LOW-DENSITY LIPOPROTEIN RECEPTORS IN

SIGNALING MODULATION AND DEVELOPMENT

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DEDICATION

I would like to dedicate this thesis to my parents.

I am indefinitely grateful for your support.

LOW-DENSITY LIPOPROTEIN RECEPTORS IN SIGNALING MODULATION AND DEVELOPMENT

by

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DISSERTATION

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The Low-Density Lipoprotein Receptor gene family is a group of ancient membrane receptors. Originally implied in cargo transport and development of atherosclerosis, the number of members and the diversity of functions have been greatly expanded. LRP1, LRP1b and LRP4 are gene family members that are implicated in the regulation of signaling pathways at the intracellular, extracellular and transcriptional level. These regulations confer viability, control the cellular proliferation at several molecular steps, and allow for proper organ formation by moderating and integrating cellular signaling pathways.

The use of knockin mutant mice has, for the first time, implicated the extracellular domains of LRP1b and LRP4 in signaling modulation in development. While the complete knockout of either receptor is embryonically lethal, the expression of a truncated receptor, spanning only the extracellular domain, confers viability and only a mitigated phenotype.

For LRP4, the difference is most visible in the kidney. The present LRP4 extracellular preserves thresholds critical for organogenesis, yet, the complete absence displays a subpenetrant phenotype of kidney agenesis. In this thesis work, results demonstrate the ability of the LRP4 extracellular domain to not only bind a broad variety of soluble ligands in the extracellular space, but further to influence the Wnt, and possibly others, signaling pathways that are required for kidney development.

In an osteoblast-specific model of LRP1 knock-out, the relationship between the LRP1 and the PDGF receptor has been further investigated. LRP1 is known to negatively regulate the PDGF receptor. However, the exact mechanism(s) are not fully understood. In the wild-type, PDGF receptor beta binds directly to LRP1 upon ligand stimulation. LRP1 knockout leads to significant upregulation of the PDGF receptor beta at the protein level. The stimulation of the receptor with PDGF-BB, its corresponding ligand, leads to overactivation of the signaling pathway with both increased turnover and phosphorylation/activation of the receptor, demonstrated by cellular proliferation and p21 downregulation. *In vivo*, the LRP1 knockout leads to a bone-derived hyperproliferation with formation of tumors at the epiphysis. The in vitro experiments are supporting evidence, combined with previously published literature, to imply the LRP1/PDGF receptor pathway.

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PRIOR PUBLICATIONS

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LIST OF ABBREVIATIONS

ACSF – Artificial Cerebrospinal Fluid

AD – Alzheimer's Disease

APOE – Apolipoprotein E

APOER2 – Apolipoprotein E Receptor 2

APP – Amyloid Precursor Protein

Aβ – Amyloid Beta Peptide

BAC – Bacterial Artificial Chromosome

BAEC – Bovine Aortic Endothelial Cells

BLRP – Biotin Ligase Recognition Peptide

BMP-4 – Bone morphogenetic protein 4

BSA – Bovine Serum Albumin

CNS – Central Nervous System

CREB – cAMP-response element binding protein

CSF – Cerebrospinal Fluid

DAB1 – Disabled

DAPT – γ-secretase inhibitor

DBP – Vitamin-D Binding Protein

EGFP – Enhanced Green Fluorescent Protein

ER – Endoplasmic Reticulum

ES cell – Embryonic Stem Cell

FACS – Fluorescent Activated Cell Sorting

FCS – Fetal Calf Serum

FH – Familial Hypercholesterolemia

GABA – γ-aminobutyric acid

GP330 – Megalin

GSK3β – Glycogen Synthase Kinase 3β

ICD – Intracellular Domain

I/O – Input/Output

IPTG - Isopropyl Thiogalactoside

JIP – JNK Interacting Protein

JNK – c-Jun N-terminal Kinase

KI - Knockin

KO – Knockout

LB – Luria Broth

LDLR – Low-density Lipoprotein Receptor

LIF – Leukemia Inhibitory Factor

LRP1 – Low-density Lipoprotein Receptor-Related Protein 1

LRP1b – Low-density Lipoprotein Receptor-Related Protein 1b

LRP2 – Megalin

LRP4 – MEGF7

MEGF7 – Multiple epidermal growth factor containing protein 7

MLS – Multiple Cloning Site

MMP – Matrix Metalloproteinase

NLS – Nuclear Localization Signal

NMJ – Neuromuscular Junction

PDGF – Platelet-Derived Growth Factor

PI3K – Phosphatidylinositol-3-kinase

PKB – Protein Kinase B

PKC – Protein Kinase C

RAP – Receptor Associated Protein

SFK – Src Family Kinase

SHH – Sonic hedgehog

UTR – Untranslated Region

VLDLR – Very-low-density Lipoprotein Receptor

WT – Wild-type

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

The LDL Receptor Family

The LDL Receptor gene family is an ancient family of transmembrane receptors (Figure 1.1). The initial discovered LDL receptor implied the transport of cholesterol and apolipoproteins as its main function (Brown and Goldstein, 1976). However, with the discovery of additional family members and the creation of the respective knock-out models, the versatility of involvement in physiological processes, in development and disease became apparent (May et al., 2007).

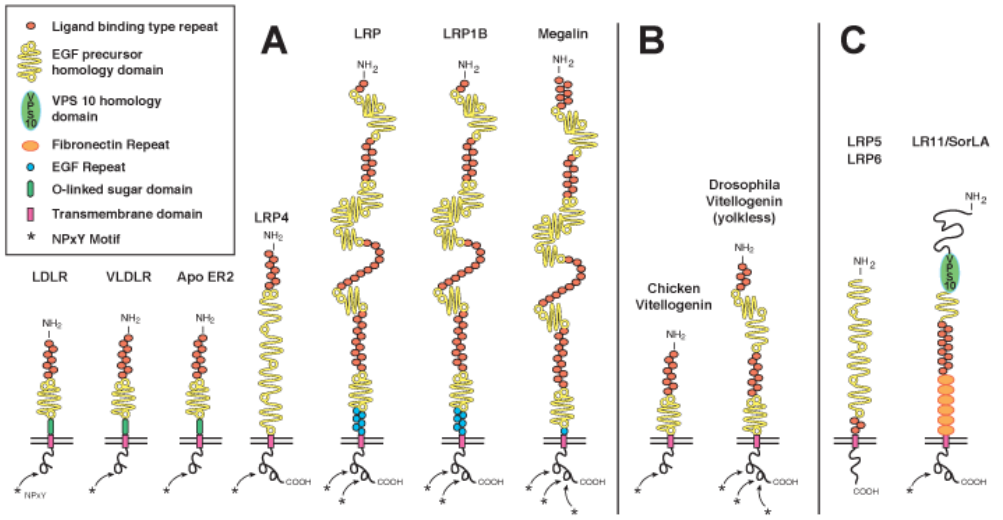


Figure 1.1: The LDL Receptor Family. (A) The seven core members of the LDL receptor family with their common structural organization. (B) Chicken Vitellogenin, Drosophila Vitellogenin. (C) The Wnt receptors LRP5, LRP6 and LR11/SorLA are more distantly related proteins, share some similarity in domain structure with the main members of the family. (Dieckmann et al., 2010)

Today, the individual members have been implicated in the development of atherosclerosis, Alzheimer's disease, appropriate formation of the nervous and musculoskeletal system, kidney formation, and synaptic plasticity and function (Herz, 2009; May and Herz, 2003; May et al., 2005).

The complexity of their features and functions requires a closer look the individual receptors. To remain within the scope of this dissertation, only the seven core members of the LDL receptor family are discussed. Those included the low-density lipoprotein receptor (Ldlr), the very-low-density lipoprotein receptor (Vldlr), the apolipoprotein E receptor 2 (Apoer2), LDL receptor-related protein 4 (originally multiple epidermal growth factor containing protein 7 (Megf7)), LDL receptor-related protein 1 (Lrp1), LDL receptor-related protein 1b (Lrp1b), and LDL receptor-related protein 2 (also known as megalin or gp330) (Figure 1.1, Panel A). All core members of the family share common structural motifs (Herz and Strickland, 2001). Those structural similarities are the identifying criterion for association to the LDL receptor gene family. On the extracellular side of the receptors are multiple ligand binding repeats. Those ligand binding domains are followed by an EGF precursor homology domain. A structural speciality specific to Ldlr, Vldlr, and Apoer2 are the O-linked sugar domains just prior to the transmembrane spanning region that have implications for protein stability, processing, and signaling function (May et al., 2003). Several serine and

threonine residues within this O-linked sugar domain contain serve as attachment sites for O-linked sugars.

The transmembrane domain is highly homogenous throughout the seven core members. In contrast, the intracellular parts of the individual receptors are highly divergent with less similarity, except for the presence of tetra-amino acid motif that the LDL receptor family members do share, the so called NPxY motif (Guttman et al., 2009). x in this case stands for any amino acid. Those intracellular tail motifs of the receptors are found up to three times within the family members. This sequence has its functions in protein interaction/signal transduction and endocytosis (Knisely et al., 2007; Martin et al., 2008). In some of the family members, endocytosis has been shown to be the primary function of the motif. This is true for the LDL receptor, where the motif is primarily responsible for endocytosis of the lipid bound receptor (Goldstein and Brown, 2009). Therefore, this motif mediates the critical cholesterol homeostasis. In other family members, particularly for LRP1, the motif has its primary functions in binding protein interaction and transmembrane signal transduction (Boucher et al., 2002; Gordts et al., 2009). Another example is the Apoer2 receptor, despite its misleading nomenclature. The endocytosis rate is very low; however the motif serves as a signal transducer across the plasma membrane (Herrick and Cooper, 2004). Its intracellular adaptor protein Disabled (Dab1) activates intracellular signaling cascades and interacts with Apoer2 through the NPxY domain.

The prominent involvement of Ldlr in lipid transport and its pivotal implications in cardiovascular disease has led to an assumed role in cargo transport for all subsequently described family members. However, the functions go much beyond this and include extracellular matrix protein clearance, cellular signaling transduction, endocytosis of a broad spectrum of ligands, including vitamins and minerals, synaptic function, brain lamination, and organ development, cellular signal transduction, endocytosis of various ligands, vitamins and other nutrients, synaptic function, and brain lamination (Blacklow, 2007; Stolt and Bock, 2006). The complexity of the functional repertoire and its phenotypical presentation, it seems appropriate to discuss the family members individually.

The LDL receptor (Ldlr) is the founding member of the gene family. The receptor is a 195kDa protein and, through its unique importance, ubiquitously expressed throughout the body, most notably in hepatocytes and the CNS (Brown and Goldstein, 1992; Hobbs et al., 1992). Ldlr plays a pivotal role for the regulation of cholesterol homeostasis throughout the vasculature, the liver, and the brain. The ligand binding domain of the receptor binds LDL particles rich in cholesterol and absorbs them from the bloodstream, subsequently clusters and participates in coated pit mediated endocytosis of the receptor and attached

lipoprotein particle (Helfand et al., 2009). Deregulation of the cholesterol homeostasis through LDL receptors leads to premature cardiovascular disturbances, including atherosclerosis, myocardial infarction and stroke (O'Donnell and Nabel, 2008). The rare but insightful condition of familial hypercholesterolemia (FH), where Ldlr function is impaired, leads to increased LDL plasma concentrations and causes early, fatal cardiovascular events (Oosterveer et al., 2009).

The Lrp4 receptor, originally termed as Megf7, is one of the newer members of the gene family (Tomita et al., 1998). First described in cardiomyocytes, it is an intermediately sized receptor with a molecular weight of about 250 kDa. Lrp4 has been found to play an important role in organogenesis (Johnson et al., 2006). Although its knock-out in mice does not lead to embryonic lethality, death occurs immediately post-partum due to the complete absence of neuromuscular junctions (Weatherbee et al., 2006). In cattle, mice, and humans the intracellular domain of Lrp4 has to be functional for proper development of the musculoskeletal system (Duchesne et al., 2006). Deletion of this receptor aspect leads to polysyndactyly (Duchesne et al., 2006), decreased bone mass and length (Choi et al., 2009), as well as an increased susceptibility to bone density reduction (Rivadeneira et al., 2009). The bone phenotype has been attributed to the ability of Lrp4 to moderate Wnt signaling *in vitro* and *in vivo*. Other organs

that require Lrp4 for appropriate organogenesis are the kidneys, where it leads to a subpenetrant phenotype of agenesis in mice, and molar teeth (Ohazama et al.), where inappropriate fusions are observed. A mutation in Lrp4 with the introduction of a premature stop codon before the intracellular domain is the cause of the bovine mulefoot disease which exhibits the fusion of hooves in cows. Recently, Lrp4 was also implicated in the orchestration of the interactions of Agrin, APP, and MuSK (Kim et al., 2008; Zhang et al., 2008). Lrp4 is now thought to act as co-receptor for the MuSK receptor complex and binding partner for Agrin.

The second described member of the family is Lrp1; a giant, ubiquitous and versatile receptor (Herz et al., 1988). It is one of the largest and most functionally complex receptors in the family. The 600 kDa receptor consists of two components: the α-chain, a 515 kDa extracellular fragment, and an 85 kDa β-chain that are non-covalently linked and cleaved by the furin protease (Willnow et al., 1996b). Lrp1 can specifically bind over 40 different ligands including the apolipoprotein E, protease/inhibitor complexes, α2-macroglobulin, PDGF-BB, and amyloid precursor protein (Guttman et al.). Through its ubiquitous expression in many tissues, including the liver, brain, and vasculature, Lrp1 carries many important functions both as an endocytic receptor and a signaling receptor. Like Ldlr, Lrp1 is involved in maintaining cholesterol homeostasis by

acting as a receptor for chylomicron remnants and lipases (Cooper, 1997). Tissue-specific knock-out in the liver has confirmed this critical contribution. Lrp1 is further known to regulate extracellular proteolytic activity by clearing matrix metalloproteases, however, MMP2 and MMP9 are activated through Lrp1 (Emonard et al., 2005). Important for the context of Alzheimer's disease is the regulation of expression of APP and the consequential production of Aβ, which implies the receptor in the disease etiology (Deane et al., 2008; Marzolo and Bu, 2009).

Lrp1b, originally termed Lrp-DIT, is a close relative to Lrp1 (Liu et al., 2000). It is also around 600 kDa in size and is approximately 59% identical to Lrp1 at the protein level (Liu et al., 2001). The structural and functional domains of Lrp1b and Lrp1 are organized in a highly similar manner. However, Lrp1b has an extra repeat in the fourth ligand binding domain, an additional 33 amino acid fragment in the cytoplasmic tail, and a slower endocytosis rate. In contrast to Lrp1, it consists of one chain only and is not cleaved by the protease furin (Li et al., 2005; Liu et al., 2007). It gets processed at the extracellular level through the metalloproteases ADAM10 and ADAM17 with subsequent cleavage through γ-secretase at the intracellular level. Its frequent deletion has suggested a role for Lrp1b as a tumor suppressor gene in several solid cancers, where it is either mutated through genetic or epigenetic silencing or deleted in about 40-45% of all non-small cell lung carcinomas and other cancers (Ding et al., 2008). In humans,

the expression is mainly restricted to the CNS and the testis and has been linked to increased APP retention at the membrane level and decreased Aβ production (Cam and Bu, 2006; Li et al., 2005).

Lrp2 (Megalin/gp330) is an equally large receptor of about 600 kDa in size(Kerjaschki and Farquhar, 1982). Like Lrp1 and Lrp1b, it is capable of binding numerous extracellular ligands, including various vitamins, nutrients, apolipoproteins, sonic hedgehog (Shh), and bone morphogenetic protein 4 (BMP-4) (Fisher and Howie, 2006). Lrp2 has a restriction expression pattern in certain epithelial cells of the kidney, lung, and intestines. It has received most attention for the endocytosis of low molecular weight proteins such as the vitamin-D binding protein (DBP) in the proximal tubules of the kidney (Nykjaer et al., 1999; Rowling et al., 2006). The Megalin knock-out leads to holoprosencephaly, a fusion defect of the frontal forebrain as a developmental defect in mice (Willnow et al., 1996a).

In this dissertation work, the regulation of cellular signal transduction has been a particular focus. Over the course of the last decade it has become abundantly clear that modulation of cellular signaling by the LDL receptor gene family is not merely an indirect phenomenon (Newton et al., 2005). In fact, most

family members are directly, i.e. physically involved in either transmitting a signal across the plasma membrane by themselves or in modulating such signals by increasing or decreasing the activity of related fundamental signaling pathways. The latter include for instance membrane receptor tyrosine kinases such as the PDGF receptor, the Wnt, TGF-β, Bone morphogenic protein, sonic hedgehog and other critical signaling pathways. In the following the main principles and mechanisms by which the different members of the family control a wide variety of diverse biological responses in the embryo and in the adult are summarized.

One mechanism by which LDL receptor gene family members can regulate signaling is regulated proteolysis. LRP1 cannot only remove active proteinases from the cell surface and extracellular space; it is also itself cleaved by cell surface metalloproteinases, resulting in the shedding of the extracellular domain (von Arnim et al., 2005). ApoER2 can be cleaved in a similar manner, which is likely to occur physiologically in response to signals that activate typical or atypical forms protein kinase C (Hoe et al., 2007). In both cases, this cleavage is regulated by the glycosylation state of the receptor (Koch et al., 2002). Shedding of the extracellular domain may affect cellular signals on the short- as well as long-range, by sequestration of regulators of cell proliferation, e.g. PDGF or TGFβ in the case of LRP1, or of the neuronal signaling protein Reelin in the

case of ApoER2 (Durakoglugil et al., 2009). Moreover, release of the extracellular domain leaves behind a short extracellular stub, the membrane spanning segment and the intracellular domain (May et al., 2002). Processing of this truncated receptor occurs constitutively through the action of γ-secretase, the same intramembraneous aspartyl protease that mediates the processing of the amyloid precursor protein involved in Alzheimer's disease (Fuentealba et al., 2007). In response to inflammatory activation, γ-secretase increased its processing of Lrp1 to facilitate a negative, anti-inflammatory feedback loop on expression of inflammatory genes (Zurhove et al., 2008). This second processing step results in the release of the intracellular domain. The tail translocates subsequently to the nucleus and modulates transcriptional activity. Lrp1b and Lrp4 are processed in a comparable two step manner. Mutational analysis revealed that expression of a truncated receptor expressing only the extracellular domain of either LRP1b or LRP4 is sufficient to preserve an apparently normal or only a mitigated phenotype with musculoskeletal involvement, respectively (Johnson et al., 2005; Marschang et al., 2004). In contrast, complete knock-out of either receptor is lethal. This suggests that the extracellular domains of LRP1b and LRP4, even in the absence of proper membrane integration, serve as signaling receptors in the extracellular space. In principle, two signaling concepts are possible. One model suggests that the extracellular domain binds and neutralizes ligands in the extracellular space.

The second model suggests that improved presentation of a Lrp4 ligand leads to an enhanced signal and preservation of a critical activation threshold.

The previously discussed signaling in the extra- and intracellular space is complemented through interaction of LDL gene family members at the membrane level. Mice lacking ApoER2 and VLDLR gave the first indication that LDL receptor family members can transmit signals directly across the plasma membrane by activation of cytoplasmic kinase cascades through binding of the extracellular ligand Reelin (Rogers and Weeber, 2008). Both receptors can bind the extracellular signaling protein Reelin. Oligomeric Reelin in the extracellular space induces clustering of the receptors, thereby facilitating transphosphorylation of Src family tyrosine kinases (SFKs) which are recruited to the cytoplasmic tail of the receptors through interactions with the adaptor protein Dab1 (Katyal et al., 2007). As mentioned earlier, this adaptor protein itself binds to 'NPxY' motifs in the receptor tails and the plasma membrane. These two interactions are necessary for Reelin signal transduction, which increases signal specificity through stringent compartmentalization. Phenotypically, VLDLR/ApoER2 double knock-out mice duplicate the Reeler mice, a model in which the signaling protein Reelin is absent (Hamburgh, 1963).

Intracellularly, activation of SFKs is the master switch that turns on a broader and diverging signaling cascade facilitating the activation of numerous, most notably cytoskeletal components such as actin and microtubules, molecular motors and ion channels, specifically the NMDA receptor (Qiu et al., 2006). The pathways that are activated by Reelin are essential in the embryo for brain development, i.e. the ordered formation of cortical layers in the neocortex and in the cerebellum, but also in the adult where they regulate synaptic transmission and synaptic plasticity (Beffert et al., 2006; Beffert et al., 2005; Beffert et al., 2004; May et al., 2004; Qiu and Weeber, 2007; Qiu et al., 2006; Sinagra et al., 2005; Weeber et al., 2002) dendrite and dendritic spine formation and neuronal survival (Frotscher et al., 2009).

Another involvement of LDL gene family members is the modulation of tyrosine kinase receptor signaling through co-receptor function. Although LDL receptor family members never harbor their own kinase domain in the short intracellular tail, they are well capable of modulating other tyrosine kinases in cellular processes. Activation by Reelin induces clustering of ApoER2 and VLDLR that can in turn directly activate tyrosine kinases through recruitment of SFKs into the complex (Qiu and Weeber, 2007; Strasser et al., 2004). LRP1 employs another mechanism to modulate the activity of a membrane tyrosine kinase receptor by binding PDGF-BB and forming a complex with the PDGF

receptor β (Takayama et al., 2005; Zhou et al., 2009). Lrp1 binds to PDGF receptor β in a ligand dependent fashion at the membrane level. This direct interaction binds the PDGF receptor and slows down its endocytosis and activation through phosphorylation. In the absence of LRP1, PDGFRβ is rapidly endocytosed in response to PDGF-BB exposure. This coincides with an increased association of PDGFRβ with the E3 ubiquitin ligase c-Cbl and a concomitant increase in ubiquitination. In the absence of LRP1, basal activity/phosphorylation of PDGFRβ is increased, possibly due to ligand-independent dimerization or autocrine ligand stimulation with decreased threshold, which is normally prevented in the presence of LRP1. This increased basal activity leads to continuous growth factor signaling with accelerated proliferation, responsible for the increased proliferation of smooth muscle cells in the media layer of the Lrp1 deficient aorta and increased migration in smooth muscle cell-specific LRP1 knockout mice. The second NPxY domain of LRP1 is phosphorylated upon ligand induced activation of PDGF receptor β and SFKs. Intriguingly, this phosphorylation event is prevented by the binding of ApoE-containing lipoproteins to LRP1, providing an attractive mechanism that could explain the powerful effect of ApoE on preventing PDGF-induced smooth muscle cell migration in vitro. It may also be relevant for explaining the atheroprotective effect of locally, i.e. macrophage produced ApoE in the vascular wall.

LDL receptor-deficient mice that are also lacking LRP1 in the smooth muscle cells of their aortas spontaneously develop striking atherosclerotic lesions and abdominal aneurysms, even when plasma cholesterol levels are low (Boucher et al., 2003). Moreover, they are extremely susceptible to cholesterol-feeding, resulting in the development of rampant atherosclerosis and death from abdominal and mesenteric vessel occlusion at cholesterol levels equal to those present in mice lacking only their LDL receptors. Blockade of PDGFR signaling with the tyrosine kinase inhibitor Gleevec prevented atherosclerosis progression upon cholesterol-feeding in the LRP1-deficient cohort, suggesting that PDGFR signals are responsible for the variations in atherogenicity at a given cholesterol level. Intriguingly, ApoE deficient mice are also more susceptible to lesion development than LDL receptor knockouts, raising the possibility that this difference could be caused by the abolished suppression of PDGF-induced LRP1 tyrosine phosphorylation by ApoE.

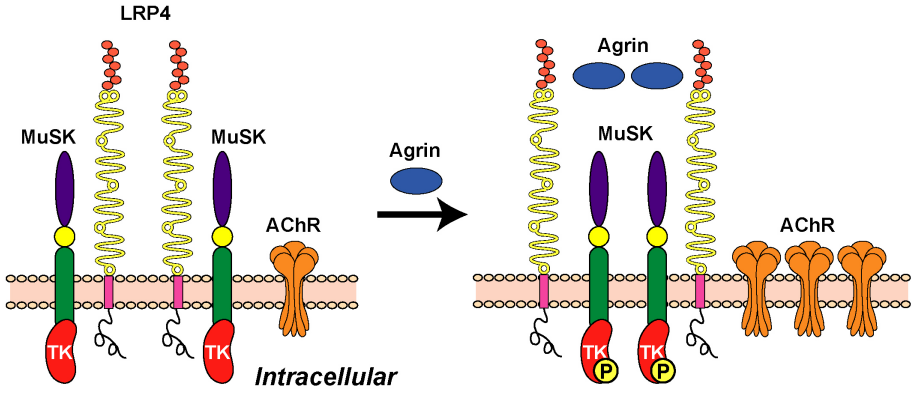


Figure 1.2: LRP4 Tyrosine Kinase Co-Receptor Function for Musk. The receptor serves as a receptor for agrin and is a co-receptor for the tyrosine kinase MUSK in the muscle. Activation of the membrane tyrosine kinase MUSK is required for the induction of acetylcholine receptor (AChR) clustering during the formation of neuromuscular junctions (endplates). MUSK forms a complex with LRP4, but does not bind agrin directly. Agrin binding to LRP4 enhances complex formation of MUSK with LRP4 and induces transphosphorylation of MUSK.

Lrp1 is capable of modulating inflammatory activity by moderating TGF β signaling (Boucher et al., 2007). Huang and colleagues identified a large TGFβ1 binding protein on the cell surface which they erroneously called TGFβ receptor V. They later identified this protein as LRP1 and showed that LRP1 also binds insulin growth factor binding protein 3 (IGFBP3) and that it was required for growth inhibition by IGFBP3 and TGFβ1 (Huang et al., 2004; Tseng et al., 2004). Signaling by TGFβ1 involves the ligand-induced heterodimerization of TGFβR-I and II. Huang and colleagues showed that LRP1 can form a complex with

TGFβR-I and proposed that the relative ratio of TGFβR-I and II is critical for partitioning of receptor-bound TGFβ between the clathrin/signaling and a caveolae/degradation pathway. This model is consistent with the presence of LRP1 in caveolae, where it can sequester TGFβR-I and prevent its association with TGFβR-II. In the absence of LRP1, TGFβ signal repression is abolished, resulting in a massive increase of p-Smad2/3 accumulation in the nucleus of LRP1 deficient smooth muscle cells.

Thrombospondin is an activator of TGFβ signaling and itself a ligand for LRP1 (Greenaway et al., 2007). IGFBP3 binding to LRP1 and TGFβR-I has also been proposed to activate a growth inhibitory cascade. Thus, LRP1 can in principle suppress the activation of TGFβ receptor-dependent signaling pathways in at least five distinct ways: direct binding of TGFβ1, possibly indirectly by removing TGFβ1 bound to α2M, endocytosis of thrombospondin, sequestration of TGFβR-1, and endocytosis of IGFBP3. The existence of multiple independent mechanisms impressively confirms the central role LRP1 has in the regulation of TGFβ function. VLDLR is also a high affinity receptor for thrombospondins that mediate growth suppression (Oganesian et al., 2008). This appears to involve a pathway that activates Akt and Erk, rather than Smads.

Conditional knockout mice lacking LRP1 only in their smooth muscle cells present with medial thickening of the aorta, which is accompanied by disruption of elastic layers and increased fibrosis. This Marfan-like syndrome is consistent with the simultaneous activation of proliferative PDGFRβ-dependent and fibrosis- and elastolysis-inducing TGFβ-dependent signaling pathways. This regulation of vascular wall maintenance, potentially also involving ApoE as a modulator *in vivo*, is an excellent example for the integration of two fundamentally distinct signaling pathways by LRP1.

Recent studies *in vitro* and *in vivo* have provided evidence that some LDL receptor family members are also involved in the regulation of at least two other morphogenetic signaling pathways, those involving Wnt and Bmp proteins. LRP1 has been reported to interact with human Frizzled-1 (Zilberberg et al., 2004), which mediates Wnt signaling together with another co-receptor, LRP5/6 (the orthologue of Drosophila *arrow*). LRP4 suppresses Wnt signaling (Fliniaux et al., 2008), probably by competing for LRP5/6 in the Wnt/Fz signaling complex. LRP4 knockout mice present with several remarkable phenotypes, including defects of kidney development, limb development, tooth development and failure to form neuromuscular junctions. Not all of these defects can be explained by deregulation of Wnt signaling alone, suggesting that LRP4 is also involved in the execution or modulation of other signaling pathways. This has now been

confirmed by the very recent discovery that LRP4 forms a complex with the muscle-specific tyrosine kinase MuSK, which is required for the formation of neuromuscular junctions and discussed in detail below.

Another member of the gene family, LRP2, participates in forebrain development by removing Bmp4 from the extracellular space. In the absence of LRP2, Bmp4 expression is increased leading to a subsequent loss of sonic hedgehog expression in the ventral forebrain. LRP2 can also bind and thereby remove Shh from the extracellular space. By thus regulating the levels of an upstream (Bmp4) as well as a downstream (Shh) morphogen in the developing brain, LRP2 participates in the integration and feed-back regulation of both signaling pathways.

Shh is palmitoylated at its amino terminus and also modified by a cholesterol-moiety at its carboxyl-terminus. Likewise, Wnts are also palmitoylated. These lipid modifications promote the association of the proteins with lipoprotein particles and this is important for regulating their short-range and long-range signals in insects. To what extent lipoprotein particles regulate Shh or Wnt signaling in higher organisms such as mammals is not yet well understood.

The functions of LRP1 as a powerful negative modulator of cellular

proliferation in smooth muscle cell proliferation in atherosclerosis and its role in the regulation of numerous growth factors with well-documented roles in cancer biology (tyrosine kinase receptors, Wnt, Shh, and TGF-β) suggest that LDL receptor family members might themselves be involved in tumor development or progression. Evidence for this was indeed found several years ago, when Lisitsyn and colleagues first demonstrated that LRP1b, the closest relative to LRP1, was frequently deleted in non-small cell lung cancer cell lines (Liu et al., 2000). In a number of descriptive follow-up studies, LRP1b has been further confirmed as a major site of mutation in head and neck cancers (Cengiz et al., 2007; Nakagawa et al., 2006), urothelial malignancies (Langbein et al., 2002), esophageal squamous cell carcinomas (Sonoda et al., 2004), gliomas (Roversi et al., 2006; Yin et al., 2009), oral cancers, cervical adenocarcinomas (Choi et al., 2007; Hirai et al., 2004), B-cell lymphomas, breast cancer (Kadota et al.) and leukemias (Taylor et al., 2007). This finding was recently confirmed in a large screen for mutated candidate tumor promoting or tumor suppressor genes in pulmonary adenocarcinomas as the fourth most common mutation only exceeded in frequency by p53, k-ras, and EGFR tyrosine kinase mutations (Ding et al., 2008). Initial evidence for a potential involvement in cancer was derived from chromosomal studies in which mutations at the LRP1b gene locus (chromsome 2q21) have been linked to decrease overall survival and reduced response to chemotherapeutic treatment (Saretzki et al., 1997). Mechanistically, LRP1b has

been implicated in the regulation of the PDGF receptor β and the urokinase receptor (Li et al., 2002; Tanaga et al., 2004). However, these two candidate targets are also negatively regulated by LRP1 which is both frequently expressed and unmutated in the aforementioned cancer types (Gaultier et al.). This functional overlap is suggestive of a distinct mechanistic involvement of LRP1b in the control of cell cycle and proliferation in the context of cancer. Another complicating factor for the involvement of LRP1b is the extremely low expression in mouse and human lung tissue (Li et al., 2005). In the mouse, expression is mainly limited to the brain and testis. In human, the distribution pattern is more ubiquitous with relatively low expression levels in the lung and other reported cancer sites with LRP1b involvement. However, previous studios have connected increased frequency of LRP1b mutations with higher grade and stage of different tumor types. Given those two factors it can be speculated that an unidentified microenvironmental factor, e.g. local inflammatory processes, triggers the upregulation of LRP1b and increases the selective pressure towards LRP1b mutant tumor cell clones. Further, the monogenetic studies of cancers with known environmental causes have posed a great challenge. In a mutant Lrp1b mouse model we created in our lab, we observed no development of pulmonary tumors (Marschang et al., 2004). This, in combination with the gene analysis data from humans, suggests that Lrp1b is rather an aggravating influence on existing tumors rather than an initiating event. Taken together, these independent findings

suggest that LRP1b probably functions in the lung in a manner analogous to that shown for LRP1 in the vascular wall, i.e. as a signal integrator of as yet undefined growth regulating signaling pathways. The molecular identification of these pathways, however, is complicated by aforementioned low expression of LRP1b in the normal lung in mice and in humans (Marschang et al., 2004)

.

While LRP1b is the only member of the LDL gene family known to harbor frequent genetic and epigenetic alterations, other members have been implicated in playing a role in cancer. Initially, LRP1 gene polymorphisms were suspected in posing an increased risk for the development of breast cancer, follow-up studies however were unable to confirm those reports (Benes et al., 2003; Jakubowska et al.). Also, the complex involvement of LRP1 logically leads to involvement in a large number of physiological functions. Inevitably, some of those will play a role in cancer. The control of angiogenic events through negative regulation of the PDGF receptor β on vascular endothelial cells and the mediation of antiangiogenic activity of thrombospondin-2 would suggest a role in a tight control of local tumor growth, however, the LRP1 mediated activation of metalloproteases 2 and 9 was reported to promote cancer cell migration and invasion (Song et al., 2009). The absence of knowledge about gene mutations or alterations of expression of the LRP1 gene indicates that these functions are part

of the physiological repertoire of the receptor rather than a specific tumor promoting or suppressing influence.

CHAPTER TWO

ECTODOMAINS OF LIPOPROTEIN RECEPTOR RELATED PROTEIN RECEPTORS LRP1B AND LRP4 IN MICE HAVE ANCHORAGE INDEPENDENT FUNCTION

Adapted from Martin F. Dietrich, Louise van der Weyden, Haydn M. Prosser, Allan Bradley, Joachim Herz, David J. Adams (2010). Ectodomains of the LDL Receptor-related Proteins LRP1b and LRP4 Have Anchorage Independent Functions in Vivo. PLoS One (Accepted for Publication).

Summary.

Background. The low-density lipoprotein (LDL) receptor gene family is a highly conserved group of membrane receptors with diverse functions in developmental processes, lipoprotein trafficking, and cell signaling. The low-density lipoprotein (LDL) receptor-related protein 1b (*LRP1B*) was reported to be deleted in several types of human malignancies, including non-small cell lung cancer. Our group has previously reported that a distal extracellular truncation of murine *Lrp1b* that is predicted to secrete the entire intact extracellular domain (ECD) is fully viable with no apparent phenotype.

Methods and Principal Findings. Here, we have used a gene targeting approach to create two mouse lines carrying internally rearranged exons of *Lrp1b* that are predicted to truncate the protein closer to the N-terminus and to prevent normal trafficking through the secretory pathway. Both mutations result in early embryonic lethality, but, as expected from the restricted expression pattern of LRP1b *in vivo*, loss of *Lrp1b* does not cause cellular lethality as homozygous *Lrp1b*-deficient blastocysts can be propagated normally in culture. This is similar

to findings for another LDL receptor family member, *Lrp4*. We provide *in vitro* evidence that Lrp4 undergoes regulated intramembraneous processing through metalloproteases and γ-secretase cleavage. We further demonstrate negative regulation of the Wnt signaling pathway by the soluble extracellular domain.

Conclusions and Significance. Our results underline a crucial role for *Lrp1b* in development. The expression in mice of truncated alleles of *Lrp1b* and *Lrp4* with deletions of the transmembrane and intracellular domains leads to release of the extracellular domain into the extracellular space, which is sufficient to confer viability. In contrast, null mutations are embryonically (*Lrp1b*) or perinatally (*Lrp4*) lethal. These findings suggest that the extracellular domains of both proteins may function as a scavenger for signaling ligands or signal modulators in the extracellular space, thereby preserving signaling thresholds that are critical for embryonic development, as well as for the clear, but poorly understood role of *LRP1b* in cancer.

Introduction.

The LDL receptor gene family is a highly conserved class of cell surface receptors (May et al., 2007) involved in various functions, including cell signaling, cargo transport, and gene regulation (Zurhove et al., 2008). *LRP1b*, initially named *LRP-DIT* (Deleted in Tumors) (Liu et al., 2000), was first described as a gene that was frequently inactivated in non-small cell lung cancer. It was subsequently also shown to be mutated in urothelial (Langbein et al., 2002), head and neck (Cengiz et al., 2007; Nakagawa et al., 2006), esophageal tumors (Sonoda et al., 2004) and in B-cell lymphomas (Rahmatpanah et al., 2006). The specific deletion of *LRP1b* in certain tumors through genetic and epigenetic silencing suggests a role as a tumor suppressor. However, the exact mechanism by which *LRP1b* functions in this manner remains elusive. *LRP1* and *LRP1b* share 86 percent mRNA and 52 percent amino acid identity. Previously reported mechanisms of action for *LRP1b*, including the regulation of the urokinase (uPAR) and platelet-derived growth factor (PDGF) receptor trafficking at the membrane level (Boucher et al., 2002; Loukinova et al., 2002), overlap with the functions of expressed and unmutated *LRP1* in tumor tissues. We have previously reported that mice that express of a truncated allele lacking both the transmembrane and intracellular domains of Lrp1b is viable (Marschang et al., 2004).

Here, we extend our earlier findings by demonstrating embryonic lethality of two lines of mice carrying null alleles of *Lrp1b.* Interestingly, similar observations were made with the *Lrp4* knockout mice (Johnson et al., 2005). While *Lrp4* knockout mice fail to develop neuromuscular junctions and succumbed to respiratory failure post-natally (Weatherbee et al., 2006), a truncated allele lacking the transmembrane and intracellular domains displays a mitigated phenotype compatible with postnatal survival (Johnson et al., 2005; Johnson et al., 2006). The common feature of the truncated *Lrp1b* and *Lrp4* alleles is that they secrete an intact and apparently physiologically functional extracellular domain (Marschang et al., 2004).

All members of the LDL receptor gene family harbor at least one structurally highly similar extracellular ligand binding domain consisting of a series of negatively charged cysteine-rich Ca^{2+}-chelating repeat modules that bind numerous ligands (Croy et al., 2003). These ligand binding domains have numerous and partially overlapping functions in cell signaling and cargo transport (Ohazama et al., 2008). The ability of the extracellular domain (ECD) to rescue embryonic or perinatal lethality suggests a functional role for the isolated ECDs.

We therefore propose a model in which the ECDs of LRP1b and LRP4 may modulate cellular signaling by scavenging and neutralizing extracellular ligands, thereby preserving signaling thresholds that are critical for proper

embryonic development. The same mechanisms could impact on the development and progression of some malignancies.

Materials and Methods.

Generation of Lrp1b-deficient mice.

Mice carrying two different *Lrp1b* alleles were generated. These lines were termed *Lrp1b*^{tm1wtsi} and *Lrp1b*^{tm2wtsi} and carry N-terminal and C-terminal duplications of exons of *Lrp1b*, respectively. Targeting vectors were obtained from the MICER collection. *Lrp1b*^{tm1wtsi} mice carry an internal duplication of exons 6-8 of *Lrp1b* while mice with the *Lrp1b*^{tm2wtsi} allele carry an internal duplication of exon 69 of *Lrp1b*. Both alleles are predicted to cause frameshift mutations.

10 µg of the linearized targeting vectors were electroporated into AB2.2 embryonic stem (ES) cells (from mouse strain 129S5/SvEvBrd). The ES cells were cultured on a lethally irradiated SNL76/7 feeder layer and picked into 96-well plates after 7 days of drug selection in G418 (180 µg/ml). To check for homologous recombination, genomic DNA was analyzed by Southern blotting. For the *Lrp1b*^{tm1wtsi} allele, a 359 bp 3' external probe was used (generated by PCR from AB2.2 genomic DNA, using the primers: forward, 5'- AAA AAA TCT TCC TTG AAG GCT CTT GTA AG GTC -3'and reverse, 5'- ATG CAT ATG GAA TGC CAG GGG GAT GTT CAC AC -3') and hybridized with *EcoR*V-digested DNA to identify restriction fragments of 18.2 kb for the wild-type and a 11.3 kb for the targeted allele. For the *Lrp1b*^{tm2wtsi} allele, a 500 bp external probe was used

(generated by PCR from AB2.2 genomic DNA, using the primers: forward, 5'-GAA AGT GAT CAA ATG AAC ATA TTC AAA TCC TTC-3' and reverse, 5'-CTT GAT CAC AGC TTT CTC TCA ATG GAC TTT AC-3') on *Bam*HI-digested DNA to identify a 10 kb wild-type and a 20 kb targeted allele.

Correctly targeted ES cell clones were injected into C57BL/6J blastocysts and germline transmission of the targeted (mutant) allele was demonstrated by Southern blot analysis of tail DNA. Mice were maintained on a mixed 129/C57 background and husbandry was in compliance with Home Office regulations (United Kingdom). All animal work was conducted according to the relevant national and international guidelines and in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." All animal experiments were reviewed and approved by the Institutional Committees on Animal Use and Care at UT Southwestern Medical Center.

Isolation and in vitro culture of mouse blastocysts.

6-8 week old male and female heterozygous *Lrp1b* mice were intercrossed (with each mouse carrying a different *Lrp1b* allele, such that homozygote embryos could be detected as those carrying both the $Lrp1b^{tm1wtsi}$ and $Lrp1b^{tm2wtsi}$ alleles). The females were inspected twice daily for signs of a plug (which was taken as embryonic day 0.5), and three days later the females were sacrificed and their uteruses collected and flushed to harvest the blastocysts (embryonic day

3.5). The blastocysts were then cultured in Knockout Dulbecco's modified Eagle medium (Invitrogen Ltd, Paisley, UK) supplemented with 10% fetal calf serum, 1 mM L-glutamine, 50 units penicillin/100 µg streptomycin per mL, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and overlaid with mineral oil in a humidified incubator containing 5% CO_2 at 37ºC for up to 1 week. After culture, each embryo was placed in 20 µL lysis buffer consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/mL gelatin, 0.45% Tween-20, 0.45% NP-40 and 1 mg/mL proteinase K. The lysis was carried out at 55ºC for 5 hr, followed by 95ºC for 15 min. The lysate was then used to perform three separate PCR reactions: to detect the *Lrp1b*^{tm1wtsi} allele (forward: 5'-AAA CCG CCT CTC CCC GCG CGT TGG C-3' and reverse: 5'-GAT GAA TAC ACT GGG TGT GAA ACA CAG CTA AA-3'), the *Lrp1b*^{tm2wtsi} allele (forward: 5'-TGT TTT CAG ACT AGA TAG GCA TTG GGT CTA TA-3' and reverse: 5'-GCG CCC AAT ACG CAA ACC GCC TCT CCC CG-3') and an unrelated allele for quality control of the lysate (forward: 5'-GAA GAT GGC TTA GTC GGC CAT CAT TGG GAA GA-3' and reverse: 5'-GAT GAA TAC ACT GGG TGT GAA ACA CAG CTA CC-3'). The PCR was performed in 50 µL reactions using 45 µL of Platinum PCR Supermix (Invitrogen) and 100 ng of each primer pair with the following PCR cycle profile: 1 cycle at 94°C for 2 min followed by 30 cycles at 94°C for 30 sec, 55°C for 1 min (or 65°C for the *Lrp1b*^{tm2wtsi} allele primer pair), and 72°C for 30 sec with a final cycle of 72°C for 10 min. The

resulting PCR products were visualized on an ethidium bromide-stained 2% agarose gel.

Lrp4 in vitro assays and Western Blotting.

Subconfluent HEK293T cells were grown in 10% FCS / DMEM High Glucose (Cellgro Mediatech Inc.). On day 1, pcDNA3.1 constructs expressing either the extracellular domain or the full length of murine Lrp4 were transfected into the cells using FuGene6 (Roche Laboratories) according to the manufacturer's protocol. Briefly, 6 µg DNA and 2 µL FuGene6 reagent were suspended (1:3 ratio of FuGene6:DNA) in a volume of 600 µL of serum and incubated for 30 min at room temperature before addition to the cell culture dish. The cells were incubated overnight in 10% serum and then switched to serum free DMEM High Glucose /0.2% bovine serum albumin for two days. Cells were collected, washed three times in ice-cold PBS and lysed in 1% Triton-X lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, with an EDTA-free protease inhibitor cocktail (Complete Mini EDTA-free Protease Inhibitor Cocktail, Roche Laboratories).

The supernatants were centrifuged at 4,000 rpm for 15 min to remove detached or dead cells. Media was then concentrated using 100 kDa size exclusion spin concentrators from Millipore (Amicon Ultra Centrifuge Device 100.000 MWCO). 50 µg cellular extract and 50 µL concentrated media

(concentration ~ 200:1) were run on 4-15% gradient gels and subsequently analyzed by Western blot for expression of both the intracellular and extracellular domains of Lrp1b and Lrp4. All antibodies were used at a 1:1,000 dilution in 5% milk/PBS-Tween, and were generated as described below. The bands were visualized by chemiluminescence (Thermo Scientific Pierce ECL Western Blotting) according to the manufacturer's instructions.

Accumulation of ICD was verified with γ-secretase inhibitor DAPT (10 μM, Sigma Aldrich). Treatment was initiated 24 hrs after transfection of the Lrp4 full length construct (murine, pcDNA3.1 vector) and treated for 16 hrs overnight. Cells were then lysed in 1% Triton-X buffer as described previously and 20 μg per lane subjected to Western blotting.

Antibody generation.

The intracellular domain antibodies for Lrp1b (4594 rabbit polyclonal) and Lrp4 (3600 rabbit polyclonal) have been described previously. The extracellular domain antibodies for Lrp1b (575C rabbit polyclonal) and Lrp4 (584c rabbit polyclonal) were generated by transfecting the murine full ligand binding domain (LBD 2 in case of Lrp1b) into maltose binding protein (MBP) bacterial expression systems. Briefly, DH5α *E. coli* cells were transformed and induced with IPTG (0.5 μM) overnight for 16 hrs. Cells were then spun down and exposed to osmolaric shock environment. Protein was then column-purified and injected

subcutaneously into 3 months old rabbits. The immunization was repeated every four weeks until serum positivity was reached.

TOP-Flash Assay.

HEK-293 cells were plated at 400 000 cells/well in 6-well plates and grown to 50–80% confluency in 10% FBS/DMEM. Cells were transfected with the TOP-Flash reporter system and the indicated expression plasmids for Wnt1, Dkk1, Lrp4 ECD, Lrp5 and Lrp6 in pcDNA3.1 backbones. To account for the different amounts of transfected plasmids, an empty pcDNA 3.1 vector construct was co-transfected. Transfections were performed with the FuGene6 using the manufacturer's protocol. Cells were lysed 48 hrs after transfection and lysates were assayed for firefly and renilla luciferase activities using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. All transfections and measurements were performed in triplicate.

Results.

Absence of Lrp1b in Mice Results in Early Embryonic Lethality.

We generated two different *Lrp1b* null alleles – the first targeting the N-terminus with duplication of exons 6-8 (*Lrp1b*^{tm1wtsi} mice), and the second targeting the C-terminus with duplication of exon 69 (*Lrp1b*^{tm2wtsi} mice); both resulting in premature termination through the generation of frameshift mutations. ES cells carrying these alleles were used to generate chimaeras, which transmitted the targeted alleles to their progeny. Heterozygous mice (*Lrp1b*$^{tm1wtsi/+}$ and *Lrp1b*$^{tm2wtsi/+}$) were healthy at birth and both males and females were fertile. However, no homozygous mice of either allele were observed at weaning.. Using the *Lrp1b*^{tm2wtsi} allele, a total of 146 mice were genotyped at weaning (4 weeks old). No homozygous *Lrp1b*^{tm2wtsi} mice were detected, suggesting that homozygous *Lrp1b* mice were not viable. We then isolated embryos at E8.5 and E10.5 for genotyping by Southern hybridization but did not find any homozygous *Lrp1b* embryos at these timepoints indicating that *Lrp1b* disruption caused early embryonic lethality.

	LRP1b				
Age	+/+	+/-	-/-	TOTAL	Fisher's exact test
4 weeks	45	101	0	146	p<0.0001
E10.5	5	22	0	27	p<0.01
E8.5	4	15	0	19	p<0.05
E3.5	11	11	3	25	NS

Table 2.1: Analysis of Genotypes in LRP1b Crosses.

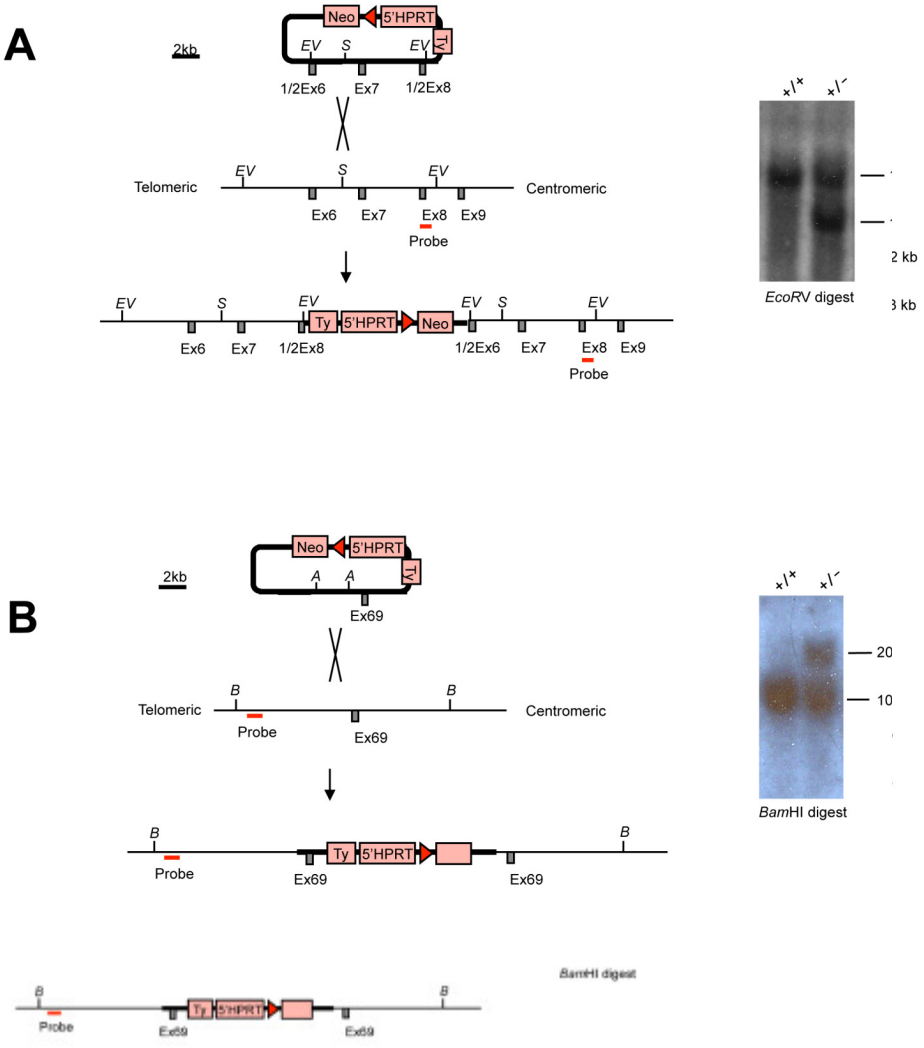


Figure 2.1: Generation of *Lrp1b* null alleles. (a) Duplication of N-terminal exons 6-8 to generate the *Lrp1b*^{tm1wtsi} allele and Southern blot hybridization after *Eco*RV digestion of embryonic stem cell genomic DNA to verify targeting of the allele. **(b)** Duplication of C-terminal exon 69 to generate the *Lrp1b*^{tm2wtsi} allele and Southern blot hybridization after *BamH*I digestion of embryonic stem cell genomic DNA to verify targeting of the allele. *A*, *Afl*III; *B*, *BamH*I; *EV*, *Eco*RV; *S*, *Swa*I.

Lrp1b-deficient blastocysts are viable.

Pre-implantation embryos do not provide sufficient material for Southern analysis and PCR genotyping is not able to distinguish homozygous embryos for each of the mutant alleles individually from heterozygous embryos. Therefore in order to narrow the timepoint when embryos in which *Lrp1b* had been disrupted we intercrossed *Lrp1b*^{tm1wtsi} and *Lrp1b*^{tm2wtsi} mice, flushed blastocysts at E3.5 and cultured these to form blastocyst outgrowths. In total, 25 blastocyst outgrowths were analyzed by PCR for the presence of both mutant alleles which would indicate homozygous null embryos.

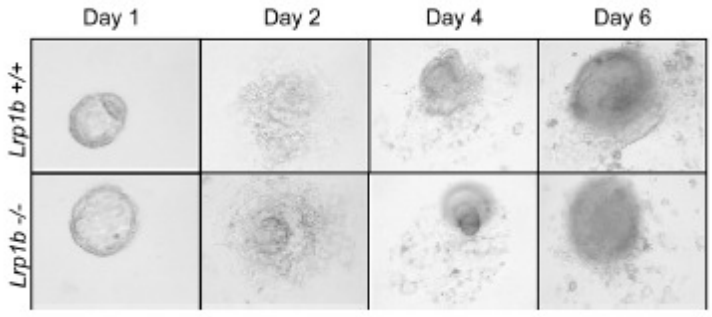


Figure 2.2: Blastocyst Outgrowth Assay. Time course of *Lrp1b* wildtype (*Lrp1b*$^{+/+}$) compared to *Lrp1b* knockout (*Lrp1b*$^{tm1wtsi/tm2wtsi}$) trophoblast explant growth, showing expansion of inner cell mass and trophoblast formation. Images were taken on days 1, 2, 4, and 6.

Three of these were genotyped as homozygotes ($p>0.1$). As shown in Figure 2 these blastocysts showed normal morphology with a time-and size-appropriate expansion of the inner cell mass and outgrowth of trophoblast structures. This result suggests that loss of *Lrp1b* does not result in a cell lethal phenotype.

Extracellular Domains are expressed in truncated models for Lrp1b and Lrp4.

The expression of the extracellular domains (ECDs) in the previously reported knockout models of *Lrp1b* and *Lrp4* was predicted but never confirmed. To confirm the expression of Lrp1b and Lrp4 ECDs, we utilized whole brain lysates and antibodies against the extracellular and intracellular ligand binding domains. For Lrp1b, only a slight size difference was noted between the wild-type and the Lrp1b truncation model (Figure 3a). However, an intracellular domain was only detectable in the wild-type. For Lrp4, the size difference confirmed the expression of the predicted 180 kDa Lrp4-extracellular domain protein in the absence of an intracellular domain (Figure 3b). The signals for extra- and intracellular domain were present at the same size in the wild-type. We therefore confirmed our prediction that the extracellular domains remain expressed in both models.

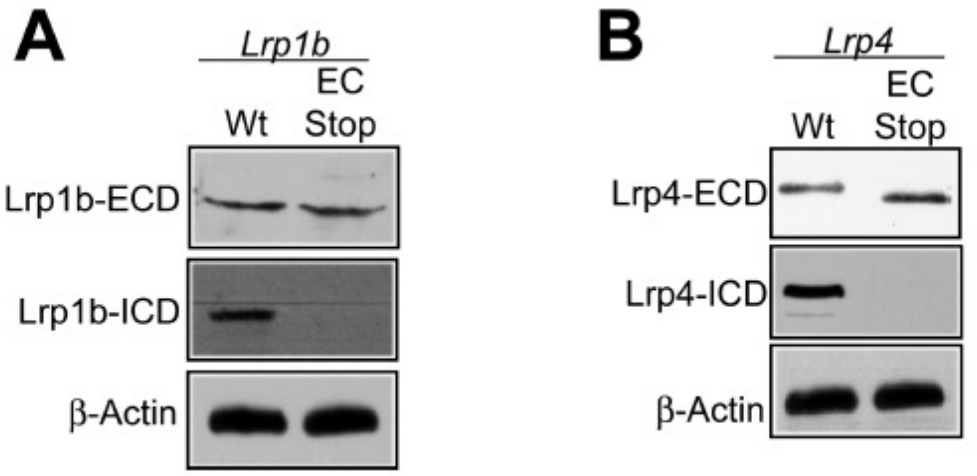


Figure 2.3: Expression of Lrp1b and Lrp4 Extracellular Domains. Whole brain lysates (50 µg) from **(a)** Lrp1b and **(b)** Lrp4 'truncation models' were analyzed for the preserved expression of the truncated receptor extracellular domain (ECD). For our *Lrp1b* truncation model ("Lrp1b EC Stop"), the ECD is expressed at approximately the same size like the full-length receptor ("Wt"). However, the intracellular domain (ICD) is only present in the wild-type. For our *Lrp4* truncation model ("Lrp4 EC Stop"), there is a significant shift in size for the ECD signals compared to the full-length receptor. Comparable to the Lrp1b findings, no signal is detected for the ICD in the truncation model. ß-Actin is used as a loading control.

Lrp4 undergoes regulated intramembranous processing in vitro.

It has previously been reported that the ECD of Lrp1b is shed into the extracellular space in an *in vitro* model and the ICD is released by γ-secretase activity(Liu et al., 2007). To investigate whether Lrp4 is similarly processed and the extracellular domain shed into the extracellular space, the supernatants of Lrp4-transfected cells were analyzed for potential shedding by Western blot using an antibody against the ECD of Lrp4 (Figure 2.3A). The Lrp4 extracellular domain construct was used as a positive control and cell lysates were used to verify transfection efficiency. As expected, the truncated *Lrp4* receptor expressing only the extracellular domain was secreted into the extracellular space (Figure 2.3A, lane 2). No shed ECD was detected in the supernatant from cells that had been transfected with the full length Lrp4 construct only (lane 3). The Adam10 metalloproteinase was co-transfected with Lrp4 to facilitate cleavage of the extracellular domain. Under these transfection conditions Lrp4-ECD was released from the cell and became detectable in the culture supernatant as a protein of approximately 160 kDa (lane 5).

Further, transfection of Lrp4 reveals bands of ~20 kDa, 75 kDa and 250 kDa (Figure 2.3B, lanes 2 and 4); while the 250 kDa band represents full length Lrp4, the two smaller bands appear to be processing products of the receptor. No bands were detected in the untransfected conditions (Figure 2.3B, lanes 1 and 3). In analogy to other members of the LDL receptor gene family, the processing of

Lrp4 includes extracellular domain cleavage by metalloproteases and a release of the ICD by γ-secretase activity. Inhibition of γ-secretase by DAPT leads to accumulation of the ~20 kDa band.

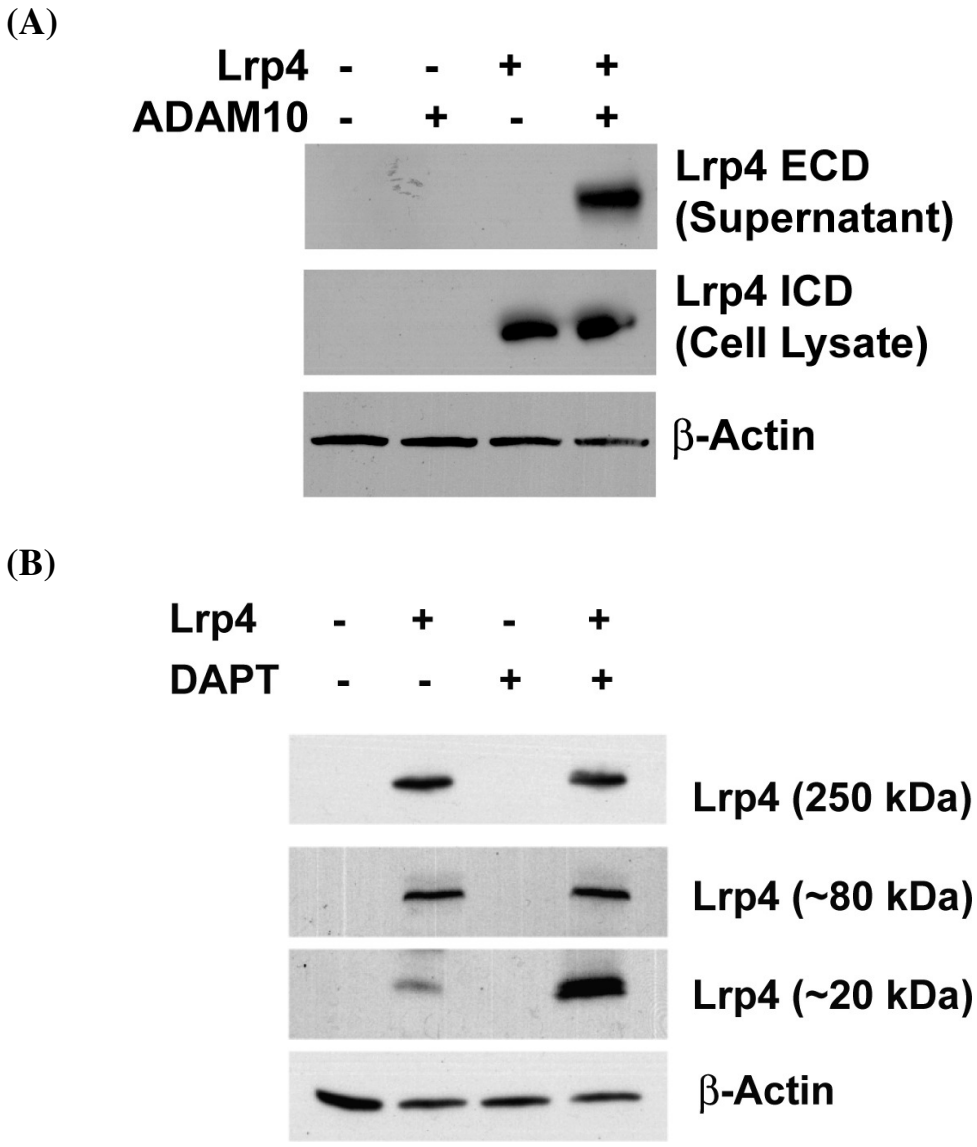


Figure 2.4: Lrp4 receptor undergoes regulated intramembraneous processing. (A) The Lrp4 ECD is shed *in vitro*. 50 µL of concentrated supernatant **(i)** and 50 µg of cell lysate **(ii)** were analyzed with antibodies detecting either Lrp4 extracellular (Lrp4 ECD antibody) or intracellular domain (lower panel). The extracellular domain is present in the supernatant after transfection with Lrp4 and co-transfection with metalloprotease Adam10 (lane 5), but not in the absence of Adam10 (lane 3). The extracellular domain fragment of Lrp4 serves as positive control. β-Actin was detected to demonstrate equal loading. **(B) Lrp4 ICD is cleaved by γ-secretase.** Lrp4 expression in 293T cells reveals bands at 20, 75, and 250 kDa (lanes 2 and 4). The protein levels of the upper and middle are independent of DAPT treatment. The suspected ICD at 20 kDa accumulates in the presence of γ-secretase inhibitor DAPT.

The Lrp4 ECD negatively regulates Wnt signaling.

Lrp4 has been reported to be a negative regulator of Wnt signaling. To investigate whether the ECD contributes to this inhibition, we used a TOP-Flash assay system and measured the β-catenin promoter activity *in vitro*. Wnt1 was used to activate signaling at the extracellular level. Dickkopf-1 (Dkk1) has been reported to be a negative regulator of Wnt signaling [103] and a Lrp4 binding partner [26]. As expected, Lrp4 and Dkk1 do not repress Wnt signaling in a non-activated stage (Figure 5, columns 3 and 4). However, in a Wnt1 activated situation both Dkk1 (Figure 5, column 5) and Lrp4 (Figure 5, column 6) can individually decrease Wnt signaling significantly. When transfected together, Dkk1 and Lrp4 display an additive effect of Wnt inhibition (Figure 5, column 7).

Figure 2.5: Lrp4 ECD Inhibits Wnt signaling *in vitro*. HEK-293 cells were transfected using the TOP-Flash reporter system in the presence of the indicated plasmids. Dkk1 and Lrp4 inhibit activated Wnt signaling (lane 5 and 6). Inhibition of Wnt1 induced activation by co-transfection of Lrp4 ECD and Dkk-1 is additive (lane 7). Lrp5 and Lrp6 are co-receptors of the frizzled complex and required for Wnt1 mediated activation, however, HEK-2 93T cells do not express Lrp5 or 6 endogenously, thus need to be co-transfected [11]. Transfected plasmid amounts were compensated by irrelevant plasmid.

Discussion.

In this study, we have presented evidence for an essential role of *Lrp1b* in embryonic development. Using two different *Lrp1b* null alleles, no viable offspring or embryos were obtained. Although blastocyst outgrowths appeared normal we were unable to identify viable embryos homozygous for *Lrp1b* mutant alleles at or beyond the E8.5 timepoint, suggesting that loss of *Lrp1b* causes early embryonic lethality and underscoring the importance of this gene in development. We have previously reported that mice carrying a truncated form of *Lrp1b* exclusively expressing a secreted ECD, are born at normal Mendelian ratios and are phenotypically unremarkable (Marschang et al., 2004). In this earlier study, we had used insertion of a 'neomycin-stop' cassette to replace the transmembrane domain at exon 88 of *Lrp1b,* resulting in the truncation of the receptor and the secretion of a fully folded and functionally intact ECD. Under physiological conditions, *LRP1b* is anchored through its transmembrane domain in the cell membrane where it can undergo regulated intramembrane proteolysis (RIP). The ECD of *LRP1b* is cleaved by several metalloproteinases, including ADAM17 and other members of the ADAM family, in the initial step of receptor processing and leads to shedding into the extracellular space where its function has not been determined (Liu et al., 2007). Subsequently, γ-secretases release the intracellular domain for further processing from the membrane. LRP1 and other members of

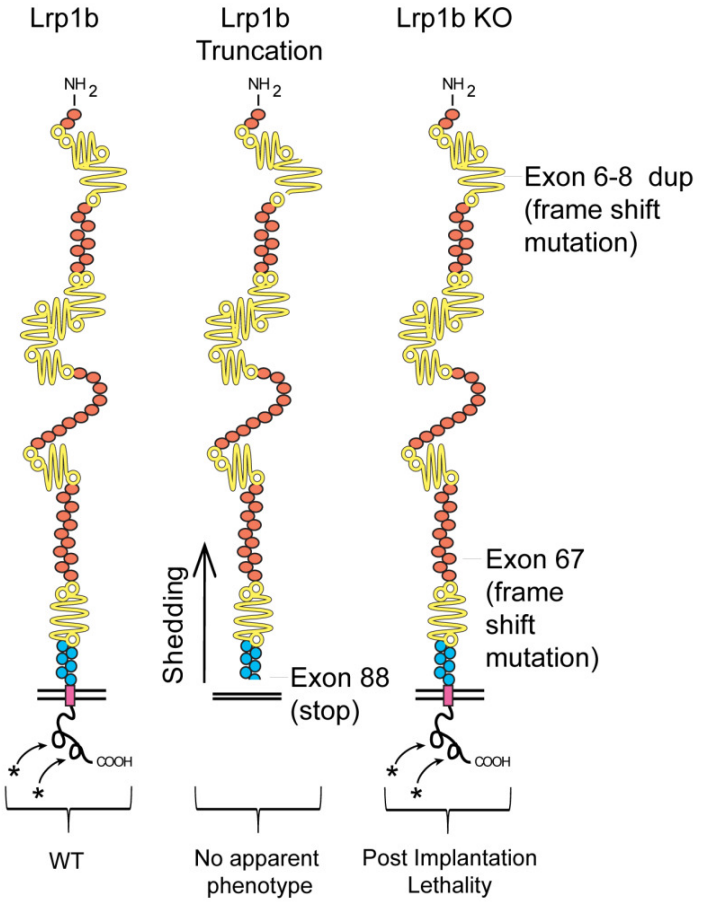
the LDL receptor gene family are known to bind a broad variety of ligands, including growth factors, membrane receptors, the amyloid precursor protein, bacterial toxins, and other proteins. Given their structural similarities, *LRP1b* is likely to bind a comparable variety of ligands. In fact, the amyloid precursor protein, Pseudomonas exotoxin A and some other ligands have already been reported to also bind to the ECD of LRP1b (Cam et al., 2004; Pastrana et al., 2005). Our gene targeting study to disrupt *Lrp1b* by duplicating internal exons of the gene suggests that the ECD can function independently from the membrane anchored receptor to regulate critical developmental processes required for embryonic viability. The shedding of the ECD into the extracellular space might therefore serve as a soluble ligand scavenger. This event presumably preserves a critical signaling threshold at an early stage of embryonic development. For other members of the LDL receptor gene family, it has been demonstrated that the cleavage of the extracellular domain equally occurs in the native receptor. Interestingly, we have found a comparable rescue of a severe perinatally lethal phenotype by a truncated form of *Lrp4,* where only the ECD remains expressed.

We could confirm Lrp4 ECD expression in this previous model and present *in vitro* evidence that Lrp4 undergoes regulated intramembraneous processing (RIP) by cleavage and shedding of the ECD by metalloproteases and ICD release after γ-secretase cleavage. Both steps have important physiological

functions in other LDL gene family members including signaling modulation and transcriptional inhibition. Further, our *in vitro* results suggest that Lrp4 ECD can negatively modulate Wnt signaling. Whether this happens through cooperation with inhibitory ligands or scavenging of activating ligands extracellularly remains to be determined. However, it remains unclear whether shedding occurs *in vivo* and how it impacts physiological processes. However, anchorage-independent modulation, at least for Lrp4, does seem to play a crucial role in preserving a signaling threshold for proper cellular functioning. No mechanisms of Lrp1b signaling modulation are currently known. This hypothesis requires further confirmation once its molecular function is revealed. Deletion of *Lrp4* causes perinatal death due to a failure to form neuromuscular junctions and subsequent respiratory failure (Weatherbee et al., 2006). This phenotype is mitigated in the truncated *Lrp4* receptor expressing only the ECD, allowing the animal to breathe and move, despite general muscular weakness and hypotrophy. Another prominent phenotype, involving abnormal distal limb development, appears to be identical in the null and hypomorph (Johnson et al., 2005; Johnson et al., 2006; Weatherbee et al., 2006).

There are several reports of *LRP1b* being deleted or epigenetically silenced in a variety of human tumors (Choi et al., 2007; Langbein et al., 2002; Liu et al., 2000; Nakagawa et al., 2006; Roversi et al., 2006; Taylor et al., 2007; Yin et al., 2009). The exact mechanistic involvement of *LRP1b* in tumor

suppression and development has remained elusive. The previously reported functional insights into tumor suppression at the molecular level overlap with its close relative *LRP1*. They include the regulation of uPA, uPAR and PDGF receptor tyrosine kinase. However, the lack of mutations in *LRP1* indicates important functions that have diverged from those of *LRP1b*. These differences could be attributed to the distinct selective pressure on the *LRP1b* gene in the process of tumor development. It is thus likely that unknown mechanisms of *LRP1b* are involved in tumorigenesis and the regulation of extracellular signaling. While the release of the intracellular domain and its effect on inflammatory signaling and proliferation has been described for both *LRP1b* and *LRP1*, no such independent function has been described for the isolated ECDs of either receptor. Our data, obtained from two distinct mouse models suggests that the ECD of *Lrp1b* can function in maintaining signaling homeostasis even in the absence of proper membrane integration. In analogy to LRP1, this might occur through binding of soluble ligands in the extracellular space. In summary, we report an essential role for *Lrp1b* in embryonic development and propose a novel role for the *Lrp1b* and *Lrp4* members as signal modulators through ligand scavenging. Elucidating the molecular functions of the LRP1b and LRP4 ECDs has the potential to provide functionally significant insights into the role of *LRP1b* in embryogenesis and tumor development.


Lrp1b
Lrp1b Truncation
Lrp1b KO
NH2
NH2
NH2
Exon 6-8 dup (frame shift mutation)
Shedding
Exon 88 (stop)
Exon 67 (frame shift mutation)
COOH
COOH
WT
No apparent phenotype
Post Implantation Lethality

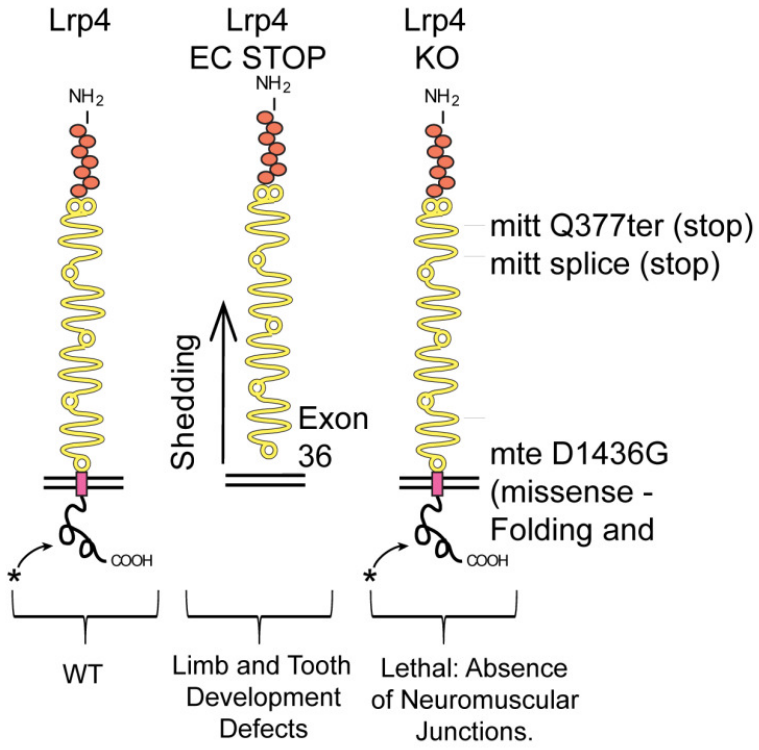


Figure 2.6: Summary of known mutations and their respective phenotypes. The known mutations in murine models for *Lrp1b* and *Lrp4* are shown. The presence of the extracellular domain (ECD) rescues the lethality caused by the complete functional null mutation.

CHAPTER THREE

LRP4 REGULATES INITIATION OF URETERIC BUDDING AND IS CRUCIAL FOR KIDNEY FOR MATION IN MAMMALS

Adapted from Courtney M. Karner, Martin F. Dietrich*, Eric B. Johnson*, Natalie Kappesser, Christian Tennert, Ferda Percin, Bernd Wollnik, Thomas Carrol, Joachim Herz. Lrp4 Regulates Initiation of Ureteric Budding and Is Crucial for Kidney Formation in Mice. PLoS One (Submitted for Publication).*

Summary.

Background. Development of the kidney is initiated when the ureteric bud (UB) branches from the Wolffian duct and invades the overlying metanephric mesenchyme (MM) triggering the mesenchymal/epithelial interactions that are the basis of organ formation. Multiple signaling pathways must be integrated to ensure proper timing and location of the ureteric bud formation.

Methods and Principal Findings. We have used gene targeting to create an Lrp4 null mouse line. The mutation results in early embryonic lethality with a subpenetrant phenotype of kidney agenesis. Ureteric budding is delayed with a failure to stimulate the metanephric mesenchyme in a timely manner, resulting in mesenchymal apoptosis and subsequent failure of kidney formation in the mouse as well as kidney hypoplasia in humans.

Conclusion. Lrp4 is a multi-functional receptor implicated in the regulation of several molecular pathways, including Wnt and Bmp signaling. Lrp4-/- mice

show a delay in ureteric bud formation that results in unilateral or bilateral kidney agenesis. These data indicate that Lrp4 is a critical regulator of UB branching and lack of Lrp4 results in congenital kidney malformations in humans and mice.

Introduction.

The definitive kidney forms as a result of inductive interactions between the metanephric mesenchyme and the UB (Shakya et al., 2005). In the mouse, signals from the metanephric mesenchyme stimulate the ureteric bud to branch from the Wolffian duct around embryonic stage E10.5 (Saxen and Sariola, 1987). The UB subsequently invades the overlying metanephric mesenchyme and produces signals that are necessary for survival, proliferation and differentiation of the mesenchyme (Maas et al., 1994). The timing and location of ureteric budding are critical factors in kidney organogenesis. Genetic and surgical manipulations have revealed that the mesenchyme is only competent to respond to signals from the bud for a narrow time window (Lipschutz, 1998). Failure of the bud to reach the mesenchyme in this narrow window results in apoptosis of the mesenchyme and subsequent kidney agenesis (Gluecksohn-Schoenheimer, 1943; Gluecksohn-Schoenheimer, 1945).

Defects in secondary branching of the ureteric bud can result in a range of phenotype, ranging from congenital anomalies like hypoplastic kidneys to cystic dysplasia (Lu et al., 2006). Defects in kidney formation constitute some of the most common birth defects in humans (Bates, 2000). Multiple signaling pathways have been implicated in UB branching. The GDNF/Ret, FGF and Wnt signaling

pathways are necessary for normal branching while the BMP pathway appears to act as a branching inhibitor (Kispert et al., 1996; Kuro-o, 2006; Lipschutz, 1998).

As would be expected, tight regulation of these pathways is essential to insure the proper timing and location of branching. Although we have gained a great deal of information on the molecular mechanism regulating ureteric bud branching in mice, there has been surprisingly little correlation between these major pathways and congenital defects in man (Searle et al., 1989).

Lrp4 is a member of the low-density lipoprotein (LDL) gene family (Tomita et al., 1998). Mutations in this membrane receptor have been implicated in neuromuscular junction, limb and tooth development where it appears to integrate signaling from multiple pathways including Wnts and Bmps (Johnson et al., 2005; Johnson et al., 2006; Kim et al., 2008; Ohazama et al.; Rivadeneira et al., 2009; Weatherbee et al., 2006; Zhang et al., 2008). Here, we describe an additional role for Lrp4 in the formation of the UB. Loss of Lrp4 results in a delay in UB formation and a subpenetrant kidney agenesis phenotype. We also identified mutations in Lrp4 in humans with congenital kidney defects. These studies establish Lrp4 as a critical regulator of ureteric budding in both mice and humans.

Materials and Methods.

Mouse Strains.

The Lrp4 knockout (KO) mouse was generated by replacing the first exon with a neomycin resistance cassette using techniques described previously [15]. The long arm of homology upstream of the first exon of Lrp4 was generated by PCR using primers

MEJ24 (5'-CCACCACCGCCTCATGGTGCTGCGGCCGCC-3'). and

MEJ23 (5'GCGGCCGCCAGGTCATGAAGTGAGTGCTGAGCCACTGGG-3')

The short arm of homology downstream of the first exon of Lrp4 was generated by PCR amplification using the primers

MEJ33 (5'-CTCGAGGAGCGGTCTGCAGATCCTGGCGATTCACGG-3') and

MEJ35 (5'-CTCGAGGGTTACAGACTCTGCAACTGCTCTACCTCATTG-3').

The long arm and short arm of homology were cloned into pJB1 using the NotI and XhoI restriction sites, respectively. Mice were maintained on a mixed 129/C57 background. All animal work was conducted according to the relevant national and international guidelines and in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research" (no primates were used in this study). Animal experiments conducted in Dallas were also reviewed and approved by the Institutional Committee on Animal Use and Care (IACUC) at UT Southwestern Medical Center.

Genotyping .

KO Mice were genotyped by PCR as follows:

MEJ358 (5'-ACTATATTCACCCGCCGGCTTTTCCACGTG-3') and

KOT12 (5'-AGCAGCTTTCAGAAGCACCTCTTCAGGACC-3')

were used to selectively amplify the wild-type allele and

Neo36 (5'-CAGGACAGCAAGGGGGAGGATTGGGAAGAC-3')

and KOT12 were used to amplify the knockout allele. The HoxB7Cre allele was amplified using the primers 5'-CCATGAGTGAACGAACCTGG-3' and TGATGAGGTTCGCAAGAACC to give a 400 base pair band using the conditions previously described. The β-catenin exon3flox allele was amplified using the primers:

5'-AACTGGCTTTTGGTGTCGGG-3' and

5'-TCGGTGGCTTGCTGATTATTTC-3'.

Using a 55°C extension temperature, the wild type allele yields a 291 base pair band while the exon 3 floxed allele yields a 400 base pair band.

In situ hybridization.

Whole-mount *in situ* hybridization was performed as previously described. Briefly embryos were harvested and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Embryos were treated with 10μg/ml proteinase K in PBST for 20 minutes at room temperature and hybridized overnight at 72°C with digoxigenin-

UTP labeled probes. Embryos were then incubated overnight at 4°C with alkaline phosphatase coupled anti-digoxigenin antibody (Roche Applied Science). Color reaction was developed using BM Purple (Roche).

H&E histology.

Kidneys from P0 pups were emersion fixed with 10% formalin and embedded in paraffin. The kidneys were then sectioned and stained with H&E using standard techniques.

Whole mount antibody staining.

Embryonic day 10.5 embryos were dissected in PBS and staged according to somite number. Embryos at the 38 somite stage were fixed overnight in 4% PBS (Electron microscopy services) overnight at 4° C. After fixation embryos were dehydrated and rehydrated through a graded ethanol series. Embryos were then washed four times for 30 minutes at room temperature with heavy agitation in PBS + 0.1% Triton-X (PBStx). Embryos were blocked for at least 3 hours at room temperature in 10% FBS/PBStx. Embryos were incubated with antibodies to E-Cadherin (Rat 1:400 Zymed) and Pax2 (Rabbit 1:400 Covance) overnight at 4 degrees Celsius, then washed six times 30 minutes each wash at room temperature in PBStx. Embryos were incubated with fluorescently coupled secondary antibodies (Molecular probes) overnight at 4° C followed by extensive washing in

PBStx. Wolffian ducts were then dissected away from the embryo and imaged on a Zeiss NeoLumar stereoscope using an Olympus DP71.

Results.

Lrp4 is required for kidney formation.

We have previously generated mice that harbor a null allele of Lrp4. In the examination of post-partum Lrp4$^{-/-}$ mice (n=156) we found 51 percent bilateral and 22 percent unilateral kidney agenesis (Fig. 3.1, b, d, e). This distribution was gender independent and involved only structures derived from the UB and metanephric mesenchyme (MM) (Fig. 3.1, a-d). The small number of kidneys that did form in Lrp4 knockouts were indistinguishable from wild-type at both the histological and molecular level. Functional analysis was not possible due to the immediate post-partum lethality caused by neuromuscular junction defects (Weatherbee et al., 2006).

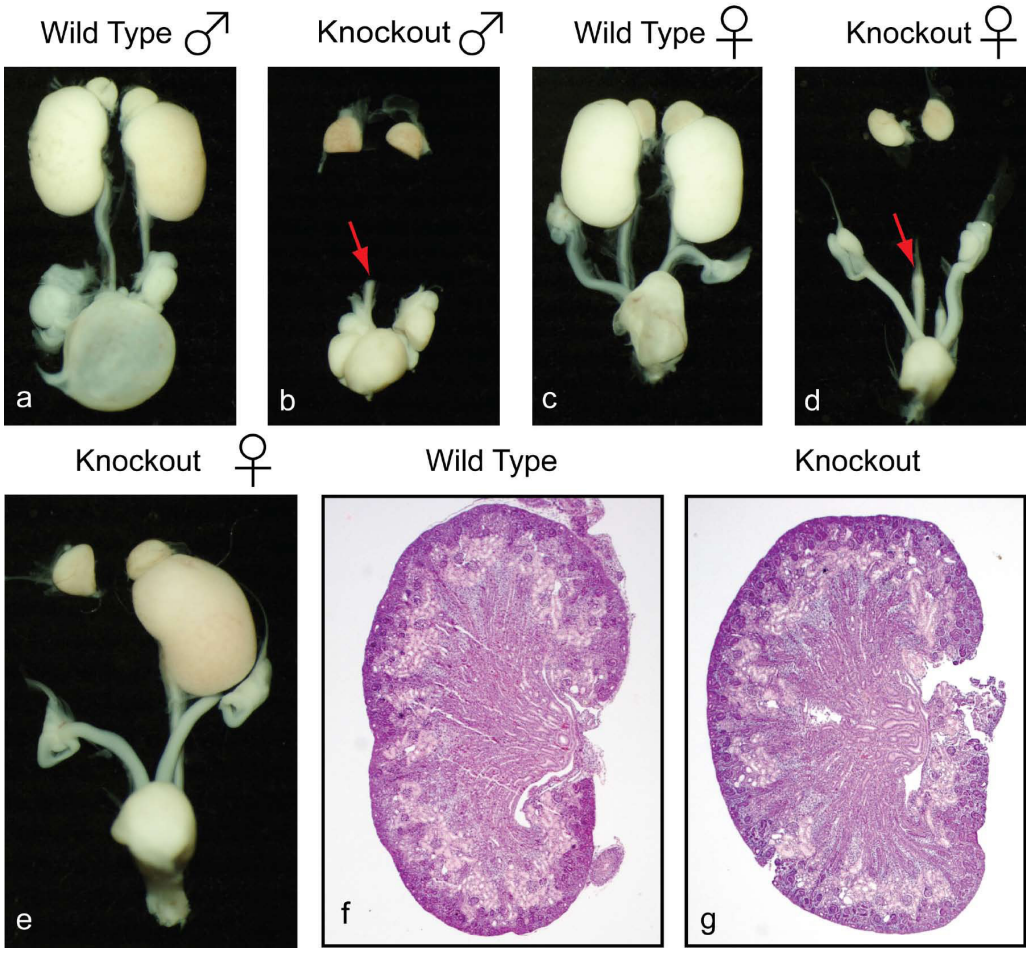


Figure 3.1. Unilateral and bilateral kidney agenesis in LRP4 knockout mice. Kidney agenesis in the Lrp4 knockout (b,d,e). Bilateral (b,d) or unilateral (e) kidney agenesis with rudimentary ureters (red arrows). The lower urinary and genital systems of males and females remain intact. Histological analysis (Hematoxylin-Eosin stain) does not reveal morphological defects in the kidneys that form in Lrp4 knockout animals (g) compared to the wild-type kidneys (f).

Lrp4 is widely expressed in the kidney during development.

To better understand its contribution to kidney formation, we investigated the expression of Lrp4 during development. Beginning at embryonic day E10.5, Lrp4 mRNA is visible in the mesonephric tubules and the Wolffian duct adjacent to the MM (Fig. 3.2, a). At E11.5, Lrp4 is expressed throughout the ureteric epithelium and the adjacent pretubular aggregates (Fig. 3.2, b). Lrp4 continues to be expressed in the ureteric bud derived epithelia and the pre-tubular aggregates/renal vesicles throughout the embryonic period (Fig. 3.2, a-d).

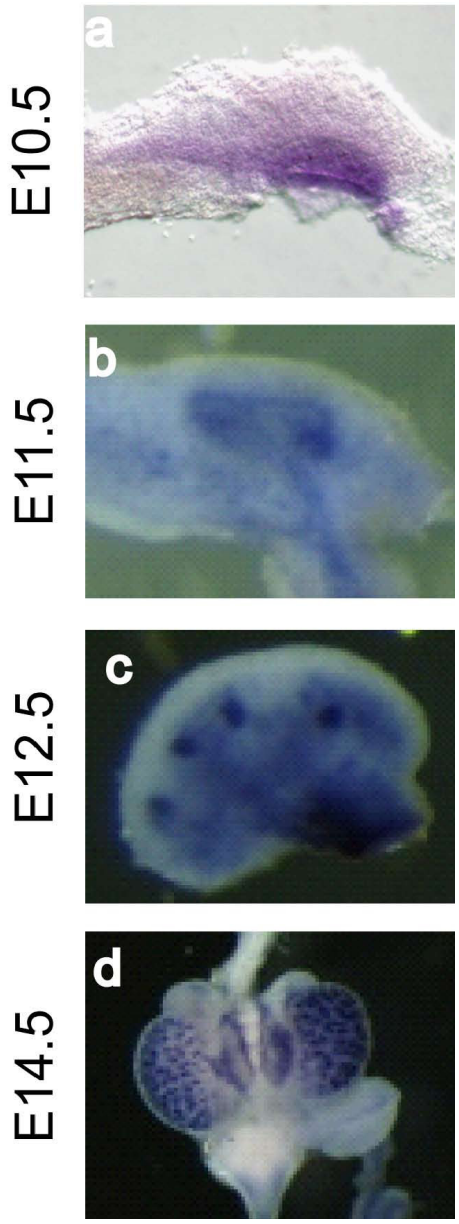


Figure 3.2. Expression of Lrp4 in the developing kidney. At E10.5 Lrp4 is expressed throughout the Wolffian duct and the ureteric bud (a). At E11.5, Lrp4 is expressed in the ureteric bud and the pre-tubular aggregates (b). At E12.5 and E 14.5, Lrp4 expression is maintained in the ureteric bud and the renal vesicles (c and d, respectively).

Pax2 signaling remains intact in the absence of Lrp4.

To gain insight into the nature of the mutant defect, we compared the earliest stages of kidney development in wild type and mutants. Pax2 is a critical regulator of kidney branching that is normally expressed in the Wolffian duct, the ureteric bud/collecting ducts and the metanephric mesenchyme throughout the developmental period. The expression levels in the Wolffian duct and metanephric mesenchyme are comparable between wild type and knockout mice at E10.5 and 11.5 (Fig. 3.3, a-d), although at E11.5 the mutant ureteric bud appears to have not contacted the mesenchyme and has not formed a T-shape (Fig. 3.3, d). By E12.5, the mesenchymal expression of Pax2 is lost (Fig. 3.3, f).

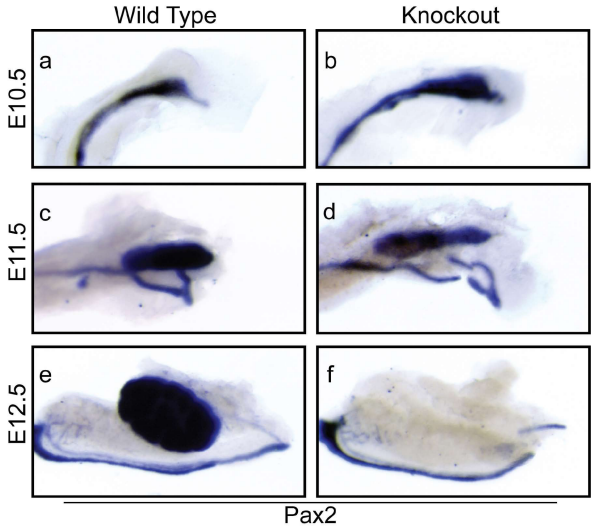


Figure 3.3. Mesenchymal Pax2 expression is lost prematurely in Lrp4 Knock-out Mice. Pax2 is expressed normally in the metanephric mesenchyme and the ureteric bud at E10.5 in the wild type and Lrp4 knockout mice (a and b). At E11.5, Pax2 is expressed normally in both the ureteric bud and metanephric mesenchyme of wild type (c) and Lrp4 knockout animals (d). However, the ureteric bud fails to invade the

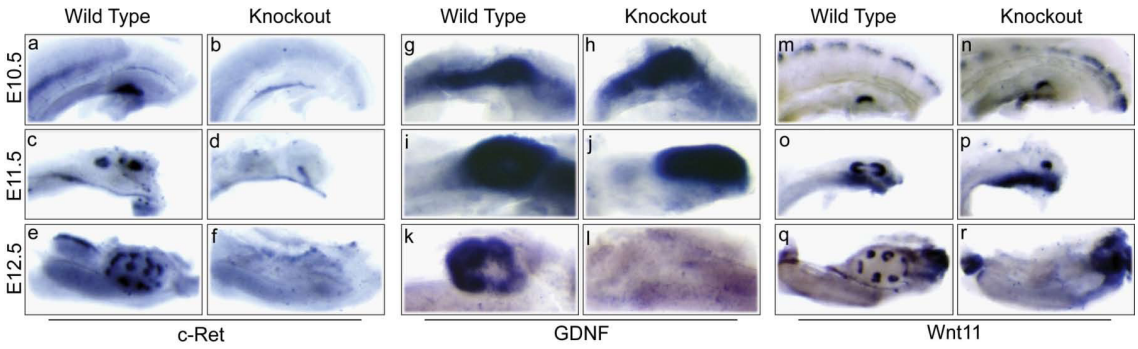
metanephric mesenchyme and undergo secondary branching in the Lrp4 knockout (d). Mesenchymal expression of Pax2 is subsequently lost in the kidney mesenchyme at E12.5, while expression in the ureteric bud and Mullerian duct is maintained compared to wild type animals (e and f).

The GDNF/Ret/Wnt11 signaling network is unaffected by Lrp4.

The Lrp4 mutant kidney defects are similar to those seen in mice with defects Ret signaling (Sampogna and Nigam, 2004). Glial cell derived neurotrophic factor (GDNF) is a ligand for Ret and a co-receptor, GFRα1 (Costantini and Shakya, 2006). Mutations in each of the three genes result in partially penetrant kidney agenesis. To examine potential defects in the Ret pathway, we first examined the expression of Ret and GDNF mRNA. At E10.5, c-Ret is expressed in the ureteric bud at equivalent levels in the Lrp4 knockout mice compared to their wild type counterparts (Fig. 3.4, a and b). At E11.5 the upregulation of the receptor at the tip of the ureteric bud occurs only in wild type animals while baseline levels are maintained in the knockout (Fig. 3.4, c and d). As expected, glial-derived neurotrophic factor (GDNF) is expressed in the metanephric mesenchyme at normal levels at E10.5 and E11.5 (Fig. 3.4, g-j).

As was seen with Pax2, by E12.5 mesenchymal expression of GDNF is completely lost (Fig. 3.4, k and l). To test whether Ret/GDNF signaling is intact, we examined the expression of Wnt11. Wnt11 is a GDNF-inducible downstream target of c-Ret (Majumdar et al., 2003). Wnt11 expression is upregulated in the tips of the bud at E10.5 and 11.5. However, Wnt11 expression is completely lost

by E12.5, presumably due to the loss of mesenchymal GDNF (Fig. 3.4, a-f). As the Lrp4 mutant defect does not appear to be the result of defects in Ret/GDNF signaling, we examined the activity of other pathways involved in ureteric bud branching. Lrp4 has been implicated in the activity of both Bmp and Wnt signaling and both of these pathways play roles in normal branching morphogenesis. To test for defects in Bmp signaling, we investigated the expression of phosphorylated Smads. We were unable to detect differences in either the level or location of p-Smad staining in either the mesenchyme or ureteric buds of Lrp4 mutants at either E10.5 or 11.5 (data not shown). To assay Wnt signaling, we examined the expression of Axin2 mRNA in the Wolffian duct and ureteric bud. Similar to the situation with the p-Smads, we were unable to



detect significant differences in transcript levels (data not shown).

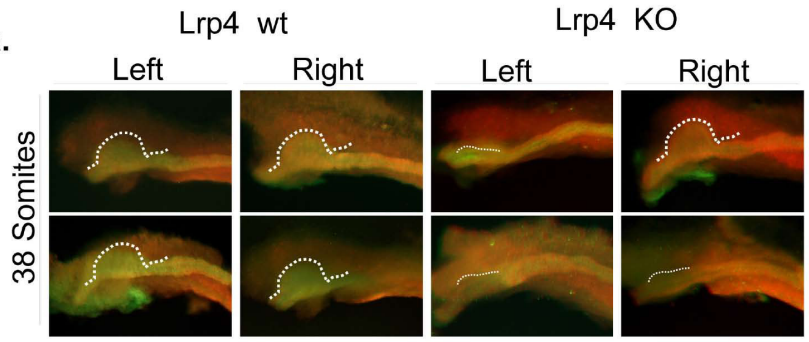
Figure 3.4. Expression of branching regulators in Lrp4 mutants. Expression of c-Ret (a-f),GDNF (g-l) and Wnt11(m-r) in E10.5 (a,b,g,h,m and n), E11.5 (c,d,i,j,o and p), and E12.5 (e,f,k,l,q,r) in wild-type (a,c,e,g,i,k,m,o, and q) and Lrp4 knockout

(b,d,f,h,j,l,n,pand r) kidneys. C-Ret is expressed in the ureteric bud at basal levels in the Lrp4 knockout mice at E10.5 (a,b). At E11.5, the Lrp4 knockout ureteric bud fails to bifurcate or upregulate c-Ret expression at the tip of the ureteric bud (d) compared to wild-type embryos (c). At E12.5, the signal is greatly reduced in the knockout kidney (f). GDNF is expressed normally in the metanephric mesenchyme at both E10.5 and 11.5 in wild type and Lrp4 knockout animals (g-j). By E12.5, GDNF expression is completely lost from the Lrp4 knockout metanephric mesenchyme (k and l). Wnt11 is expressed normally at the tips of the ureteric bud at both E10.5 (m and n) and 11.5 (o and p) in Lrp4 mutants compared to wildtype. By E12.5 Wnt11 is absent from the ureteric bud of Lrp4 knockout animals (q and r).

Ureteric budding is delayed in Lrp4 null mice.

The absence of metanephric mesenchyme at E12.5 indicates a failure of the UB to reach these cells and provide survival signals. This could be due either to defects in growth of the bud or a delay in formation of the bud. The complete lack of a phenotype in some mutants seemed more in line with a delay in bud invasion. To investigate this possibility, we examined bud formation at E10.5. Stage and somite matched embryos were stained for the epithelial markers Pax2 and E-cadherin to assess UB formation. Interestingly, although we noticed at least a partial ureter in all newborn Lrp4 mutants, we found that the UB had formed in only 12.5% (1/8) of 38 somite stage Lrp4 mutants (compared to 100% of cases for wild type controls) (Fig. 3.5, a and b). These data indicate that ureteric bud formation is delayed in mutants, and that failure of many delayed buds to reach the mesenchyme in time to support normal growth/survival is the cause for the frequent uni- or bilateral kidney agenesis.

a.



b.

E10.5 38 Somites	Total Kidneys	UB Present	
Lrp4 +/+	10	10	p(10/10)<0.01
Lrp4 -/-	8	1	p(1/8)<0.01

Figure 3.5. Ureteric Budding is delayed in Lrp4 Mutants. 38 somite stage E10.5 embryos were stained with the epithelial markers Pax2 (red) and E-cadherin (green) to label the Wolffian duct and developing ureteric bud. In the wild-type (a-d), ureteric buds appear as expected while there is a frequent delay in ureteric bud outgrowth in the Lrp4 mutants (e,g,h). One Lrp4 mutant animal is shown with a unilateral outgrowth (f). In total, all 10 expected buds are formed at the 38 somite stage in the wild-type background while only 1 out of 8 predicted buds is present in the knock-out (Panel b).

Wnt overexpression in the ureteric bud leads to kidney agenesis.

Lrp4 is a negative regulator of the Wnt signaling pathway. We therefore tested whether expression of a constitutively active β-catenin transgene would result in a phenotype comparable to a presumed overactive Wnt signaling pathway in the absence of Lrp4. Expression of this transgene under the control of a HoxBCre promoter, which is restricted to the epithelium indeed resulted in a comparable kidney agenesis phenotype (Fig. 3.6, a-c). The formation of the Wolffian duct and distal ureters as well as bladder and adrenal glands remained unaffected. The similarity of these two distinct animal models is consistent with deregulated Wnt/β-catenin signaling in the Lrp4 knockout.

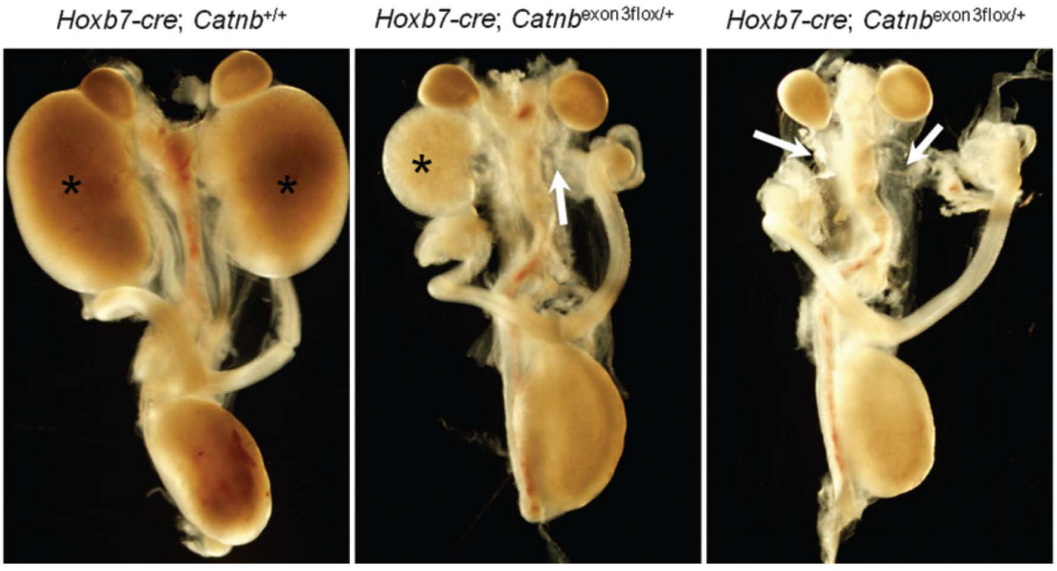


Figure 3.6. Wnt Overexpression in the Ureteric Bud Leads to Kidney Agenesis. Expression of a stabilized allele of β-catenin (Catnbexon3flox) in the Wolffian duct using HoxB7Cre to activate transgene expression phenocopies the Lrp4 knockout phenotype with both uni- and bilateral kidney agenesis (a-c). The formation of the Wolffian duct and distal ureters as well as bladder and adrenal glands remained unaffected. The asterisks (a and b) indicate the position of regular kidneys. The arrows (b and c) indicate the predicted position of kidneys that have not formed.

Lrp4 binds Gremlin1, a positive regulator of ureteric budding.

Lrp4 has been established as a regulator of both the Wnt and Bmp signaling pathways. This involves, at least in part, the binding of signal modulating ligands to the extracellular domain. We tested Gremlin1, a facilitator of ureteric budding, as a possible candidate. Previously, Gremlin1 has been reported to antagonize Bmp4 signaling and its deletion in mice results in a renal phenotype with skeletal involvement similar to the Lrp4 knockout. In co-immunoprecipitation experiments, Gremlin1 binds to Lrp4 (Fig. 3.7). Although we failed to detect a direct difference in Bmp pathway activation at the protein level, Lrp4 might function by facilitating the presentation or integration of Gremlin1 into a signaling complex that mediates the activation of ureteric budding.

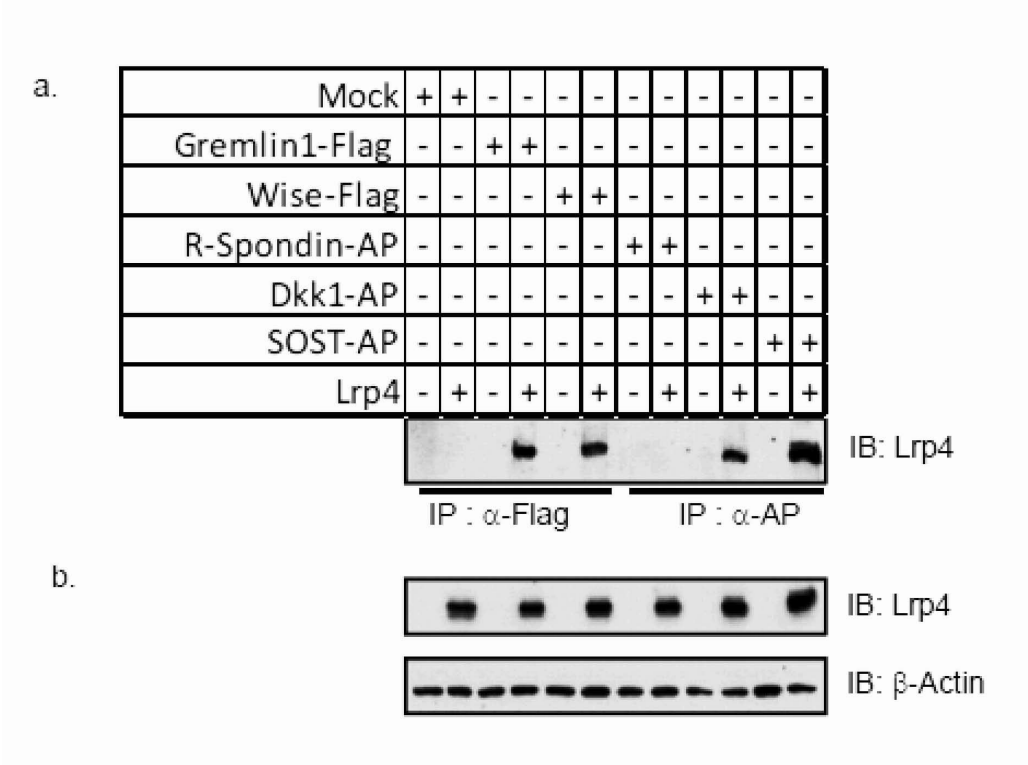


Figure 3.7. Lrp4 binds the Bmp4 antagonist Gremlin1 *in vitro*. Lrp4 has been implicated in modulating the Bmp signaling pathway through binding of the Wnt and Bmp modulator Wise. Co-immunoprecipitation reveals Gremlin1 binding to Lrp4 *in vitro* (Panel A lane 4); we further confirmed the Lrp4 binding partners Wise, Dkk1 and SOST (Panel A, lanes 6, 10 and 12). The Wnt agonist R-spondin 2 did not interact with Lrp4 (Panel A lane 7 and 8). Transfection efficiency was confirmed by immunoblot analysis (Panel B).

Lrp4 mutations cause renal malformations in humans.

In a cooperative effort, Li et al. identified homozygous *LRP4* mutations in patients with Cenani-Lenz syndrome (CLS), a congenital syndrome mainly characterized by distal limb malformations. Interestingly, we observed congenital kidney abnormalities such as renal agenesis and kidney hypoplasia in more than half of the investigated families, which was hitherto unknown. Imaging and functional studies of a CLS patient of the CL-6 family described by Li et al. reveals ectopic and hypoplastic kidneys on both sides (Fig. 3.8, a-d). Dynamic-static renal scintigraphy with Tc-99m DTPA reveals hypofunction of the right kidney, which contributed 26% vs. 74% (left kidney) to total renal function (Fig. 3.8, e). Static renal cortical scintigraphy with Tc-99m DMSA revealed increased background activity (Fig. 3.8, f). Creatinine in this patient was elevated at 1.2 mg/dL. Both of these findings indicated impaired renal function. Clinical variability of phenotypic expression suggests that additional modifying factors that affect budding, branching morphogenesis and organ maturation contribute to this phenotype in humans.

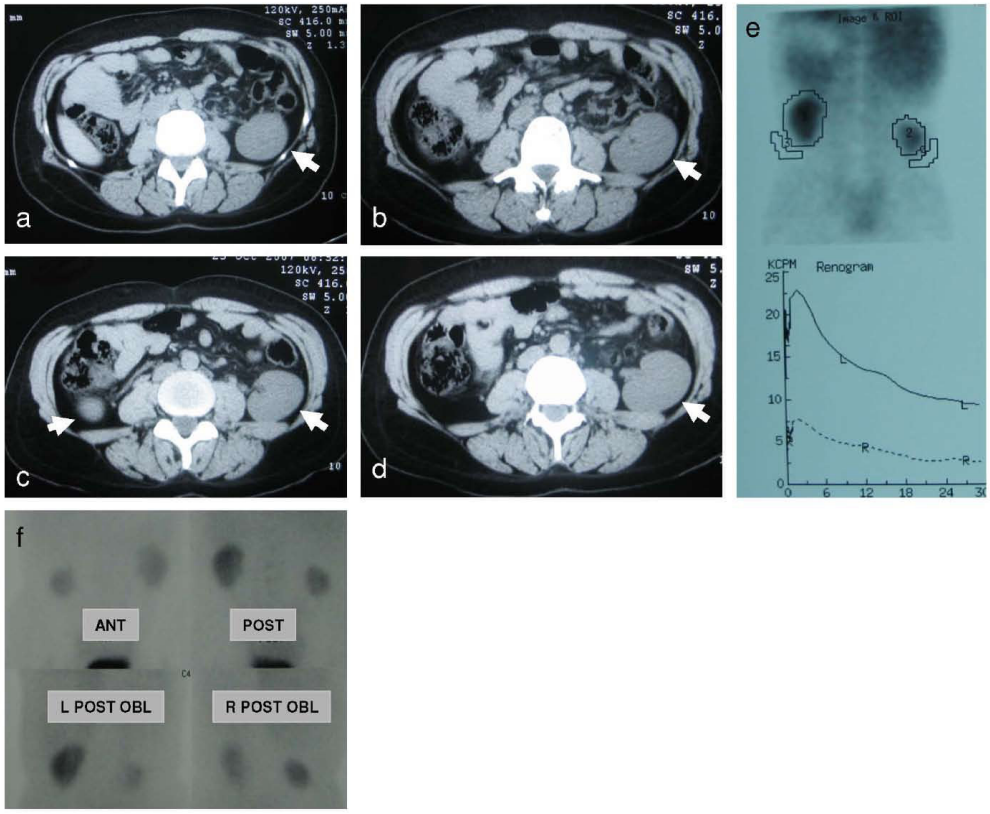


Figure 3.8. Hypoplastic and Hypofunctional Kidney in Human Lrp4 Mutations. CT scan reveals a severely hypoplastic kidney on the right and mild hypoplasia on the left side (a,d). Both kidneys are ectopic with caudal and lateral shifts (a-d). Dynamic-static renal scintigraphy with Tc-99m DTPA suggest right kidney dysfunction (e). Global renal functional participation; right kidney 26% and left kidney 74%. Static renal cortical scintigraphy with Tc-99m DMSA background activity of radiopharmaceutical is higher than expected (f).

Discussion.

We have shown that Lrp4 functions as a critical regulator of kidney development in both mouse and human. In mice, complete absence of functional Lrp4 leads to uni- or bilateral kidney agenesis caused by a delay in the formation of the ureteric bud. In other mouse models, e.g. the limb deformity (*ld*) mutation or *Danforth's short tail* (*Sd*) mice (Phelps and Dressler, 1993), delayed invasion of the ureteric bud into the receptive mesenchyme results in mesenchymal apoptosis and kidney agenesis (Gluecksohn-Schoenheimer, 1943; Gluecksohn-Schoenheimer, 1945).

The fact that normal kidneys do develop in a subset of Lrp4 null embryos suggests that the signaling capacity of the bud and the receptivity of the mesenchyme is unaffected by loss of this gene. However, the range of phenotypes observed in humans, from complete agenesis to hypoplasia, along with the expression of Lrp4 mRNA in multiple cell types of the kidney throughout the embryonic period suggest this molecule may have additional roles in kidney development, or that other factors exist, which can modify the phenotype. The precise mechanism for Lrp4 action during kidney development is still unclear. During kidney development, tissue-tissue interactions between the metanephric mesenchyme and the UB are critical and rely on the integration and regulation of

several signaling pathways. Wnt signaling is crucial for UB branching and has been shown to be regulated by Lrp4 in other systems (Johnson et al., 2005; Johnson et al., 2006; Ohazama et al., 2008). Intriguingly, a mouse model with UB specific overexpression of activated β-catenin presents with a very similar phenotype to the Lrp4 mutant (Fig. 3.6). However, analysis of the Wnt pathway activity has failed to reveal significant changes in Lrp4 mutants, possibly due to high baseline activity in the wildtype.

An alternative yet equally plausible scenario is that Lrp4 is involved in the modulation of Bmp signaling. We have found that, like other members of the LDL receptor gene family, Lrp4 is capable of modulating TGF-β related signaling (Ohazama et al., 2008). In this study, we have confirmed novel binding partners for Lrp4 including the Bmp regulating ligand Gremlin1 (Fig. 3.7). As Gremlin1 knockout mice display a phenotype of bilateral kidney agenesis (reportedly due to ectopic Bmp4 activity) (Michos et al., 2007), an attractive model is that Lrp4 cooperates with Gremlin to inhibit Bmp4 activity. However, similar to the case with β-catenin signaling, we were unable to detect significant changes in the expression of the Bmp targets, pSmad1, 4 and 8. It is therefore possible that Lrp4 acts through an unrelated pathway or perhaps through only partial modulation and integration of both Bmp and Wnt signaling.

Normal kidney formation occurs in a hypomorphic Lrp4 mutant, where only a secreted extracellular domain is expressed, and adding additional insight into the mechanism of Lrp4 during ureteric budding (Johnson et al., 2005; Johnson et al., 2006). These findings suggest that whatever factor Lrp4 is normally interacting with in the kidney, it is occurring extracellularly and most likely does not require endocytosis of the receptor. Possible mechanisms include quenching of Wnt and BMP modulators, such as Gremlin1 (Fig. 3.7) by the secreted extracellular domain.

In summary, we have identified Lrp4 as a critical factor for UB outgrowth and kidney formation in the mouse. We have also shown that mutations in Lrp4 lead to developmental malformations in human, further underscoring the importance of Lrp4 for human genetics and medicine.

CHAPTER FOUR

LRP1 CONTROLS OSTEOBLASTIC PROLIFERATION THROUGH REGULATION OF THE PLATELET DERIVED GROWTH FACTOR RECEPTOR β

Abstract.

LRP1 is a member of the low-density lipoprotein (LDL) receptor gene family, a group of highly conserved membrane receptors with diverse involvement in development, lipoprotein trafficking, and cellular signaling. In this current study, we have used a gene targeting approach to create an osteoblast-specific deletion of LRP1 under Ranx2-Cre promoter control to evaluate the role of LRP1 in bone forming cells. Serial radiological studies of the skeletal system revealed the development of diaphysal masses with a halo-like, radiodense appearance and central radiolucency. Histological analysis established the diagnoses of periosteal, or juxtacortical, chondromas that presented with increasing frequency to full penetrance at one year of age. However, the etiology of these benign tumors and predisposing risk factors are unknown. LRP1 has previously been reported to control proliferation in smooth muscle cells in the vascular wall through negative regulation of the Platelet-derived growth factor (PDGF) receptor β. We therefore investigated whether the interaction between these two membrane receptor plays a role in the development of chondromas and

tried to clarify the relationship between LRP1 and PDGF receptor β on a molecular level. In summary, our results emphasize the crucial role for LRP1 in control of cellular proliferation and maintenance in the bone.

Introduction.

LRP1 is the second member of the LDL receptor gene family and was originally cloned in the liver. Its original role was thought to be a lipid/cargo transporter, much like the LDL receptor itself. Since then, conditional knock-out models have implicated LRP1 in vascular integrity, neuronal plasticity, cargo transport and endocytosis for over 40 ligands. It has been further demonstrated that LRP1 is capable of modulating the activity of the Platelet-derived Growth Factor (PDGF) receptor β. Binding of the ligand PDGF-BB through its correspoding receptor phosphorylates LRP1 in its second NPxY domain. However, the functional significance of this phosphorylation remains vastly elusive. Conversely, the presence of LRP1 slows the trafficking of PDGFR β and therefore prevents its overactivation. In the bone, both receptors are implied in the maintenance of bone metabolism and formation. In osteoblasts, LRP1 is a predominant receptor for the uptake of Vitamin K, required for γ-carboxylation of osteocalcin, and dietary lipids to promote bone formation. The PDGF receptor β is expressed on osteoblasts and is chemotactically attracted by activated macrophage-like osteoclasts, known to secrete significant amounts of the PDGF-BB ligand. The PDGFR β / PDGF-BB axis therefore plays an important role in the communication between bone forming and bone resorbing cells and a pivotal player in balancing the ongoing process of bone remodelling. There are a variety of primary bone tumors. In our osteoblast-specific LRP1-/- model, we observe the

formation of periosteal chondromas at twelve weeks, with progression to full penetrance around one year of age. These benign tumors are of chondroid origin and arise under or in the periosteum on the surface of cortical bone. As the term already implies, the site of origin is the periosteum with no connection to the inner, bone marrow cavity side of the bone. Although Liechtenstein and Hall described this histological entity already in 1952, little is known about the etiology of the tumor. To the best of our knowledge, this is the first report of a model of chondroma formation in the mouse. To better understand its molecular origins, we hypothesized that LRP1 mediated growth control through regulation of the PDGF receptor β is implicated. We further attempted to improve the understanding of the receptors' mutual interaction.

Materials and Methods.

Mouse Strains.

The Ranx2-Cre mice, generating an osteoblast-specific knock-out model of LRP1 deletion, were studied in collaboration with Dr. Andreas Niemeier at the University of Hamburg, Germany. The mice were maintained in full accordance with the rules and regulations at the aforementioned institution and the Weatherall report. The radiological studies were performed at the University of Hamburg Imaging Facility.

LRP1 in vitro assays and Western Blotting.

The Western Blot Analysis, also known as immunoblot, is a method to detect protein in a cellular or histological extract. The name derives from the DNA hybridization method known after its inventor Edwin Southern. Samples are being homogenized using sample lysis buffer, incubated on ice for 30 minutes, and then centrifuged in a pre-chilled Eppendorf apparature at 13,200 rpm for 20 minutes. The supernatant, the so called "whole cell extract", will be mixed in running buffer, boiled at 95° C in a 1.5 ml Eppendorf tube and subjected to gel electrophoresis. The insoluble fraction, containing of both chromosomal and membranous parts, can be analyzed depending on the cellular location of the desired protein. This can be applicable for both transmembranous and nuclear proteins. A separation using the negative surface charge of proteins through an SDS page gel (7.5 to 15%) using constant vertical voltage is being performed. The separation will utilize both the

charge and size of proteins, thus smaller proteins will migrate at higher rates of speed through the gel, therefore establishing a ladder of proteins sorted by size. The proteins are then horizontally transferred from the SDS page gel onto a PVDF membrane using constant electric current. During the transfer, the buffer needs to be externally chilled. After the transfer, the membranes will be blocked in blocking milk for one hour, and then the incubation will take place in PBS-T with 7% milk blotting powder and the appropriate antibody concentration. After incubation with a secondary antibody and subsequent washing, the horseradish peroxidase is visualized using the ECL Plus luminescence system and read out on x-ray film. For the quantitative analysis, Scion Image for Windows was used.

Here, either subconfluent HEK293T or mouse embryonic fibroblast cells were grown in 10% FCS / DMEM High Glucose (Cellgro Mediatech Inc.). On day 1, pcDNA3.1 constructs expressing either the extracellular domain or the full length of murine LRP1 were transfected into the cells using FuGene6 (Roche Laboratories) according to the manufacturer's protocol. Briefly, 6 µg DNA and 2 µL FuGene6 reagent were suspended (1:3 ratio of FuGene6:DNA) in a volume of 600 µL of serum and incubated for 30 min at room temperature before addition to the cell culture dish. The cells were incubated overnight in 10% serum and then switched to serum free DMEM High Glucose /0.2% bovine serum albumin for two days. Cells were collected, washed three times in ice-cold PBS and lysed in

1% Triton-X lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, with an EDTA-free protease inhibitor cocktail (Complete Mini EDTA-free Protease Inhibitor Cocktail, Roche Laboratories).

Freezing and Thawing of cell lines.

Before freezing, cells should be growing in healthy state, preferably in logarithmic growth phase. The cells should be counted, collected and pelleted in a 15 ml Falcon test tube. After discard of the supernatant, the pellet will be resuspended in freezing media with a maximum final density of 56 cells / ml. The total volume is aliquoted in freezing vials of 1 ml each, then kept at 80° C for 24 hours before long-term storage in liquid nitrogen. For optimal results, 1ml of freezing media will be needed for 56 cells / ml. To thaw cells from liquid nitrogen storage, vials will be transferred immediately to a 37° C water bath. During the thawing process, the vial should be carefully agitated to allow thorough distribution of frozen to non-frozen compartments. When cell suspension is completely thawed, the cells will be transferred into a 15 ml Falcon tube and 10 ml of warm media appropriate for the individual cell type will be added. The mixture will be spun down at 1.000g for 5 minutes and the supernatant will be decanted. The cells will then be seeded in an appropriate container at a medium density. Alternatively, a vial of cells can be immediately transferred into a flask of

medium (about 20 ml medium per 1 ml freezing solution), be grown for 24 hours with a subsequent removal of supernatant and addition of fresh medium.

Maintenance of Cell Lines in Cell Culture Conditions.

The cell lines are frozen and thawed as described previously. As for suspensive leukemia cells, cell lines will be grown at counts ranging from 15 to 55 per ml. The cells will then be split at levels from 1:1 to 1:3 using the medium appropriate for the respective cell line. Adherent cell lines are grown based on their confluency. Upon approaching full confluency, cells will be detached using a Trypsin solution (0.25%) for 5 minutes at 37° C incubator conditions for optimal efficiency. The detachment will then be stopped either using Fetal Calf Serum or FCS containing media. Both contain trypsin inhibitors and neutralize its proteolytic effect within the culture flask.

Counting of Cell Numbers.

Cells are harvested in suspension using an appropriate dilution with PBS, mixed in a 1:1 ratio with Trypan Blue to determine cell viability among the total cell number. Trypan Blue does not enrich within healthy cells, but necrotic and late apoptotic cells passively incorporate it. Under the microscope, these cells will appear blue stained. The dilution will then be pipetted into a Neubauer chamber and counted using a light microscope.

Results.

Osteoblast-specific LRP1 knock-down leads to formation of periosteal chondromas.

LRP1 has been implicated in the regulation of several pathways involved in bone metabolism. As the bone is a continuously remodelling matrix, the diverse functions of LRP1 could lead to a variety of potential phenotypes. The osteoblast LRP1 knock-out mice and wild-type LRP1 littermates were subjected to serial radiographic analysis from 4 weeks of age until over one year of age. The plain X-ray images of the wild-type mice revealed appropriate growth and development of skeletal structures. In contrast, the LRP1 deficient mice displayed a radiodense halo with a radioluscent center at the diaphysal part of the tibia bone. With increasing size and age, the growth of the bone structures leads to a reactive curvature of tibia and fibula. Histological analysis reveals a subperiostal growth of undifferentiated cells of the cartilagenous line. The growth has a normal ratio of mitotic cells, normal nuclear and perinuclear sizes and also no other signs of malignant transformation. The lack of access to the bone marrow cavity and its origin near the cortical structures of the bone assures the diagnosis of a periosteal, or juxtacortical, chondroma.

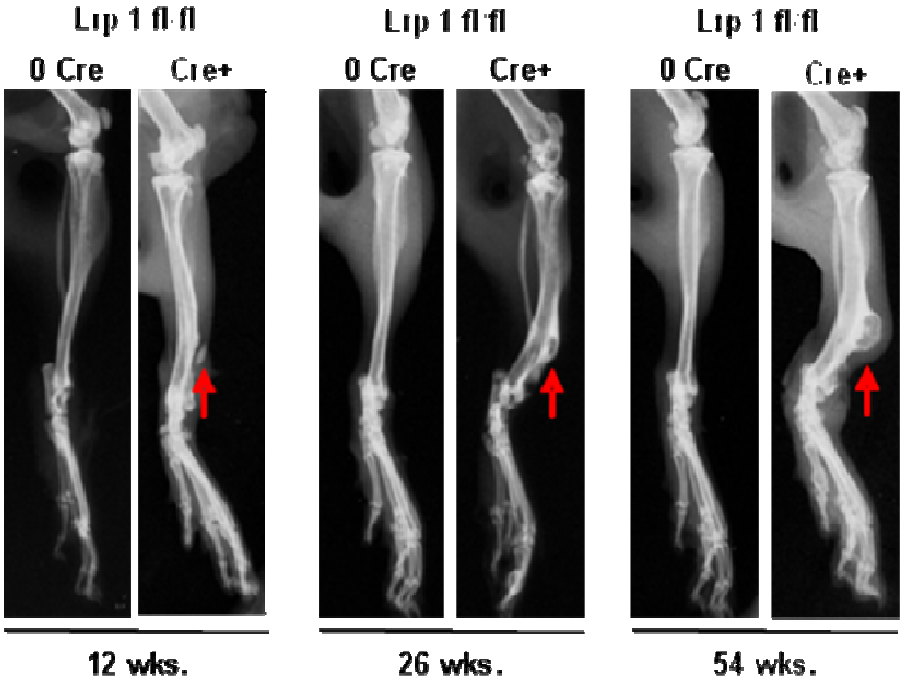


Figure 4.1. LRP1 knock-out in osteoblasts causes periosteal chondromas. X-ray analysis revealed the formation of hyperproliferative masses at the periosteal margin of the diaphysis. The tumors first appear around 8-12 weeks of age and continuously progress in size. The growth leads to a reactive bone deformation into a dorsal curvature for tibia and fibula bone.

Periosteal Chondromas occur with near full penetrance at one year.

The formation of chondromas in the LRP1-deficient osteoblast model follows a time-dependent incidence. Until 8 weeks of age, no formation of chondromas is detectable by X-ray screening. However, at 12 weeks a percentage of already fourty percent present with visible proliferation of the periosteal matrix with a vaculuous X-ray appearance. Around one year of age, the tumors have almost reached full penetrance.

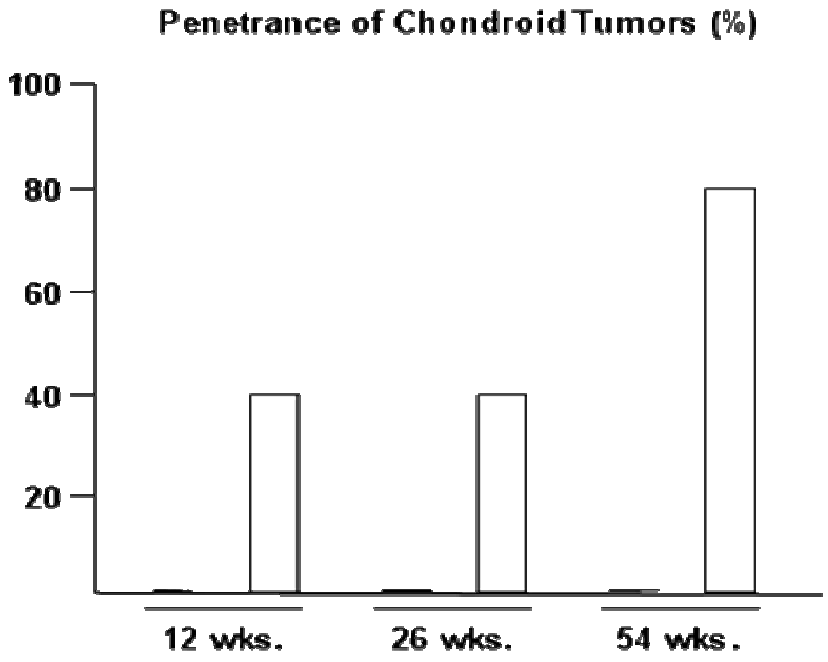


Figure 4.2. Time-dependent incidence of chondromas in the LRP1-deficient animals. Chondromas are present in fourty percent of animals at 12 and 26 weeks of age. At one year of age, about 90 percent of animals show signs of chondroma formation. No radiological changes are noted the wild-type litter mates.

PDGF receptor b is expressed on human osteoblasts and increased in LRP1 knockouts.

Culture from mesenchymal cells in the bone were differentiated along the osseal cell lineage for six and twelve days and analyzed for expression of the PDGF receptor β. In LRP1 wt and knock-out mice, the PDGF receptor is expressed. However, there is an evident upregulation of the PDGF receptor in the LRP1-deficient cells. Although the knock-out is only near complete, there seems to be an inverse relationship in expression between the two receptors.

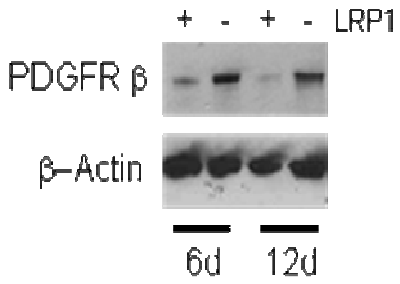


Figure 4.3. The PDGF receptor β is expressed on osteoblasts. Chondromas are present in fourty percent of animals at 12 and 26 weeks of age. At one year of age, about 90 percent of animals show signs of chondroma formation. No radiological changes are noted the wild-type litter mates.

LRP1 binds the PDGF receptor β in a dose dependent fashion.

Previous publications have indicated a co-receptor function for LRP1 towards the PDGF receptor b. To demonstrate direct physical interaction, co-immunoprecipitation between the two potential binding partners was performed. Indeed, LRP1 binds to the PDGF receptor in a dose-dependent fashion. This effect is enhanced through the addition of DSP as cross-linking agent. Under steady state conditions with 10 percent serum growth, a moderate amount of receptor co-precipitates. This is the first evidence that these two receptors interact directly, and that LRP1 prevents, through direct, physical interaction, the PDGF receptor from endocytosis and activation.

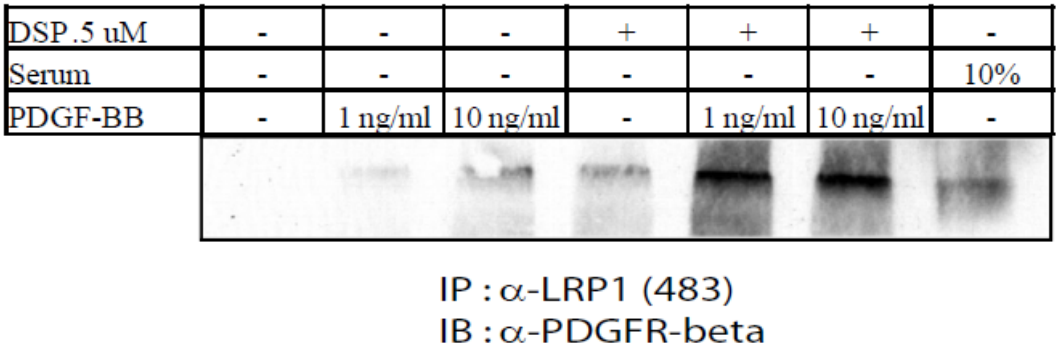


Figure 4.4. LRP1 binds the PDGF receptor in a dose-dependent fashion. Treatment with PDGF-BB leads to direct association of the PDGF receptor to LRP1. This assimilation is dose dependent and enhanced by the addition of DSP. A dish grown under normal serum conditions is used as a positive control.

LRP1 does not interfere with PDGF receptor β dimerization.

A critical step in the activation of the PDGF receptor is the formation of actively signaling dimers. To test whether the presence of LRP1 interferes with this dimerization, cells were starved for sixteen hours overnight, treated for 10 minutes with appropriate ligand and then analyzed in cross-linking experiments. MEF wt (lanes 1-4) show a comparable pattern to MEF LRP1 KO (lanes 5-8). The addition of ligand leads to dimerization (lanes 2, 4, 6, and 8). The dimerization is stabilized by DSP .5 μM for 15 minutes at room temperature (lanes 3-4, 7-8). In conclusion, LRP1 does not seem to interfere with this step of PDGF receptor β activation.

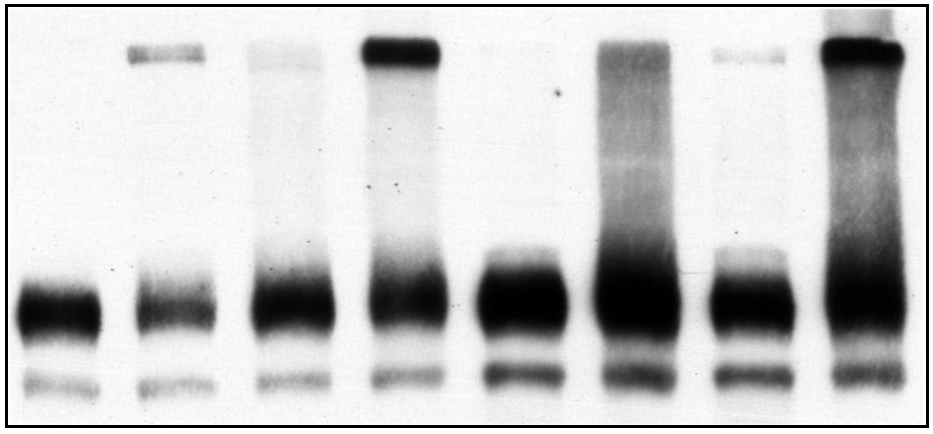


Figure 4.5. LRP1 does not interfere with PDGF receptor β dimerization. In cross-linking experiments, PDGF receptor dimerization was tested. There is no difference between LRP1 wt and ko cells. Auto-dimerization in the absence of PDGF-BB ligand is not visibly different. The signaling appears enhances through cross-linking with DSP.

Absence of LRP1 causes increased turnover of the PDGF receptor β

LRP1 has been previously reported to affect the trafficking kinetics of the PDGF receptor β. In the absence of LRP1, PDGF receptor β turn over is accelerated and removed from the surface. This is in accordance with previous reports and consisted with the other obtained data, i.e. that activation is prevented by direct, ligand-dependent binding.

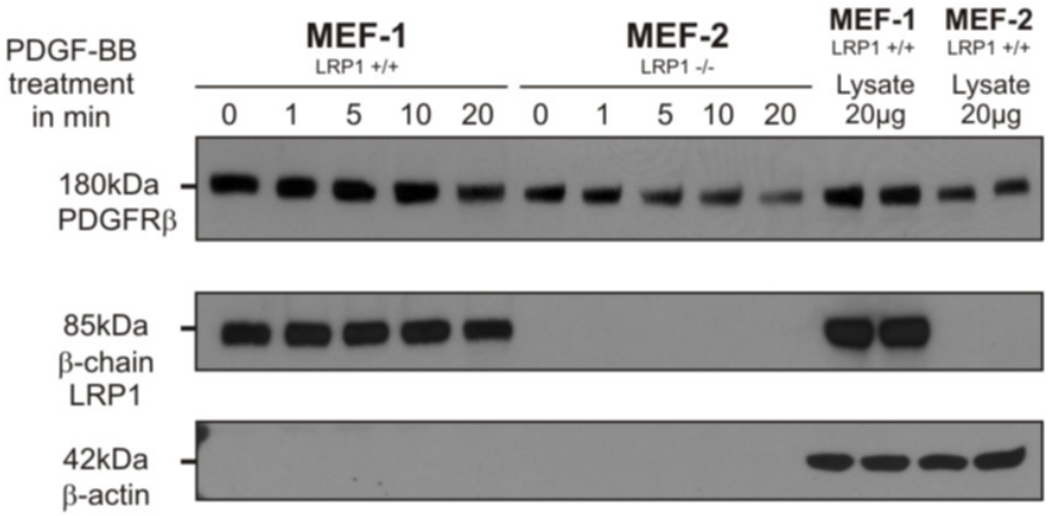


Figure 4.6. LRP1-deficiency accelerates PDGF receptor trafficking. In a surface biotinylation experiment, the accelerated turnover of PDGF receptor is shown. While the levels at the membrane decrease only moderately in the wild-type MEF1, there is a dramatic decrease in the MEF2 with LRP1 deficiency.

Absence of LRP1 causes increased activation of the PDGF receptor β.

Starved cells were treated with PDGF-BB (10 ng/ml) for 0, 2.5, 5 and 10 minutes and compared between LRP1 wild-type and LRP1 deficient mouse embryonic fibroblasts. The total levels support the previous finding of increased turnover of the total receptor levels. For the activation, the phosphorylation of tyrosine site 751 was used. Here, baseline phosphorylation in unstimulated conditions was noted only in the knock-out cells and and increased activation through phosphorylation.

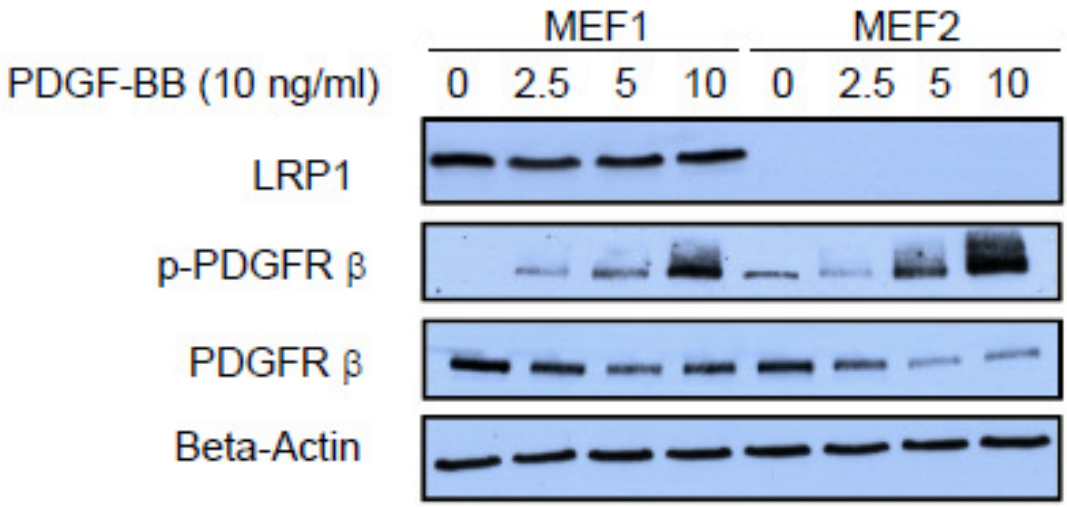


Figure 4.7. LRP1-deficient MEF cells are hyperresponsive to PDGF-BB stimulation. In a time course experiment, LRP1 wt and deficient cells were compared towards their phosphorylation/activation by ligand stimulation. There is a strong difference in both unstimulated and stimulated conditions, with increased activation in the knock-out cells.

LRP1 regulates p21 through growth factor signaling.

p21 is the inhibitor of the cyclin-dependent kinase and a marker of slowed down cell cycle progression. In LRP1 wt mouse embryonic fibroblasts, p21 is solidly expressed. In LRP1 deficient cells, p21 expression is continuously decreased (Figure 4.7, upper panel). There are no changes in expression levels of the p21 regulating transcription factor p53 (data not shown). In a second cellular background of smooth muscle cells, the same difference in p21 expression is observed. However, a second LRP1 deficient cell line carrying a mutation (F2/F2) that interferes with PDGF receptor coupling to PI-3-Kinase displays wild-type comparable levels of p21 again. There is also no difference in p53 expression, strongly suggesting the growth factor signaling mediated downregulation of p21 in the absence of LRP1.

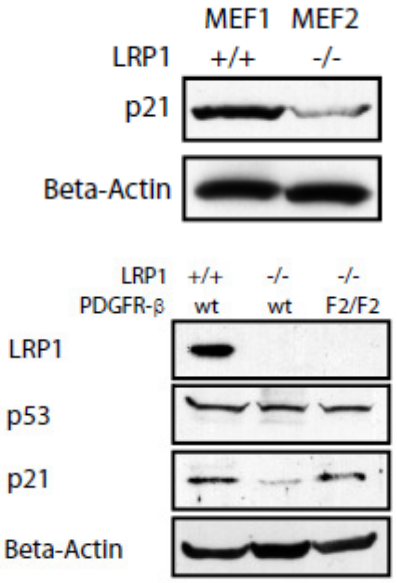


Figure 4.8. LRP1 regulates p21 by modulation of signaling.

Discussion.

In this project, we were able to demonstrate the proliferation control function of LRP1. Although LRP1 is one of the most diverse members of the LDL receptor gene family, the embryonic lethality has made the discovery of individual phenotype contributions difficult. The use of conditional knock-outs, here under the Ranx2-Cre promoter control, may be the only elegant way to successfully unravel the mysterious about the contributions of LRP1 towards different disease etiologies. Our laboratory was previously able to demonstrate the negative regulation of PDGF receptor by LRP1 in atherosclerosis model. This SM22 Cre model has demonstrated cytoskeletal changes, hypercellularity in the vascular wall, activation of TGF-beta and PDGF signaling pathways and aortic elongation. To the best of our knowledge, this is the first report of a model reliably presenting with chondromatous tumors of benign origin. Those exostotic modulations occur in humans at a low frequency and there is no understanding to its molecular pathology.

We have further clarified the exact molecular and cellular mechanism of LRP1/PDGFR interaction. The PDGF receptor activation occurs in a sequence of three steps. The ligand activation, in contrast to the EGF receptor, is required for PDGF receptor dimerization at the membrane level. The dimerization is the first step in its activation process, the second step is the phosphorylation of intercellular domains, the recruitment of signal transducers at the cytoplasmic end

of the receptor and finally its endocytosis. It is not fully understand how the individual steps are contributing to the mitogenic effect, however, we were able to evaluate the influence of LRP1 on these three steps. In dimerization experiments, we were unable to detect differences in the ability to form dimers, suggestive of no involvement of LRP1 in this step. However, co-immunoprecipitation shows the formation of heterodimers between LRP1 and PDGF receptor in a dose-dependent fashion. This seems to be the missing link in understanding the control of PDGF receptor activation by LRP1. This presumably leads to a lack of endocytosis and prevents the overshooting phosphorylation and activation of downstream targets. The effect on phosphorylation seems to be an indirect one. There are reports that suggest the necessity of endocytosis initiation for phosphorylational activation. If endocytosis is prevented from occurrence, phosphorylation might be inhibited.

In summary, we present the first tumor model for periosteal chondromas and additional insight into the critical role for LRP1 as co-receptor for PDGF receptor functioning. Further pharmacological and genetic studies as well as analysis of human specimen are needed to ascertain our findings.

CHAPTER FIVE

DISCUSSION

In this thesis, I attempted to explore the involvement of several LDL gene family members in cellular signaling and regulation of molecular pathways. Lrp4 has a significant role in development throughout different mammalian species, and it s involvement in various cellular signaling pathways is evident. Involvement of Lrp4 in disease mechanisms has yet to be described. The variety of functions indicate this to be of high likelihood. Lrp1 and Lrp1b, whose enormous size makes the exploration of mechanistic functions challenging, are also of particular interest for the modulation of signaling pathways. Often falsely belittled as ‘co-receptors’, their presence has significant implications for neuronal development and function, vascular integrity, and control of proliferation and apoptosis. To fully understand the mechanisms of these receptors, conditional knockout models and correlative genetic studies in humans may lead the way. Their evolutionary age and their versatility indicate that surprises in pathophysiological contexts can be expected.

Chapter 2 - Ectodomains of Lipoprotein Receptor Related Protein Receptors LRP1b and LRP4 in Mice have anchorage-independent function

The aim in Chapter 2 was to study the significance of different receptor components in the mouse. It has been previously known that Lrp4 has several phenotypes, including teeth and bone malformations and agenesis of neuromuscular junctions. A powerful genetic approach was the truncation of the Lrp4 receptor to the extracellular domain only. The interesting observation was that the neuromuscular junction phenotype was abrogated while the bone and tooth phenotype of syndactyly and fused molars persisted. Equally, a frameshift mutation leading to a premature stop of Lrp4 receptor expression is responsible for mule-foot disease in cattle. This implies the intracellular domain in the regulation of bone metabolism, while the extracellular domain is needed for the appropriate formation of neuromuscular junction and therefore viability. We demonstrated the persistent expression of the extracellular domain of Lrp4 in vivo with newly generated antibodies against the ligand binding domain of Lrp4. The levels were approximately equal to the wild-type receptor expression, reducing the possibility of a negative auto-feedback loop by the Lrp4 intracellular domain. I was further able to describe the processing of Lrp4 at the membrane level, providing significant hints towards the elucidation of Lrp4 regulated cellular

signaling. Upon integration in the membrane, Lrp4 is being cleaved by the extracellular metalloprotease ADAM10 and secreted into the extracellular space.

Through our two main study models, the kidney and the neuromuscular junction, we hypothesize that Lrp4 can serve in two different directions within the pericellular space. For the kidney, Lrp4 seems to serve as a scavenger receptor, trapping ligand and neutralizing their effect on Wnt activating signaling pathways. Therefore, Lrp4 preserves a critical threshold to prevent Wnt overactivation in the ureteric bud and warrants appropriate organogenesis.

Conversely, Lrp4 at the neuromuscular junction seems to enhance signaling off the extracellular ligand Agrin and induces clustering around the tyrosine kinase receptor MuSK. This clustering is necessary to induce and facilitate the accumulation of acetylcholine receptors and appropriate innervation of skeletal muscle in a murine background. Further, we were able to demonstrate *in vitro* that the extracellular domain of Lrp4 alone suffices to suppress hyperactive Wnt signaling. It has been previously reported that Lrp4 can counteract the Wnt activating functions of Lrp5 and Lrp6 through competition within the Frizzled complex. This seems to be complemented by the post-cleavage remnant of the extracellular domain, either through scavenging or improved presentation, but most likely a combination of both. Interestingly, our observations in Lrp4 were replicated in a murine model of Lrp1b mutations.

While the complete Lrp1b null mouse is lethal at an early embryonic stage around E7.5, the expression of the expression of the Lrp1b extracellular domain warrants a phenotypically unremarkable survival. This raises the question of the function of the intracellular domain for both receptors, we have demonstrated for Lrp4 and others have demonstrated for the human Lrp1b receptor that the intracellular domain is released in a γ-secretase dependent manner. The intracellular domains in both receptors carry phosphorylation sites for which the exact function is undetermined.

The suspicion has been raised that, very much like Lrp1, the ICDs can translocate to the nucleus and regulate transcriptional activation. It is still surprising that the Lrp1b mutant animals have a normal life span and no sign of tumor development. Other reports have indicated the necessity for extracellular cleavage of Lrp1b by metalloproteases ADAM10 and ADAM17 to release the growth suppressing Lrp1b ICD. There was no inhibition of proliferation when the suspected cleavage site was mutated so that processing at the extracellular level is no longer possible. An unexplored yet likely mechanism is the regulation of a tyrosine kinase receptor at the membrane, comparable to Lrp1 regulation of the PDGF receptor β. A gene expression profile shows the mutual exclusion of mutations in Lrp1b and the epidermal growth factor receptor.

Some of the lessons within the LDL gene family were learned through mechanistic analogies and transfers between the related receptors. While it is

tempting to speculate about the mechanism of Lp1b mediated inhibition of proliferation and tumor control, there are likely several components of the receptor involved that lead to a versatile regulation of several downstream targets from the membrane level.

Chapter 3 – Lrp4 Regulates Initiation of Ureteric Budding and Is Crucial for Kidney Formation in Mammals

Lrp4 is a versatile receptor and is best known for its involvement in development of neuromuscular junctions and the musculoskeletal system. However, another phenotype emerges in the retroperitoneum. In the absence of Lrp4 in our murine model, kidneys fail to form in a majority of cases, either uni- or bilaterally. Several mouse models, including Danforth's short tail mice, present comparably with a combination of skeletal and renal defects in a subpenetrant fashion. However, simulating kidney development in the interplay of epithelial and mesenchymal components *in vitro* is virtually impossible, unless you investigate a single known factor and its effect on one, but not both tissue components. The *in vivo* investigation is complicated by the small tissue amounts under investigation and the unpredictability of whether the investigated organ is actually going to develop. The tested patterns of the known kidney development factors, including the Pax2/8 branching modulators, the c-ret/GDNF/Wnt11 system and both TGF-β and Wnt signaling pathway readouts were all unchanged. This implies a previously unknown pathway in the renal agenesis phenotype. The ureteric budding delay in the Lrp4 mutants can potentially be attributed for the observed phenotype, however, the discrepancy between the observed delays and the actual formed kidneys remains a disturbing disconnect.

The model of ureteric budding delay is further complicated by the observation of several different human phenotypes. These renal defects range from complete agenesis to hypofunctional, hypoplastic, and ectopic kidneys. A budding defect/delay alone, leading to insufficient stimulation of the metanephric mesenchyme, should present in a comparable all-or-nothing fashion like in the murine background. The expression pattern of Lrp4, including the epithelial components of the UB and the renal vesicles could be the answer to that question.

With the technical tools available for this dissertation work, a sufficient answer to the exact mechanism is not possible. Conditional knock-outs with tissue specific deletion of Lrp4 could be one way to approach this task. Regardless of the exact mechanism, Lrp4 seems to have broader implications in humans. As shown in a paper from a genetics group from Germany, none of the described human receptor actually reach the surface. Even though surface biotinylation might not be the most sensitive way, the analysis of the exact mutations show unpaired cysteins that are known to cause the receptor to get trapped in the endoplasmic reticulum. Even more confusing is the question why those humans actually live and breathe, when their Lrp4 is functionally unavailable for the clustering of acetylcholine receptor. As with any research project, for every answered questions several new problems appear at the horizon. The functional versatility of Lrp4 and its involvement in very complex physiological processes will provide food for thought for many dissertations to follow.

Chapter 4 – LRP1 controls osteoblastic proliferation through regulation of the Platelet Derived growth Factor receptor β

This project provided an additional functional insight into the regulation of the PDGF receptor, and provides the first chondroma model in a mouse. Although those chondrocytic tumors are relatively frequent in occurrence in humans, its benign nature and surgical accessibility prevented thorough molecular investigations from understand the exact disease mechanisms. By observation, the knock-out of LRP1 provides a characteristic duplication of this human entity, with radiological and histological similiarities. It is further known that the continuous bone turn over is coordinated between bone resorbing osteoclast and bone forming osteoblast through the PDGF-BB / PDGF receptor axis. The osteoclasts secrete PDGF-BB to attract and activate bone forming osteoblasts. This sensitive cellular homeostasis seems impaired in the absence of LRP1 as a negative modulator of PDGF receptor β.

Additional insight into the molecular function and *in vivo* confirmation is needed to ascertain our suggested mechanism. Currently, investigations are underway to treat these conditions in mice through the small molecule antagonist of PDGF receptor signaling by STI571 (Gleevec). In analogy to a previous study in a LRP1 atherosclerosis model, it is expected that preventative treatment might abrogate or reduce the occurrence of these chondromas. Additional insight will be

gained, for LRP1 and other LDL gene family members, through the use of cell-type specific deletions of the individual gene. While it is exciting to provide a potential mechanism for a human disease entity, it is plausible to assume a broader involvement of several family members in disease context. Genetic correlation studies demonstrate the importance of this, for example the relationship between LRP1b and adenocarcinomas of the lung. With every scientific investigation more questions are asked than solved. It is going to be exciting to now appreciate the cellular signaling function and disease contributions.

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