA4 => COUP-TFs => Laminin-111 and collagen IV synthesis => BM assembly => CDC42 and RhoC => ectoderm polarization.

Gene expression studies of transcriptional changes are another way to understand the development of multicellular organism. Several studies emerged during the past years elucidating the dynamic changes of the transcriptome in developing embryos and differentiating EBs (among else: (Hailesellasse Sene et al.,

2007; Hamatani et al., 2006; Sharova et al., 2007). These studies revealed the complex inter-relationship between distinct gene interactions, and with the help of computerized analytical tools the gene expression data could be classified and positioned into specific morphological and biological classes. However, the narrow time-window when EBs differentiates and forms the endoderm and BM has not been elucidated.

Specific aims of the thesis

- Identify genes that are influenced by impaired FGF/FGFR signalling during mouse ESC differentiation using microarray analysis. (Paper I)

- Analyze gene expression changes during four days of normal ESC differentiation. (Paper III)

- Elucidate the spatial expression pattern of the uncharacterized gene *1110032E23Rik* (named *Ened*) during embryonic development. (Paper II)

3. Results and discussion

3.1. Global gene expression analysis of differentiating dnFGFR2 and wild-type mouse EBs (Paper I and III)

What kind of biological and molecular processes are present during the early differentiating events of EBs? And what is failing when a mutant FGFR is introduced into these cells? To answer these questions, we set out to study the most significant gene expression changes during wild-type (WT) as well as dnFGFR2 expressing EB differentiation during four days. The four-day time period corresponds to mouse embryonic developmental stages from about E3.5 to E6, when the endoderm and BM form and the ectoderm polarization is initiated.

3.1.1. Gene expression changes in differentiating dnFGFR2 EBs (Paper I)

Previous work demonstrated that differentiating mouse EBs expressing dnFGFR2 failed to develop the two characteristic cell layers of EBs: the endoderm and the ectoderm. In addition, due to the impaired FGF/FGFR signalling, the mutant EBs failed to synthesize laminin-111 and collagen IV, the main building blocks of BM protein network (Chen et al., 2000; Li et al., 2001b). Nevertheless, the mutant EBs survived for weeks during the cultivation (Chen et al., 2000). A less extensive microarray analysis comparing the WT to dnFGFR2 EBs showed that loss of FGFR function inhibited a number of endoderm specific transcripts (Li et al., 2004).

However, the global gene expression changes between the WT and mutant EBs remained unrequited. Also, the fact that mutant EBs survived for an extensive period during EB culture (Chen et al., 2000) raised our curiosity on the molecular events taking place within these cell structures.

In Paper I, the global gene expression profiles of EBs expressing dnFGFR2 were elucidated by the use of Affymetrix GeneChip microarray platform (See Experimental procedures, Paper I). The goal was to identify genes that were markedly influenced by FGF/FGFR signalling during four days of EB culture. By setting the fold-change > 2 and a *p*-value < 0.05, only the most significantly down- and up-regulated genes were obtained from the microarray analysis. Comparison of dnFGFR2 to WT EBs at day 0, 1, 2 and 4 of EB culture resulted in an extensive catalogue of differentially expressed genes for each day. A large number of down-regulated genes was observed at day 0, 1, 2 and 4 (183, 252, 513 and 887, respectively). Because of the quantity, only the top 20 down-regulated genes are presented for a summarizing view of gene expression over the four days of EB culture (Table 1, with the fold-change of the genes indicated for each day; Complete lists in Paper I, Supplemental Tables 1, 3, 5 and 7).

TABLE 1. Top 20 Down-regulated Genes during dnFGFR2 EB Differentiation.

Day 0		Day 1		Day 2		Day 4	
Gene symbol	FC*	Gene symbol	FC	Gene symbol	FC	Gene symbol	FC
Sox17	-32.24	**Dab2**	-24.37	**Sox17**	-25.68	S100g	-24.42
Prg1	-18.78	**Prg1**	-24.17	Dkk1	-19.35	**Sox17**	-18.66
Flrt3	-13.36	**Sox17**	-21.04	**Ttr**	-19.13	**Lama1**	-18.34
Foxq1	-10.37	Tcfec	-18.55	**Cubn**	-18.91	**Gata6**	-18.06
Lama1	-10.14	**Nostrin (Daip2)**	-15.61	**Amn**	-17.87	Flrt3	-17.04
Gata6	-9.58	1200002N14Rik	-11.56	Tcfec	-17.51	Aqp8	-16.52
Col4a1	-8.36	Bmper	-11.02	**Sox7**	-17.47	**Pdgfra**	-15.81
Foxa2 (Hnf3β)	-8.17	Foxq1	-10.68	**Lama1**	-16.92	**Sox7**	-15.25
Lamb1-1	-7.63	Grb10	-10.41	4933402E13Rik	-16.79	**Afp**	-14.75
1110032E23Rik	-6.55	**Gata6**	-10.24	Flrt3	-16.74	Pga5	-14.59
Spink3	-6.53	**Sox7**	-9.97	Ctsh	-15.72	Grb10	-14.48
Col4a2	-6.51	Clic6	-9.00	Clic6	-15.04	Enpep	-14.14
Glipr1	-6.06	**Col4a1**	-8.48	**Dab2**	-14.81	**Cubn**	-14.10
Ctsc	-5.75	**Lamb1-1**	-8.46	**Nostrin (Daip2)**	-14.02	**Foxa2 (Hnf3β)**	-13.59
Mael	-5.52	**Pdgfra**	-7.33	Pga5	-13.77	**Cited1**	-13.14
MGC107533	-5.41	**1110032E23Rik**	-7.00	Spink3	-13.54	4933402E13Rik	-12.82
Car2	-5.40	Pthr1	-6.61	**Amot**	-13.34	Klb	-12.60
Efhc2	-5.40	**Foxa2 (Hnf3β)**	-6.45	Grb10	-13.24	Ctsh	-12.54
1200002N14Rik	-5.34	4933402E13Rik	-6.22	**Prg1**	-13.08	Lgals2	-12.04
Gja1	-5.32	Tspan8	-6.19	Car4	-12.85	Foxq1	-11.98

Genes in bold style are related to endoderm and BM processes
*Fold-change

Additional analysis of the down-regulated gene lists with the gene ontology (GO) annotation system (an analysis tool for molecular functions, biological process, and cellular components; (Harris et al., 2004), confirmed the findings that the most significantly down-regulated cellular component was BM at each time point. This outcome was surprising, since we anticipated that other genes related to processes such as FGF/FGFR signalling pathway, mitogenesis and migration would emerge as profoundly down-regulated transcripts due to deficient FGF/FGFR signalling. Overall, approximately 50% of the genes were related to either endodermal or BM related processes at each day (Table 1, genes denoted in bold).

3.1.2. Major impact of dnFGFR signalling on endoderm and BM regulatory genes (Paper I)

Already at day 0 of EB culture, when the ESC aggregates were transferred to bacterial culture dishes in the absence of LIF, a down-regulation of endoderm and BM related genes was observed and this tendency was maintained throughout four days of EB culture. Highly down-regulated genes from day 0 to day 4 were *Afp, Amn, Amot, Cited1, Col4a1, Col4a2, Cubn, Dab2, Foxa2 (Hnf3β), Gata6, Lama1, Lamb1-1, Nostrin (Daip2), Pdgfra, Prg1, Sox7, Sox17* and *Ttr*. All of these genes have been linked to endoderm and BM development (Abe et al., 1996; Choi et al., 2005; Drake et al., 2004; Dunwoodie et al., 1998; Esner et al., 2002; Futaki et al., 2003; Kalantry et al., 2001; Lee et al., 2005; Li et al., 2004; Li et al., 2001a; Li et al., 2001b; Miner et al., 2004; Morrisey et al., 2000; Niimi et al., 2004; Poschl et al., 2004; Shimono and Behringer, 2003; Sinner et al., 2004; Wang and Song, 1996; Yang et al., 2002), although only *Afp, Col4a1, Col4a2, Gata6, Lama1, Lamb1-1, Sox7* and *Sox17* expression has been shown to be influenced by FGF/FGFR signalling (Li et al., 2004; Li et al., 2001a; Li et al., 2001b; Murakami et al., 2004). In addition, several uncharacterized genes appeared in the most significantly down-regulated gene lists (*1110032E23Rik, 1200002N14Rik* and *4933402E13Rik*).

The expression of *1110032E23Rik*, elucidated in Paper II, appears to be located in epithelial cells and in the developing peripheral nervous system. The expression pattern of *1200002N14Rik* and *4933402E13Rik* is yet to be determined; however the human orthologue of *1200002N14Rik*, DRAM, was shown to be involved in autophagy and apoptosis in carcinoma cell lines (Crighton et al., 2007). *Amot, Cited1* and *Ttr* have been shown to be expressed in endoderm during

embryonic development (Abe et al., 1996; Dunwoodie et al., 1998; Shimono and Behringer, 2003). *Pdgfra* encodes a cell surface RTK that sends signals through among else the MAPK pathway, was shown to be expressed in the lens epithelium (Reneker and Overbeek, 1996), where FGF/FGFR signalling has an important role during embryonic lens development (Lang, 2004). In addition, in F9 embryonic carcinoma cells GATA4 induced the expression of *Pdgfra* (Wang and Song, 1996). The expression of *Gata4* is regulated by GATA6, and *Gata6* expression has been shown to be induced by FGFR signalling (Li et al., 2004; Morrisey et al., 1996) Thus, it is tempting to speculate that FGFR and PDGFR signalling pathways are converged through the MAPK pathway and the abrogation of FGFR signalling down-regulates the gene expression of PDGFR related components.

Another convergence can be drawn between the expression of *Dab2* and *Gata6*, *Cubn* and *Dab2*, and *Amn* and *Cubn*. DAB2 is a mitogen-responsive endocytic adaptor protein and a downstream target of GATA6 in the visceral endoderm (Morrisey et al., 2000). In order to form the first epithelial layer of endoderm, cells need to localize to the outer surface of the spherical structure of EBs. DAB2 mediates the directional transport of vesicles and was shown to be essential for surface sorting and positioning of endoderm cells in developing EBs (Rula et al., 2007). *Cubn* encodes an endocytic multiligand scavenger receptor that is located at the apical pole of epithelial cells, and requires DAB2 for correct localization in the cells (Maurer and Cooper, 2005). In addition, studies in AMN -/- mouse embryos showed that *Amn* is necessary for *Cubn* expression in epithelial cells (Strope et al., 2004). *Nostrin* (also called *Daip2*) is involved in the intracellular trafficking of eNOS (Icking et al., 2005) and its interaction with DAB2 has been shown in retinoic acid induced F9 embryonal carcinoma cell differentiation (Choi et al., 2005). Furthermore, the endodermal expression of *Prg1* (also called *Serglycin*) was demonstrated during embryonic development (Keith Ho et al., 2001). *Prg1* encodes a secreted proteoglycan that might be involved in vesicle cargo packaging and secretion (Schick et al., 2001). It is unclear how these genes are linked to the FGF/FGFR pathway, although it is tempting to hypothesize that FGFR downstream signalling activates gene expression related to cell positioning, subsequent polarization and vesicle trafficking within the developing EBs.

3.1.3. Genes previously not associated with endoderm or BM development (Paper I)

Other genes among the top 20 down-regulated lists were related to transcription and translation (*Foxq1, Mael, MGC107533, Tcfec*), enzymes (*Car2, Car4, Ctsh, Enpep, Pga5, Spink3*), receptor signalling (*Bmper, Dkk1, Flrt3, Grb10, Klb, Pthr1*), ion and water transport (*Aqp8, Clic6*), plasma membrane (*Glipr1, S100g, Tspan8*) and cellular processes (*Efhc2, Gja1, Lgals2*).

How can these findings be linked to deficient FGFR signalling, and is there any role for these genes in endoderm and BM development? Dissecting the roles for individual genes upon depletion of a central regulatory pathway (*i.e.* the FGF/FGFR pathway) is complex, because of extensive cross-talk and convergence between different pathways. However, several of these genes have been shown to be connected to FGFR signalling and endoderm development in some way. For example, *Flrt3* codes for a fibronectin leucine rich transmembrane protein that forms a complex with FGFR and thereby positively regulates FGFR signalling (Bottcher et al., 2004) (Figure 1B). *Flrt3* was found to be widely expressed during mouse embryonic development and its expression was induced by FGF/FGFR signalling (Haines et al., 2006). Interestingly, another member of the FLRT family, *Flrt2*, was also down-regulated (although not to the same extent as *Flrt3*) in dnFGFR2 mutant EBs with a negative fold-change of 2.96 at day 4 (Supplemental list 7). Recent findings showed that targeted gene deletion of *Flrt3* did not exert any effects on FGF/FGFR signalling during early embryonic development, although mutant embryos displayed impaired headfold fusion and visceral endoderm migration (Maretto et al., 2008). These findings suggest that *Flrt3* has important roles in cell adhesion and early morphogenesis during development.

Gja1 (also called *Connexin43*) codes for a gap junctional protein that is expressed in the lens epithelium, and studies showed that its expression is positively regulated by FGFs (Le and Musil, 2001). In addition, recently in has been shown that *Klb*, which encodes a type I membrane protein, serves as a vital subunit in the FGF21-FGF21R synexpression group (Kharitonenkov et al., 2008), and that *Tspan8* (also called *Tm4sf3*) expression might be a target of FGF10 during lung morphogenesis (Lu et al., 2005). Hence, the decrease of these genes can directly be linked to the deficient FGF/FGFR signalling and thus indirectly to endoderm and BM related gene expression.

On the other hand, down-regulation of negative regulators of the BMP and WNT pathways also was observed. During early development, FGF/FGFR signalling modulates the activity of among else the BMP and WNT pathways, thereby promoting differentiation. *Bmper* codes for a secreted protein that binds BMPs and inhibits their signalling, thereby modulating cell differentiation (Moser et al., 2003), while *Dkk1*, that codes for a WNT signalling antagonist, was shown to be down-regulated in FGFR1-/- mouse embryos (Hajihosseini et al., 2004). The decrease of *Bmper* and *Dkk1* could be due to active BMP and WNT signalling that inhibit their repressor´s activity. This points out the importance of FGF/FGFR signalling that must repress BMP and WNT signalling components for correct development. Parallels in the down-regulation of *Dkk1* and *Foxa2* were implicated by a study in *Otx2* transgenic mice where it was shown that *Foxa2* expression, which is crucial for endoderm specific gene regulation, was also necessary for the induction of *Dkk1* expression (Kimura-Yoshida et al., 2007). The significant down-regulation of genes encoding enzymes, ion and water transport and membrane associated molecules further indicates that the main machinery for the proper cell sorting and positioning, cargo trafficking and cellular homeostasis appears to be blocked by deficient FGF/FGFR signalling.

Hence, our microarray study substantiates the notion that impaired FGF/FGFR signalling abrogates endodermal development and the subsequent secretion of BM components.

3.1.4. Insufficient FGF/FGFR signalling allows the activity of other signalling pathways (Paper I)

In the early stages of embryonic development, the primitive ectoderm induces the adjacent cells to form mesodermal structures by the process of EMT (Thiery and Sleeman, 2006). During the differentiation of WT EBs, the BM subsequently undergoes regional degradation, thereby promoting the mesodermal differentiation from the primitive ectodermal cells (Coucouvanis and Martin, 1995). Different pathways, such as the WNT and FGF/FGFR signalling pathways, work in parallel and in an orchestrated fashion to accomplish these morphogenetic events (Lonai, 2005; Zorn and Wells, 2007). As mentioned previously, upon differentiation dnFGFR2 ESCs were able to aggregate and survive for an extended period of time in EB culture without disintegrating. Although studies pointed toward the fact that dnFGFR2 EBs

were composed of undifferentiated homogenous stem cell aggregates, it has been shown that the outer cell layer of dnFGFR2 EBs were able to recognize external differentiation signals, such as laminin-111, and induced polarization of the underlying cells to form ectodermal structures (Li et al., 2004; Li et al., 2001b).

Indeed, our microarray analysis confirmed the lack of gene transcription related to endoderm and BM; however the up-regulated genes presented by the microarray data indicated an active gene transcription in the mutant EBs. The number of the up-regulated genes was fewer than the down-regulated ones with 62, 43, 90 and 212 genes at day 0, 1, 2 and 4, respectively. The up-regulated genes in dnFGFR2 EBs throughout four days of EB differentiation were related to different signalling routes, where several components were affected and emerged in clusters within the activated pathways. These genes were related to the Hedgehog (*Hhip, Smo* and *Gli2*), TGFβ (*Lefty2, Arid5b, Tgfbi*), NOTCH (*Dtx1, Notch4*), BMP (*Smad1*), VEGF (*Vegfc*), EGF (*Hbegf*), IGF (*Igf2bp3*) and WNT (*Sfrp1, Mitf, Ski*) signalling pathways. Other genes observed with significant up-regulation were *Mest (Peg1), Mras, Nnat (Peg5), Odz3* and *Trps1*. In addition, genes related to the maintenance of ICM pluripotency in the embryo and undifferentiated ESC, such as *Esrrb, Nanog, Nr5a2* and *Zfp42* were detected (Complete lists in Paper I, Supplemental Tables 2, 4, 6 and 8; Collection of up-regulated genes in Table 2).

TABLE 2. Collection of Genes with Up-regulated Expression during dnFGFR2 EB Differentiation.*

Extracellular matrix / Remodeling	Spp1, Matn2, Mmp2, Mmp11
Signaling / Receptor signaling related / Factors	Arid5b, Dtx1, Dusp16, Emp1, Erbb2ip, Foxp1, Gli2, Hbegf, Hck, Hhip, Ifitm1, Ifitm2, Ifitm3, Igf2bp3, Inhbb, Mitf, Mras, Notch4, Rai3, Rasgrp2, Schip1, Sfrp1, Ski, Smad1, Smo, Tcf4, Tcl1, Tgfbi, Tm4sf10, Tmem45a, Trsp1, Vax2, Vgefc, Zfp521
Cytoskeleton / Cell junctions related	Cobl, Epb4.1l3, Fnbp1, Gja7, Jam2, Myl9, Myo1f, Myo5a, Sntb2, Tmod2
Pluripotency related	Gdf3, Esrrb, Nanog, Nr5a2, Zfp42
Development	Lefty2, Mest, Nnat, Odz3

*Complete gene lists in Paper I, Supplemental Tables 2, 4, 6 and 8.

These results were intriguing, since they indicated that dnFGFR2 EBs up-regulated genes related to mesodermal differentiation without passing through the obligatory endodermal and ectodermal stages that would be the case during gastrulation and EB development. In addition, the presence of genes related to pluripotency indicates that dnFGFR2 EBs are composed of mixed undifferentiated cell populations and cells that due to impaired FGF/FGFR signalling launched the transcription of genes expressed in later stages of development. This clearly

demonstrates the importance of a correctly functioning FGF/FGFR signalling, where its actions converge with other signalling pathways to promote normal development in the organism.

How can these observations be explained? Lung development is an excellent model to unfold at least a part of the mystery with the up-regulation of the observed genes. Early lung organogenesis is controlled by several transcription factors and signalling pathways, delicately cooperating in a sequential manner to establish the bases for epithelial-mesenchymal interactions (EMI) to promote lung branching morphogenesis and create a working blood-gas interface in the lung alveoli (Kumar et al., 2005). Initiation of epithelialization that leads to EMI requires FOXA2 and GATA factor signalling, and branching morphogenesis is initiated primary by FGF10 whose activity is regulated or repressed by among else BMP, EGF, Hedgehog, TGFβ and PDGF signalling components. It seems that due to the deficient FGF/FGFR signalling in dnFGFR2 EBs, the components of these pathways are activated prematurely and the lack of negative feed-back from FGF/FGFR signalling allows the continuous activity of these genes.

This hypothesis is supported by a study in ESCs, in which deficient FGF/FGFR signalling failed to attenuate BMP signalling and hence increased the expression of mesodermally related markers (Willems and Leyns, 2008). The phenomenon displayed in dnFGFR2 EBs has also been observed in EBs that lacked the γ1-chain of laminin-111 (LAMC1-/- EBs), where EBs formed endoderm structures but failed to induce the deposition of the underlying BM (Fujiwara et al., 2007). The absence of the BM led to activation of mesodermally related genes in the mutant EBs, just like in dnFGFR2 EBs, although in the latter model the absence of endoderm (that forms before the BM) caused accelerated transcription of mesodermally related genes. During normal EB differentiation around day 7, the BM subsequently undergoes regional degradation and promotes the subsequent differentiation of mesoderm from the ectodermal cells through EMI. Thus, the presence of the BM is significant in the formation of the second epithelial layer, *i.e.* the ectoderm of EBs, although its presence is not prerequisite for mesodermal differentiation, which is also demonstrated by our study in dnFGFR2 EBs.

3.1.5. Analysis of the sequential gene expression changes during four days of WT EB differentiation (Paper III)

The molecular mechanisms associated with early developmental events occurring during implantation throughout gastrulation are believed to be sequential modulations of distinct sets of genes enabling cell differentiation.

In Paper III, the temporal gene expression changes during four days of spontaneous ESC differentiation were investigated. The focus of this study was to concentrate on the narrow time period from the emergence of the first germ layer of endoderm, the deposition of the underlying BM and the initiation of the polarization of the second germ layer of ectoderm. These differentiation events mimic the early embryonic development from the blastocyst stage to the beginning of the egg-cylinder stage (from about E3.5 to E6).

Differentiation of ESCs was investigated on five different time points: 0, 1, 2, 3 and 4 (undifferentiated ESC, day 0, day 1, day 2 and day 4 of EB culture, respectively). To obtain only the most significant differentially expressed genes for the five different time points, the microarray raw-data was stringently filtered by ANOVA test using a *p*-value = 0.000001 (See Experimental procedures, Paper III). From this filtration, a list of 429 genes emerged displaying difference in at least one of the time points. These genes were classified into six clusters to characterize their temporal transcription patterns. Each cluster represents genes with similar expression behavior over the observed time period (Complete lists in Paper III, Supplemental Tables 1 to 6). A selection of genes from each cluster is shown in Figure 3, divided into their functional groups according to the GO annotation system.

Gene expression changes are modulated through transcription factors and regulators that appeared to be significantly represented in all clusters analyzed. Both down-regulated (*Bcl3, E2f4, Lmnb2, Nfib, Nr5a1, Nup98, Taf8*) and up-regulated (*Bach1, Creb, Ets2, H1f0, Pcgf6, Rnf12, Yaf2, Morf4l2*) genes related to DNA-binding and transcription was found as significantly induced during EB differentiation.

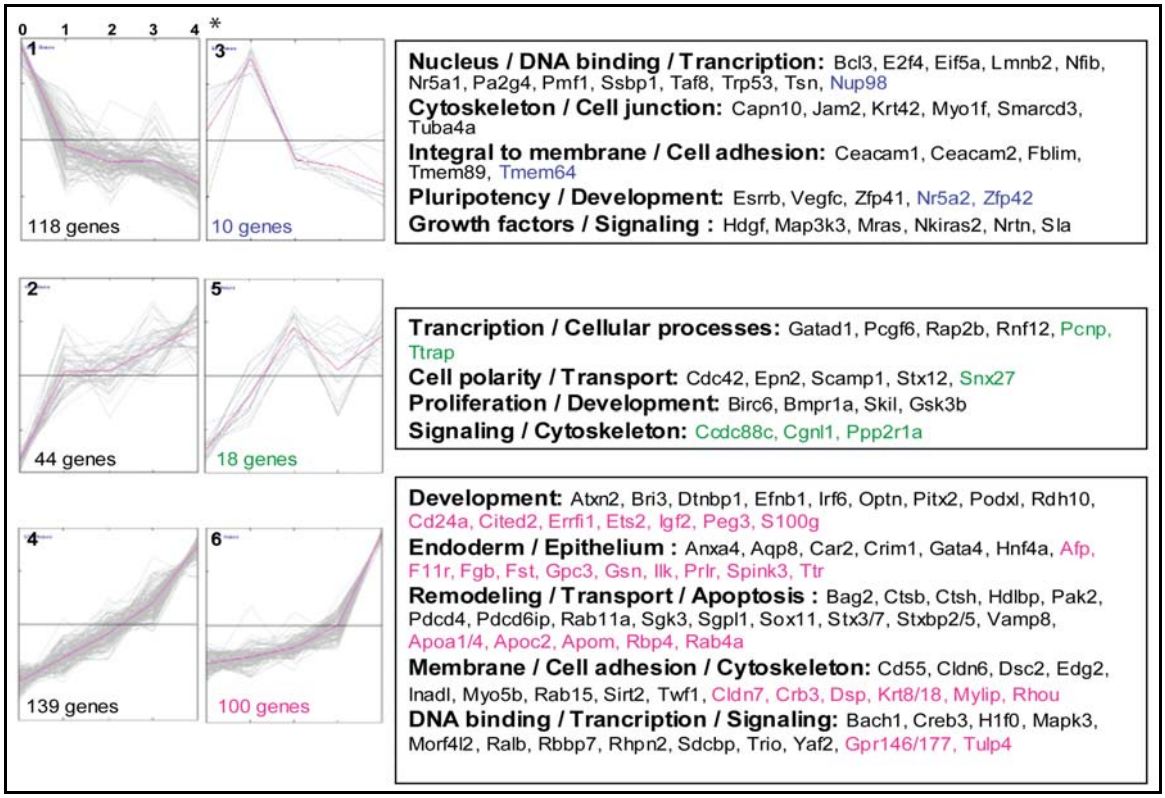

Figure 3. Selection of significantly expressed genes during four days of WT EB differentiation. After filtration of the microarray raw-data, the resulting 429 significantly expressed genes were clustered according to their temporal transcription pattern. Each cluster contains genes with similar expression behavior over the observed time period. Genes found in cluster 3, 5 and 6 are color coded in each box. *(Time point 0 = Undifferentiated ESCs; Time point 1 = Day 0 of EB culture; Time point 2 = Day 1 of EB culture; Time point 3 = Day 2 of EB culture; Time point 4 = Day 4 of EB culture).

The most dramatic gene expression changes occurred from time point 0 to 1 (undifferentiated ESC to day 0 of EB culture) when pluripotent WT ESC were transferred to bacterial culture dishes in suspension culture media without LIF. In order to engage differentiation events, ESCs need to initiate the down-regulation of certain genes related to pluripotency (*Essrb, Nr5a2, Zfp42*). The interplay of *Essrb, Nr5a2* and *Zfp42* with the core transcription factors *Oct4, Nanog* and *Sox2* is important in the maintenance of pluripotency in ESCs (Kim et al., 2008; Zhou et al., 2007). On the other hand, up-regulation of genes controlling cellular differentiation, remodelling and development of the first epithelial layer, *i.e.* the endoderm (among else *Afp, Bmpr1a, Car2, Ctsb, Ctsh, F11r, Gata4, Hnf4a, Irf6*) is necessary to commence differentiation. The GATA factors *Gata4* and *Gata6* are master regulatory genes for endoderm development (Fujikura et al., 2002), which was demonstrated by GATA6-/- EBs that failed to express early and late endodermal markers such as HNF4, GATA4 and AFP (Morrisey et al., 1996). In addition, the endodermal expression of *F11r* and *Bmpr1a* (Davis et al., 2004; Thomas et al., 2004) has been

shown during embryonic development. E-cadherin has an important function during compaction of the ICM in the early blastocyst (Ohsugi et al., 1996), and during aggregation of ESCs (Larue et al., 1994). E-cadherin signalling is connected to the β-catenin/GSK3/WNT pathway (Huber et al., 1996), where GSK3 was shown to be activated by β-catenin and inhibited by WNT signalling (Katoh and Katoh, 2007). In addition, WNT signalling promoted the maintenance of ESC pluripotency when *Gsk3* was inhibited (Sato et al, 2004). These facts are reflected in our study, where *Gsk3b* was gradually up-regulated (cluster 5) from the undifferentiated ESCs (time point 0) toward day 4 (time point 4) of EB differentiation.

The next task during EB differentiation is to initiate the secretion of the underlying BM between time point 2 and 3 (day 1 and 2 of EB differentiation). The MAPK pathway has been shown to be important in the modulation of ESC linage commitment (Binetruy et al., 2007). This pathway can be activated through RTKs such as FGFRs that in turn interact with small Rho GTPases (Schiller, 2006). The importance of FGF/FGFR signalling and its downstream targets, the MAPK and PI3K/AKT pathway has been shown to be necessary for activation of BM related genes, such as laminin-111 and collagen IV (Chen et al., 2000; Li et al., 2001b). Although no transcripts coding for BM proteins occurred among the most significantly represented genes in the six clusters, the up-regulation of genes related to the MAPK pathway and Rho GTPases (*Cd24a, Mapk3, Rab4a, Rab11a, Rab15, Ralb, Rhpn2, Trio*) and genes related to vesicle transport and secretion (*Apoa1, Apoa4, Apoc2, Apom, Hdlbp, Stx3, Stx7, Stxbp2, Stxbp5, Vamp8*) were observed. *Mapk3* is a downstream target of FGF/FGFR signalling and was up-regulated gradually from time point 0 to 4 (cluster 4). Interestingly, it has been proposed that FGF/FGFR signalling was required for undifferentiated ESCs to exit self-renewal and initiate differentiation events (Kunath et al., 2007), which strengthen the findings in Paper I, where the significance of FGF/FGFR signalling was discussed during endoderm and BM development. In addition, the gene encoding the Rho GTPase *Rhou*, that is a component in the non-canonical WNT pathway (Katoh and Katoh, 2007), was gradually up-regulated from time point 0 toward time point 3, and its expression rapidly increased to time point 4 (cluster 6). Rho GTPases regulate the assembly and organization of the cytoskeleton (Bishop and Hall, 2000) and are important in the coordination of vesicle transport and endo/exocytosis (Lanzetti, 2007).

When the BM components are secreted, the initiation of the polarization of the second epithelial layer, *i.e.* the ectoderm takes place from time point 3 to 4 (between days 2 and 4 of EB differentiation). During this time period, the up-regulation of genes related to polarization (*Cdc42*) and morphogenesis and organogenesis (*Bri3, Cited2, Dtnbp1, Efnb1, Optn, Pitx2, Podxl, Rdh10, Igf2, Peg3*), and down-regulation of genes related to later developmental processes (*Vegfc, Zfp41*) were observed. The role of the small Rho GTPase *Cdc42* was demonstrated during the establishment of the epithelial polarity in CDC42 -/- EBs (Wu et al., 2007) and its link to the BM has been demonstrated during ectoderm polarization (Li et al., 2004). The overlapping expression of *Cited2* and *Pitx2* was demonstrated during heart development (Weninger et al., 2005), while the expression of the imprinted genes *Igf2* and *Peg3* was shown to be important for normal embryonic development (Deng et al., 2007). In addition, the expression of *Rdh10* that is involved in the retinoic acid signalling (Cammas et al., 2007) and *Efnb1* (Davy et al., 2004) has been shown to be expressed during embryonic development. Also, genes coding for apoptosis related processes that are necessary for cavitation during EB differentiation (Coucouvanis and Martin, 1995) were launched at this stage (*Bag2, Birc6, Pak2, Pdcd4, Pdcd6ip, Sgk3, Sgpl1*). Apoptosis is initiated around day 5 to 6 of EB development, when the centrally located cells within the EB start to form the cavity equivalent to the proamniotic cavity in embryos. Although apoptosis is related to later events, the transcription of genes encoding apoptosis related proteins was activated in the EBs.

Taken together, in Paper III we provide a catalogue of genes that were significantly expressed during the formation of the first epithelial layer (the endoderm), the BM and the second epithelial layer (the ectoderm) during EB differentiation. The results indicated that ESCs need to rearrange their transcriptional machinery and rapidly down-regulate their pluripotency genes to could continue the differentiation events leading to the formation of endoderm, the subsequent secretion of BM components and the polarization of ectoderm.

3.1.6. Common gene expression observed in WT and dnFGFR2 EBs (Paper I and III)

Interestingly, there were several genes that were present in both the significantly expressed gene lists of WT EBs and the down-regulated list of dnFGFR2 EBs (Table 3, genes marked red). The conclusion of this outcome could be the following: (i) In

order to initiate differentiation, ESCs need to activate transcription of among else small GTPases (*Rhpn2, Sirt2, Trio*) to carry out cytoskeletal rearrangements (*Crb3, Gsn, Krt18, Mylip*) for relocalization of ESCs - destined to become endodermal cells - to the outer rim of the EB structure. (ii) Then, through up-regulation of genes encoding proteins involved in vesicle transport and uptake of nutrition (*Apoa1/4, Apoc2, Apom, Rab11a, Rbp4, Stx3/7, Tulp4, Vamp8*), formation of endoderm is undertaken (*Aqp8, Afp, Car2, Cldn6, F11r, Gata4, Hnf4a*). (iii) The endoderm initiates the secretion of BM and ECM components, and remodeling of ECM (*Ctsh, Fst, Sdcbp, Spink3*), polarization of ectoderm (*Ilk, Igf2, Irf6, Rhou*), morphogenesis (*Efnb1, Gpc3, Krt8, Optn, Podxl, Rdh10, S100g*) and apoptosis (*Bag2, Peg3*) begins. These genes appear to be important for early morphological events, since they were observed in both normal and FGFR-deficient EB differentiation systems.

TABLE 3. Collection of Down-regulated Genes Observed throughout Four Days of dnFGFR2 EB Differentiation and Their Correlation to Genes during WT EB Differentiation

Endoderm related	Afp, Amot, Cited1, Cited 2, Cldn6, Cubn, Dab2, Flrt2, Flrt3, Foxa2, Foxq1, Gata4, Gata6, Hnf4a, Nostrin, Sox7, Sox17, Tcf2, Ttr, Pdgfra
Basement membrane / Extracellular matrix / Remodeling	Adamts1, Adamts9, App, Col4a1, Col4a2, Cthrc1,Ctsc, Ctsh, Ctsl, Ctsz, Dpp4, Enpep, Fst, Gpc6, Habp2, Has2, Hs3st1, Kitl, Lad1, Lama1, Lamb1-1, Lamc1, Lcn7, Leprel1, Lgmn, Lrpap1, Nid1, P4ha1, Pep4, Plod2, Pros1, Prss11, Rbp4, Sdcbp, Serpina3m, Serpine2, Serping1, Serpinh1, Sparc, Spink3, Thbs1, Timp, Timp3, Tmprss2
Epithelium / Epithelial-Mesenchymal transition related	Aqp8, Bmp2, Car2, Dnmt3a, **1110032E23Rik**[#], Ets1, F11r, Glipr1, Glipr2, Idb3, Ilk, Igf2, Irf6, Marveld2, Pdlim1, Pou3f1, Procr, Rhoc, Snai1, Tspan8, Vil1
Signaling / Receptor signaling related / Factors	Akap2, Anxa3, Arhgap18, Arhgef3, Arhgef5, Arhgef16, Calm1, Car4, Chn2, Crim1, Cst3, Dkk1, Dusp9, Edg2, F2r, Fgb, Fgf5, Fgf15, Frzb, Gas6, Gpr126, Grb10, Hey2, Irs1, Lgals2, Lgals9, Pem, Pik3ap1, Pik3r1, Plaur, Plk2, Ptpn14, Pthr1, Rab20, Ramp2, Rbp1, Rhoc, Rhou, Rhpn2, Rragc, Rras2, Runx1, Sirt2, Spry4, Tax1bp3, Tgfbr2, Tgfbr3, Tmem20, Tmem27, Tmem30b, Tnk1, Tfpi, Trio, Vwf
Morphogenesis / Organogenesis related	Bicc1, Cryab, Cyp26a1, Cxadr, Edg7, Efnb1, Efnb2, Fhl1, Fndc3b, Folr1, Gpc3, Hesx1, Idb2, Ihh, Klf6, Krt8, Lyn, Nedd9, Neurod, Nr6a1, Optn, Pcdh19, Podxl, Prickle1, Rdh10, S100g, Sycp3, Tnfrsf19
Homeostasis / Secretion / Endocytosis / Exocytosis / Granulas	Apoa1, Apoa4, Apob, Apoc2, Apom, Car12, Clcn5, Clic5, Clic6, Efhc2, Fcgr3, Ghr, Lmna, Ly6a, Napa, Neu1, Nxf7, Prg1, Prkwnk3, Rab11a, Rab11fip5, Rbp4, Ripk4, Scarb2, Sepw1, Stx3, Stx7, Synj1, Tcfec, Tulp4, Vamp3, Vamp8
Cytoskeleton / Cell junctions related	B2m, Catnal1, Cipp, Cotl1, Crb3, Csrp1, Dcs2, Dsg2, Eps8, Esam1, Eva1, Gja1, Gjb5, Gsn, Krt18, Lpp, Mylip, Myo6, Myo7a, Nef3, Nefl, Pard6b, Rhoq, Shrm, Smarca2, Stmn2, Tmod3, Zyx
Apoptosis related	Atf3, Bag2, Bcl2l11, Clu, Dap, Itm2b, Peg3, Pim2, Rnf130, 1200002N14Rik (Dram)

*Complete gene lists in Paper I, Supplemental Tables 1, 3, 5 and 7 and Paper III, Supplemental Tables 1 to 6.
[#]1110032E23Rik (Ened: Expressed in Nerve and Epithelium during Development; Expression elucidated during embryonic development in Paper II).

3.2. Analysis of the uncharacterized gene *1110032E23Rik* (Paper II)

3.2.1. Sequence and homology analysis of 1110032E23Rik, and its new alias: Ened

Microarray analysis of the global gene expression changes during dnFGFR2 expressing mouse EB differentiation resulted in an extensive catalogue of genes that were influenced by FGF/FGFR signalling (Paper I). The study provided an opportunity to elucidate the expression and function of hitherto uncharacterized genes during embryonic development. From the lists of significantly down-regulated genes in dnFGFR2 EBs (Table 1), the gene *1110032E23Rik* was chosen for further studies because of its potential connection to FGF/FGFR signalling.

The expression of *1110032E23Rik* was highly down-regulated at day 0 and 1 during dnFGFR2 EB development, with 6.55- and 7-fold decrease in gene expression, respectively, when compared to WT EBs. Although *1110032E23Rik* was not among the top 20 down-regulated genes at day 2 and 4, its expression remained low with a decreased fold-change of 5.14 and 6.42 respectively, during dnFGFR2 EB culture. A preliminary search on *1110032E23Rik* in the Ensemble database (See Experimental procedures, Paper II) showed that the transcript codes for a 517 long amino acid sequence and contains a putative transmembrane domain.

The *1110032E23Rik* genomic sequence is located on chromosome 3E3, and contains 4 exons (Figure 4A). Searching the GenBank database, one cDNA clone was found (I.M.A.G.E. clone MGC:66815; GenBank Accession No. BC056975) to contain the complete coding sequence of *1110032E23Rik*. Sequencing revealed a transcript of 3538 base pairs that were deposited to the GenBank database (Accession No. pending). Nucleotide BLAST search of *1110032E23Rik* showed no homology to other transcripts in mouse, however when comparing the transcript against the GenBank EST database, results showed that *1110032E23Rik* had considerable identity to several other vertebrate transcripts, such as human (83%), horse (83%), opossum (72%) and chicken (61%) (GenBank Accession No. NP_001026870.1, XP_001500550, XP_001366268.1 and XP_420382.2), and multiple alignment of predicted amino acid sequences for these species demonstrated near homology (Figure 4B). To further validate the sequence comparison, we identified an orthologous EST sequence in *Xenopus laevis* (African clawed frog). The EST clone

(I.M.A.G.E. clone 8070075) contained a 1.5 kb insert and was partially sequenced (Accession No. pending). The predicted transmembrane domain for *1110032E23Rik* amino acid sequence was confirmed by the TMpred hydrophobicity search algorithm. The putative transmembrane domain, spanning from amino acids 38-56, appeared to be conserved between mouse, human, horse, opossum and chicken (Figure 4B; box).

Next, the spatial expression of *1110032E23Rik* was elucidated by non-radioactive whole mount and radioactive in situ hybridization analysis during mouse and frog embryonic development. Intriguingly, the expression of *1110032E23Rik* was observed at sites with known FGF/FGFR signalling activity and BM location, such as the developing peripheral nervous system and epithelium. From the observed expression pattern in mouse embryos, *1110032E23Rik* was thereby given its new designation ***Ened*** **(Expressed in Nerve and Epithelium during Development)**.

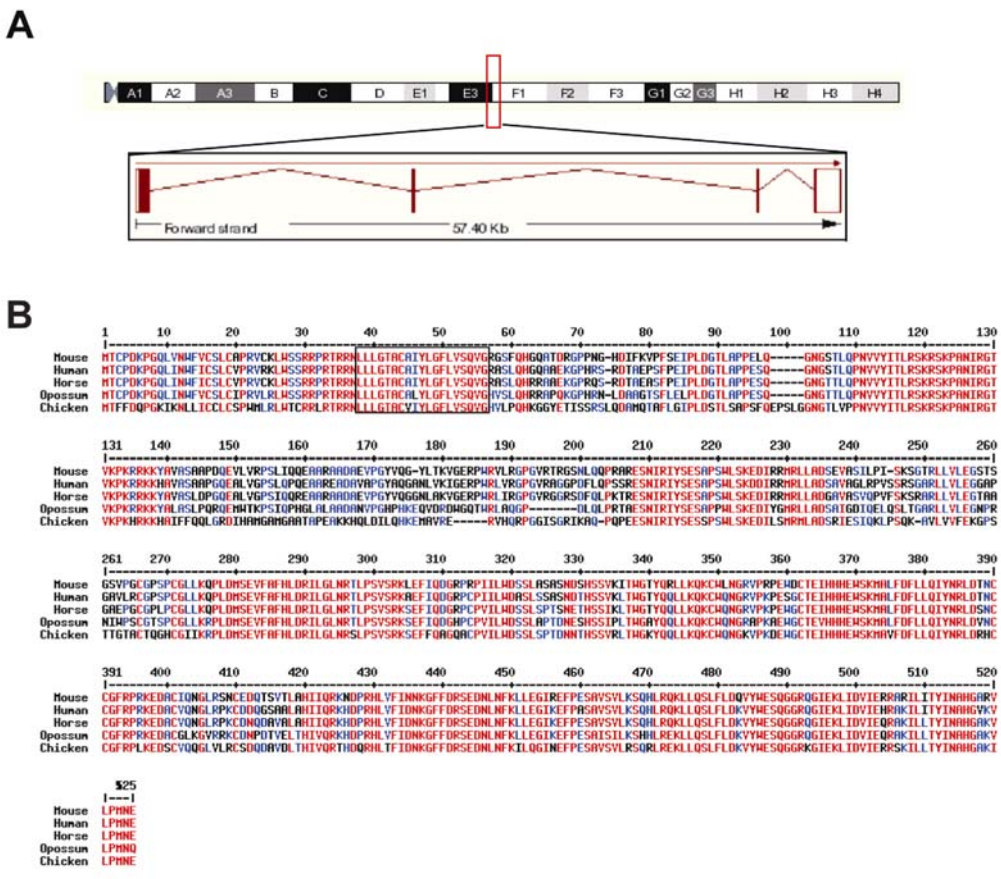

Figure 4. Chromosomal location and homology analysis of *1110032E23Rik*. (A) *1110032E23Rik* gene is located on the mouse chromosome 3 domain E3, and its transcript contains 4 exons. (B) Multiple alignment of the 517 amino acid bases long *1110032E23Rik* sequence with homologous sequences of other vertebrates. The transmembrane domain from amino acids 38-56 is marked with an open box.

3.2.2. Epithelial expression of Ened at early organogenesis from E9.5 to E14.5 in mouse embryos

During gastrulation at around E6.5 to E7.5, major cell fate decisions and cell linage allocations take place in mouse embryos. As cells migrate, they pass through the primitive streak and allocate to either to the endodermal or the mesodermal cell layer. At around E9, the gut tube closes, the lateral mesoderm condenses over the endoderm and EMI further patterns the gut and regulates the specification and budding of different organs, such as lung, pancreas and liver (Stainier, 2005). At these stages, the epithelial lining of the future gut expresses FGFR2 that cooperates with FGF10 from the mesenchymal layer (Roberts, 2000). Also, FGF4 has been shown to regulate the anterior-posterior patterning of the early gut tube (Dessimoz et al., 2006). The BM underlies epithelial cells in the developing gut and is an important component in the regulation of EMI (Simo et al., 1992). The mesodermal layer will give rise to the heart muscle, and positive signalling most prominently from the endoderm induces mesodermal cells to become cardiogenic. These signals include BMP2, FGF8, and WNT11 (Abu-Issa and Kirby, 2007). In addition, GATA4 is required for the morphogenesis of the endoderm of foregut and hindgut (Roberts, 2000), and it was demonstrated that GATA4 -/- mouse embryos lacked foregut structures and had defect cardiac development due to the lack of signals from the disrupted endoderm (Molkentin et al., 1997). Also, the presence of FGFR1 and FGFR2, and BM components adjacent to the endocardium has been shown at this stage in the heart (Nakajima et al., 1997).

Ened expression appeared at E9.5 in the inner epithelial lining of heart atrium and ventricle, and in the midgut (Figure 5). At this stage, the heart has already undergone the initial stages of morphogenesis, and continues to receive further inductive signals from the adjacent foregut endoderm emanating from TGFβ, BMP, VEGF and FGF/FGFR pathways. The restricted expression of *Ened* observed at this stage could be due to morphogen gradients of FGF8 and FGF10 from the overlying mesenchyme, and the adjacent BM that might have modulator activity on *Ened* expression.

From E10.5, *Ened* expression was observed in the mesenteric lining of the gut, stomach and duodenum, and in addition in the urogenital ridge and midline dorsal aorta (Figure 5). The spreading expression pattern of *Ened* in the gastrointestinal tract indicated a gradient dependent induction from the foregut mesoderm on the adjacent

epithelium. Such a gradient could be exerted by retinoic acid that induces the expression of FGF10 in the foregut mesoderm (Desai et al., 2004), which in turn sends a signal to the endodermal layer of the foregut and hence may induce the expression of *Ened*.

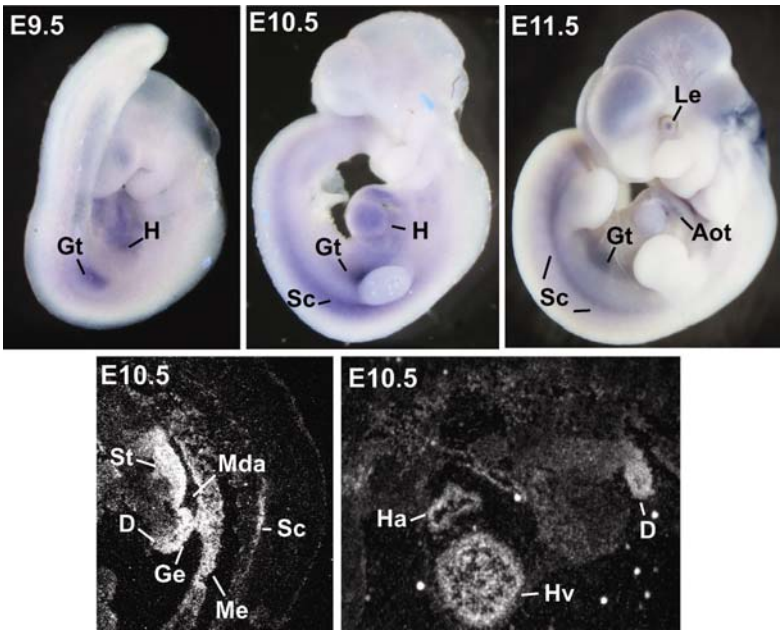

Figure 5. *Ened* expression from E9.5 to E11.5 mouse embryos. Upper panel: lateral view of mouse embryos at E9.5, E10.5 and E11.5, whole mount in situ hybridization with *Ened* probe. At E9.5 *Ened* expression was seen in heart and midgut, at E10.5 additional expression appeared in the sympathetic chain. At E11.5 expression was observed in the lens epithelium. Lower panel: radioactive in situ hybridization on sagittal sections of E10.5 mouse embryos. Expression was detected in the epithelial lining of the stomach, duodenum, mesentery, genital eminence and midline dorsal aorta. Additional magnified view of the thoracic area showing labeling in the heart atrium and ventricle and in the duodenum. Aot, aortic outflow tract; D, duodenum; Ge, genital eminence; Gt, gastrointestinal tract; H, heart; Ha, heart atrium; Hv, heart ventricle; Le, lens epithelium; Mda, midline dorsal aorta; Me, mesentery; Mg, midgut; Sc, sympathetic chain; St, stomach.

The mammalian mesonephros develops from the mesodermal region called aorta–gonad–mesonephros zone. The gonad develops within the midsection, and the future kidney will arise from the posterior section of the urogenital ridge. This development is regulated by among else SPROUTY2, SOX9, WNT4 and FGF9 (Capel, 2000; Chi et al., 2004; Stark et al., 1994; Yoshioka et al., 2005) and the BM underlying the mesenchyme (Kuure et al., 2000). The midline dorsal aorta is adjacent to the gut tube and receives inductive signals from both the gut and notochord through the VEGF, Hedgehog and BMP signalling pathways, and might also be induced by

the BM secreted from the epithelial cells destined to be endothelium (Risau and Lemmon, 1988), however it is not clear if the midline dorsal aorta has any role in cell migration of the developing gonads and mesonephrose. The primitive gut is enveloped by the mesentery, which is derived from the mesoderm and contains a BM. Later during development it will form the peritoneum and functions as a suspender for the gut from the dorsal wall of the foregut to the hindgut, and was suggested to have role in primordial germ cell migration to the gonads (Molyneaux et al., 2001), during which FGFs have a regulatory role (Kawase et al., 2004). It is thus tempting to hypothesize that the progressive expression of *Ened* could be due to the expanding epithelia that receive FGF signals from the adjacent mesenchyme and that its expression is maintained or regulated by the adjacent BM.

At E13.5 expression appeared in the epidermis of the skin (Figure 7). Interestingly, *Ened* expression coincided with the appearance of laminin-111 and collagen IV in the BM underlying the epidermal epithelium and not with laminin-511 that is deposited already at E7 of epidermal morphogenesis (Fleischmajer et al., 1998). In addition, FGF/FGFR signalling was shown to be necessary for the proper epidermal development through the signalling of FGF10 to its receptor FGFR2 (Petiot et al., 2003).

At E14.5 *Ened* expression was seen, additionally to the gastrointestinal tract and mesentery, in the epithelial lining of developing organs such as lung, kidney, testis and submandibular gland (Figure 7). The lung is derived from the foregut endoderm and the submandibular gland from the oral epithelium, while the kidney and testis are derived from the urogenital ridge. What is the common developmental denominator between these organs that could induce *Ened* expression?

At mid-gestational stages from around E11, FGF/FGFR signals through FGF8, FGF10 and FGFR2, and the BM with its component laminin-111 were demonstrated to be necessary in the maintenance of a proper reciprocal signalling between the epithelium and mesenchyme during lung, kidney and submandibular gland morphogenesis (Arman et al., 1999; Bates, 2007; Durbeej et al., 1996; Ekblom et al., 1998; Kadoya et al., 1995; Steinberg et al., 2005). Also, *Shh* from the endoderm and *Bmp4* from the mesoderm controls the early events of gut patterning. The lung is derived from the foregut, and its development is induced by *Shh* signals from the foregut endoderm that induce GLI signalling from the mesenchyme. This in turn will induce FGF10, produced by the mesoderm that regulates branching morphogenesis

via its receptors on the lung epithelium (Warburton et al., 2000). In addition, during testis development, the expression of FGFR1 and FGFR2 (Cancilla et al., 2000) and the presence of laminin-111 have been shown (Sasaki et al., 2002). In frogs, FGF8 expression has been detected in the developing kidney (Christen and Slack, 1997) and FGF signalling was shown to be required for the condensation of the pronephric primordium from intermediate mesoderm and the epithelialization of mesenchyme into pronephric nephrons (Urban et al., 2006).

These results indicate that for *Ened* to be expressed, the epithelium needs the induction from the mesenchyme in form of FGFs that induce EMI through FGFRs expressed by the epithelium that in turn up-regulates *Ened*. It is intriguing that during the morphogenesis of these different organs, the common denominators perpetually are the FGF/FGFR signalling and the presence of a BM.

3.2.3. Expression of Ened in the lens epithelium from E11.5

From E11.5 *Ened* expression was observed in the lens epithelium (Figure 5 and 6). This finding was exciting, since it confirmed the notion that *Ened* could be regulated by FGF/FGFR signalling.

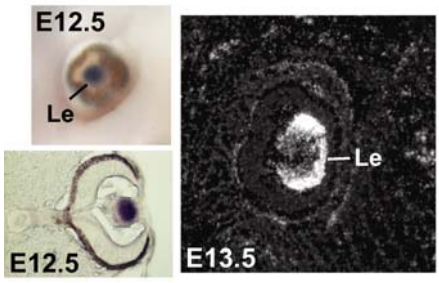

Figure 6. *Ened* expression in the lens epithelium. Magnified views of the eye at E12.5 showing whole mount in situ hybridization with *Ened* probe. Upper left panel: image captured of whole embryo, and lower panel showing sagittal section of whole embryo at the eye level. Lens of the eye at E13.5, right panel (radioactive in situ hybridization) depicting the expression in the lens epithelium. Le, lens epithelium.

The lens arises from the head ectoderm and is positioned in close association with the optic vesicle (Lovicu and McAvoy, 2005). ECM containing laminins and fibronectin form the lens vesicle around E10 and affects the migration and differentiation of the lens cells (Parmigiani and McAvoy, 1991). At around E11 the

lens pit has deepened, the lens starts to form and divide into the anterior undifferentiated lens epithelium cells and the posterior differentiated lens fiber cells. A morphogenetic gradient of increasing FGF concentration originating from the vitreous humor provides an anterior-posterior direction pattern promoting proliferation, migration and differentiation of the lens epithelial cells (Lovicu and McAvoy, 2005). At around E12 the lens vesicle will divide into two different cell structures, the posterior part that forms the differentiated lens fibers and the anterior part that forms the undifferentiated lens epithelium (Lovicu and McAvoy, 2005). The lens retains a BM that contains among else laminin-111, and produces ECM components that are excreted into the vitreous humor (Halfter et al., 2008). Lens fiber cells express FGFR1, FGFR2 and FGFR3 that appear to have overlapping functions in lens development (Robinson, 2006). Interestingly, *Ened* expression was also observed in the developing eyes of frog tailbud stage embryos (Paper II, Figure 5). Frogs express three FGFRs: XFGFR1, XFGFR2 and XFGFR4, and all these receptors are present in the frog eye (Golub et al., 2000).

The observation that mouse *Ened* was expressed in the lens epithelium may suggest that the epithelial cells received signals from the FGF gradient in the forming vitreous, and through FGFR signalling up-regulated the expression of *Ened* upon lens formation. In addition, *Ened* expression appeared when the lens pit broke away from the overlying ectoderm, the lens epithelium started to differentiate and acquired a polarity with posteriorly migrating lens fiber cells. This could also up-regulate *Ened* expression that might be modulated or initiated by the lens BM.

3.2.4. Expression of Ened in the peripheral nervous system

The first signs of *Ened* expression in the future peripheral nervous system was detected at E10.5 in the sympathetic chain at the ventral side of the spinal cord, indicating neural crest cells migrating to form the dorsal root ganglions (Figure 5, lower panel). In addition, labelling was present in the floor plate of the neural tube and weakly in the notochord (Paper II, Figure 2). By E13.5 to E14.5, expression of *Ened* was seen in the facial-, spinal- and head peripheral nerves, and in the dorsal root ganglions and NCCs migrating between the somites (Figure 7; Paper II, Figure 3).

NCCs are ectoderm-derived cells that originate from the dorsal region of the fusing neural tube. Neural tube closure is completed at around E10 in mouse, and this coincided with expression of *Ened* in the rostral part of the somites. At that stage,

NCCs have already been induced by BMP and WNT signals, and after undergoing EMT, they start to migrate from the dorsal neural tube. Trunk NCCs produce cells of the dorsal root ganglion, enteric neurons that take a ventral pathway, and melanocytes that take a dorsolateral pathway (Marmigere and Ernfors, 2007).

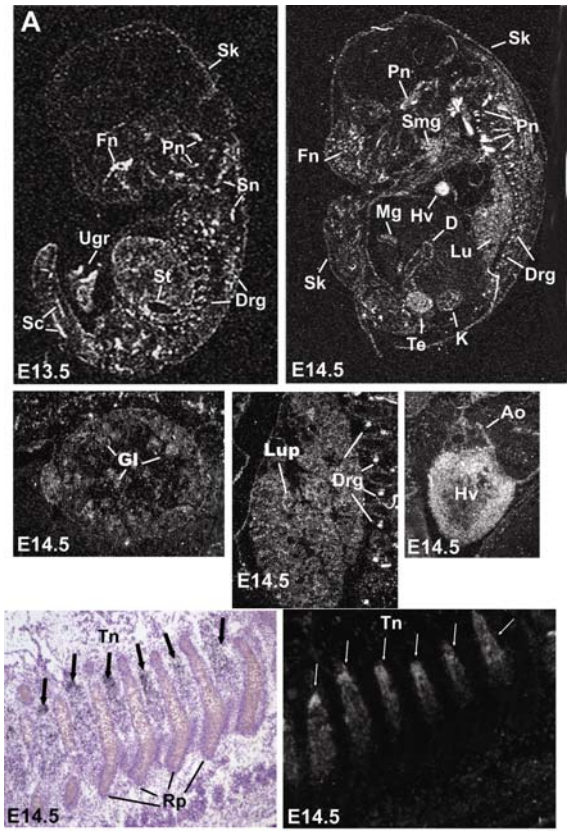

Figure 7. *Ened* expression in epithelial and peripheral nervous tissues. Radioactive in situ hybridization with *Ened* probe on midline sagittal sections of E13.5 and E14.5 mouse embryos. Upper panel showing overview of *Ened* expression in the whole embryo, with distinct labelling in skin, facial and peripheral nerves in the head and thorax, dorsal root ganglia, heart, and in the epithelial lining of organs such as submandibular gland, lung, gastrointestinal tract, kidney and testis. Middle panel shows enhanced views on kidney with labelling in the glomeruli, lung parenchyma, heart ventricle and aortic outflow tract at E14.5. Lower panel shows *Ened* expression in the thoracic nerves emerging from between the rib primordial. Light microscopy capture revealed labelled thoracic nerve cells between the ribs (black arrows), and dark field capture of the same area depict signals exclusively in thoracic nerves (white arrows). Ao, aorta; D, duodenum; Drg, dorsal root ganglions; Fn, facial nerve; Gl, glomerulus; Hv, heart ventricle; K, kidney; Lu, lung; Lup, lung parenchyma; Mg, midgut; Pn, peripheral nerve; Rp, rib primordial; Sc: sympathetic chain; Smg, submandibular gland; Sk, skin; Te, testis; Tn, thoracic nerve; Ugr, urogenital ridge.

Migration involves extensive interactions of NCCs with ECM components such as laminin-111 and fibronectin and requires cytoskeletal reorganization, *i.e.* Rho GTPases, and up-regulation of adhesion molecules, *i.e.* integrins in NCCs (Henderson and Copp, 1997). Also, migrating NCCs express GATA4 and GATA6 (Pilon et al.,

2008). A subset of NCCs develops into Schwann cells that will surround the peripheral axons at around E14. FGF2 induces Schwann cell maturation and inhibits the sensory neuron differentiation in NCC (Ota and Ito, 2006). In addition, FGF2 produced by the mandibular mesenchyme, serves as a chemo-attractant for NCCs migrating from the mesencephalon (Kubota and Ito, 2000). Interestingly, another study showed that FGF2 promoted the proliferation of trunk NCCs that were cultured *in vitro* (Murphy et al., 1994).

Ened expression was maintained in the migrating NCCs and was seen in the condensating dorsal root ganglions from E11.5 (Paper II, Figure 3). The migration from between the somites was clearly visible at E13.5. Also, the same pattern of NCC migration was observed in chicken embryos (Kasemeier-Kulesa et al., 2005). At E14 *Ened* expression was evident in the thoracic nerves emerging between the rib primordium that was negative for *Ened* expression (Figure 7). It has been shown that FGF/FGFR signalling is important during bone and cartilage development and mutations in this signalling system result in bone abnormalities (Jackson et al., 2006). However *Ened* was not detected in bone tissues, which further indicates that *Ened* expression is restricted to epithelial and peripheral nerve cells. In addition, NCCs will enervate the gastrointestinal tract to form the enteric nervous system. This event is promoted by laminin expressed in abundance in the gut, where NCCs interact with the BM and differentiate into enteric neurons (Gershon et al., 1993). It is thus possible that additionally to the epithelial expression of *Ened* detected in the gut, the populating NCCs also could display an up-regulated *Ened* expression.

From E13.5 *Ened* expression was observed in head peripheral nerves (trigeminal ganglions) that originate from the trigeminal placodes that form at the neural plate border just lateral to the neural crest (Figure 7). Interestingly, trigeminal ganglions have a mixed origin containing neurons from both NCCs and placodes (Baker and Bronner-Fraser, 2001). It is not clear why *Ened* expression was delayed two days in the head region when compared to *Ened* expression in the trunk region, however it might be that neurons contributing to trigeminal ganglions need to exit the ectodermal milieu and encounter the BM (laminin), which could induce the expression of *Ened*. These data would indicate that *Ened* expression observed in migrating trunk and trigeminal NCCs is induced by the interactions with the ECM, possibly laminin-111, and the chemo-attractant actions of FGF2. In addition, it cannot

be ruled out that pre-mature Schwann cells (that are induced by FGF2), at around E14.5 settled around both trunk and head peripheral nerves and expressed *Ened*.

Ened expression in the heart could also be ascribed to the colonization from cardiac NCCs, which delaminate from the dorsal neural tube and migrate ventrally to form a component of the vascular smooth muscle of the aortic outflow tract and the aortico-pulmonary and ventricular septum. FGF8 that is produced by the heart mesoderm has been shown to be necessary for the survival of cardiac NCCs (Abu-Issa et al., 2002). *Ened* expression was detected throughout the endocardial lining of heart atrium and ventricle, although strong expression was also detected in the aortic outflow tract from E11.5, which could indicate infiltration of NCCs that contribute to the fibrous tissue in the vessels and to the cardiac ganglions during heart development (Figure 5 and 7).

Also, *Ened* expression was detected in the notochord and hypochord (that lies beneath the notochord) in frog embryos (Paper II, Figure 5). The notochord is a prominent site of FGF4 (eFGF) expression, and the BM component laminin was shown to be expressed around the notochord and neural tube during *Xenopus* embryogenesis (Fey and Hausen, 1990; Isaacs et al., 1995). In addition, weak expression was seen in E10.5 mouse embryos in the notochord and in the floor plate of the neural tube (Paper II, Figure 2). The floor plate and notochord express laminin-111 at this stage (Anderson et al., 2007). This could indicate the inductive up-regulation of *Ened* through the FGF/FGFR signalling pathway in frogs, and that up-regulation of *Ened* might be modulated by the expression of laminin-111 at least in mouse embryos.

4. Conclusion and future perspectives

This thesis was focused on two main topics:

- To elucidate the gene expression changes during both WT and FGFR deficient mouse EB differentiation.
- To elucidate the spatial expression pattern of the uncharacterized gene *1110032E23Rik*, which is now denominated as *Ened*.

The gene expression changes observed during EB differentiation produced extensive catalogues of genes that were influenced by impaired FGF/FGFR signalling. In addition, through stringent filtration of the microarray data, a catalogue of significantly expressed genes was obtained during WT EB differentiation. These observations encompass four days of EB differentiation, the time window that is equivalent to mouse embryogenesis from E3.5 to E6.

Surprisingly, the top 20 most significantly decreased genes due to deficient FGF/FGFR signalling were related to endoderm and BM regulatory genes, and an increase in mesoderm related genes. It can also be concluded that dnFGFR2 EBs consist of a homogenous population of both undifferentiated and differentiated ESCs, since they also failed to down-regulate some of their pluripotency related genes. These findings substantiate the importance of a correctly functioning FGF/FGFR signalling cascade during both early embryonic and EB development. In addition, by comparing the gene lists during WT EB differentiation with gene lists of down-regulated genes in dnFGFR2 EB differentiation, we noted several common transcripts that are mainly involved in cellular processes related to cytoskeletal rearrangements, vesicle transport and secretion, and morphogenesis. This comparison indicated that these genes have important roles in early developmental processes. The gene lists obtained in the two studies could provide a solid base for future studies on genes involved in early embryonic development and potentially in FGFR signalling.

Accordingly, in the next study we elucidated the expression pattern of the hitherto uncharacterized gene *1110032E23Rik* in both mouse and frog embryos. This gene was significantly down-regulated in dnFGFR2 EBs when compared to WT EBs. The expression of *1110032E23Rik* was restricted to epithelial and peripheral nervous tissues during mouse embryonic development, hence the name *Ened* (Expressed in Nerve and Epithelium during Development).

Ened expression appeared in mouse embryos at E9.5 and was restricted to heart epithelium and the midgut region, and was successively up-regulated in the epithelial lining of the gastrointestinal tract, urogenital tract, eye and in the developing peripheral nervous system. From the in situ hybridization results it can be concluded that *Ened* is not expressed in bone and cartilage, and no expression was seen in the central nervous system (CNS), *i.e.* brain and spinal cord. The lack of *Ened* expression in the CNS is supported by another microarray study comparing gene expression in CNS and DRG form adult mice. LeDoux and colleagues (LeDoux et al., 2006) demonstrated that the expression of *1110032E23Rik* (*Ened*) was more than 4-fold higher in DRGs compared to that of CNS. To further substantiate the findings in mice, the expression of the mouse orthologue *Ened* was also elucidated in frog embryos. It can be concluded from these two studies that mouse and frog *Ened* transcripts share several expression domains, including the eye, heart, notochord, kidney, and epidermis.

Ened was expressed in epithelia, in migrating NCCs and later on in DRGs and peripheral nerves. In these tissues FGF/FGFR signalling and the presence of the BM have important roles during embryonic development. Hence, it is tempting to hypothesize that *Ened* expression was induced by FGF/FGFR signalling. Interestingly, by adding Matrigel to differentiating dnFGFR2 EBs, we have observed an increase in *Ened* expression when compared to control dnFGFR2 EBs (unpublished results). Matrigel is derived from Engelbreth-Holm-Swarm sarcoma cells, and it is a reconstituted BM extract that is rich in laminin-111 (about 60%), growth factors, nidogen, collagen IV (about 30%), and perlecan. The presence of growth factors in the Matrigel could have induced the expression of *Ened*, although it cannot be excluded that *Ened* expression might be modified or even induced by the BM protein laminin α1-chain.

Yet, these theories need further testing. To elucidate how *Ened* expression is induced and maintained, and to determine *Ened* functions, several approaches could be used:

- **Inhibition of *Ened* expression in differentiating ESCs.** To repress *Ened* expression in WT ESCs, transfection with siRNA containing a green fluorescent protein-tag, could be used. After selection of positively transfected

ESCs, the effects of *Ened* inhibition could be evaluated by observing the EB´s phenotype during development.

- **Over-expression of *Ened* in ESCs.** Another approach could be to transfect ESCs with a construct that causes over-expression of *Ened* in ESCs, and evaluate if excessive *Ened* expression affect ESCs proliferation and/or EB development.
- **Knock-out *Ened* in mouse.** The most exciting approach would be to remove *Ened* expression completely in mouse embryos, and study the effect during embryonic development. By this approach, *Ened* deficient ESCs could also be obtained by generation of ESCs lacking both *Ened* alleles. In this way, EBs completely lacking *Ened* could be derived and analysed.
- **Binding studies of ENED.** The transmembrane domain of *Ened* indicated that it could be localized to a membrane. To evaluate if the protein form of ENED has binding properties, a construct expressing a fusion protein of ENED in bacterial or mammalian cells could be used. The purified fusion protein product could than be used in for example solid binding assays to elucidate if ENED actually bind to laminin-111 or another BM/ECM component.
- **Functional studies in frogs.** In addition to functional studies in mouse and ESCs, the function of *Ened* orthologue in frogs could be examined by injecting frog embryos with so called morpholinos, which inhibits the production of the target protein. In addition, the *Ened* orthologue could be over-expressed by *Ened* mRNA injection in frog embryos to evaluate its function during development.

Populärvetenskaplig sammanfattning på svenska

Bakgrund till studien

Under fosterutvecklingen sker vissa kritiska moment som innefattar förändringar i genuttryck i cellen och signalering mellan cellerna. I normala fall utvecklar musfostren först runt den tredje dagen efter befruktningen ett epitelcellslager som heter trofektoderm. Inom dessa väggar vilar fosterstamcellerna och väntar på signaler som ska tala om hur de ska utvecklas vidare. När dessa signaler kommer formar fosterstamcellerna (som är pluripotenta och kan bilda alla sorters celler i fostrets kropp) en nytt epitelcellslager som kallas endoderm. Detta kommer i sin tur att utsöndra ett så kallat basalmembran, vilket är viktigt för att det tredje epitelet, som kallas ektoderm, ska kunna utvecklas. Själva fostret bildas helt och hållet från ektodermet. Denna tidiga fosterutveckling kan reproduceras utanför modern, då man isolerar den inre cellmassan från fostret och odlar dessa i odlingskålar. Dessa celler behåller sin förmåga att dela sig oändligt och förbli pluripotenta, men kan också bilda så kallade embryoida kroppar när de induceras. Embryoida kroppar bildar först ett yttre endoderm som utsöndrar basalmembranet under sig. Basalmembranet inducerar i sin tur de underliggande cellerna att forma ektoderm. Det har tidigare visats att en signaleringsväg som styrs av fibroblast tillväxtfaktorer och dess receptorer (FGF och FGFR) är viktiga för att endodermet ska kunna utvecklas. Om inte endodermet kan bildas, beroende på en genförändring, en så kallad mutation i FGFR, kan inte heller basalmembranet formas och således förhindras utvecklingen av ektodermet.

Genuttryck under vanlig stamcellsutveckling

Vi har undersökt förändringar i genuttrycket under den vanliga stamcellsutvecklingen med hjälp av så kallad microarray analys, som kan visa genuttrycks-förändringar i stor skala. Tidsperioden sträckte sig över fyra dagar, vilket motsvarar den tidiga fosterutvecklingen från dag 3.5 - 6 hos möss. Endast de gener som mest markant uttrycktes blev kvar efter filtreringen av microarray rådatan. Analysen resulterade i en katalog av gener som kan ha viktiga roller under den normala utvecklingen. Resultaten visade på en snabbt nedgående trend för de gener som ansvarar för att hålla stamcellerna i pluripotent tillstånd och en snabbt uppgående trend för de gener som ansvarar för att stamcellerna ska kunna utvecklas. Vi har även kunnat identifiera vissa

gener som styr omdirigeringen av cellernas inre skelett och bestämmer cellens poler; gener som kodar för signalerings-molekyler som styr vilka celltyper den embryoida kroppen ska producera, och gener som ansvarar för att celler i den centrala delen av embryoid kroppen ska självdö. Dessa gener verkar vara viktiga för den normala utvecklingen i embryoida kroppar och kan även spela stor roll vid normal fosterutveckling.

Genuttryck i embryoida kroppar som inte har FGFR signalering

Förändringar i genuttrycket har även undersökts i stamceller med en FGFR mutation. Rådatan har filtrerats på ett sätt att endast de gener som markant uttrycktes över eller under 2 (siffran här anger signifikansnivån) var kvar i genlistorna. Gener som var under nivån 2 betraktades som markant nedreglerade. Vi valde att analysera de gener som toppade listorna. Förvånansvärt många gener hörde till klassen som reglerar igångsättandet av det första epitelet, endodermet, i embryoida kroppar. Vi har även observerat en markant nedreglering av gener som kodar för basalmembranproteiner. Detta var överaskande, eftersom vi hade förväntat oss att gener som är inblandade i FGF/FGFR signalering, celldelning eller cellrörelse skulle dyka upp som markant nedreglerade. Även gener som blev uppreglerade, det vill säga hade över 2 i uttryck, dök upp i listorna. Dessa gener tillhör gruppen som sätter igång formandet av mesodermala strukturer i embryoida kroppar och borde alltså inte vara uttryckta förrän det första epitelet (endodermet), basalmembranet, och det andra epitelet (ektodermet) är utvecklade. Dessa resultat visade på att FGF/FGFR signaleringen är viktig för att de första epitelen och basalmembranet ska kunna bildas, och att denna signalväg behövs för att hålla andra signaleringsvägar vilande så att dessa inte går igång för tidigt. Med denna microarray studie har vi också erhållit listor på gener som kan vara inblandade i FGFR signalering. Vi har även observerat flera hittils okända och okarakteriserade gener, som kan vara potentiella mål för FGF/FGFR signalering

Studie i uttrycksmönstret av den hittils okända genen *1110032E23Rik*

Vi valde att klarlägga uttrycket av en gen (*1110032E23Rik)*, vars uttryck var starkt nedregelerat i FGF/FGFR mutanta embryoid kroppar, under utvecklingen av både mus och grodfoster. Vi har kunnat se ett mycket intressant uttrycksmönster i dessa foster, som vi har följt under flera dagar av fosteruvecklingen. *1110032E23Rik* var uttryckt på platser i kroppen som är kända för en aktiv FGF/FGFR signalering,

dessutom observerades uttrycket på ställen som innehåller mycket basalmembran. *1110032E23Rik* uttrycket observerades från dag 9 hos musfostren i hjärtat och i mag- och tarmkanalen. Detta uttryck spred sig successivt till andra ställen som är täckt med ett epitellager, det vill säga i inre tarmväggarna, lungan, njuren, testiklarna, på ögats lins, och under huden. Även det perifera nervsystemet uppvisade uttryck av *1110032E23Rik*. De nyformade nervknutorna längs ryggraden och de perifera nervtrådarna i bålen och huvudet visade starkt uttryck av *1110032E23Rik*. Uttrycket i grodfostren liknade det i möss.

Eftersom ingen gen ska gå utan namn, har vi bestämmt oss för att döpa *1110032E23Rik* baserat på dess uttryck. Från och med nu heter *1110032E23Rik* Ened, som står för Expressed in Nerve and Epithelium during Development (vilket betyder ”Uttryckt i Nerv och Epitelium under Utvecklingen”).

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