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REGULATION OF CELL GROWTH, DEATH, AND POLARIZATION BY *ERBB4*

by

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ABSTRACT

ErbB4 is a member of the epidermal growth factor receptor (EGFR, ErbB) family. The other members are EGFR, ErbB2 and ErbB3. ErbB receptors are important regulators for example in cardiovascular, neural and breast development but control key cellular functions also in many adult tissues. Abnormal ErbB signaling has been shown to be involved in various illnesses such as cancers and heart diseases. Among the ErbBs, ErbB4 has been shown to have unique signaling characteristics. ErbB4 exists in four alternatively spliced isoforms that are expressed in a tissue-specific manner. Two of the isoforms can be cleaved by membrane proteases, resulting in release of soluble intracellular domains (ICD). Once released into the cytosol, the ICD is capable of translocating into the nucleus and participating in regulation of transcription. The functional differences and the tissue-specific expression patterns suggest isoform-specific roles for ErbB4 isoforms. However, the abilities of ErbB4 isoforms to differently regulate cellular functions were discovered only recently and are not well understood.

This study aimed to determine the expression patterns of ErbB4 in normal and diseased tissue, and to define whether the cleavable and non-cleavable isoforms could regulate different target genes and therefore, cellular functions. In this study, a comprehensive ErbB4 expression pattern in several normal tissues, various cancers and non-neoplastic diseases was determined. In addition, the data demonstrated that the cleavable and non-cleavable ErbB4 isoforms could regulate different cellular functions and target genes. Finally, this study defined the cellular responses regulated by ErbB4 during kidney development.

Key words: EGFR, ErbB4, isoform-specific signaling, kidney development

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***ERBB4*-välitteinen solujen kasvun, kuoleman ja polarisaation säätely**
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TIIVISTELMÄ

ErbB4 kuuluu EGFR-kasvutekijäreseptoriperheeseen, jonka muut jäsenet ovat EGFR, ErbB2 ja ErbB3. ErbB4:n on osoitettu olevan tärkeä sydämen, hermoston ja rintakudoksen kehityksen säätelyssä. Lisäksi ErbB4 välitteisellä signaloinnilla on ehdotettu olevan merkitystä syövän ja sydänsairauksien muodostumisessa. ErbB4 silmukoituu kudospesifisesti neljäksi eri alamuodoksi. Kaksi näistä alamuodoista voidaan katkaista proteaasi-välitteisesti, josta seuraa liukoisen ICD:n (intracellular domain) irtoaminen. Katkaistu ICD kykenee siirtymään tumaan, jossa se osallistuu geenien luennan säätelyyn. Kudosspesifinen ErbB4 alamuotojen ilmentyminen sekä niiden toiminnalliset erot viittaavat siihen, että eri alamuoto-välitteiset signaalit voivat johtaa erilaisiin tai jopa päinvastaisiin solutason vasteisiin. Erot ErbB4 alamuotojen toiminnassa on kuitenkin havaittu vasta viime vuosina, eikä niiden merkitystä vielä täysin ymmärretä.

Tutkimukseni tarkoituksena oli määrittää ErbB4:n ja sen alamuotojen ilmenemistä normaali- ja tautikudoksissa. Tutkimuksessa tarkasteltiin myös ErbB4 alamuotojen välisiä signalointieroja ja havaittiin, että eri alamuodot säätelevät eri kohdegeenejä ja solutason vasteita. Lisäksi osoitettiin ErbB4 signaloinnin säätelevän tärkeitä epiteelisolujen toimintoja munuaisen kehityksen aikana.

Avainsanat: EGFR, ErbB4, alamuotospesifinen signalointi, munuaisen kehittyminen

TABLE OF CONTENTS

ABBREVIATIONS

aa	Amino acid
ADAM	A disintegrin and a metalloproteinase
ADPKD	Autosomal dominant polycystic kidney disease
AP-2	Activator protein 2
AR	Amphiregulin
ARPKD	Autosomal recessive polycystic kidney disease
ATP	Adenosine triphosphate
BH3	Bcl-2 homology domain 3
BIO	5'-bromoindirubin-3'-oxime
BTC	Betacellulin
cDNA	Complementary DNA
CYT	Cytoplasmic
DAPI	4',6-diamidino-2-phenylindole
DBA	*Dolichos biflorus* agglutinin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECD	Ectodomain
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
EPG	Epigen
EPR	Epiregulin
ERK	Extracellular-regulated kinase 1,2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GHR	Growth hormone receptor
GST	Glutathione *S*-transferase
HA	Hemagglutinin
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HMGB1	High mobility group protein B1
ICD	Intracellular domain
IHC	Immunohistochemistry
JM	Juxtamembrane
kD	Kilodalton
KRAB	Kruppel-associated box
LTL	*Lotus tetragonolobus* lectin
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPK kinase kinase
MDCK	Madine-Darby canine kidney
MET	Mesenchymal to epithelial transition
MMP	Matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole
NSCLC	Non-small cell lung cancer
NCC	Thiazide-sensitive NaCl transporter
NCoR	Nuclear receptor corepressor
NLS	Nuclear localization signal
NMDAR	*N*-methyl-d-aspartate receptor
NRG	Neuregulin
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFRA	Platelet-derived growth factor receptor alpha
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKD	Polycystic kidney disease
PMA	Phorbol 13-myristate 12-acetate
PTB	Phosphotyrosine-binding
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RCC	Renal cell carcinoma
RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
RPGN	Rapidly progressive glomerulonephritis
RT-PCR	Reverse-transcriptase PCR
RTK	Receptor tyrosine kinase
SH2	Src homology-2
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1
STAT	Signal transducer and activator of transcription
TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein
TACE	Tumor necrosis factor-alpha converting enzyme
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TKI	Tyrosine kinase inhibitor
TUNEL	TdT-mediated dUTP nick end labeling
VEGF	Vascular endothelial growth factor
WT1	Wilms tumour-suppressor gene 1
Wwox	WW domain-containing oxidoreductase
YAP	Yes-associated protein
ZO-1	Zonula occludens-1

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–III.

I Veikkolainen, V., Vaparanta, K., Halkilahti, K. Iljin, K., Sundvall, M., and Elenius, K. Function of *ERBB4* is determined by alternative splicing. *Cell Cycle.* 10 (16): 2647-2657, 2011.

II *Sundvall, M., *Veikkolainen, V., Kurppa, K., Salah, Z., Tvorogov, D., van Zoelen, E.J., Aqeilan, R., and Elenius, K. Cell death or survival promoted by alternative isoforms of ErbB4. *Molecular Biology of the Cell*. 21: 4275-4286, 2010.
*Equal contribution

III *Veikkolainen, V., *Naillat, F., Railo, A., Chi, L., Manninen, A., Hohenstein, P., Hastie, N., Vainio, S., and Elenius, K. ErbB4 modulates tubular cell polarity and lumen diameter during kidney development. *Journal of American Society of Nephrology*. 2011. In Press.
*Equal contribution

In addition, this thesis contains some unpublished data.

1. INTRODUCTION

The ErbB receptor family is one of the most well characterized tyrosine kinase families. It consist of four members, epidermal growth factor receptor (EGFR, ErbB1), ErbB2, ErbB3 and ErbB4, and interestingly, has structural homology to avian erythroblastic leukemia viral oncogene (*v-erbB*). The receptors can be activated by several EGF-related growth factors that induce receptor homo- or heterodimerization and activation of intracellular signaling cascades. Each ligand and receptor dimer pair has differing characteristics to initiate downstream pathways providing for a very complex and versatile signaling network. The ErbB signaling system can therefore regulate several important cellular functions including proliferation, survival, differentiation and migration. All ErbB receptors have been indicated crucial roles during development and they promote and maintain important cellular processes also in several adult tissues.

Excessive ErbB signaling is often observed in malignancies and typically associates with an unfavorable clinical outcome. Intensive research on the role of ErbB receptors in cancer has resulted in several therapeutic applications that aim to inhibit their overactivity. To date, the main focus of research has been on monoclonal antibodies and tyrosine kinase inhibitors that target EGFR or ErbB2. Some of the ErbB targeting pharmacological drugs, such as EGFR tyrosine kinase inhibitors erlotinib (Tarceva) and gefitinib (Iressa), and ErbB2-targeting antibody trastuzumab (Herceptin), have demonstrated significant clinical effect in cancer patients. In addition to cancer, altered ErbB expression, localization or signaling has been implicated in non-neoplastic diseases. Recent findings have raised interest in developing applications to target ErbB signaling for example in cardiovascular, psychiatric, and kidney diseases.

Compared to other members of the EGFR family, the functions of ErbB4 in normal tissues and diseases are less well known. This is at least partially due to the alternative splicing of the *ERBB4* gene into four functionally distinct isoforms. Two of the isoforms can, in addition to conventional receptor tyrosine kinase signaling, signal in a more direct manner, via regulated intramembrane proteolysis (RIP). RIP results in detachment of an intracellular domain (ICD) from the receptor and translocation of the ICD into the nucleus where it participates in transcriptional regulation. These functional differences suggest significantly different biological roles for ErbB4 isoforms.

The aim of this study was to address the distribution and specific signaling characteristics of the ErbB4 isoforms. In addition, to better understand the biology of ErbB4 in the kidney, both gain- and loss-of-function mouse models were used to address the cellular processes regulated by ErbB4 signaling *in vivo*. A comprehensive knowledge of these processes is essential if ErbB4 is to be utilized as a prognostic marker or a therapeutic target.

2. REVIEW OF THE LITERATURE

2.1. Receptor tyrosine kinases

Protein phosphorylation is a major regulator of cell signaling pathways by changing functional states of the signaling proteins. Phosphorylation is mediated by protein kinases, enzymes that transfer a phosphate group from adenoside triphosphate (ATP) to specific amino acids of target proteins. The human genome encodes more than 500 protein kinases, over 90 of which phosphorylate tyrosines (protein tyrosine kinases, PTK) (Blume-Jensen and Hunter 2001; Manning et al. 2002). The PTKs contain a family of 58 receptor tyrosine kinases (RTK), transmembrane receptor proteins that mediate extracellular signals forward by phosphorylating intracellular substrates (Lemmon and Schlessinger 2010). RTKs are divided into 20 subfamilies and act as key regulators of cellular events, such as proliferation, differentiation and migration (Lemmon and Schlessinger 2010).

2.2. ErbB family of receptor tyrosine kinases

2.2.1. Structure

ErbB or epidermal growth factor (EGF) receptor subfamily of RTKs is composed of four members: epidermal growth factor receptor (EGFR; or ErbB1/HER1) (Ullrich et al. 1984), ErbB2 (Neu/HER2) (Schechter et al. 1984; Coussens et al. 1985), ErbB3 (HER3) (Kraus et al. 1989; Plowman et al. 1990) and ErbB4 (HER4) (Plowman et al. 1993a). ErbB receptors are transmembrane type 1 glycoproteins, approximately 180 kDa in size. They all have a glycosylated extracellullar ligand-binding domain, a single transmembrane-spanning domain, and a cytoplasmic tyrosine kinase domain with a carboxy-terminal (C-terminal) tail region (Figure 1). Extracellular domain consists of four subdomains, I, II, III and IV (Lax et al. 1988), of which subdomains I and III are needed for ligand binding (Garrett et al. 2002; Ogiso et al. 2002), and II and IV for receptor dimerization (Berezov et al. 2002). The main function of the short transmembrane domain is to anchor the receptor to the membrane but it has also been shown to participate in receptor dimerization (Weiner et al. 1989). The cytoplasmic kinase domain consists of two lobes, NH_2-terminal (N-lobe) and C-terminal (C-lobe), which are separated by an ATP binding cleft. The activity of the kinase enzyme can be regulated through inactive and active conformations enabled by division of important catalytic elements among the two lobes (Stamos et al. 2002). The C-terminal tail of the receptors contains several tyrosine residues that upon phosphorylation act as docking sites for enzymes and adaptor proteins that facilitate the initiation of the intracellular signal transduction.

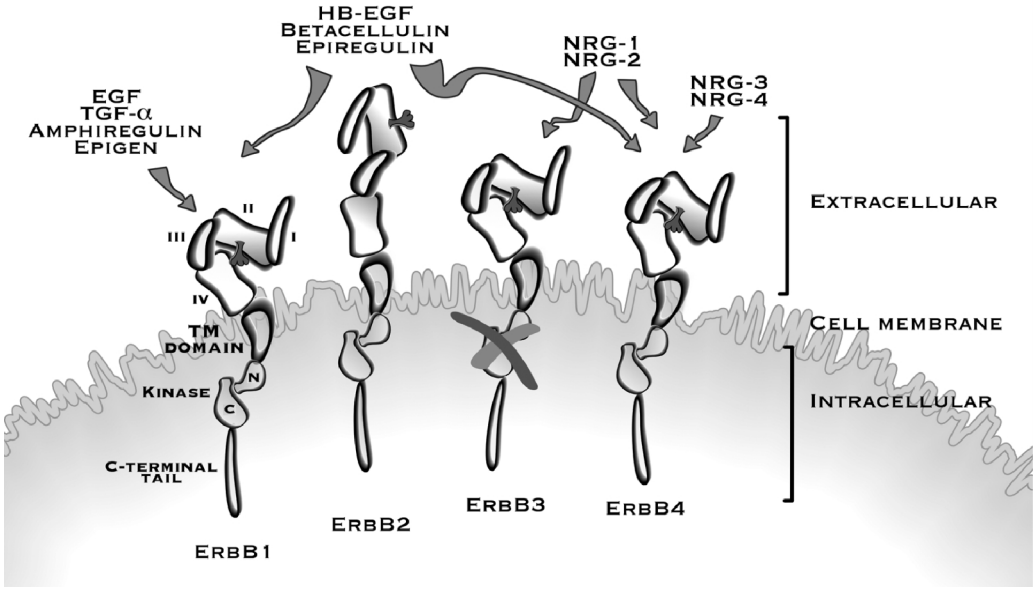

Figure 1. ErbB receptor family and ligands. The extracellular domain of ErbB2 is in an active conformation and cannot bind a ligand. The kinase domain of ErbB3 is not fully functional.

2.2.2. The EGF-related ligand family

ErbB receptors are activated by EGF-related growth factors. Proteolytic cleavage of cell membrane-anchored growth factor precursors produces soluble ligands into extracellular matrix for auto- and paracrine signaling (Kataoka 2009). The ligands can be divided into three groups based on their binding affinity (Figure 1). Epidermal growth factor (EGF) (Cohen 1962), transforming growth factor-α (TGF-α) (Derynck et al. 1984), amphiregulin (AR) (Shoyab et al. 1989) and epigen (EPG) (Strachan et al. 2001) constitute the first group of ligands as they only bind to EGFR. Ligands of the second group, betacellulin (BTC) (Sasada et al. 1993; Riese et al. 1996), heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama et al. 1991; Elenius et al. 1997b) and epiregulin (EPR) (Toyoda et al. 1995; Komurasaki et al. 1997), can activate both EGFR and ErbB4 receptors. The third group is composed of four neuregulins (NRG): NRG-1 (Holmes et al. 1992; Peles et al. 1992; Plowman et al. 1993b), NRG-2 (Carraway et al. 1997; Chang et al. 1997), NRG-3 (Zhang et al. 1997), NRG-4 (Harari et al. 1999). The NRGs can either bind both ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4). Furthermore, alternative splicing of NRGs adds to the intricacy of NRG signaling as it results in numerous differently expressed and functionally distinct NRG isoforms (Falls 2003). The ErbB2 is an orphan receptor as its ectodomain structure differs from other ErbBs and is not capable of binding a ligand (Garrett et al. 2003).

In addition to conventional, soluble ligand-mediated activation of receptors on nearby cells (paracrine signaling), cells further away (endocrine) or on the surface of the ligand expressing cell (autocrine), unprocessed membrane-bound EGF-related

ligand precursors are also capable of activating receptors of neighboring cells (juxtacrine signaling) (Anklesaria et al. 1990; Singh and Harris 2005). Moreover, some shed EGF-related ligands can be further processed to produce intracellular fragments that are capable of signal mediation (back signaling) (Bao et al. 2003; Iivanainen et al. 2007; Hieda et al. 2008).

2.2.3. Mechanism of activation

According to most the commonly accepted theory, inactive ErbB receptors reside at the cell membrane as monomers. The unligated monomer receptor resides in an autoinhibited (tethered) conformation due to interaction between subdomains II and IV (Cho and Leahy 2002; Ogiso et al. 2002; Bouyain et al. 2005). This interaction constrains the ectodomain structure keeping the ligand-binding subdomains I and III exposed but apart, and masking the dimerization modules in subdomains II and IV. Ligand binding disrupts the tethered conformation and allows the ectodomain to adopt its extended active conformation (Garrett et al. 2002; Ogiso et al. 2002; Ferguson et al. 2003). In the active conformation, the exposed subdomain IV and dimerization arm of subdomain II enable dimerization with another activated receptor monomer (Garrett et al. 2002) (Figure 2). Interestingly, the structure of ErbB2 ectodomain differs from other ErbB receptors and resembles the extended active conformation explaining its potency to form heterodimers as well as its inability to bind a ligand (Garrett et al. 2003). The altered ectodomain structure also weakens the competency of ErbB2 to form homodimers (Garrett et al. 2003). Therefore, in normal cells the signal transmitting capabilities of ErbB2 depend on the availability to heterodimerize with other activated ErbB receptors.

In an ErbB receptor monomer, the tyrosine kinase enzyme is also in an inactive conformation, thus preventing the phosphorylation of the receptor's own C-terminal tail and initiation of intracellular signal transduction. The kinases of a dimer adopt active conformations due to dimerization-induced allosteric changes in the N-and C-lobes (Stamos et al. 2002; Zhang et al. 2006). Consequently, the active kinases phosphorylate the C-terminal tyrosines of the dimer partner initiating the downstream signaling pathways (Figure 2). Interestingly, ErbB3 cannot form homodimers upon ligand activation but needs other ErbB receptors as dimerization partners (Berger et al. 2004). Furthermore, mutations (Guy et al. 1994) and structural differences (Jura et al. 2009) of the ErbB3 kinase domain were thought to completely abolish its kinase activity and the kinase domain was therefore proposed to have no role in ErbB3 heterodimer signaling. However, the recent discovery that the purified intracellular domain of ErbB3 has sufficient kinase activity for autophosphorylation suggests significance also for ErbB3 kinase in ErbB signaling (Shi et al. 2010).

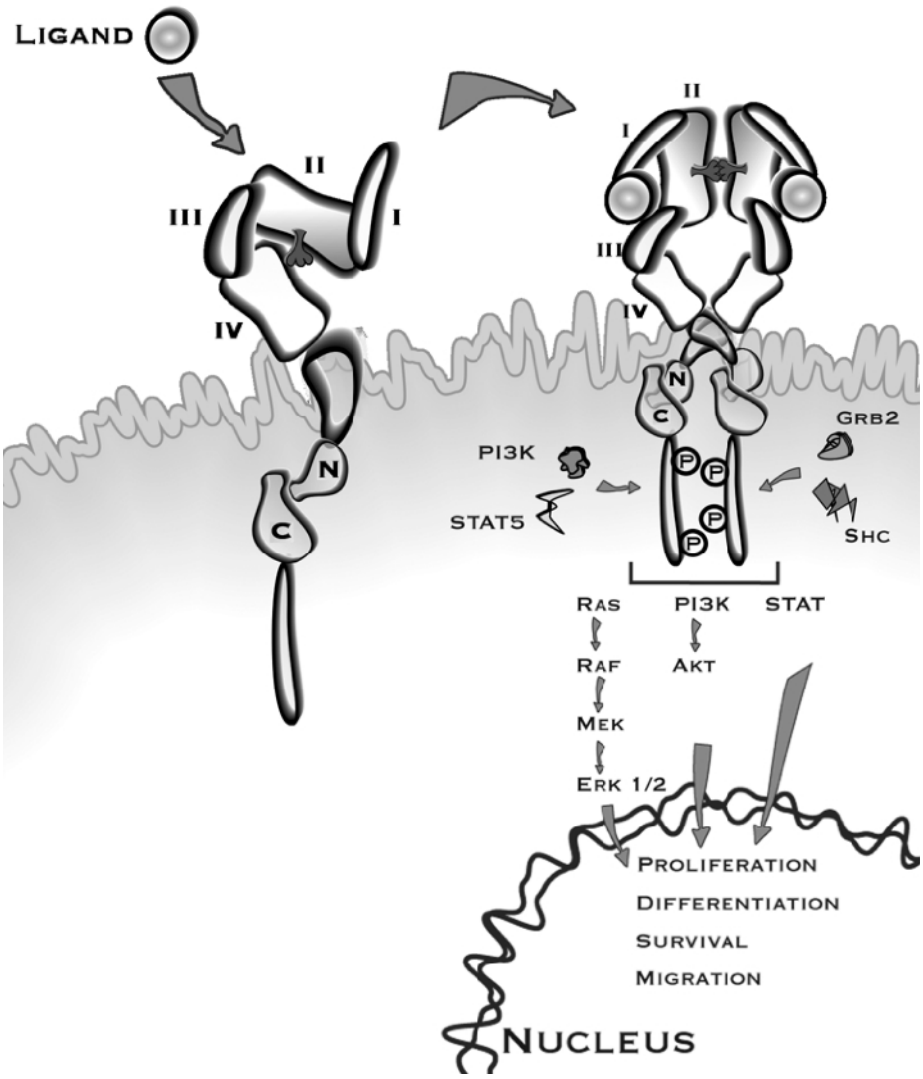

Figure 2. ErbB receptor activation and signaling. Ligand binding to subdomains I and III of EGFR, ErbB3 or ErbB4 changes the receptor to active conformation exposing subdomains II and IV needed for dimerization. The dimerization results in activation of kinase domains, autophosphorylation of the C-terminal tyrosines and binding of signaling molecules. The combination of different dimers and ligands determine the pattern of phosphorylated tyrosines. The phosphorylated tyrosines in turn determine the composition of bound signaling molecules and therefore the activated downstream signaling pathways.

2.2.4. Signaling

The various ligands signaling via numerous ErbB homo- and heterodimer combinations provide the complexity and diversity of ErbB signaling in mammals. Diversity is important to achieve the ErbB-mediated regulation of many vital cellular responses, including cell proliferation, survival, differentiation and migration (Burgess 2008). The importance of ErbB family signaling diversity for mammals is also evident

from interspecies comparisons; for example less complex species such as the nematode (*Caenorhabditis elegans*) or the fruitfly (*Drosophila melanogaster*) have one EGFR receptor homolog which is activated by one or five ligands, respectively (Chang and Sternberg 1999; Shilo 2003).

Phosphorylated C-terminal tyrosines of the activated receptor dimers act as initiator sites for intracellular signaling pathways and provide a branching point for various signaling networks (Figure 2). Cytoplasmic signaling molecules containing Src homology-2 (SH2) and phosphotyrosine-binding domains are recruited and bound to the phosphorylated tyrosines either directly or indirectly via an adaptor protein (Lemmon and Schlessinger 2010). There are several known interacting partners for phosphorylated ErbB tyrosines including such molecules as Grb2, Shc, phosphoinositide 3-kinase (PI3K), signal transducer and activator of transcription 5 (STAT5), and intracellular kinase Src (Schulze et al. 2005). Each ErbB receptor has a unique set of phosphorylated C-terminal tyrosines and therefore attracts a different array of signaling molecules (Schulze et al. 2005). Moreover, the phosphorylation status of the tyrosines on each activated receptor depends on the bound ligand and the dimerization partner (Olayioye et al. 1998; Sweeney et al. 2000). Thus, each ErbB receptor dimer and ligand combination has a different binding partner interactome and a disposition to initiate certain downstream signaling pathways.

2.2.4.1. Signaling pathways

The ErbB downstream signaling can progress through relays of multiple mediators, *e.g.* utilizing the Ras/Raf/mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, or, through a direct activation of a transcription factors such as the STATs (Holbro and Hynes 2004; Burgess 2008) (Figure 2). The MAPKs are signal mediators transforming extra- and intracellular stimuli into cellular responses. The Ras/MAPK pathway is one of the best-known MAPK pathways involved in regulation of proliferation, differentiation, survival and migration (Raman et al. 2007). All ErbBs are capable of initiating the Ras/MAPK pathway by binding Grb2 or Shc (Schulze et al. 2005). Grb2-associated guanitide nucleotide exchange factor Sos activates Ras, which in turn launches a three-leveled kinase cascade by activating the first kinase, Raf (MAP kinase kinase kinase, MAPKKK) (Figure 2). Active Raf phosphorylates MEK (MAPKK), which subsequently phosphorylates Erk-1 and Erk-2 (extracellular signal-regulated kinases, MAPKs). Activation of Erks results in their translocation to the nucleus where they regulate transcription by phosphorylating transcription factors. Of the other ErbB pathways, PI3K/Akt is involved in regulation of survival, proliferation and migration (Manning and Cantley 2007), and the STAT-mediated target gene regulation is important for controlling differentiation, apoptosis and progression of the cell cycle (Calò et al. 2003).

ErbB signaling is further diversified by cross-talk between different signaling networks that enable ErbB signaling either influence, or be influenced, by other signaling systems (Burgess 2008). A classical example of this is EGFR phosphorylation, and consequent MAPK pathway initiation, via growth hormone receptor (GHR) activated Janus tyrosine kinase 2 (Yamauchi et al. 1997).

2.2.4.2. Nuclear signaling

In addition to the conventional transmembrane roles of RTKs in receiving and mediating extracellular signals, several RTKs or their intracellular fragments have been shown to act as signaling molecules in the nucleus (Wells and Marti 2002). All ErbB receptors have nuclear localization signals (NLS) and have been reported to translocate to the nucleus (Hsu and Hung 2007; Wang and Hung 2009). EGFR (Lin et al. 2001), ErbB2 (Wang et al. 2004) and ErbB3 (Offterdinger et al. 2002) are transported to the nucleus as full-length receptors, and suggested to mainly utilize endosome-mediated translocation (Giri et al. 2005; Lo et al. 2006). ErbB4 in turn is translocated as an ICD released via RIP (Figure 3) (Ni et al. 2001; Lee et al. 2002).

As the ErbBs do not have the ability to bind DNA, they need to associate with transcription factors to regulate gene expression. EGFR cooperates, for example, with STATs and E2F1 in regulating genes such as *iNOS* (Lo et al. 2005), *B-MYB* (Hanada et al. 2006), *Aurora-A* (Hung et al. 2008) and *COX-2* (Lo et al. 2010). In addition to regulating transcription, the kinase of translocated EGFR has been shown to be capable promoting cell proliferation and DNA repair through proliferating cell nuclear antigen (PCNA) phosphorylation (Wang et al. 2006). Nuclear ErbB2, ErbB3 and ErbB4 have also been demonstrated to regulate transcription. ErbB2 has, for example, been shown to associate with the *COX-2* promoter and stimulate its transcription (Wang et al. 2004). The functional significance of nuclear ErbB3 has remained unclear until recently. It was suggested that nuclear ErbB3 participates in myelination of Schwann cells and is capable of regulating promoter activity of *ezrin* and *high mobility group protein B1* (HMGB1) (Adilakshmi et al. 2011). Interestingly, the findings were shown to result from expression of a previously unreported 50 kDa nuclear transcript variant of ErbB3 (nuc-ErbB3). The functions of the ErbB4 ICD in the nucleus are discussed in more detail in Chapter 2.3.2.

2.2.4.3. Termination of ErbB signaling

Efficient signal termination is needed to ensure appropriate signal strength and duration. There are several negative feedback mechanisms such as dephosphorylation by protein tyrosine phosphatases (PTP) or internalization of activated receptors that control the RTK signaling networks (Lemmon and Schlessinger 2010). Depending on the receptor and/or the ligand, internalized RTKs can continue to activate intracellular signaling pathways. However, the internalized receptors eventually become dephosphorylated and ubiquitinated, destined for lysosomal degradation or recycling back to the cell membrane (Lemmon and Schlessinger 2010).

The endocytosis of EGFR is very robust involving several redundant mechanisms, and although widely studied, is not fully understood (Sorkin and Goh 2009). The downregulation of activated EGFR is, however, known to be mainly achieved through clathrin-mediated endocytosis, the fastest internalization pathway of integral membrane proteins (Sorkin and Goh 2009). Downregulation of activated ErbB2 and ErbB3 is less well known but clearly very different from EGFR as they have been observed to internalize at much slower rate (Sorkin et al. 1993; Baulida et al. 1996). Whereas ErbB2 seems to be truly endocytosis-impaired and can also influence EGFR internalization by dimerization (Sorkin and Goh 2009), activated ErbB3 has been

shown to be downregulated through ligand-induced receptor ubiquitination and subsequent proteosomal degradation (Cao et al. 2007). Downregulation, endocytosis, and ubiquitination of ErbB4 have been shown to be different for alternatively spliced ErbB4 isoforms (Sundvall et al. 2008b).

2.2.5. ErbB functions during embryonic development

Genetic studies on mice and zebrafish have demonstrated a wide range of developmental defects in animals lacking ErbB receptors or their ligands. Deletion of *Egfr* in mice results in defective development of multiple organs and tissues, particularly where branching morphogenesis occurs (Table 1) (Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995). Depending on the genetic background the mutant mice die before implantation or can survive up to three weeks after birth. Interestingly, whereas the initial *Egfr* knockout studies reported no heart defects, mice with reduced *Egfr* kinase activity or overexpressing a human kinase-dead *EGFR* demonstrate defective heart valve development (Chen et al. 2000; Sibilia et al. 2003). In addition, inhibition of *egfr* kinase function with kinase inhibitors or mRNA levels with morpholinos in zebrafish induces defective cardiovascular development (Goishi et al. 2003).

Table 1. ErbB expression in major organs. Expression levels: no (–), low (+), moderate (++), or high (+++). Both adult and fetal expression is shown. ND, not determined/not applicable; PT, proximal tubule; DT, distal tubule; CD, collecting duct. The table is based on data reported by the following references: [1](Gusterson et al. 1984; Damjanov et al. 1986; Partanen and Thesleff 1987; Hormi and Lehy 1994; Bernardini et al. 1996; Peng et al. 1997; Gresik et al. 1998; Cupp and Skinner 2001; Iwamoto et al. 2003; Jackson et al. 2003; Sibilia et al. 2003) [2](Press et al. 1990; Iwamoto et al. 2003), [3](Prigent et al. 1992), and [4](Srinivasan et al. 1998; Carver et al. 2002; Junttila et al. 2005; Zeng et al. 2007).

Organ	**EGFR[1]** adult/fetal	**ErbB2[2]** adult/fetal	**ErbB3[3]** adult/fetal	**ErbB4[4]** adult/fetal
Skin, epidermis	++/+++	++/++	++/+++	++/++
Brain	+/++	–/–	+++/–	+++/+++
Salivary gland, ducts	++/+++	+/ND	++/ND	+++/ND
Stomach	–/+	+/++	++/++	++/ND
Intestines	–/++	+/++	+/+	–/++
Lung bronchi	++/++	++/++	++/++	++/++
Breast	+++/ND	++/ND	+/ND	+/ND
Heart	+/+	+/–	+/++	++/+++
Kidney, glomeruli	–/–	+/+	–/–	–/–
Kidney, PT	+/+	++/+	–/++	++/++
Kidney, DT	+/+	++/+	++/+++	+++/+++
Kidney, CD	+/+++	++/+++	++/++	++/++
Pancreas, ducts	+++/+	+/++	–/–	++/++
Liver	++/++	–/–	++/++	–/–
Testis	++/++	–/–	++/++	+++/+++
Prostate	+/ND	+/ND	+/ND	+/ND
Ovary	–/ND	+/–	++/++	+/++
Uterus	++/ND	+/++	++/++	+/ND

ErbB2 is crucial for cardiac and neural development. Mice lacking (Lee et al. 1995; Erickson et al. 1997), or expressing only kinase-dead *Erbb2* (Chan et al. 2002) die by mid-gestation at embryonic day 10.5 (E10.5) due to defective heart development. The ventricles of the mutant mice lack muscular structures called trabeculae. Consistently, defective trabeculation has been seen in zebrafish *erbb2* mutants (Liu et al. 2010). A detailed examination of the *erbb2* mutant fish also suggested roles for ErbB2 in cardiomyocyte proliferation and in trabeculation initiating cardiomyocyte migration. Mice with conditional *Erbb2* deletion only in ventricular myocardium are viable but after birth develop cardiomyopathy and enlarged ventricular chambers with thinned walls (Crone et al. 2002; Ozcelik et al. 2002). In addition to cardiac defects, ErbB2 has been indicated important roles in neural crest cell development, spinal cord oligodendrocyte differentiation and Schwann cell migration and myelination (Wieduwilt and Moasser 2008; Birchmeier 2009).

Similar to *Erbb2* mutant mice, *Erbb3* and *Erbb4* knockout mice die due to cardiac abnormalities at E13.5 and around E11, respectively (Gassmann et al. 1995; Erickson et al. 1997; Riethmacher et al. 1997). Whereas *Erbb3* deficient mice have defects in cardiac cushions that form the valves and normal trabeculae, the *Erbb4* mutants have defective trabeculae. In addition, *Erbb3* and *Erbb4* deficient mice manifest neural defects, of which the *Erbb3* phenotype demonstrating abnormal cerebellum, cranial ganglia and Schwann cell development is strikingly similar to the phenotype of mice with *Erbb2* deletion (Gassmann et al. 1995; Erickson et al. 1997; Riethmacher et al. 1997). The developmental roles of ErbB4 are discussed more thoroughly in Chapter 2.3.3.1.

2.2.6. ErbBs in carcinogenesis

The oncogenic potential of ErbB receptors was first discovered in 1980's when avian *v-erbB* (Downward et al. 1984) and rat *c-neu* (Schechter et al. 1984; Coussens et al. 1985) oncogenes were indentified as *EGFR* and *ERBB2* homologues, respectively. In the past three decades, abnormal ErbB signaling has been implicated in several cancers (Hynes and MacDonald 2009). The research has shown that ErbB-mediated malignant transformation results from constitutive receptor activation caused by overexpression, mutations or excessive availability of ligands. The best-known examples include *EGFR* activating mutations in 10-15% of Caucasian and 30-40% of Asian non-small cell lung cancer (NSCLC) patients (Lynch et al. 2004; Paez et al. 2004; Engelman and Jänne 2008), and *ERBB2* amplification in approximately 20% of breast cancers (Slamon et al. 1987; Berger et al. 1988).

The impact of ErbB receptors in malignant growth has led to development of several drugs either approved as cancer drugs, or being currently tested in clinical trials (Hynes and MacDonald 2009; Hollmén and Elenius 2010) (www.clinicaltrials.gov). These therapeutics include small molecule tyrosine kinase inhibitors, capable of crossing cell membrane and blocking ErbB kinase activity, and antibodies that target the extracellular domain of the receptor. Examples include the EGFR tyrosine kinase inhibitors erlotinib (Tarceva; Genentech) and gefitinib (Iressa; AstraZeneca), approved as treatments for NSCLC patients with *EGFR* mutations (Hynes and MacDonald 2009), and the humanized monoclonal anti-ErbB2 antibody, trastuzumab (Herceptin;

Genentech) (Fendly et al. 1990), which increases survival of breast cancer patients with *ERBB2* amplification (Hudis 2007).

Whereas the oncogenic activity of ErbB3 and ErbB4 (Chapter 2.3.3.2.) has been less studied, they too have been linked with several types of cancer (Baselga and Swain 2009; Hollmén and Elenius 2010). ErbB3 has been indicated key functions in oncogenic signaling of various cancer types, mainly as a EGFR or ErbB2 heterodimerization partner (Baselga and Swain 2009). Interestingly, ErbB3 has also been shown pivotal roles in development of resistance to ErbB targeted drugs by enabling bypassing of EGFR or ErbB2 inhibition (Engelman et al. 2007; Sergina et al. 2007; Hynes and MacDonald 2009). Recent findings about the roles of ErbB3 and ErbB4 in cancer suggest, that in addition to EGFR and ErbB2, they could also serve as candidates for cancer drug targets.

2.3. ErbB4

2.3.1. Alternative spliced isoforms

Unlike genes encoding the other members of the ErbB family, *ERBB4* is alternatively spliced. Alternative splicing is an essential process between gene transcription and mRNA translation where a single pre-mRNA product is spliced to produce two or more mRNA products encoding different proteins, typically with distinct functional properties. Alternative splicing contributes significantly to the phenotypic complexity of organisms (Wang et al. 2008) and is an indispensable tool for evolution (Keren et al. 2010). In addition, defective splicing is associated with cancer progression (David and Manley 2010) and has been associated with over 60% of human diseases caused by mutations (López-Bigas et al. 2005).

The ErbB4 isoforms differ either at the extracellular juxtamembrane domain by mutually exclusive exons (JM-a and JM-b isoforms), or at the intracellular cytoplasmic domain by exon skipping (CYT-1 and CYT-2 isoforms) (Figure 3A) (Elenius et al. 1997a; Elenius et al. 1999). The JM-a isoforms include a 23 amino acid sequence (exon 16) that contains a tumor necrosis factor-alpha converting enzyme (TACE) cleavage site and can produce a shed extracellular ErbB4 ectodomain (Rio et al. 2000; Cheng et al. 2003). Instead of sequence encoded by exon 16, the JM-b isoforms have a 13 amino acid sequence (exon 15b) that lacks the TACE cleavage site (Elenius et al. 1997a; Tan et al. 2010). Additional isoforms that lack (JM-c) or contain (JM-d) both of the JM exons 16 and 15b have also been reported to be present in developing and neoplastic tissues (Gilbertson et al. 2001; Gilmour et al. 2001; Zeng et al. 2009c).

The ErbB4 CYT isoforms differ near the C-terminus by one exon coding for 16 amino acids (exon 26, SEIGHSPPPAYTPMSG) (Elenius et al. 1999). This exon is included in CYT-1 but absent from CYT-2 isoforms and has been shown to contain binding sites for PI3K (YTPM) (Kainulainen et al. 2000) and WW domain-containing proteins (PP*X*Y) (Komuro et al. 2003).

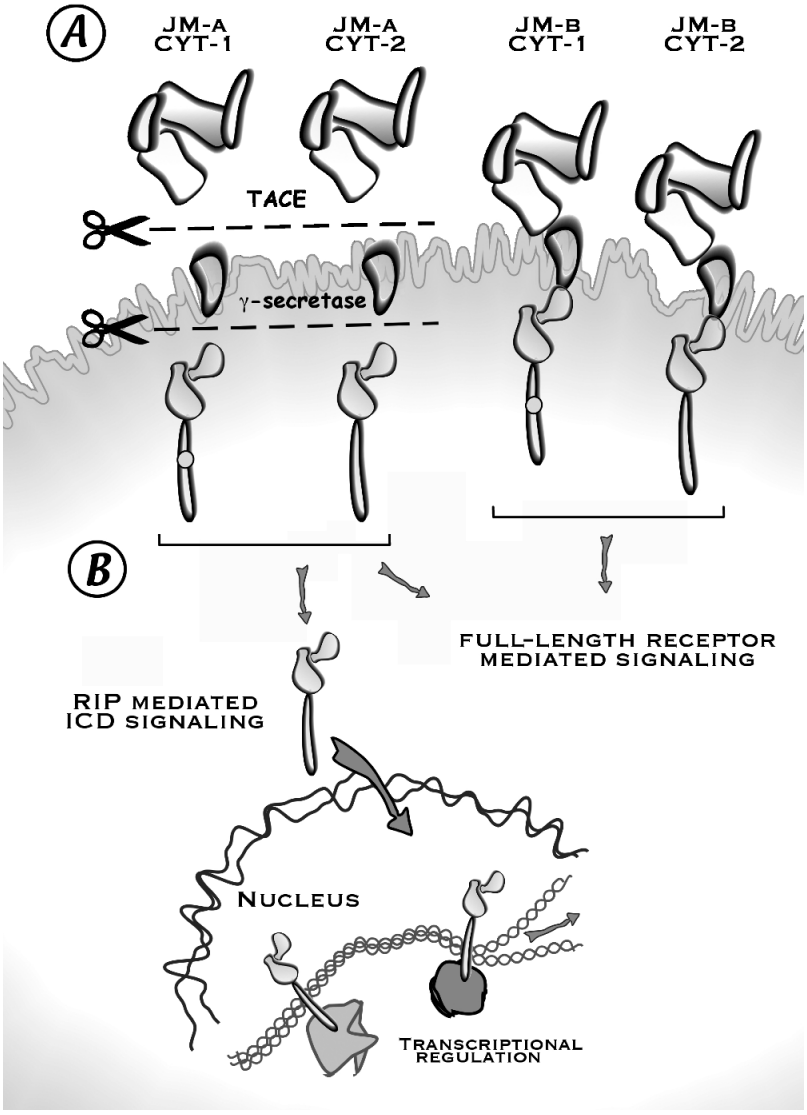

Figure 3. ErbB4 isoforms and signaling. (A) Alternative splicing of ErbB4 generates functionally different isoforms. The JM-a isoforms can signal as full-length receptors or via regulated intramembrane proteolysis (RIP), where the receptor is proteolytically processed by TACE and γ-secretase. The JM-b isoforms can only signal as full-length receptors. The CYT isoforms differ near the C-terminal tail. The CYT-1 isoforms include a unique sequence with WW-domain and PI3K binding sites. (B) RIP results in production of a soluble intracellular domain (ICD) of the receptor, which is capable of nuclear translocation and regulation of transcription through interactions with transcription factors.

2.3.1.1. Distribution of isoforms

Immunohistochemical and RT-PCR analyses have demonstrated ErbB4 expression in several mammalian adult and fetal tissues including brain and heart, and epithelium of many organs (Table 1) (Elenius et al. 1997a; Srinivasan et al. 1998; Junttila et al.

2005). Highest expression levels have been observed in the brain, heart, kidney and the salivary gland (Elenius et al. 1997a; Junttila et al. 2005). The splicing of ErbB4 juxtamembrane isoforms seems to be regulated in a tissue-specific manner as RT-PCR data demonstrates that epithelial tissues (and cell lines) exclusively express the cleavable JM-a isoform, while neural and mesenchymal tissues express both JM-a and JM-b or predominantly JM-b (Elenius et al. 1997a). Consistently, isoform-specific real-time RT-PCR data have shown that different cell types predominantly express either cleavable or non-cleavable ErbB4 isoforms (Junttila et al. 2003; Junttila et al. 2005; Zeng et al. 2009c; Richards et al. 2010). The more rare JM-c and JM-d have been observed in medulloblastoma (Gilbertson et al. 2001; Zeng et al. 2009c), pilocytic astrocytoma (only JM-c) (Zeng et al. 2009c), ovarian cancer (only JM-d) (Gilmour et al. 2001), and in fetal cerebellum (Gilbertson et al. 2001). However, JM-c or JM-d has not been reported to be present in normal adult tissues.

In contrast to the JM isoforms, both CYT isoforms seem to be present in all tissues expressing ErbB4 (Elenius et al. 1999; Junttila et al. 2003; Junttila et al. 2005; Zeng et al. 2009c). However, the relative CYT ratios may vary substantially. For example, the proportion of CYT-1 in testis is 90%, but only 10% in the adrenal gland (Junttila et al. 2005).

2.3.2. Signaling

ErbB4 can be activated by at least seven ligands (Chapter 2.2.2) and signal via ErbB4 homodimers or by heterodimerizing with other ErbB family members (Holbro and Hynes 2004). Activation of both JM-a and JM-b isoforms can trigger conventional intracellular signal transduction pathways such as the PI3K/Akt and Ras/MAPK cascades (Kainulainen et al. 2000) (Figure 3B), or can lead to interactions with WW domain-containing proteins, such as tumor suppressor WW domain-containing oxidoreductase (Wwox) and ubiquitin ligases through three intracellular PP*X*Y motifs (Komuro et al. 2003; Aqeilan et al. 2005; Omerovic et al. 2007; Sundvall et al. 2008b; Feng et al. 2009; Zeng et al. 2009b). In addition to activating the indirect signaling cascades, the JM-a isoforms of ErbB4 can signal via RIP (Ni et al. 2001; Lee et al. 2002) (Figure 3B). The RIP of ErbB4 is conducted by consecutive proteolytic cleavage events. TACE releases the ectodomain (ECD) (Rio et al. 2000; Cheng et al. 2003), and γ–secretase releases the soluble ICD (Ni et al. 2001). The ICD of ErbB4 can translocate to the nucleus (Ni et al. 2001) and regulate transcription as a co-activator or co-repressor. Currently, transcriptional regulators shown to cooperate with the nuclear ErbB4 ICD include Yes-associated protein (YAP) (Komuro et al. 2003), STAT5A (Williams et al. 2004), ETO2 (Linggi and Carpenter 2006), estrogen receptor (ER) α (Zhu et al. 2006), the TGF-beta activated kinase 1/MAP3K7 binding protein 2 and nuclear receptor corepressor (TAB2-NCoR) complex (Sardi et al. 2006), and KRAB (Kruppel-associated box)-associated protein 1 (Kap1) (Gilmore-Hebert et al. 2010).

2.3.2.1. Cellular responses promoted by full-length and RIP-dependent ErbB4 signaling

The different signaling properties of the ErbB4 JM-a and JM-b isoforms have also been shown to translate into abilities to promote distinct cellular functions. *In vitro*, overexpression of the cleavable JM-a CYT-2 isoform, but not the non-cleavable JM-b CYT-2 isoform, promotes ligand-independent receptor activation and cell growth (Määttä et al. 2006). While overexpression of all ErbB4 isoforms in Madine-Darby canine kidney (MDCK) epithelial cells cultured in collagen gels seem to suppress both proliferation and colony formation, only the cleavable JM-a CYT-2 isoform is able to promote tubule formation (Zeng et al. 2007).

In addition, several investigations have described functions specific for the soluble ErbB4 ICD indicating differences between signaling induced by full-length ErbB4 and RIP-released ErbB4 ICD. For example in breast cancer cells, the ErbB4 ICD has been shown to interact with ER-α in the nucleus and promote ER signaling (Junttila et al. 2005; Zhu et al. 2006). Moreover, the soluble ICD has been shown to promote apoptosis either through its intrinsic BH3 (B-cell lymphoma 2 homology domain 3)-like proapoptotic domain (Naresh et al. 2006) or by interacting with the p53 regulator Hdm2 (Arasada and Carpenter 2005).

Despite the many *in vitro* findings clearly suggesting that JM isoforms mediate different cellular responses, to date only one study has examined the JM isoform-specific functions *in vivo*. In that study overexpression of full-length JM-a but not JM-b in developing astrocytes of *Erbb4* null mice was shown capable of rescuing the improper astrocyte differentiation (Sardi et al. 2006).

2.3.2.2. Cellular responses promoted by different CYT isoforms

The CYT isoforms have also been shown to affect ErbB4 signaling, mediated either through full-length receptors or soluble ICDs. It was initially shown in an ErbB-deficient fibroblast model that the PI3K binding domain (YTPM) within the CYT-1-specific sequence enabled JM-b CYT-1 but not JM-b CYT-2 to mediate survival and chemotaxis (Kainulainen et al. 2000). Also, in human medulloblastoma cells only JM-a CYT-1 promotes PI3K-dependent survival (Ferretti et al. 2006). However, all ErbB4 isoforms can initiate PI3K signaling through heterodimerization with ErbB3 when ErbB3 is present in the cellular context (Gambarotta et al. 2004).

In addition to the PI3K binding site, the CYT-1-specific sequence has been shown to harbor a functional WW domain-binding motif (PP*X*Y). Instead of the three PP*X*Y motifs in CYT-1, the CYT-2 isoforms only have two and seem to interact less efficiently with such WW domain-containing proteins as YAP that can utilize the CYT-1-specific PP*X*Y motif (Komuro et al. 2003; Omerovic et al. 2004; Feng et al. 2009). In contrast, Wwox has been shown to interact with ErbB4 primarily through a PP*X*Y motif present in both CYT-2 and CYT-1 (Aqeilan et al. 2005; Aqeilan et al. 2007). Therefore, Wwox interaction, shown to prevent ErbB4 ICD nuclear translocation and suppress its transactivation activity, affects both CYT isoforms similarly. Recently, the CYT-1-specific PP*X*Y motif has also been shown to couple with WW domain-containing E3 ubiquiting ligases, such as Itch, Nedd4 and WWP1, selectively enhancing the degradation of the CYT-1 isoforms (Omerovic et al. 2007;

Sundvall et al. 2008b; Feng et al. 2009; Zeng et al. 2009b). This difference between the stability of the isoforms, could partially explain why the CYT-2 ICD has been often observed to localize more efficiently to the nucleus when compared to the CYT-1 ICD (Määttä et al. 2006; Sundvall et al. 2007; Zeng et al. 2007; Muraoka-Cook et al. 2009; Zeng et al. 2009b).

The potential of the different CYT isoforms to induce even opposing cellular responses has been demonstrated *in vivo* with an eloquent use of transgenic mice (Muraoka-Cook et al. 2009). The mouse model enabled inducible overexpression of soluble ErbB4 ICD of CYT-1- or CYT-2-type in the developing mammary epithelium. The mice overexpressing CYT-1 ICD manifested promotion of epithelial differentiation and decreased proliferation, while hyperplasia and increased proliferation was observed in the mammary epithelium of the mice overexpressing CYT-2 ICD. Consistently, overexpression of full-length cleavable ErbB4 JM-a CYT-1 and JM-a CYT-2 in mouse mammary epithelial cells *in vitro* were shown to inhibit and promote growth, respectively (Muraoka-Cook et al. 2009). Interestingly, the CYT-1 mediated growth suppression was shown to be dependent on the CYT-1-specific WW domain-binding PP*X*Y motif.

2.3.3. Functions of ErbB4 in normal tissues and diseases

2.3.3.1. ErbB4 during development and in adult tissues

Erbb4 deficient mice die of defective heart development at mid-embryogenesis (Gassmann et al. 1995). Similarly to the *Erbb2* mutant mice, *Erbb4* knock-out mice fail to form heart trabeculae and demonstrate defective development of the central nervous system. Consistently, *Erbb4* knock-out mice rescued with heart-specific expression of *ERBB4* demonstrate abnormal development of the nervous system with migration defects in cranial neural crest and alterations in cerebellum neuronal composition (Tidcombe et al. 2003). Interestingly, the only known case of ErbB haploinsufficency in humans, a patient with one disrupted *ERBB4* allele, has been reported to suffer from myoclonic encephalopathy, a severe degenerative brain disorder (Backx et al. 2009). In addition, NRG-1/ErbB4 signaling has been indicated regulatory roles in the adult heart. For example, NRG-1 has been shown to affect myocardial contractility (Lemmens et al. 2004) and promote cardiomyocyte proliferation and survival both *in vitro* and *in vivo* (Zhao et al. 1998; Bersell et al. 2009). A conditional deletion of *Erbb4* in ventricular muscle has been shown to induce various cardiac defects such as dilated cardiomyopathy (García-Rivello et al. 2005).

A role for ErbB4 in mammary gland development was initially suggested as robust induction of ErbB4 phosphorylation was observed during terminal differentiation of the mammary epithelium (Schroeder and Lee 1998). Indeed, defective differentiation of lobuloalveolar epithelium and impaired lactation has thereafter been demonstrated in mouse mammary glands expressing dominantly negative *ERBB4* (Jones et al. 1999), in conditional mammary gland-specific *Erbb4* knock-out mice (Long et al. 2003), and in the heart expression-rescued *Erbb4* mutants (Tidcombe et al. 2003). More specifically, the functions of ErbB4 in lactation have been shown to involve RIP signaling and STAT5A-mediated transcriptional regulation (Long et al. 2003; Williams et al. 2004).

The ErbB4 ICD interacts with STAT5A, is needed for nuclear translocation of STAT5A, and can regulate STAT5A target gene transactivation (Long et al. 2003; Williams et al. 2004; Muraoka-Cook et al. 2006). These data suggest that during lactation ErbB4 regulates STAT5A-mediated milk gene expression, and that the ICD might function as a nuclear chaperone or a co-factor for STAT5A.

The ErbB4 ligands also have crucial roles during development. Targeted mutation of *Nrg-1* results in embryonic lethality with defective development of heart trabeculae, cardiac cushions, Schwann cells and cranial ganglia (Meyer and Birchmeier 1995). As a ligand for both ErbB3 and ErbB4, these developmental defects are consistent with phenotypes of mice lacking *Erbb2*, *Erbb3* and *Erbb4* (Gassmann et al. 1995; Lee et al. 1995; Erickson et al. 1997). In addition, a specific removal of *Nrg-1*α isoform, expressed for example in the mammary gland, does not result in embryonic lethality but in defective lobuloalveolar development and lactogenesis (Li et al. 2002), as also seen in mice with *Erbb4* deletion in mammary glands (Jones et al. 1999; Iwamoto et al. 2003; Long et al. 2003).

Interestingly, mice deficient for *Hb-egf*, a ligand for EGFR and ErbB4, demonstrate cardiac phenotypes that are different from *Nrg-1* deletion. The mice lacking *Hb-egf* die early after birth and manifest dilated ventricular chambers, enlarged cardiac valves and overall diminished cardiac function (Iwamoto et al. 2003). As mentioned above, these findings are similar to *Erbb4* deficiency-associated cardiomyopathy (García-Rivello et al. 2005), enlarged ventricular chambers and cardiomyopathy observed in mice with ventricular-specific *Erbb2* deletion (Crone et al. 2002; Ozcelik et al. 2002), and with valve defects associated with altered *Egfr* activity (Chen et al. 2000; Sibilia et al. 2003) and *Erbb3* deficiency (Erickson et al. 1997). These observations suggest that all ErbB receptors are involved in HB-EGF-mediated heart development.

Taken together, the intertwined roles of ErbB4, its ligands, and other ErbBs in developing and adult mammalian tissues suggest specific functions for each ligand-receptor pair and receptor heterodimer, and demonstrate the importance of ErbB signaling network intricacy.

2.3.3.2. ErbB4 in cancer

The role of ErbB4 signaling in tumor initiation and progression has remained poorly understood. Both enhanced and reduced ErbB4 expression has been associated with various cancer types. Overexpression of ErbB4 has been reported in ovarian (Steffensen et al. 2008) and colorectal cancer (Leung et al. 2008), and in brain malignancies (Gilbertson et al. 1997; Gilbertson et al. 2002; Ferretti et al. 2006). In contrast, downregulation of ErbB4 has been reported in meningioma (Laurendeau et al. 2009), and in kidney (Thomasson et al. 2004), thyroid (Wiseman et al. 2008), and pancreatic (Thybusch-Bernhardt et al. 2001) cancers. Both up- and downregulation of ErbB4 have also been observed in bladder (Junttila et al. 2003; Kassouf et al. 2008) and breast cancer (Sundvall et al. 2008a). In addition to changes in expression levels in cancer, functional assays have demonstrated both oncogenic and tumor suppressor activities for ErbB4, often in breast cancer-related investigations (reviewed below). However, oncogenic functions of ErbB4 have been studied in other models as well. For

example, fibroblasts overexpressing ErbB4 stimulated tumor growth when transplanted into nude mice (Alaoui-Jamali et al. 2003).

The role of ErbB4 in breast cancer is particularly controversial. In all, up- and downregulation, presence and lack of *ERBB4* amplification, and association with both favorable and poor prognostic indicators have been observed (Bièche et al. 2003; Witton et al. 2003; Junttila et al. 2005; Muraoka-Cook et al. 2008). In addition, the ErbB4 ligand NRG-1 has contradictory roles in breast cancer. NRG-1 has been shown to induce carcinogenesis in mouse mammary glands (Krane and Leder 1996), promote cancer cell proliferation and metastasis both *in vivo* and *in vitro* (Atlas et al. 2003; Tsai et al. 2003), and in contrast, to suppress breast cancer cell growth for example by inducing apoptosis or differentiation (Ni et al. 2001; Sartor et al. 2001; Naresh et al. 2006). Consistently with breast cancer cell growth inhibitory functions, NRG-1 has also been shown to promote differentiation in normal mammary epithelial cells (Muraoka-Cook et al. 2006). Nevertheless, the majority of studies on ErbB4 inhibition in breast and other cancer cells with specific antibodies (Hollmen et al. 2009), siRNAs (Määttä et al. 2006), or ErbB kinase inhibitors (Prickett et al. 2009) have reported reduced cancer cell growth and survival. Moreover, the few studies that have addressed the *in vivo* functions of ErbB4 in mammary tumor formation or in normal mammary epithelium are in line with the role of ErbB4 in promoting cancer cell growth: (i) no tumor suppressor functions for ErbB4 were observed in *Neu*-driven mammary tumors (Jackson-Fisher et al. 2006), (ii) downregulation of ErbB4 in breast cancer cell xenografts significantly inhibited tumor growth (Tang et al. 1999), and (iii) the expression of soluble ErbB4 CYT-2 ICDs was shown to increase mammary epithelial cell proliferation (Muraoka-Cook et al. 2009).

Interestingly, somatic mutations of *ERBB4* have been found in 19% of metastatic melanoma (Prickett et al. 2009), 5% of non-small cell lung cancer (NSCLC) (Davies et al. 2005; Soung et al. 2006; Ding et al. 2008), 1% of breast cancer, 2% of gastric cancer (Soung et al. 2006), and 3% of colorectal cancer (Parsons et al. 2005; Soung et al. 2006; Greenman et al. 2007) patients.

2.3.3.3. ErbB4 in non-neoplastic disease

Altered ErbB4 signaling has, in addition to cancer, been indicated roles in non-neoplastic diseases, mainly in cardiovascular diseases (Pentassuglia and Sawyer 2009; Xu et al. 2010) and schizophrenia (Mei and Xiong 2008; Banerjee et al. 2010).

Consistent with the abovementioned regulatory functions in adult cardiomyocytes, the NRG-1/ErbB4 signaling has been shown to promote cardiomyocyte proliferation and survival in response to heart injuries (Zhao et al. 1998; Kuramochi et al. 2004; Bersell et al. 2009). In addition, downregulation of ErbB4 expression in the myocardium has been associated with heart failure (Rohrbach et al. 1999; Rohrbach et al. 2005). These findings have encouraged experimentation on treating heart diseases with intravenous administration of recombinant NRG-1. Indeed, NRG-1 treatment has been shown to improve cardiac performance and survival in mouse, rat and dog models of cardiomyopathy (Liu et al. 2006). Moreover, three recent phase II clinical trials have shown beneficial effects for intravenously administered recombinant NRG-1 in treating chronic heart failure patients (Xu et al. 2010; Jabbour et al. 2011).

Aberrant NRG-1/ErbB4 signaling has also been suggested to predispose to schizophrenia. Initially it was discovered that mice with heterozygous deletions of either *Nrg-1* or *Erbb4* demonstrated similar hyperactive behavior as schizophrenia model mice (Stefansson et al. 2002). The schizophrenia mouse models include a model characterized by a reduced number of *N*-methyl-d-aspartate receptors (NMDAR) (Mohn et al. 1999), hypofunction of which is recognized as the leading hypothesis to cause cognitive dysfunction in schizophrenia (Kantrowitz and Javitt 2010). Consistently, the forebrains of mutant *Nrg-1* mice expressed significantly fewer functional NMDAR receptors. Moreover, several single nucleotide polymorphisms (SNPs) in both *NRG1* and *ERBB4* genes have been shown to associate with an increased risk for schizophrenia (Mei and Xiong 2008). Recently, it has been suggested that ErbB4 expression is increased in the brains of schizophrenia patients (Silberberg et al. 2006; Law et al. 2007; Chong et al. 2008). More specifically, selective overexpression of ErbB4 isoforms JM-a and CYT-1 has been observed in dorsolateral prefrontal cortex (DLPFC) and hippocampus, regions known to be important in schizophrenia-associated cognitive dysfunction (Silberberg et al. 2006; Law et al. 2007). Interestingly, this selective overexpression of specific ErbB4 isoforms in DLPFC and hippocampus has been associated with the *ERBB4* SNPs that predispose to schizophrenia (Law et al. 2007) indicating that changes in the splicing of the *ERBB4* gene may also have significance in pathogenesis of diseases.

The molecular mechanisms by which aberrant NRG-1/ErbB4 signaling is involved in the pathophysiology of schizophrenia have remained unknown until very recently. Most interestingly, NRG-1/ErbB4 signaling was shown to suppress the function of NMDAR (Pitcher et al. 2011). In addition, the soluble ICD of ErbB4 has been shown to mediate transcriptional activation of schizophrenia-associated genes in hippocampus, indicating a role for RIP-mediated ErbB4 signaling in schizophrenia (Allison et al. 2011).

2.4. Mammalian kidney organogenesis

The mammalian kidney originates from the intermediate mesoderm through three phases: development of pronephros, mesonephros and metanephros (Gilbert 2006). The pronephros is initiated as the intermediate mesoderm interacts with the paraxial mesoderm to form the pronephric duct (Mauch et al. 2000). The cells of the pronephric duct migrate and induce the adjacent mesenchyme, nephrogenic mesenchyme, to form the primary kidney structure, the pronephros (Figure 4A). The pronephros forms the permanent kidney in fish and amphibians but it is not known to have a functional purpose in mammals, reptiles or birds. In mammals most of the pronephros degenerates but the remaining part forms the nephric (Wolffian) duct (Saxén and Lehtonen 1987). The consequential nephric structure, the mesonephros, is also formed from the nephrogenic mesenchyme and sections of the pronephric duct (Figure 4B) (Sainio and Raatikainen-Ahokas 1999). In some mammals the mesonephros filtrates urine but not for example in mice. In humans the possible filtration function of the mesonephros remains unknown. Most of the mesonephric tubules degenerate but some contribute to the vas deferens and efferent ducts of the testis and act as a source for hematopoietic stem cells.

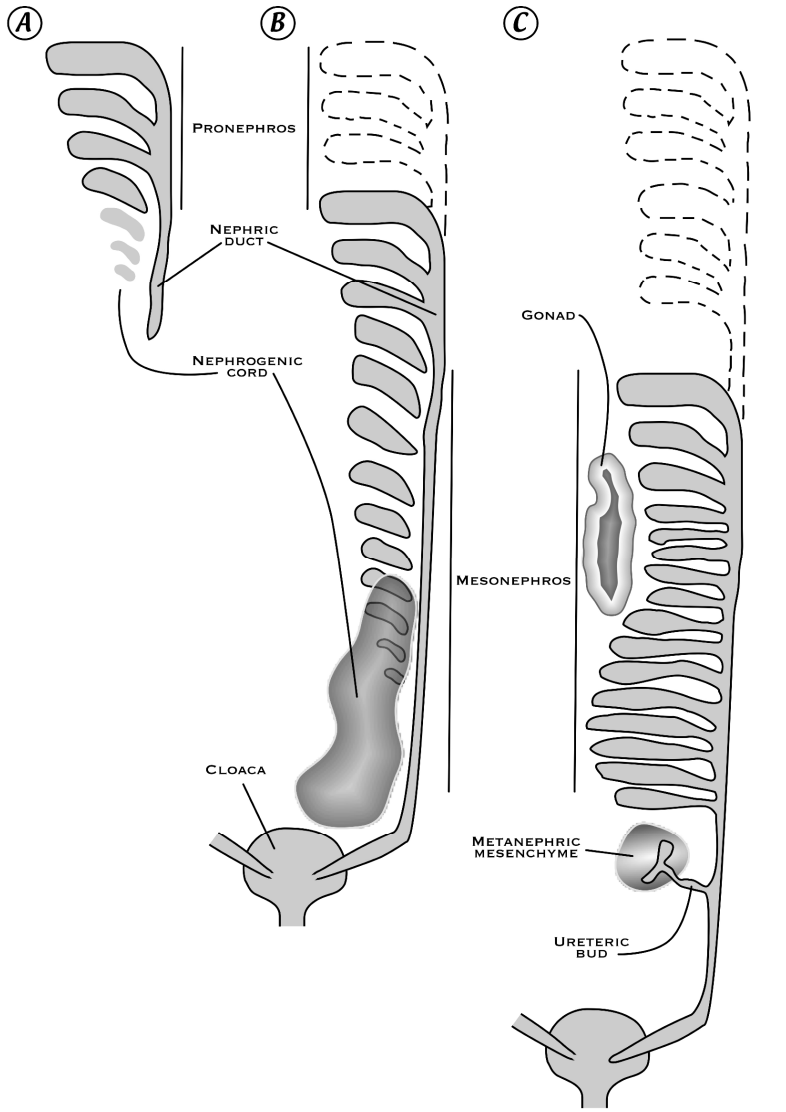

Figure 4. Development of mammalian kidney. (A) The Pronephros is formed from nephrogenic mesenchyme and nephric duct. (B) After degeneration of the pronephros, nephric duct and nephrogenic mesenchyme generate the mesonephros. (C) The metanephros is formed from metanephric mesenchyme and the ureteric bud that branches from the nephric duct. Modified from Saxen 1987.

The permanent mammalian kidney, the metanephros, is constructed partially from the same components as pro- and mesonephros, around day 35 in human embryos (E10.5-11.0 in mice) (Figure 4C) (Saxén and Lehtonen 1987; Shah et al. 2004; Gilbert 2006). The metanephric mesenchyme forms from intermediate mesoderm and induces each of the two nephric ducts to branch and create ureteric buds (Figure 5A). Reciprocal induction between ureteric bud and metanephric mesenchyme is a central mechanism in development of the metanephros and the mature nephrons. This interaction between mesenchyme and epithelial tissues occurs for the first time as the

ureteric bud grows out from the nephric ducts, invades the metanephric mesenchyme and induces the mesenchymal tissue to condensate around the bud (Figure 5B-C). The condensated mesenchyme differentiates as it undergoes mesenchymal to epithelial transition (MET) and in turn induces the ureteric bud to branch and grow. The mesenchyme-induced epithelium differentiates to construct the blood and urine filtrating units of the kidney, the nephrons. In turn, the branching ureteric epithelium forms the collecting duct system and the ureter that are responsible for transporting the filtrated urine to the bladder.

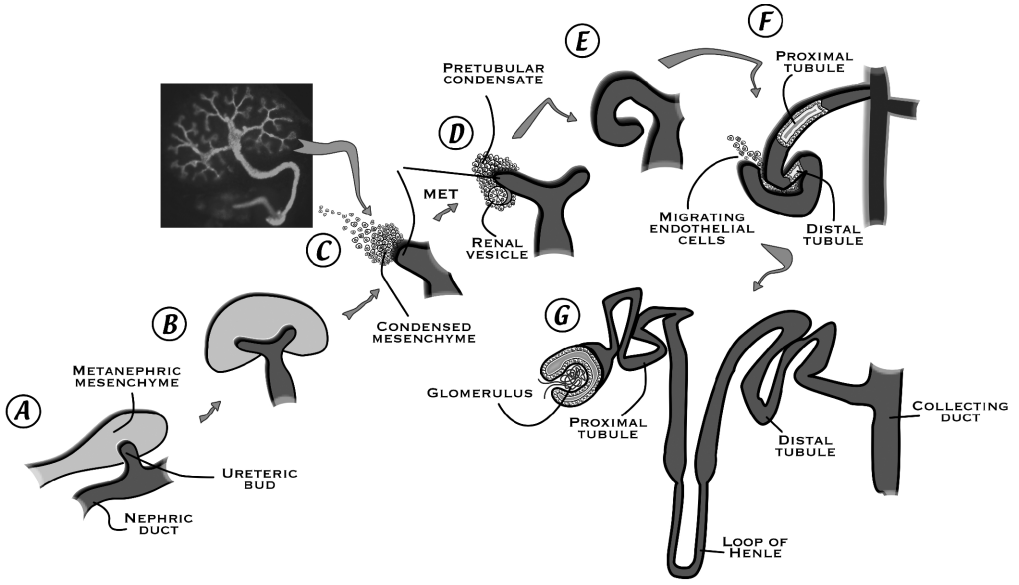

Figure 5. Development of the metanephros. (A, B) Metanephric mesenchyme induces ureteric bud to invade the mesenchyme and branch. (C) The ureter induces the mesenchyme to condensate at its tips. (D) The condensed mesenchyme undergoes mesenchymal to epithelial transition (MET) and forms the renal vesicle. The renal vesicle grows into comma- (E) and s-shaped bodies (F) and the cells eventually differentiate to generate a nephron. (F) Podocyte precursors induce migration of endothelial cells from dorsal aorta to form the blood capillaries of the glomerulus. (G) The mesenchyme-derived epithelium generates the different components of the nephron while the ureteric epithelium forms the urine transport system, the collecting ducts and the ureter.

2.4.1. Nephrogenesis

Nephrogenesis is initiated at each tip of the branched ureter (Saxén and Lehtonen 1987). Branched ureter induces mesenchyme to form caps of cell aggregates around the tips while the ureter itself continues to grow and branch (Figure 5C) (Vainio and Lin 2002; Dressler 2006). The cells in the aggregate undergo MET, become polarized and generate structures called renal vesicles (Figure 5D). Other mesenchymal cells retain their mesenchymal characteristics and form the stromal tissue of the kidney. While the renal vesicle grows and forms a prenephrogenic tubular structure called comma-shaped body (Figure 5E), the other end remains in contact with the ureteric epithelium. The comma-shaped body further elongates into an s-shaped body (Figure

5F). Cells of the s-shaped body differentiate into specific nephron cell types, such as the podocytes, and the cells of proximal and distal tubules and the loop of Henle. A continuous epithelial tubule is formed as the distal end of the s-shaped body fuses with the ureteric epithelium by breaking down its basal lamina. The development of the nephron advances and proximal and distal tubules elongate, convolute, and become separated by the loop of Henle (Figure 5G).

The development of the blood filtration unit of the nephron, the glomerulus, is initiated when precursor cells at the proximal end of the s-shaped body acquire podocyte cell characteristics (Dressler 2006). The podocyte precursors secrete vascular endothelial growth factor A (VEGFA) and attract endothelial cells to migrate from the dorsal aorta and invade the vascular cleft (Figure 5F) (Klanke et al. 1998; Eremina et al. 2003; Gilbert 2006). The main components of a functional glomerulus are constructed of capillary blood vessels formed from the endothelial cells, and the podocytes that wrap around the capillaries to generate a filtration barrier between the blood and the urinary fluid.

2.4.2. Molecular mechanisms regulating kidney development

Various signaling events are needed to achieve and control the reciprocal interactions between mesenchymal and epithelial tissues (Gilbert 2006). These inductive signals are repeated numerous times during kidney development in a temporally and quantitatively regulated manner, and in correct order to eventually generate the 300 000 to one million functional nephrons of one human kidney (Nyengaard and Bendtsen 1992).

Expression of transcription factor forkhead-winged-helix genes (*Foxc1* and *Foxc2*) (Kume et al. 2000), homeobox (*Hoxa11 and Hoxd11*) genes (Patterson et al. 2001; Wellik et al. 2002) and Wilms' tumor suppressor gene (*WT1*) (Kreidberg et al. 1993) initiate the development of the metanephros. They regulate the formation and position of the metanephric mesenchyme and its capability to respond to signals from the ureteric bud. In addition, these transcription factors regulate pivotal signal molecules such as paired box 2 (Pax2), Eya1 and Six2. Activation of these signaling molecules is needed for the metanephric mesenchyme to secrete glial cell-line derived neurotrophic factor (GDNF) (Vainio and Lin 2002). Secreted GDNF activates GDNF receptors (receptor tyrosine kinase Ret and glycosylphosphatidylinositol-linked co-receptor GFRα1) expressed in the nephric duct inducing the ureteric bud outgrowth and subsequent invasion of the mesenchyme (Costantini and Shakya 2006).

Within the mesenchyme, the ureteric bud secretes factors, such as fibroblast growth factors (FGF) (Perantoni et al. 1995) and Wnts (Itäranta et al. 2002; Carroll et al. 2005) that induce the aggregation of mesenchymal cells at the tips of the ureteric buds. In turn, the aggregated mesenchymal cells begin to secrete Wnt4, which by autocrine induction completes their epithelialization (Stark et al. 1994; Kispert et al. 1998). The crucial role of Wnt4 in MET has been demonstrated with transgenic mice lacking active *Wnt4* (Stark et al. 1994). The kidneys of the *Wnt4* deficient mice do not develop tubules due to incomplete MET of the aggregated mesenchyme.

Following the ureteric bud invasion and induction of the mesenchyme, mesenchyme-secreted factors induce the ureteric bud to branch. The branching morphogenesis is complex and crucial for normal kidney development. To generate a

properly branched ureter, key branching morphogenesis events need to progress in a tightly regulated manner. For example, the number of adequate branching events and correct direction of branching need to be determined (Costantini and Kopan 2010). Moreover, to create the complex branching patterns, branch remodeling and utilization of different branching types, *e.g*. terminal or lateral branching, are needed. These processes are under tight regulation by several signaling factors. Factors that promote branching include GDNF (Sainio et al. 1997), hepatocyte growth factor (HGF) and EGF (Ishibe et al. 2009), while transforming growth factor-beta 1 (TGF-β1) (Ritvos et al. 1995) and bone morphogenic protein 4 (BMP4) (Miyazaki et al. 2000) have been shown to inhibit branching. In addition, the processes of ureter growth and differentiation must be coordinated with branching. For example, differential rates of cell proliferation in the ureter tip and trunk regions are needed depending on whether the ureter is branching or elongating (Michael and Davies 2004; Karner et al. 2009).

Regulation of ureter growth and differentiation is not completely understood. However, some of the same molecules that regulate other steps of kidney organogenesis, such as GDNF and Ret, are known be involved (Shakya et al. 2005). Interestingly, other RTKs, MAPK and PI3K/Akt pathways, and NRG, have also been suggested to control growth and differentiation of the ureter (Sakurai et al. 2005; Costantini and Kopan 2010).

Taken together, studies on the molecular mechanisms of kidney development have provided a significant amount of important knowledge on organogenesis. Even though these complex processes and their regulatory machinery are currently only partly understood, they portray an overwhelmingly sophisticated and beautiful process of how kidneys develop.

2.4.3. ErbB expression and functions in kidney development

All four ErbB receptors and many of their ligands have been shown to be expressed both in adult and developing kidneys (Zeng et al. 2009a). The ErbB receptors are mainly expressed in the epithelial compartments, the tubular epithelium of the nephrons and the collecting ducts (Table 1) (Gusterson et al. 1984; Damjanov et al. 1986; Partanen and Thesleff 1987; Press et al. 1990; Prigent et al. 1992; Srinivasan et al. 1998; Zeng et al. 2007). Interestingly, among the ErbBs, the expression levels of ErbB4 are most abundant in the kidney when compared to other tissues (Table 1).

ErbBs and their ligands have also been examined in the developing kidney but the studies have almost exclusively concentrated on EGF, TGF-α, HB-EGF and their receptor EGFR (Zeng et al. 2009a). They have been shown to be expressed early on during development and capable of influencing tubulogenesis and branching morphogenesis *in vitro* (Barros et al. 1995; Takemura et al. 2001; Yosypiv et al. 2006). However, the exact roles of the ligands in kidney development *in vivo* have remained unknown as the transgenic mice lacking *Egf*, *Tgf-α*, *Hb-egf* do not display significant kidney abnormalities (Luetteke et al. 1999; Zeng et al. 2009a). The specific functions of EGFR in the developing kidney have also been difficult to determine. For example, only one of the three independent studies on *Egfr* deficient mice reported defects in the kidney (Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995). However, the observed structural alteration in collecting duct epithelium of the mutant

mice (Threadgill et al. 1995) together with the abovementioned *in vitro* findings (Barros et al. 1995; Takemura et al. 2001; Yosypiv et al. 2006) suggests that EGFR has functional significance in the ureteric epithelium. More specifically, the data indicate a role for EGFR signaling during the later stages of ureteric epithelial development, in branching and differentiation (Threadgill et al. 1995; Zeng et al. 2009a). EGFR seems to regulate these processes through cross-talk with other signaling networks, such as the Met RTK and angiotensin type 1 receptor (Yosypiv et al. 2006; Ishibe et al. 2009).

The functions of other ErbB receptors and ligands during the development of the kidney have not been widely studied but recent findings suggest that ErbB4-mediated signaling could have a role in kidney organogenesis. For example, metanephric mesenchyme has been demonstrated to express NRG-1 and NRG-1 has been shown to promote growth and differentiation of the ureter tissue *in vitro* (Sakurai et al. 2005). Similarly, NRG-1 has been shown to induce growth of cultured MDCK epithelial kidney cells overexpressing ErbB4 (Zeng et al. 2009a).

2.4.4. ErbBs in kidney disease

ErbB signaling has also been implicated in renal injury, polycystic kidney diseases and renal cell cancer. Following acute renal injury, expression of EGFR and its ligands have been shown to increase rapidly (Sakai et al. 1997; Hise et al. 2001). Furthermore, administration of EGF, TGF-α or HB-EGF accelerates recovery from ischemia-induced renal injury in rats (Humes et al. 1989). Consistently, *waved-2* mice, which have a point mutation in *Egfr* and significantly decreased EGFR kinase activity, showed slower recovery after acute renal injury (Wang et al. 2003). Interestingly, HB-EGF/EGFR signaling has been recently suggested significant roles in glomerular diseases such as rapidly progressive glomerulonephritis (RPGN) (Bollée et al. 2011). It was shown that HB-EGF-activated EGFR drives formation of cellular crescents, histological patterns that result from glomerular capillary damage during glomerulonephritis, and renal failure in RPGN mouse models. Furthermore, EGFR inhibition prophylactically or after induced renal injury effectively prevents or reduces renal damage and failure, respectively.

Deregulated activity or mislocalization of ErbB receptors has also been associated with polycystic kidney diseases (PKD). Especially the roles of EGFR and ErbB2 in PKD have been broadly studied. Alterations in EGFR and ErbB2 activity or localization have been observed in human autosomal recessive PKD (ARPKD) (Sweeney and Avner 1998; Nakanishi et al. 2001) and autosomal dominant PKD (ADPKD) (Du and Wilson 1995) as well as in rodent models of ARPKD (Sweeney and Avner 1998; Sweeney et al. 2008) and ADPKD (Wilson et al. 2006). The significance of ErbB4 signaling in PKDs has been studied less but elevated ErbB4 expression has been observed in the kidneys of an ARPKD mouse model (Nemo et al. 2005). Interestingly, decreased ErbB4 expression has also been associated with kidney cysts in a patient with a deletion of an *ERBB4* allele (Backx et al. 2009). These data have inspired a hypothesis that targeting ErbB receptors could be used to treat PKDs. Indeed, ErbB2 kinase inhibitors have been demonstrated to reduce renal cyst formation in a PKD mouse model, and ErbB kinase inhibitors or ErbB2-specific siRNAs to

rescue the impaired migration of epithelial cell lines isolated from patients with ADPKD (Wilson et al. 2006).

Finally, deregulated signaling or altered expression of EGFR (Prewett et al. 1998; Lee et al. 2008) and ErbB4 (Thomasson et al. 2004) has been associated with renal cell carcinoma (RCC). Moreover, targeting EGFR signaling *in vitro* has been shown to sensitize RCC cells for cancer drugs (An and Rettig 2007). However, clinical trials with the EGFR targeting antibody cetuximab (Motzer et al. 2003) or the EGFR kinase inhibitors genifinib (Drucker et al. 2003) and erlotinib (Bukowski et al. 2007) have not indicated anti-tumor activity.

3. AIMS OF THE STUDY

The specific aims of this study were:

1) To determine the expression levels and distribution of ErbB4 and its isoforms in normal tissues, cancer, and non-neoplastic diseases.

2) To determine the target genes and cellular responses promoted by different ErbB4 isoforms.

3) To determine the expression pattern and function of ErbB4 during kidney development.

4. MATERIAL AND METHODS

Reagents and methodology are presented in detail in the original publications (I-III).

4.1. Methods

Method	Used in
Analysis of protein phosphorylation	II
Cell culture	I–III
3D- Matrigel cell culture	I, III
cDNA microarrays	II
Cell cycle analysis	II
Molecular cloning	II, III
Confocal microscopy	I–III
DNA extraction	II, III
Generation of transgenic mouse lines	III
Genotyping and breeding of mouse lines	III
GST pull-down assay	II
Histology	III
Immunofluorescence and DAPI staining	I–III
Immunohistochemistry	III
Immunoprecipitation	II
In silico analysis	I
In situ hybridization	III
MTT mitochondrial activity assay	II
Organ collection, organ- and tissue culture	III
PCR, RT-PCR and Real-Time RT-PCR	II, III
PDGFRA promoter assay	II
Proliferation analysis	I–III
RNA interference	II
RNA isolation and cDNA synthesis	II, III
Soft agar colony formation assay	II
Southern blotting	III
Statistical analyses	I–III
Transfection	I–III
TUNEL staining	II
Western blotting	II, III

4.2. Reagents

Compound	Application	Source	Used in
AG 1478	Inhibition of ErbB kinases	Calbiochem	II, III
AG 1296	Inhibition of PDGFRA kinase	Calbiochem	II
BIO	GSK3 inhibition/mesenchyme induction	Calbiochem	III
DAPI	Staining of nuclei	Sigma	I–III
DBA-rhodamin	Collecting duct marker	Vector Labs.	III
LTL-fluorescein	Proximal tubule marker	Vector Labs.	III
LY294002	PI3K inhibition	Calbiochem	II
NRG-1-β1	ErbB4 ligand	R&D	I–III
PD98059	Mek1 inhibition	Calbiochem	II
PDGF-BB	PDGFRA ligand	Pepro Tech EC	II
TRITC-Phalloidin	Actin filament staining	Sigma	III
Alexa555-Phalloidin	Actin filament staining	Invitrogen	I
Retinoic acid	PDGFRA promoter activation	Sigma	II
TUNEL	DNA damage visualization	Roche	II

4.3. Primers and Probes

Name	5'->3' Sequence	Method	Reference	Species	Used in
AP-2α fw	TAAAGCTGCCAACGTTACCC	Real-Time RT-PCR		h	II
AP-2α rev	AAGTCCCTGGCTAGGTGGA	Real-Time RT-PCR		h	II
AP-2α probe	Universal Probelibrary probe #7	Real-Time RT-PCR		h	II
AP-2α fw	AGTTCACAGTTTTTCAGCTATGGA	Real-Time RT-PCR		m	II
AP-2α rev	GCGCTGGTGTAGGGAGATT	Real-Time RT-PCR		m	II
AP-2α probe	Universal Probelibrary probe #104	Real-Time RT-PCR		m	II
*att*B1-A2	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGAGTCGACGCCACCATG	attB cloning		h	III
*att*B2-A2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACACCACAGTATTCCGGTGTCT	attB cloning	(Junttila et al. 2003)	h	III
β-actin-fw	ATCTGGCACCACACCTTCTACAAT	Real-Time RT-PCR	(Junttila et al. 2003)	h,m	I–III
β-actin-rev	CCGTCACCGGAGTCCATCA	Real-Time RT-PCR	(Junttila et al. 2003)	h,m	I–III
β-actin probe	TGACCCAGATCATGTTTGAGACCTTCAACAC	Real-Time RT-PCR	(Junttila et al. 2003)	h,m	I–III
Cre-fw	GCACGTTCACCGCATCAAC	Genotyping	(Jokela and Vainio 2007)	-	III
Cre-rev	CGATGCAACGAGTGATGAGGTTC	Genotyping	(Jokela and Vainio 2007)	-	III
Cre2-fw	GTTTTGCCGGGTCAGAAAA	Real-Time RT-PCR		-	III
Cre2-rev	GGCGCGAGTTGATAGCTG	Real-Time RT-PCR		-	III
Cre2 probe	Universal Probelibrary probe #87	Real-Time RT-PCR		-	III
ErbB1 fw	GCCACGCCAACTGTACCTAT	Real-Time RT-PCR		m	III
ErbB1 rev	GCCACACTTCACATCCTTGA	Real-Time RT-PCR		m	III
ErbB1 probe	Universal Probelibrary probe #107	Real-Time RT-PCR		m	III
ErbB2 fw	TCAACTGCACCCACTCATGT	Real-Time RT-PCR		m	III
ErbB2 rev	CTGGCTCTCTGCTCTGCTG	Real-Time RT-PCR		m	III
ErbB2 probe	Universal Probelibrary probe #85	Real-Time RT-PCR		m	III
ErbB3 fw	CTTGCCTACAGGAACGCTTAC	Real-Time RT-PCR		m	III
ErbB3 rev	CCAAAACCGCAGAATCCA	Real-Time RT-PCR		m	III
ErbB3 probe	Universal Probelibrary probe #106	Real-Time RT-PCR		m	III

Name	5'->3' Sequence	Method	Reference	Species	Used in
ErbB4 cDNA1	TGGAAACAGAGTTGGTGGAA	Genotyping		h	III
ErbB4 cDNA2	TCCAACATTTGACCATGACC	Genotyping		h	III
ErbB4 CYT-fw	CAACATCCCACCTCCCATCTATAC	Real-Time RT-PCR	(Junttila et al. 2003)	h	I, II
ErbB4 CYT-rev	ACACTCCTTGTTCAGCAGCAAA	Real-Time RT-PCR	(Junttila et al. 2003)	h	I, II
ErbB4 CYT-fw	TCCTCCCATCTACACATCCAGAA	Real-Time RT-PCR		m	III
ErbB4 CYT-rev	GGCATTCCTTGTTGTGTAGCAA	Real-Time RT-PCR		m	III
ErbB4 CYT-1 probe	TGAAATTGGACACAGCCCTCCTCCTG	Real-Time RT-PCR	(Junttila et al. 2003)	h, m	I–III
ErbB4 CYT-2 probe	AATTGACTCGAATAGGAACCAGTTTGTATACCGAGAT	Real-Time RT-PCR	(Junttila et al. 2003)	h	I, II
ErbB4 CYT-2 probe	AATTGACTCCAATAGGAATCAGTTTGTGTACCAAGATG	Real-Time RT-PCR		m	I, III
ErbB4 Flox P20	CAAATGCTCTCTCTGTTCTTTGTGTCTG	Genotyping	(Long et al. 2003)	m	III
ErbB4 Flox P22	TTTTGCCAAGTTCTAATTCCATCAGAAGC	Genotyping	(Long et al. 2003)	m	III
ErbB4 Flox P23	TATTGTGTTCATCTATCATTGCAACCCAG	Genotyping	(Long et al. 2003)	m	III
E4ICD-FW	CGGGTCGACTCAGAAGGAAGAGCATCAAAAAGAAAAG	ICD-GST cloning		h	II
E4ICD-REV	TATGCGGCCGCTTACACCACAGTATTCCGGTGTCTG	ICD-GST cloning		h	II
E4ICD-N-FW	CGGGTCGACTCAGTCCAAATGACAGCAAGTTCTTTCAGAAT	ICD-GST cloning		h	II
E4ICD-C-REV	TATGCGGCCGCTTAGGGAAGCTTCATACGATCATCACC	ICD-GST cloning		h	II
ErbB4-*insitu*1	GTTTGGAGCTATGGCGTCAC	*In situ* cloning		m	III
ErbB4-*insitu*2	CACCACAGTATTCCGGTGTC	*In situ* cloning		m	III
ErbB4 JM-fw	CCACCCATGCCATCCAAA	Real-Time RT-PCR	(Junttila et al. 2003)	h	I, II
ErbB4 JM-rev	CCAATTACTCCAGCTGCAATCA	Real-Time RT-PCR	(Junttila et al. 2003)	h	I, II
ErbB4 JM-fw	TTGCCATCCAAACTGCACC	Real-Time RT-PCR		m	III
ErbB4 JM-rev	TCCAATGACTCCGGCTGC	Real-Time RT-PCR		m	III
ErbB4 JM-a probe	CATGGACGGGCCATTCCACTTTACCA	Real-Time RT-PCR	(Junttila et al. 2003)	h, m	I–III
ErbB4 JM-b probe	TTCAAGCATTGAAGACTGCATCGGCCT	Real-Time RT-PCR		m	III
ErbB4 JM-b probe	CTCAAGTATTGAAGACTGCATCGGCCTGAT	Real-Time RT-PCR	(Junttila et al. 2003)	h	I, II
ErbB4 total fw	TCAAGCATTGGATAATCCCGA	Real-Time RT-PCR	(Junttila et al. 2005)	h	II, III
ErbB4 total rev	AGTGGCTCATTCACATACTCATCCT	Real-Time RT-PCR	(Junttila et al. 2005)	h	II, III
ErbB4 total probe	TATCACAATGCATCCAATGGTCCACCC	Real-Time RT-PCR	(Junttila et al. 2005)	h	II, III
Erk fw	CAAAGAACTAATTTTTGAAGAGACTGC	Real-Time RT-PCR		h	II

Name	5'->3' Sequence	Method	Reference	Species	Used in
Erk rev	TCCTCTGAGCCCTTGTCCT	Real-Time RT-PCR		h	II
Erk probe	Universal Probelibrary probe #20	Real-Time RT-PCR		h	II
Mek1 fw	CATTGCTGTAATAAAAGGCCTGA	Real-Time RT-PCR		h	II
Mek1 rev	ATGTTGGAGGGCTTGACATC	Real-Time RT-PCR		h	II
Mek1 probe	Universal Probelibrary probe #7	Real-Time RT-PCR		h	II
Mek1 fw	GTGGGCACGAGATCCTACAT	Real-Time RT-PCR		m	II
Mek1 rev	TCCGACTGCACAGAGTAGTGA	Real-Time RT-PCR		m	II
Mek1 probe	Universal Probelibrary probe #109	Real-Time RT-PCR		m	II
NRG-1-fw	TGGGACCAGCCATCTCATAAAG	Real-Time RT-PCR		m	III
NRG-1-rev	TGAGGGGTTTGACAGGTCCTT	Real-Time RT-PCR		m	III
NRG-1 probe	AACTTTCTGTGTGAATGGAGGCGAGTGCTT	Real-Time RT-PCR		m	III
Oct-1 fw	AGGAGCAGCGAGTCAAGATG	Real-Time RT-PCR		h	II
Oct-1 rev	GCTCTTCTTACTTTTCCAGTCTGC	Real-Time RT-PCR		h	II
Oct-1 probe	Universal Probelibrary probe #74	Real-Time RT-PCR		h	II
PDGFRA fw	AGTTCCTGCATCCATTTTGG	RT-PCR		m	II
PDGFRA rev	GAACTGCGATGGTCTCTTCC	RT-PCR		m	II
PDGFRA fw	CACCCTGCGTTCTGAACTCAC	Real-Time RT-PCR		h	II
PDGFRA rev	TTTCTGTTTCCAAATGACAACCAG	Real-Time RT-PCR		h	II
PDGFRA probe	TGCAGTCCTGGTGCTGTTGGTGATTG	Real-Time RT-PCR		h	II
ROSA prom	CCTAAAGAAGAGGCTGTGCTTTGG	Genotyping	(Soriano 1999)	m	III
SA-2	CATCAAGGAAACCCTGGACTACTG	Genotyping	(Soriano 1999)	-	III
Slc3A1-fw	CCATGTCAACGGTGTAACCA	Real-Time RT-PCR		m	III
Slc3A1-rev	GCCAGCTGGAGTTTCCATAC	Real-Time RT-PCR		m	III
Slc3A1 probe	Universal Probelibrary probe #102	Real-Time RT-PCR		m	III
Sp-1 fw	ACCCCCTTGAGCTTGTCC	Real-Time RT-PCR		h	II
Sp-1 rev	CCACAGCTGTCATTTCATCC	Real-Time RT-PCR		h	II
Sp-1 probe	Universal Probelibrary probe #87	Real-Time RT-PCR		h	II

Probes labeled with TAMRA. Abbreviations: h = human, m = mouse.

4.4. Expression vectors

Vector	Source	Used in
pBABE-puro	Addgene	I
pBABE-puro*ErbB4JM-aCYT-1*		I
pBABE-puro*ErbB4JM-aCYT-2*	(Tvorogov et al. 2009)	I
pBABE-puro*ErbB4JM-bCYT-1*		I
pBABE-puro*ErbB4JM-aCYT-2*		I
pcDNA3.1	Invitrogen	II
pcDNA3.1*ErbB4JM-aCYT-2*	(Määttä et al. 2006)	II, III
pcDNA3.1*ErbB4JM-bCYT-2*	(Määttä et al. 2006)	II
pcDNA3.1*ICD2*	(Sundvall et al. 2007)	II
pSLA4*PDGFRaluc–441/+118*	(Afink et al. 1995)	II
pSLA4*PDGFRaluc–944/+118*	(Afink et al. 1995)	II
pSLA4*PDGFRaluc–1253/+118*	(Afink et al. 1995)	II
pEGFP-C3	Clontech	II
pcDNA3.1*ICD2-HA*	(Sundvall et al. 2007)	II
pcDNA3.1*ICD2*	(Sundvall et al. 2007)	II
pcDNA3.1*ErbB4JM-bCYT-2-HA*	(Sundvall et al. 2007)	II
pCMV*HA-AP-2α*	(Aqeilan et al. 2004a)	II
pCMV*HA-AP-2γ*	(Aqeilan et al. 2004a)	II
pCMV*Myc-AP-2α*	(Aqeilan et al. 2004a)	II
pCMV*Myc-AP-2γ*	(Aqeilan et al. 2004a)	II
pCMV*Myc-WWOX*	(Aqeilan et al. 2004b)	II
pRL-TK *Renilla* luciferase	Stratagene	II
pGEX6p-1	GE Healthcare	II
pGEX6p-1*ICD-GST*		II
pGEX6p-1*ICD-ΔN-GST*		II
pGEX9p-1*ICD-ΔC-GST*		II
pDONR-221	Invitrogen	III
pDONR-221*hE4A2*		III
pROSA26*DEST*	(Hohenstein et al. 2008)	III
pROSA26*DEST-hE4A2*		III

4.5. Antibodies

Antigen	Name/Source	Species/Type	Application	Used in
Acetylated α-tubulin	T6793 Sigma-Aldrich	Mouse monoclonal	IF	III
Akt	sc-1618 Santa Cruz	Goat polyclonal	W	II, III
AP-2α	sc-25343 Santa Cruz	Mouse monoclonal	IF	II
Aquaporin 1	Chemicon Int.	Rabbit polyclonal	IF	III
Aquaporin 2	Sigma-Aldrich	Rabbit polyclonal	IF	III
β-actin	sc-1616 Santa Cruz	Goat polyclonal	W	I–III
c-Myc	Invitrogen	Mouse monoclonal	W, IP	II
GAPDH	Chemicon Int.	Mouse monoclonal	W	III
E-cadherin	Dr. Kai Simons	Mouse monoclonal	IF	III
ErbB4	sc-283 Santa Cruz	Rabbit polyclonal	W, IP, IHC	II, III
ErbB4	E200 Abcam	Rabbit monoclonal	W	I–III
Erk1/2	9102 Cell Signaling Tech.	Rabbit polyclonal	W	II, III
HA	3F10 Roche	Rat monoclonal	W	II
HIF-1α	H1alpha67 Abcam	Mouse monoclonal	IHC	III
NCC	Chemicon Int.	Rabbit polyclonal	IF	III
Ki-67	Invitrogen	Mouse monoclonal	IF	III
P38	Zymed	Mouse monoclonal	W	II
Pax-2	Covance	Rabbit polyclonal	IF	III
PCNA	Invitrogen	Mouse monoclonal	IHC	III
PDGFRA	3164 Cell Signaling Inc.	Rabbit polyclonal	W	II
Phospho-Akt	9271 Cell Signaling Tech.	Rabbit polyclonal	W	II, III
Phospho-histone H3	Millipore	Rabbit polyclonal	IF	III
Phospho-ErbB4	4757 Cell Singaling Tech.	Rabbit monoclonal	W	II, III
Phospho-Erk1/2	4284 Cell Signaling Tech.	Rabbit monoclonal	W	II, III
Phospho-p38	Zymed	Rabbit polyclonal	W	II
Phosphotyrosine	4G10 Upstate Biotech. Inc.	Mouse monoclonal	W, KA	II
Podocalyxin	Dr. Kai Simons	Mouse monoclonal	IF	III
Troma1	Developmental Studies Hybridoma Bank	Rat monoclonal	IF	III
ZO-1	Invitrogen	Rabbit polyclonal	IF	III

Abbreviations: IF = immunofluorescence microscopy, IP = immunoprecipitation, IHC = immunohistochemistry, KA = kinase assay, W = Western blotting.

4.6. siRNAs

Target	Target sequence	Source	Species	Used in
AP-2α	CCGGGTATTAACATCCCAGAT	Qiagen	human/mouse	II
Non-silencing control	AATTCTCCGAACGTGTCAGCT	Qiagen	-	II
Erk1/2	AAGTTCGAGTAGCTATCAAGA	Qiagen	human	II
ErbB4 JM-a	ACTGAGCTCTCTCTCTGAC	Eurogentec	human	II
ErbB4 JM-b	GTATTGAAGACTGCATCGG	Eurogentec	human	II
Mek1	TTGTGAATAAATGCTTAATAA	Qiagen	human/mouse	II
Oct-1	CAGGATCTTCAACAACTGCAA	Qiagen	human	II
Sp1	CAGCAAGTTCTGACAGGACTA	Qiagen	human	II

4.7. Cell lines

Cell line	Cell type	Species	Used in
COS-7	kidney fibroblast-like cells	monkey	II
HEK-293T	embryonic kidney cells	human	II
HC11	mammary epithelial cells	mouse	I
MCF-7	breast cancer cells	human	II
MDCK	kidney epithelial cells	dog	III
NR6	fibroblasts	mouse	II
PC12	pheochromocytoma	rat	I
SK-N-MC	neuroblastoma cells	human	II

4.8. Mouse lines

Mouse line	Used for	Used in
R26ERBB4/Pax8-Cre	ErbB4 overexpression during kidney development	III
E4 Flox/Pax8-Cre	ErbB4 kidney knock-out	III
CD-1	Wild-type control	III
ROSA26LacZ	Localization of Pax8-Cre expression in the kidney	III

5. RESULTS

5.1. Distribution of ErbB4 isoforms in normal tissues (I)

Immunohistochemical staining has demonstrated ErbB4 expression in several adult and fetal human tissues and organs including brain, heart, breast, kidney and testis (Srinivasan et al. 1998). Similarly, RT-PCR analyses have shown highest levels of ErbB4 expression in the brain, heart, kidney and salivary gland (Elenius et al. 1997a; Junttila et al. 2005). To comprehensively analyze *ERBB4* mRNA expression in normal and disease samples, the In Silico Transcriptomics (IST) database (Kilpinen et al. 2008) was used. Consistently with earlier data, the analysis demonstrated most abundant *ERBB4* expression in the nervous system, kidney, salivary gland, and the testis (Figure 6) (I, Fig. 2A). In addition, relatively high expression levels of *ERBB4* were observed in rather unexpected tissues and cell types, such as the reticulocytes, bone marrow lymphoid cells and B-lymphocytes (I, Fig. 2A).

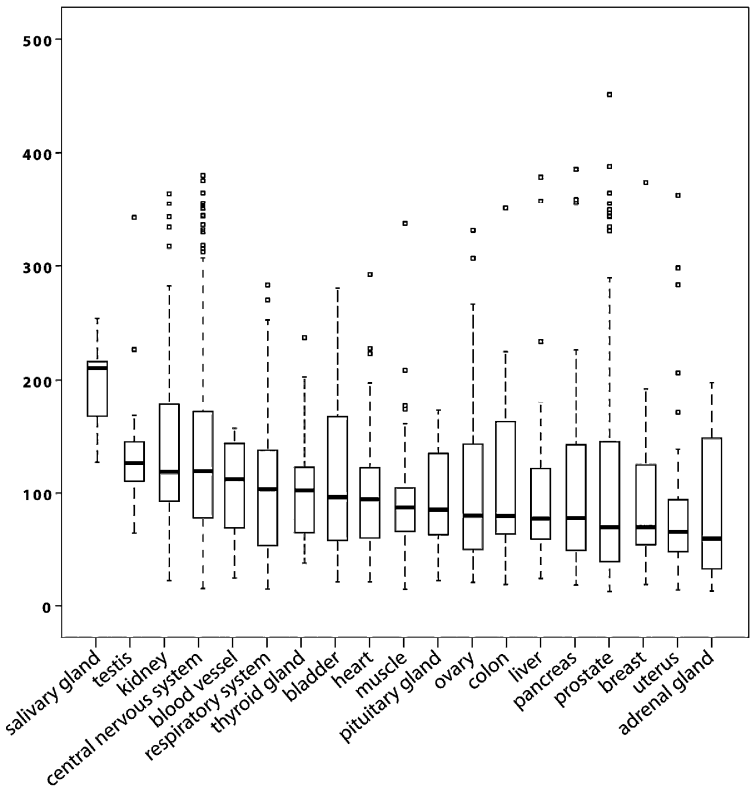

Figure 6. ErbB4 expression in various normal human tissues. The bottom whiskers indicate the lowest 25 percentage of the samples, the box outlines the middle 50 percent of the samples with the median value marked by the thick line, the top whiskers represent the highest 25 percentage, and the small squares denote the outlier samples (I, Fig. 2).

A meta-analysis of all published isoform-specific RT-PCR (Gilbertson et al. 2002; Junttila et al. 2003; Thomasson et al. 2004; Junttila et al. 2005; Law et al. 2007; Zeng et al. 2009c) (I, Fig. 3) indicated that the ErbB4 isoforms are, at least partially, spliced in a tissue-specific manner. Especially, the main JM isoforms, JM-a and JM-b, displayed exclusive splicing patterns (I, Fig. 3A). Epithelial tissues and cell lines seemed to only express the cleavable JM-a, while neural and mesenchymal tissues either expressed both isoforms, or predominantly JM-b (Elenius et al. 1997a). However, the only published JM isoform-specific *in situ* hybridization experiment suggested that different cell types of the same tissue preferentially express either JM-a or JM-b (Elenius et al. 1997a). The more rare JM isoforms, JM-c and JM-d, were only observed in fetal cerebellum (Gilbertson et al. 2001) and some cancer samples (I, Fig 3A) but not in normal adult samples. In contrast to the JM isoforms, it seems that splicing of CYT isoforms is not exclusive. Both CYT isoforms were present in all ErbB4 expressing tissues often at similar quantities (I, Fig. 3B). However, there are tissues, such as the testis, adrenal gland or lung, where one of the CYT isoforms was clearly preferred.

These observations support the hypothesis that different cell types preferentially express either cleavable or non-cleavable ErbB4 isoforms. In contrast, the overall isoform distribution in *ERBB4* expressing tissues does not suggest a clear preference for either CYT isoform.

5.2. Distribution of ErbB4 isoforms in developing kidney (III)

The expression of *Erbb4* throughout kidney development was determined from mouse embryos (E10.5 to birth) by real-time RT-PCR. Of the four ErbB4 isoforms only the two cleavable JM-a isoforms were expressed, starting at E12.5 and increasing until birth (Figure 7A) (III, Fig. 1B). The initiation and pattern of *Erbb4* expression coincided with nephrogenesis and suggested epithelial localization for ErbB4. Previous publications had reported epithelial localization for ErbB4 in later stages of developing kidney, and in adult kidney (Srinivasan et al. 1998). Expectedly, immunohistochemistry (Figure 7B), *in situ* hybridization, and tissue recombination experiments with cultured kidney mesenchyme demonstrated ErbB4 expression in the epithelial structures of the kidney (III, Fig. 1C-F). More specifically, ErbB4 was expressed in both epithelial compartments of the kidney, the mesenchyme- and ureteric-derived epithelium. These two epithelial compartments develop into tubules of the nephron and ducts of the urine collecting system, respectively. In tissue recombination experiments, E11.5 kidney mesenchymes were separated from their natural inducer tissue, ureteric bud, and nephrogenesis was activated with a heterologous inducer tissue, a piece of spinal cord (Kispert et al. 1996). Importantly these experiments showed that ErbB4 was not expressed in the kidney mesenchyme but that its expression was activated in the induced pretubular aggregates and therefore associated with epithelialization of the mesenchymal cells. Most prominent expression was observed in the developing proximal tubules of the nephron and collecting ducts. Thus, the data strongly suggest a role for ErbB4 in development of kidney epithelium.

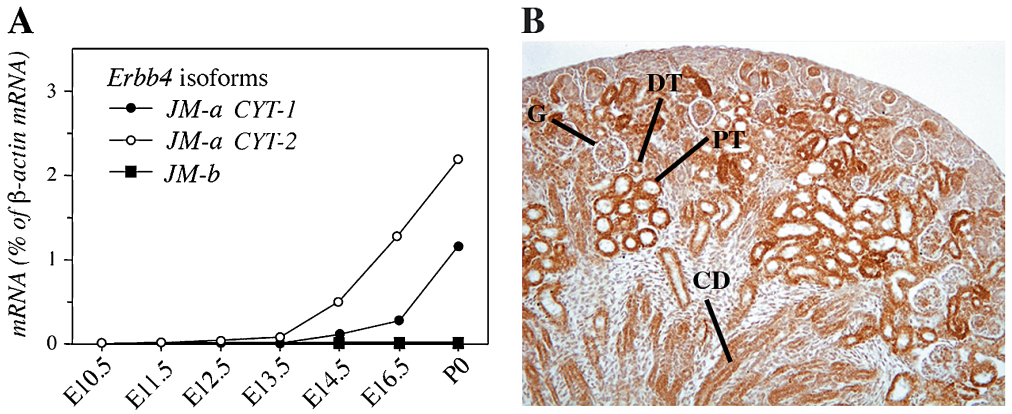

Figure 7. *Erbb4* expression during kidney development. (A) Expression of all four *Erbb4* isoforms was analyzed during mouse kidney development by real-time RT-PCR. Only isoforms JM-a CYT-1 and JM-a CYT-2 were detected. Expression levels were normalized with β-actin. (B) Immunohistochemical analysis of E17.5 mouse kidney revealed immunoreactive ErbB4 (brown staining) in developing epithelial structures. CD, collecting duct; DT, distal tubule; G, glomerulus; PT, proximal tubule.

5.3. Distribution of ErbB4 isoforms in cancer (I)

Consistently with previous findings on ErbB4 expression in different cancers (Chapter 2.3.3.2.), the *in silico* analysis also demonstrated both significant overexpression and downregulation of *ERBB4* in various cancers (I, Fig. 2B). Significant ErbB4 overexpression was observed in lymphomas (lymph node samples), esophageal adenocarcinomas and yolk sac tumors (I, Fig. 2B; data not shown). In addition, while substantial variation was observed between individuals, the median *ERBB4* expression was upregulated in the tumors of the ovary, uterus and breast. These findings were consistent with earlier reports (Junttila et al. 2005; Ejskjaer et al. 2007; Steffensen et al. 2008). However, significant downregulation of *ERBB4* in cancer was observed more frequently than overexpression. The *in silico* analysis showed significant downregulation of *ERBB4* in cancers of the salivary gland, kidney, testis, central nervous system (CNS), and lung (I, Fig. 2B). In addition, ErbB4 was downregulated in muscle sarcomas, and interestingly, in various samples of malignant blood lymphoid cells. Importantly, when subtypes of malignancies with significant *ERBB4* downregulation were analyzed, several subgroups with abnormally high expression were discovered. Within the CNS malignancies, *ERBB4* was downregulated only in astrocytoma and glioblastoma, but consistently with previous reports (Gilbertson et al. 1997; Ferretti et al. 2006), increased expression was observed in medulloblastoma (I, Fig. 2B; data not shown). Moreover, analysis of subtypes of the blood lymphoid cell neoplasias demonstrated that the aberrantly highly expressing outliers consisted mostly of samples from chronic lymphoid leukemia patients (I, Fig. 2B; data not shown). In contrast, the overall downregulation of *ERBB4* in the blood lymphoid cell malignancies was due to significant downregulation observed in a subgroup of B-cell lymphomas (I, Fig. 2B; data not shown). Similar observations were made from testicular cancers. While

all other testis tumor subgroups, such as seminomas, teratomas and germ cell tumors demonstrated significant *ERBB4* downregulation, the yolk sac tumors expressed very high levels of *ERBB4*.

The microarray data of the IST database does not discriminate between different *ERBB4* isoforms. However, a few real-time RT-PCR-based studies have investigated the expression of different *ERBB4* isoforms in cancer. A compilation of these reports showed altered JM isoform ratio in brain tumors (I, Fig. 3A). Compared to JM-a levels in normal brain tissues (40%, average of cerebellum, total brain, dorsolateral prefrontal cortex [DLPFC] and hippocampus), the JM-a proportion was clearly increased in pilotic astrocytoma (approx. 59%) and ependymoma (98%).

The examination of published real-time RT-PCR data also demonstrated that similarly with normal tissues, both CYT isoforms were present in all malignant tissues (I, Fig. 3B). Interestingly, altered CYT isoform expression ratio was seen in breast, brain and bladder cancers. The proportion of CYT-1 increased from 38% to 53% in breast cancer samples, and from 51% to 65% in combined CNS malignancies (medulloblastoma, pilocytic astrocytoma and ependymoma) (I, Fig. 3B). In contrast, a decrease in the amount of CYT-1 from 59% to 42% was observed in bladder cancer samples shown to overexpress both CYT isoforms (I, Fig. 3B) (Junttila et al. 2003).

Taken together, these data indicate different ErbB4 isoform expression patterns in various cancer types.

5.4. Distribution of ErbB4 isoforms in non-neoplastic diseases (I)

Altered ErbB4 signaling has also been implicated in non-neoplastic diseases (Chapter 2.3.3.3.). However, relatively little is known about ErbB4 expression in these diseases. To further address the role of ErbB4 in non-neoplastic diseases, the IST database *ERBB4* expression information from various cardiovascular, neurologic, and inflammatory disease samples was analyzed and compared to representative normal tissues (I, Fig. 2C). Significant overexpression of *ERBB4* was observed in lung epithelium of patients suffering from chronic obstructive lung disease, in rejected transplanted kidneys, and in ventricles of heart failure patients. In contrast, significant *ERBB4* downregulation was observed in acute pancreatitis. A clear trend for decreased *ERBB4* expression was also found in myocardia of patients with arrhythmias. Unexpectedly, no difference in *ERBB4* expression was observed in samples of the two neurological disorders included in the IST database, hippocampus samples from Alzheimer's disease patients and DLPFC samples from bipolar disorder patients, when compared to normal tissues.

5.5. Different ErbB4 isoforms are capable of inducing opposing cellular responses (I, II)

The functional properties of JM isoforms were investigated in detail using various cell lines. First, NR6 fibroblasts lacking endogenous ErbB4 expression were transfected to create stable cell lines expressing a cleavable JM-a CYT-2 or non-cleavable JM-b CYT-2.

In culture medium supplemented with 10% of fetal calf serum (FCS), the NR6 cells expressing ErbB4 JM-a CYT-2 demonstrated efficient ErbB4 autophosphorylation and enhanced proliferation (II, Figs. 1B and 2A). In addition, ErbB4 JM-a CYT-2 increased anchorage-independent growth when cells were seeded in soft agar (II, Fig. 2B). No differences, however, were observed in the morphology or growth of cells expressing ErbB4 JM-b CYT-2 compared to vector control cells when cultured in presence of serum (II, Fig. 2). To examine the effect of ErbB4 isoform expression on cells induced into quiescence, they were grown in serum-deprived conditions. Under serum starvation, vector control cell numbers gradually declined but the effect was smaller on cells expressing ErbB4 JM-a CYT-2 (II, Fig. 3B). In contrast, ErbB4 JM-b CYT-2 expression promoted starvation-induced cell death as cells rapidly died (II, Fig. 3B). Activation of ErbB4 signaling by adding the ErbB4 ligand NRG-1 during starvation further enhanced the growth resistance of cells expressing JM-a CYT-2 but did not alter the faith of cells expressing JM-b CYT-2 (II, Fig. 3B). Furthermore, it was shown that a chemical ErbB kinase inhibitor AG 1478 inhibited the growth of cells expressing JM-a CYT-2 but partially rescued the cells expressing JM-b CYT-2 from starvation-induced cell death (II, Fig. 5). The observation indicated that the opposing cellular responses mediated by the two isoforms were at least partially dependent on ErbB4 kinase activity.

Consistently with the findings from experiments on NR6 fibroblasts, the cleavable and non-cleavable ErbB4 isoforms were also demonstrated to promote distinct cellular responses when overexpressed in HC11 mouse mammary epithelial cells (I, Fig. 4A and B). Expression of JM-a CYT-2 seemed to increase growth and reduce differentiation as the cells formed more colonies and less acinar structures than the vector control cells. In contrast, the cells expressing JM-b CYT-2 did not significantly differ from vector control cells (I, Fig. 4A and C).

Different functions were also indicated for the cytoplasmic isoforms (CYT-1 and CYT-2) when examining the differentiation of rat adrenal gland pheochromocytoma cells (PC12) overexpressing JM-a CYT-1 or JM-a CYT-2 (I, Fig. 4D-F). The PC12 cells overexpressing JM-a CYT-1 demonstrated enhanced proliferation, whereas JM-a CYT-2 overexpression promoted neurite outgrowth.

These results are in line with previous findings indicating that even subtle structural differences between isoforms of a single gene can result in mediation of different cellular functions (David and Manley 2010). Furthermore, the data demonstrate that the functional diversity of ErbB4 accomplished by alternative splicing can result in regulation of dramatically different, even opposing, cellular responses.

5.6. ErbB4 isoforms control different sets of target genes (II)

While the JM-a CYT-2 and JM-b CYT-2 isoforms promoted different responses in NR6 cells, they did not have significant differences in activating ErbB4 signaling-associated pathways involved in proliferation and survival, Ras/Raf/MAPK or PI3K/Akt (II, Fig. 1D). To identify novel molecular mechanisms by which these isoforms facilitate promotion of antagonistic cellular responses, the gene expression profiles of the NR6 transfectants were determined using cDNA microarrays.

Expectedly, JM-a CYT-2 and JM-b CYT-2 isoforms were found to regulate different sets of genes (II, Suppl. Table 2). The gene encoding platelet-derived growth factor receptor alpha (*PDGFRA*) was upregulated by JM-a CYT-2 but downregulated by JM-b CYT-2. This finding was corroborated by treatment the NR6 cultures with ErbB kinase inhibitor AG 1478, which reversed the isoform-specific *PDGFRA* regulation (II, Figs. 6B). In addition, in cell culture assays the PDGFRA inhibitor AG1296 reduced the number of cells expressing JM-a CYT-2 whereas the PDGFRA ligand PDGFR-BB rescued the cells expressing JM-b CYT-2 from starvation-induced cell death (II, Fig. 6F).

Taken together, the data demonstrate regulation of PDGFRA as a key mechanism of ErbB4 isoform-specific cellular behavior in the fibroblast model.

5.7. ErbB4 isoforms utilize different mechanisms to regulate transcription (II)

ErbB4-mediated regulation of endogenous *PDGFRA* was investigated using SK-N-MC neuroblastoma cells that naturally overexpress ErbB4 JM-a (II, data not shown). Targeting SK-N-MC cells with ErbB4 siRNAs was shown to reduce the amount of *PDGFRA* mRNA (II, Fig. 7A). To define the mechanisms by which ErbB4 regulates *PDGFRA* transcription, luciferase promoter assays were performed with MCF-7 breast cancer cells that endogenously express JM-a isoform. MCF-7 cells were transfected with luciferase reporter constructs encoding various lengths of *PDGFRA* promoter fragments and treated with NRG-1 to stimulate ErbB4 signaling. Expectedly, enhancing ErbB4 JM-a signaling with NRG-1 increased *PDGFRA* promoter activity (II, Fig. 7B). The NRG-1 induction was abolished with ErbB4 JM-a specific siRNAs (II, Fig. 7C). As only the longest *PDGFRA* promoter construct demonstrated significantly increased activity upon NRG-1 induction, the putative transcription factor binding sites specific for that construct were examined in more detail. siRNAs were designed to target selected transcription factors, and their effects were examined on NRG-1-stimulated *PDGFRA* promoter activation. Downregulation of AP-2α suppressed, while targeting Sp1 enhanced the NRG-1-induced promoter activity (II, Fig. 7D). Transcription factor Sp1 has been shown to suppress *PDGFRA* promoter activity in response to fibroblast growth factor-2 (FGF2) through a Mek/Erk pathway (Bonello and Khachigian 2004). Expectedly, targeting of Erk1/2 or Mek1 also resulted in enhanced NRG-1 stimulation of *PDGFRA* promoter activity (II, Fig. 7D).

To address the functional significance of AP-2 and the Mek/Erk/Sp1 pathway in promoting cellular growth downstream of the different ErbB4 isoforms, cell viability of NR6 transfectants overexpressing JM-a CYT-2 or JM-b CYT-2 was estimated by MTT assays. Consistently with the promoter assays, siRNAs targeting AP-2α suppressed the number of viable cells expressing JM-a CYT-2 but had no significant effect on cells expressing JM-b CYT-2 (II, Fig. 8E). In contrast, siRNA-mediated (Figure 8A) or chemical inhibition (Figure 8B) of Mek1 rescued the viability of cells expressing JM-b CYT-2. Moreover, increased content of total and phosphorylated Sp1 in the JM-b CYT-2 expressing cells further suggested

Mek/Erk/Sp1 pathway involvement in JM-b CYT-2-mediated repression of *PDGFRA* promoter (Figure 9).

These findings demonstrate, that while activation of ErbB4 JM-a isoforms can both enhance and repress *PDGFRA* promoter activity, the collective effect of ErbB4 JM-a signaling results in activation of the *PDGFRA* promoter. In contrast, JM-b isoform expression results in repression of the *PDGFRA* promoter via a pathway involving a Mek1-induced activation of factors such as Erk and Sp1.

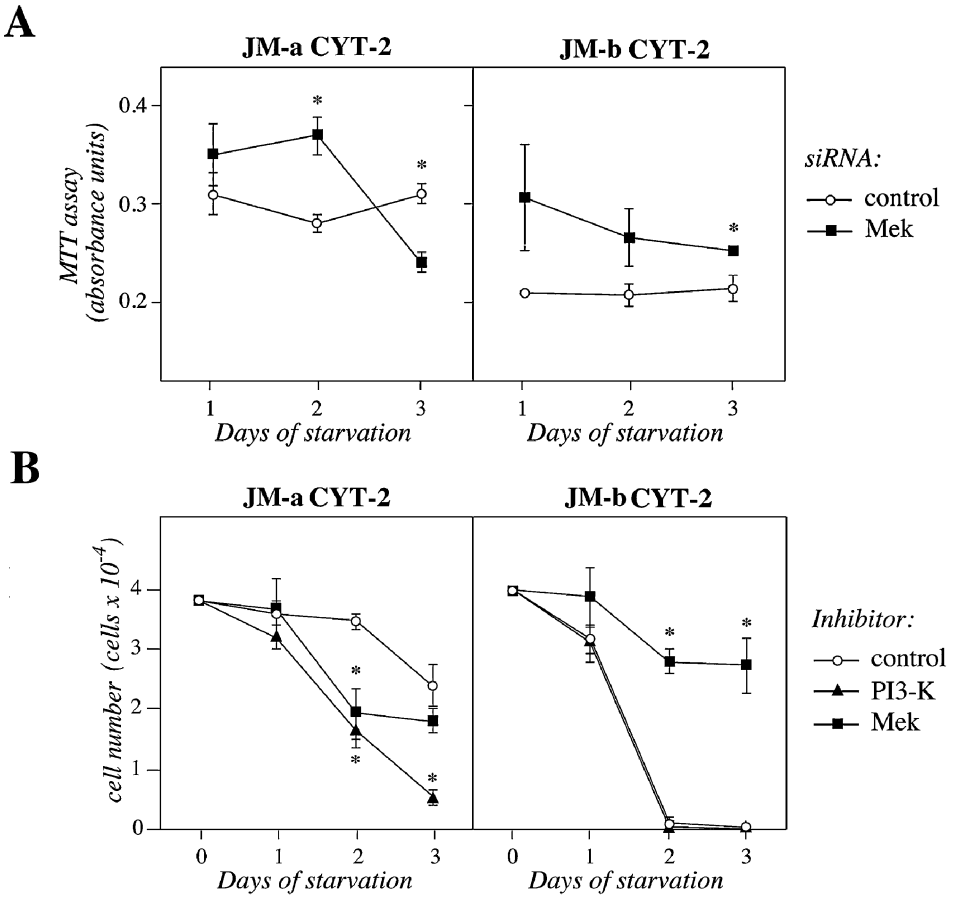

Figure 8. Effect of Mek inhibition on the growth of NR6 transfectants expressing different ErbB4 isoforms. (A) NR6 cells stably expressing JM-a CYT-2 or JM-b CYT-2 were transfected with siRNAs targeting Mek1 or with a non-silencing siRNA control. Following siRNA transfection the cells were starved for three days and the number of viable cells was determined with MTT assay. *p <0.05 for a difference between Mek and control siRNAs. (B) NR6 transfectants were plated onto 24-well plates. The next day the medium was replaced with serum-free medium containing dimethyl sulfoxide (DMSO), 20 μM of Mek1 inhibitor PD98059, or 20 μM PI3K inhibitor LY294002. Adherent cells were counted at the indicated time points with hemocytometer. *p <0.05 for a difference between Mek or PI3-K inhibitor and DMSO control.

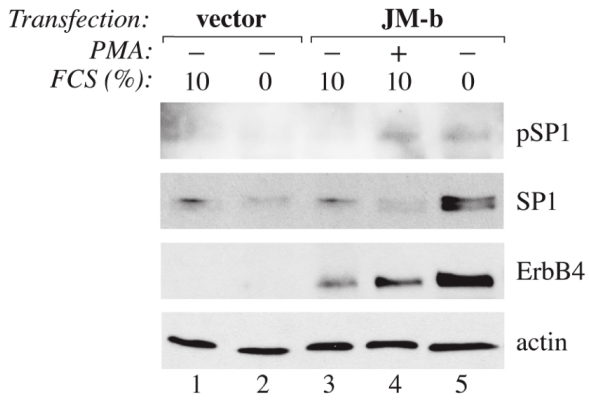

Figure 9. Analysis of Sp1 phosphorylation in cells expressing ErbB4 JM-b. NR6 transfectants cultured with or without serum for 16 h were treated with or without PMA (100 ng/ml) for 45 min and lysed. Lysates were immunoprecipitated with anti-Sp1 antibody followed by Western blotting with anti-phospho-Sp1 (Thr 453). Sp1 and ErbB4 expression were controlled by Western analysis with anti-Sp1 and anti-ErbB4 antibodies, and loading with anti-actin antibody. PMA was administered as a positive control previously reported to induce Sp1 phosphorylation (Tai and Wong 2003).

Finally, the role of RIP was examined in ErbB4 JM-a-mediated *PDGFRA* promoter activation. To address whether AP-2 interacts directly with the ErbB4 ICD, co-localization and co-precipitation experiments were performed. It was shown that AP-2 and ICD of ErbB4 co-localized in the nucleus and that the ICD but not the full-length non-cleavable ErbB4 JM-b co-precipitated with AP-2 (II, Figs. 8A and B). Consistently, in promoter assays co-expression of ErbB4 ICD and AP-2 significantly enhanced the activation of *PDGFRA* (II, Fig. 8D). Furthermore, MTT assays were used to demonstrate the importance of AP-2 in mediating ErbB4 ICD stimulated cellular responses (II, Fig. 8E). In the MTT assays with NR6 transfectants, AP-2 siRNA inhibited the growth stimulatory effect of JM-a CYT-2 expression but had no effect on the growth of cells expressing JM-b CYT-2. These data indicate that signaling via RIP-produced ICD is essential in JM-a CYT-2 isoform-mediated cellular functions and promotion of *PDGFRA* transcription. In addition, these findings suggest that the ErbB4 JM-a-induced activation of *PDGFRA* promoter is achieved through direct interaction between nuclear ICD and the transcription factor AP-2.

5.8. Functions of ErbB4 in developing kidney epithelium *in vitro* (III)

Tissue and organ culture were used to examine functions of ErbB4 in the developing tubular epithelium *in vitro*. To achieve a more progressed nephrogenesis that can be achieved with spinal cord induction, E11.5 kidney mesenchymes were induced with 5'-bromoindirubin-3'-oxime (BIO), a glycogen synthase kinase-3 (GSK-3) inhibitor. By blocking GSK-3 activity, BIO sufficiently promotes Wnt signaling to irreversibly program differentiation of pretubular aggregates into nephron structures (Figure 10) (Stark et al. 1994; Kuure et al. 2007). Treatment of the BIO-induced mesenchymes with the ErbB4 ligand NRG-1 promoted changes in the organization

of the epithelialized tubules. NRG-1 treatment enhanced comma- and S-shaped body formation and increased tubular lumen width in the differentiating renal vesicles (Figure 10B and F). The ErbB kinase inhibitor AG 1478 did not significantly affect the epithelial organization (Figure 10C and G) but neutralized the NRG-1-specific effects (Figure 10D and H). Therefore, these findings suggest that ErbB4 signaling can regulate organization and lumen formation of nephrogenic structures *in vitro*.

To further address the functions of ErbB4 *in vitro*, embryonic kidney rudiments were cultured and treated with NRG-1 or AG 1478, or both. NRG-1 induced significant thickening of the ureters (Figure 11A and B) and inhibited ureter branching (Figure 11A and C). Treatment with the inhibitor AG 1478 alone slightly decreased the thickness of the ureters, and when combined to NRG-1, significantly reversed the NRG-1 effect (Figure 11A and B). However, the ErbB kinase inhibitor AG 1478 did not rescue the branching defect (Figure 11A and C). This suggests involvement of kinase-independent mechanisms such as the RIP-mediated signaling in the regulation of NRG-1-promoted decrease in branching. These findings are consistent with the expression pattern of ErbB4 *in vivo* as well as with the mesenchyme tissue culture experiments, and suggest a role for ErbB4 signaling in the development of both the ureter- and mesenchyme-derived epithelial compartments of the kidney.

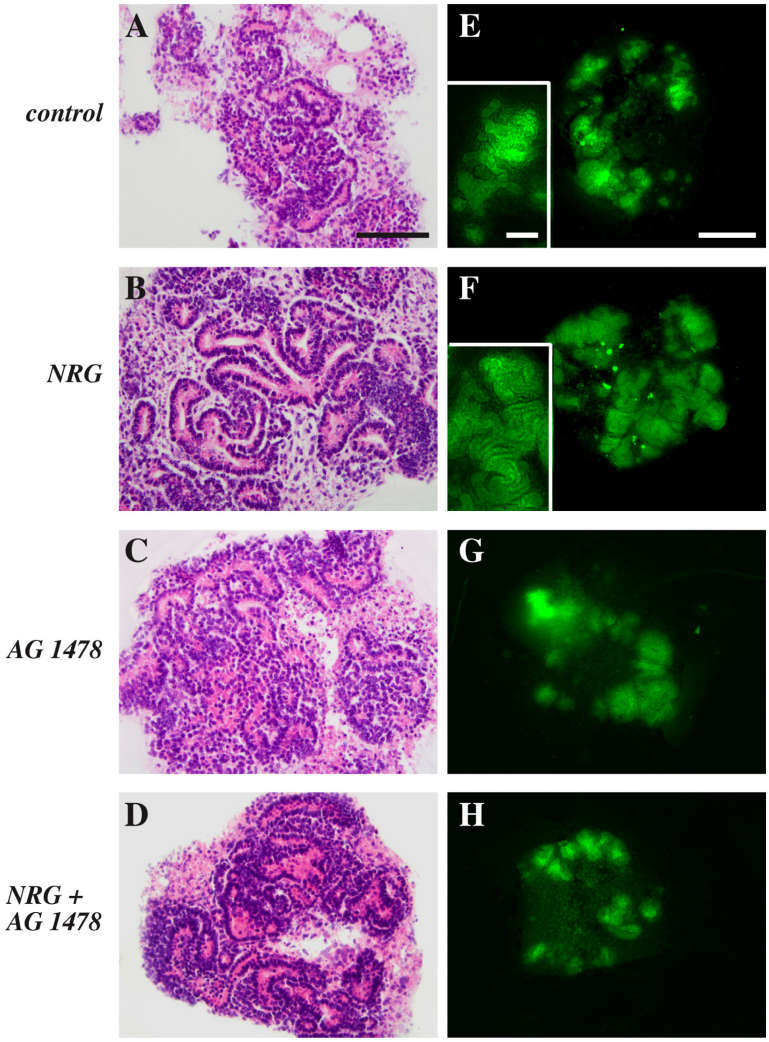

Figure 10. ErbB4 activation enhances nephrogenesis and tubular lumen formation in chemically induced kidney mesenchymes. (A-H) Separated kidney mesenchymes were induced for 20 hours with 1 μM of BIO, a GSK-3 inhibitor. After BIO induction, medium was replaced and the mesenchyme cultured for 76 hours in the presence or absence of the ErbB4 ligand NRG-1 (100 ng/ml), the ErbB kinase inhibitor AG 1478 (10 μM), or both. NRG-1 stimulation enhanced nephron precursor development and increased lumen size as observed in paraffin sections stained by hematoxylin and eosin (A-D), and in whole-mount mesenchymes stained with anti-Pax2 to visualize induced mesenchyme (E-H). AG 1478 did not significantly affect nephrogenesis alone but reversed the NRG-1-induced effects. Scale bars: (A) 100 μm; (E) 500 μm, insert 100 μm.

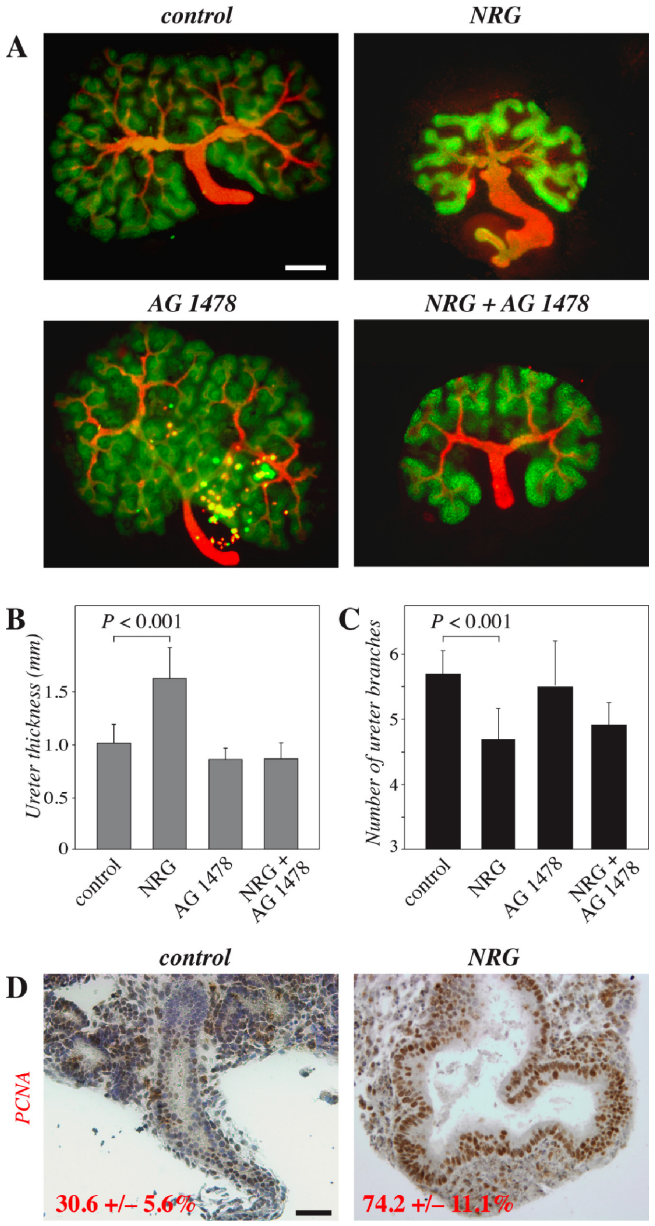

Figure 11. ErbB4 activity regulates ureter epithelium organization by controlling epithelial cell proliferation in kidney cultures. (A) Mouse kidneys (E11.5) were cultured for four days on nucleopore filters in the presence or absence of the ErbB4 ligand NRG-1 (200 ng/ml) or the ErbB inhibitor AG 1478 (20 μM). Whole-mount tissues were immunostained for Pax2 (green) and Troma1 (red) to visualize epithelium induced from the mesenchyme and the ureteric epithelium, respectively. (B and C) Columns represent quantitated data of ureter thickness (B) and degree of branching (C). Treatment with NRG-1 significantly thickened ureters and inhibited ureter branching. AG 1478 normalized the ureter thickness but did not restore the defective branching. (D) Cultured kidneys were sectioned and their proliferation status determined by PCNA immunostaining (brown color indicates proliferating cells). NRG-1 significantly increased proliferation of ureteric epithelial cells (numbers denote percentage of proliferating cells). Scale bars: (A) 250 μm; (D) 50 μm.

5.9. ErbB4 regulates proliferation of kidney epithelial cells (III)

The NRG-1-induced changes in the thickness of the ureter were shown to be due to increased proliferation (Figure 11D). This suggested functions for ErbB4 in regulating cell division during development of kidney epithelium. To address this, *in vitro* and *in vivo* gain-of-function studies were conducted. MDCK kidney epithelial cells were transfected with *ERBB4 JM-a CYT-2* or empty vector. Impact of ErbB4 overexpression on epithelial organization and morphogenesis was examined by culturing the cells in 3D matrix. In 3D the MDCK cells form hollow spherical cysts that represent primitive tubular structures. ErbB4 overexpressing cells formed cysts that contained significantly more proliferating cells than the control cell cysts (III, Fig. 2B and C).

Knock-in mice (*R26ERBB4/Pax8-Cre*) were generated to overexpress human *ERBB4 JM-a CYT-2* in the developing kidney epithelium. Histological examination of mutant mouse kidneys revealed several phenotypes including irregularly structured collecting ducts. The mutant collecting ducts included significantly more proliferating cells when compared to the collecting ducts of the wild-type littermates (III, Fig 3 G-I). In addition, the epithelial cells of the mutant collecting ducts had defects in the orientation of cell division (III, Fig. 5 G-I). They demonstrated increased radial axis of mitosis, while the control mice mostly demonstrated longitudinal cell division.

Taken together, these data strongly indicate that during kidney organogenesis ErbB4 signaling controls epithelial cell proliferation.

5.10. ErbB4 regulates tubular cell polarization during kidney development (III)

A more detailed analysis of the MDCK cysts with cell polarization markers indicated that ErbB4 overexpression promoted disrupted polarization (III, Fig. 2D and E). Control cysts were usually composed of a single lumen while ErbB4 overexpressing cells frequently formed cysts with multiple lumens. Consistently, collecting ducts of *ERBB4* overexpressing mutant mice were demonstrated to have polarization defects. For example, aquaporin-2, a water-transporting molecule, was expressed apically in collecting ducts of wild-type control mice but was dispersed throughout the cytosol in the mutant collecting ducts (III, Fig. 5A and B).

In addition to collecting duct defects, the mutant mice overexpressing *ERBB4* had abnormal proximal tubules, which were reduced in number and contained less lumenal space (III, Fig. 4). Similarly with polarization defects in the collecting ducts, proximal tubule marker aquaporin-1 immunohistochemical staining demonstrated a cytosolic staining pattern in epithelial cells of the mutant proximal tubules as opposed to the apical surface localization observed in the wild-type control mice (III, Fig. 6A and B).

The role of ErbB4 in the developing kidney was also examined using mice conditionally lacking *Erbb4* from kidney epithelium. Consistently with data obtained from *ERBB4* overexpressing mice, the knock-out mice demonstrated defective polarization in epithelial cells of the collecting ducts (III, Fig. 8, A-D) and the proximal tubules (III, Fig. 8, E-H).

Interestingly, approximately 50% of the *ERBB4* knock-in mice manifested tubular kidney cysts of various sizes (III, Fig 4G). Enhanced ErbB4 expression in the epithelial

lining of the cysts was demonstrated with immunohistochemistry, but in some cysts the expression seemed to be gradually lost as the diameter of the cysts increased. These findings are consistent with other defects observed in the knock-in mice as cystogenesis is thought to involve defects in both epithelial cell proliferation and polarization (Wilson and Goilav 2007).

Taken together, these data suggest that in addition to regulating epithelial cell proliferation, ErbB4 signaling controls polarization of collecting duct and proximal tubule epithelium. Moreover, these findings indicate that deregulated ErbB4 signaling may have a role in renal cystogenesis.

6. DISCUSSION

6.1. Diversity of ErbB4 isoform signaling: clinical implications

Whereas EGFR and ErbB2 receptors are well-known oncogenes and clinically relevant cancer drug targets (Hynes and MacDonald 2009), the applicability of ErbB4 as a cancer drug target has remained unclear due to contradictory findings about its biological functions (Sundvall et al. 2008a; Hollmén and Elenius 2010). ErbB4 has been shown to have both tumor-promoting and -suppressing functions. Several findings have associated ErbB4 with promoting proliferation and tumor growth (Tang et al. 1999; Hollmen et al. 2009; Prickett et al. 2009), while many studies have suggested ErbB4 to induce differentiation (Jones et al. 1999; Sartor et al. 2001) or apoptosis (Ni et al. 2001; Arasada and Carpenter 2005; Naresh et al. 2006). Some of the contradictory findings may be explained by functional differences of the ErbB4 isoforms (Junttila et al. 2003; Määttä et al. 2006; Muraoka-Cook et al. 2009), changes in their expression patterns in some cancer types (II, Fig. 3), or the use of reagents that do not discriminate between the different isoforms.

The ambiguous role of ErbB4 in carcinogenesis was demonstrated also in this study as both overexpression and downregulation of *ERBB4* was observed in various cancer types with the *in silico* analysis (I, II). Moreover, this study demonstrated the diversity ErbB4 isoform-specific functional capabilities. Two of the ErbB4 isoforms, a cleavable JM-a CYT-2 and a non-cleavable JM-b CYT-2 were shown to promote opposing cellular responses in NR6 fibroblasts (II). Upon serum deprivation, JM-a CYT-2 expression induced cell survival in contrast to JM-b CYT-2, which promoted apoptosis. Moreover, expression of JM-a CYT-2 but not JM-b CYT-2 increased growth and reduced differentiation in HC11 mouse mammary epithelial cells (I). Consistently, JM-a CYT-2 has been previously shown to enhance survival and proliferation in myeloid and breast cancer cells (Junttila et al. 2005; Määttä et al. 2006). In addition, the functions of JM-a CYT-2 in fibroblasts are consistent with observations made from an examination of previously published isoform-specific expression information in normal tissue and tumors (I). Exclusive, or increased expression of cleavable JM isoforms in many cancers and cancer cell lines suggest that their presence would provide a growth-advantage for tumors compared to expression of non-cleavable isoforms.

The two-step proteolytic cleavage of JM-a and JM-d isoforms results in a release of a soluble ICD capable of nuclear translocation and transcriptional regulation (Gilbertson et al. 2001; Ni et al. 2001; Komuro et al. 2003). The release of the soluble ICD is the obvious candidate mechanism through which the cleavable isoforms could obtain their unique functional characteristics, *e.g.* the suggested ability to promote cancer cell growth. Accordingly, the production of ICD in the NR6 cells expressing JM-a CYT-2 was implicated as the key mechanism in mediating JM-a specific cellular responses, such as activating the *PDGFRA* promoter.

Recent findings of somatic ERBB4 mutations in 19% of metastatic melanoma and 5% of NSCLC patients (Soung et al. 2006; Ding et al. 2008; Prickett et al. 2009) have further raised interest in ErbB4 as a cancer drug target. However, the functional differences of the isoforms suggest that the targeting approaches should be based on isoform-specific reagents, such as monoclonal antibodies. This hypothesis is supported by results from isoform-specific inhibition of ErbB4 signaling. The only study to date examining an isoform-specific antibody showed that an antibody specifically recognizing the JM-a isoforms suppressed breast cancer cell growth (Hollmen et al. 2009). Consistently, data from this study demonstrated that treating the NR6 fibroblasts with a chemical ErbB tyrosine kinase inhibitor suppressed the growth of JM-a CYT-2 overexpressing cells, but promoted the growth of JM-b CYT-2 overexpressing cells (II). In addition to cancer, isoform-specific targeting may be beneficial in some non-neoplastic diseases such as schizophrenia where overexpression of certain isoforms has been associated with pathogenesis (Silberberg et al. 2006; Law et al. 2007).

To summarize, for a complete understanding of the role of ErbB4 in disease, it is important that the composition of expressed isoforms in analyzed cells and tissues is determined. This study provided important information on isoform-specific roles and expression patterns but also demonstrated the complexity of ErbB4 signaling suggesting that more isoform-specific gain- and loss-of-function studies should be conducted. This means that more isoform-specific tools, such as the JM-a CYT-2 overexpressing mice presented in this study, the mice overexpressing soluble CYT-isoforms mammary epithelium-specifically (Muraoka-Cook et al. 2009), or isoform specific conditional knock-out mice, need to be constructed. Finally, as pharmacological inhibition of different ErbB4 isoforms can lead to different or even antagonistic responses, it is essential that isoform-specific approaches, such as monoclonal antibodies, should be used when examining ErbB4 as a drug target candidate.

6.2. Mechanisms of transcriptional regulation by ErbB4

Soon after ErbB4 was discovered to also signal via RIP (Ni et al. 2001; Lee et al. 2002), the nuclear ICD was shown to function as a transcriptional co-factor (Komuro et al. 2003). ErbB4 ICD has been shown to interact with several transcription factors and participate in transcriptional regulation both as a co-activator and a co-repressor. This study aimed to determine which target genes are regulated via the full-length and RIP-mediated ErbB4 signaling in NR6 transfectants, and to elucidate the underlying mechanisms (II).

To determine the target genes of ErbB4 signaling, expression patterns of NRG-1-induced genes in NR6 cells expressing JM-a CYT-2 or JM-b CYT-2 isoforms were compared using cDNA microarrays. The two transfectants were found to mainly regulate unique sets of genes. However, the expression of some genes, such as *PDGFRA*, was affected by both isoforms. Interestingly, *PDGFRA* was regulated in opposing directions; upregulated by JM-a CYT-2 but downregulated by JM-b CYT-2. Furthermore, the observed isoform-specific regulation of *PDGFRA* was shown to be

relevant in mediating the promotion of survival or apoptosis via JM-a CYT-2 or JM-b CYT-2, respectively. Whereas NRG-1 has been shown to inhibit PDGFRA ligand PDGF-BB-stimulated cellular functions in vascular smooth muscle (Clement et al. 2007), direct ErbB4-mediated regulation of PDGF receptors has not been previously described. In a quest to determine the mechanisms by which ErbB4 isoforms specifically regulate transcription, the regulation of *PDGFRA* promoter was investigated in more detail.

First, targeting of transcription factors known to interact with *PDGFRA* promoter (Afink et al. 1995; Kawagishi et al. 1995) identified AP-2 and Sp1 as factors positively and negatively regulating *PDGFRA* transcription, respectively. ErbB4 was shown to regulate *PDGFRA* through both of these transcription factors. AP-2 was shown to associate with the soluble ICD but not the full-length ErbB4. Moreover, the ICD was shown to co-localize with AP-2 in the nucleus. Most importantly, co-expression of AP-2 and ErbB4 promoted *PDGFRA* transcription. In contrast, the full-length non-cleavable ErbB4 JM-b CYT-2 promoted accumulation of phosphorylated Sp1, an effect previously shown to repress *PDGFRA* transcription downstream of activated Mek and Erk (Bonello and Khachigian 2004). The role of the Mek/Erk/Sp1 pathway in full-length ErbB4-mediated negative regulation of PDGFRA promoter and the consequent suppression of survival was further investigated using siRNAs targeting the pathway and a chemical inhibitor of Mek1. Both siRNA targeting and chemical inhibition of Mek1 rescued cells expressing JM-b CYT-2 from starvation-induced apoptosis. Consistently, siRNAs targeting any one of the components of the Mek/Erk/Sp1 pathway increased NRG-1-induced *PDGFRA* promoter activity.

In conclusion, this study presents one mechanistic model for ErbB4 isoform-specific transcriptional regulation. This model of *PDGFRA* regulation emphasizes the role of the ICD by demonstrating how the JM-a CYT-2 isoform upregulates transcription through direct interaction between the ICD and AP-2 in the nucleus. The JM-b CYT-2 is only capable of inducing a repressive effect on survival and *PDGFRA* transcription via mechanisms involving the indirect Mek/Erk/Sp1 pathway. However, this study examined only the differences between the cleavable JM-a and non-cleavable JM-b isoforms, both coupled to a cytoplasmic CYT-2-specific sequence. The functional differences in nuclear translocation and stability between the CYT-1 and CYT-2 ICDs (Määttä et al. 2006; Sundvall et al. 2008b; Muraoka-Cook et al. 2009; Zeng et al. 2009b) suggest that the cytoplasmic isoforms may provide an additional level of ErbB4 isoform-specific transcriptional regulation.

6.3. ErbB4 controls epithelial cell proliferation and polarization during kidney development

ErbB4 JM-a CYT-2 gain-of-function increased epithelial cell proliferation during kidney development, both *in vitro* and *in vivo* (III). These findings were expected based on previous reports where endogenous ErbB4 JM-a expression (Hollmen et al. 2009) and overexpression of JM-a CYT-2 (Määttä et al. 2006) were shown to promote proliferation. In addition, the NR6 fibroblast experiments in this study demonstrated that overexpression of ErbB4 JM-a CYT-2 promoted proliferation (II).

Unexpectedly however, gain- and loss-of-function models also suggested that ErbB4 might regulate cell polarity in the developing kidney epithelium. Overexpression of ErbB4 JM-a CYT-2 in epithelial kidney MDCK cells was observed to disrupt the polarization of the hollow spherical structures the cells form in 3D culture. Consistently, examination of ErbB4 functions in mouse kidneys with Pax8-mediated ErbB4 overexpression and deletion revealed polarization defects. Both mouse models demonstrated mispolarized epithelial cells in the collecting ducts and the proximal tubules of the nephrons. Most dramatic defect was observed in the apico-basal polarization of aquaporin-2 in the collecting ducts of *ERBB4* overexpressing mice. It has been shown that aquaporin-2 can be regulated by the hydration state of the body (Takata et al. 2008). Although difference in the hydration state of newborn mice can be considered unlikely, the polarization of the collecting ducts was examined with additional markers. Indeed, the defective collecting duct polarization of mutant mice was corroborated with immunohistochemical analysis demonstrating apical surface localization of *Dolichos biflorus* agglutinin (DBA) instead of both apical and basolateral staining pattern observed in the wild-type mice, and partial disruption of tight junction polarization (ZO-1). Similar polarization defects were observed in the proximal tubules of the overexpressing mutant mice with immunohistochemical analysis of aquaporin-1 and *Lotus tetragonolobus* lectin (LTL).

Examination of the *Erbb4*^{Flox}*/Pax8-Cre* knock-out mice with the same panel of polarization markers demonstrated that lack of *Erbb4* expression also resulted in polarization defects of collecting ducts and proximal tubules. As mentioned, the *Erbb4* knock-out mice die due to defective cardiac development (Gassmann et al. 1995). Therefore, to exclude the possibility of cardiac Pax8 promoter leakage, resulting heart defects, and their impact kidney function, the *Erbb4*^{Flox}*/Pax8-Cre* mice were examined as adults. No signs of cardiac insufficiency, such as edema in their spleen or lung tissues were observed in the sacrificed mutant mice (III, data not shown). Moreover, inefficient kidney perfusion was excluded experimentally with anti-HIF-1α immunohistochemistry, which did not demonstrate increased hypoxia in the mutant kidneys. Finally, to rule out any disturbance on aquaporin-2 cellular localization due to altered hydration state or water balance disorders such as nephrogenic *diapetes insipidus* (Noda et al. 2010), body weight, plasma creatinine and electrolyte concentrations were determined (III, data not shown). No significant differences were found indicating that the hydration status of the mice was similar and that the mutant mice did not suffer from defective water retention.

These findings indicate that during kidney development, ErbB4 may, in addition to proliferation, have important roles in regulating epithelial cell polarization.

6.4. ErbB receptors in polycystic kidney disease

Renal cystogenesis is a multistep process. It is thought to involve hyperproliferation of epithelial cells accompanied with defective polarization, and disturbed fluid transport in the epithelium due to changes in ion and water channels (Wilson and Goilav 2007). These changes are also associated with increase in the diameter of the duct lumen.

Renal cysts were occasionally observed in newborn mice overexpressing ErbB4 (III). Consistently with cystogenesis, increased epithelial proliferation, mispolarization and altered lumen size were observed in the collecting ducts and proximal tubules of the ErbB4 gain- and loss-of-function mouse models (III). Intriguingly, the orientation of epithelial cell division was also disturbed in collecting ducts of the mice overexpressing ErbB4. The mutant collecting duct cells divided more along the radial axis of the duct whereas in wild-type mice longitudinal cell division was predominant. Orientation of cell division has been suggested to regulate the appropriate growth of ductal epithelium in the kidney (Fischer et al. 2006). In normal ducts epithelial cells that divide along the longitudinal axis increase the length of the duct, whereas the radial division increases the duct diameter (Yu et al. 2009). Consistently, defects in the orientation of cell division have been implicated in renal cystogenesis (Fischer et al. 2006; Saburi et al. 2008; Veland et al. 2009).

Abnormal ErbB expression or localization has been previously demonstrated in renal cystogenesis. In fact, abnormal apical localization of EGFR in cyst epithelium was one of the first cellular defects described in PKDs (Du and Wilson 1995). Since then overactivity and altered polarization of EGFR and ErbB2 signaling has been observed in human PKDs (Du and Wilson 1995; Nakanishi et al. 2001) and rodent PKD models (Wilson et al. 2006; Sweeney et al. 2008). In addition, altered ErbB4 expression has been associated with kidney cysts in a patient with one disrupted *ERBB4* allele, and in a mouse ARPKD model (Nemo et al. 2005; Backx et al. 2009).

These observations suggest that targeting of ErbB receptors and their downstream signaling pathways could have therapeutic relevance in PKDs. However, whereas ErbB2 inhibition has been reported to be beneficial in a mouse model of PKD, and in rescuing migratory defects of human ADPKD epithelial cell lines (Wilson et al. 2006), their true potential as PKD treatments remains unknown. In this study the mice overexpressing ErbB4 in the kidney (Pax8-cre recombination) were only examined during development due to the characteristics of the ROSA26 promoter. Nevertheless, the cysts observed at birth together with the various PKD-associated defects imply that in addition to EGFR and ErbB2, also ErbB4 may have roles in cystogenesis.

7. CONCLUSIONS

This study examined the distribution and function of *ERBB4* gene products both *in vitro* and *in vivo*. The following conclusions can be made from the presented results:

1) Normal tissues preferentially express either the JM-a or JM-b isoforms but produce similar levels of CYT-1 and CYT-2 isoforms.

2) The *ERBB4* expression patterns and splicing are altered in many cancer types and in some non-neoplastic diseases.

3) Different ErbB4 isoforms control different sets of target genes through unique mechanisms of transcriptional regulation. These functional differences can result in promotion of different, even opposing, cellular responses.

4) Differential regulation of *PDGFRA* by ErbB4 isoforms is indicated as one mechanism of achieving promotion of different cellular functions. While activation of ErbB4 JM-a isoforms results in activation of the *PDGFRA* promoter, JM-b expression results in repression of its transcription.

5) ErbB4 expression is initiated early on in the epithelium of developing kidney, and is expressed most abundantly in the collecting ducts and the proximal and distal tubules of the nephron.

6) ErbB4 signaling regulates epithelial cell proliferation, polarization, and orientation of cell division during kidney development.

7) Deregulated ErbB4 signaling is implicated in renal cystogenesis. The data suggests that, in addition to inhibiting EGFR and ErbB2, targeting of ErbB4 could be considered as a therapeutic strategy for treating polycystic kidney diseases.

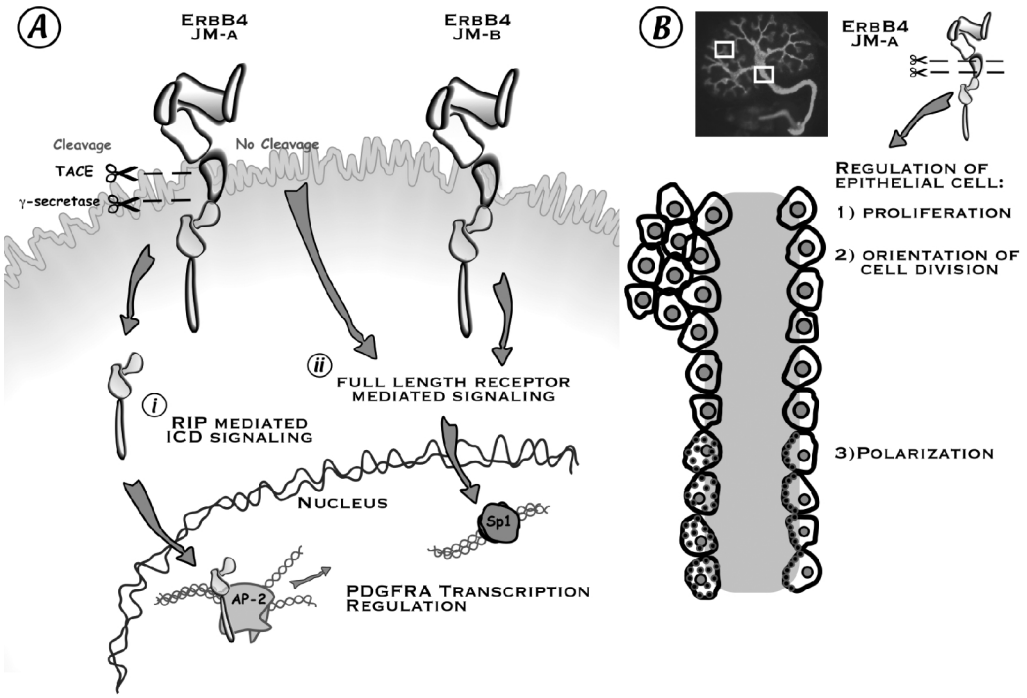

Figure 12. Key findings of the thesis work. (A) The cleavable JM-a isoforms and non-cleavable JM-b isoforms were shown to regulate different target genes and to promote even opposing cellular responses in a fibroblast model. Regulation of *PDGFRA* transcription was demonstrated to have a central role in the isoform-specific regulation of cellular functions. ErbB4 JM-a isoform is capable of signaling in two ways, via RIP (i) or as a full-length receptor (ii). The overall effect of ErbB4 JM-a signaling resulted in promotion of *PDGFRA* transcription through RIP and ICD-AP-2 interaction in the nucleus. In contrast, expression of JM-b isoforms was demonstrated to result in repression of *PDFGRA* transcription via transcription factor Sp1. (B) The developing kidney was shown to express only the cleavable JM-a isoforms, which during tubulogenesis were shown to they regulate cell proliferation, orientation of cell division and polarization of epithelial cells.

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