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Characterization of chicken LRP-2 and its role in embryonic development

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Abstract

LDL receptor-related protein-2 (LRP-2/Megalin/gp330) was initially identified as the pathogenic autoantigen in a rat kidney disease known as Heymann nephritis. Hence, research primarily focused on the role of the protein in the kidney. LRP-2 is a 600kDa member of the low-density lipoprotein (LDL) receptor family, membrane proteins that share structural properties and functional features. The receptor is expressed on the apical surface of polarized epithelial cells, which show high endocytic activity. Although LRP-2 is a classical endocytic receptor, recent findings helped to reveal its prominent role in cell signaling, vitamin homeostasis, and steroid hormone function. To exert these different tasks, LRP-2 is a promiscuous receptor for various ligands including lipoproteins, vitamin-binding proteins, hormones, and enzymes.

LRP-2, which is important for the developing and adult organism, is highly expressed in organs including mammalian kidney, lung, small intestine, and male and female reproductive tracts. During development the receptor can be found in various embryonic as well as extraembryonic tissues. Significantly, LRP-2 is highly expressed in the yolk sac, which is mostly of evolutionary importance in mammals, but is essential in avian embryogenesis. Therefore, the chicken serves as an excellent model organism not only to study lipid metabolism, but also to analyze embryogenesis. Although expression and functions of mammalian LRP-2 are well characterized, the roles of its avian homologue need further investigation.

By analyzing chicken LRP-2 expression patterns, possible receptor functions may be elucidated. Here I revealed that LRP-2 is highly expressed in the chicken yolk sac, which is composed of an outer layer of interlinked mesenchymal cells pervaded by blood vessels, and an inner single layer of large endodermal endothelial cells (EEC) facing the yolk. To localize the receptor in the yolk sac, immunohistochemistry showed that LRP-2 is expressed on the surface of EECs. Western blotting experiments confirmed these results. LRP-2 expression in the yolk sac can be observed in the first week of incubation, it culminates at the beginning of the third week and then slowly decreases. This observation correlates with the fact that transport processes operate at highest levels in the last trimester of chicken development. Therefore, LRP-2 expression in the membrane of EECs may mediate

nutrient uptake from the yolk. In contrast, LRP-2 expression steadily increases during kidney development and persists in the adult organ. It is important to note that LRP-2 expression levels in the adult kidney differ significantly between male and female animals. A possible explanation for this observation could be the influence of sex hormones. This notion is supported by my data showing increased LRP-2 expression in the rooster kidney following estrogen treatment. Future studies should provide better insights into the hormonal regulatory mechanisms of receptor expression.

The data obtained from studies on aspects of chicken development support the crucial role of the yolk sac; the underlying mechanisms are planned to be elucidated in ongoing studies.

Zusammenfassung

LRP-2 (Megalin/gp330) wurde ursprünglich als pathogenes Autoantigen in Nieren von Ratten gefunden, die an Heymann Nephritis erkrankt waren. Von diesem Zeitpunkt an war die Forschung auf die Funktionsaufklärung des Proteins in der Niere fokussiert. LRP-2 ist ein Membran-gebundenes, 600kDa großes Mitglied der LDL Rezeptor Familie, die sich durch strukturelle Gemeinsamkeiten und ein ähnliches Funktionsspektrum auszeichnet. Der Rezeptor wird an der apikalen Oberfläche von polarisierten Epithelzellen exprimiert, die eine hohe endozytotische Aktivität aufweisen. Obwohl LRP-2 hauptsächlich die Endozytose verschiedenster Liganden vermittelt, weisen neueste Forschungsergebnisse auf weitere Funktionen hin. Zu diesen neuen Wirkungsgebieten zählen Signaltransduktion, Vitamin Homöostase und Steroidhormonfunktion.

LRP-2 ist nicht nur im adulten Organismus wichtig, sondern auch für die Embryogenese essentiell. Niere, Lunge, Dünndarm und weibliche und männliche Reproduktionsorgane gehören zu den Geweben mit der höchsten LRP-2 Expression in Säugetieren. Während der Embryonalentwicklung wird LRP-2 sowohl im Embryo als auch in extra-embryonalen Geweben exprimiert. In Säugetieren ist der Dottersack, in dem hohe Mengen an LRP-2 vorhanden sind, strukturell und funktionell weitgehend von evolutionärer Bedeutung. Im Gegensatz dazu ist der Dottersack ein zentraler Bestandteil der embryonalen Entwicklung bei Vögeln. Deshalb ist das Huhn nicht nur für die Erforschung des Lipidstoffwechsels, sondern auch für Studien während der Embryonalentwicklung ein exzellenter Modellorganismus. In Säugetieren ist die Expression und Funktionsweise von LRP-2 weitgehend aufgeklärt, im Huhn jedoch bedarf es noch gründlicher Erforschung des homologen Proteins.

Die Analyse der Expressionsmuster von LRP-2 kann zur Funktionsaufklärung beitragen. Der Dottersack besteht aus zwei Schichten, einer äußeren Schicht mesenchymaler Zellen, die mit Blutgefäßen durchzogen sind und einer inneren Einzelschicht, die aus großen endodermalen Endothelzellen (EEC) zusammengesetzt ist und zum Dotter weist. Der Rezeptor wird im Dottersack stark exprimiert. Mit Hilfe von Immunhistochemie konnte LRP-2 an der Oberfläche von EECs lokalisiert

werden. Diese Ergebnisse konnten durch Western-Blot Experimente bestätigt werden. Die Expression des Rezeptors wird in der ersten Entwicklungswoche sichtbar, erreicht ihren Höhepunkt am Anfang der dritten Woche und sinkt dann langsam wieder ab. Dieses Expressionsmuster korreliert mit den intensiven Transportprozessen von Nahrungsbestandteilen, die im letzten Trimester der Entwicklung stattfinden. Die Expression von LRP-2 in der Membran von EECs ist daher wahrscheinlich an der Nährstoffaufnahme aus dem Dotter beteiligt.

Im Gegensatz zur Situation im Dottersack, kommt es zu einem kontinuierlichen Anstieg der LRP-2 Expression während der Nierenentwicklung, die im erwachsenen Tier den höchsten Level erreicht. Dabei zeigt sich, dass es signifikante Unterschiede zwischen männlichen und weiblichen Tieren gibt. Geschlechtshormone könnten eine mögliche Erklärung für diese Beobachtung sein. Es zeigt sich, dass es nach Östrogen-Injektionen zu einem starken Anstieg der LRP-2 Expression in Hähnen kommt. Weitere Studien könnten über die hormonellen Regulationsmechanismen der Rezeptor-Expression Auskunft geben.

Die Daten, die im Zuge dieser Arbeit ermittelt werden konnten, haben einige Aspekte der Embryonalentwicklung des Huhns aufgeklärt und die wichtige Rolle des Dottersacks aufgezeigt. Die Mechanismen, die diesen Prozessen zugrunde liegen, sollen in weiteren Studien untersucht werden.

Chapter 1

Introduction

1.1 The Low-Density Lipoprotein (LDL) Receptor Family

1.1.1 Overview

The evolutionary conserved LDL receptor family of closely related cell-surface receptors can be found in mammals and other organisms as well. Initially identified as endocytic receptors, recent findings indicate new roles for this ancient protein family. LDL receptor relatives (LRs) mediate the binding, internalization, and degradation of various ligands, a process called receptor-mediated endocytosis [Nykjaer and Willnow, 2002]. Besides the regulation of lipid metabolism, LRs are involved in embryonic development, nutrient and vitamin transport, and signal transduction. Latest evidence suggests significant novel physiological roles of individual LRs, which have yet to be fully elucidated [May et al., 2007].

1.1.2 Structural Properties of the LDL Receptor Family

The members of the LDL receptor family are trans-membrane glycoproteins, which comprise large extracellular and relatively small intracellular domains. This protein family shares structural motifs and functional features [Hussain, 2001].

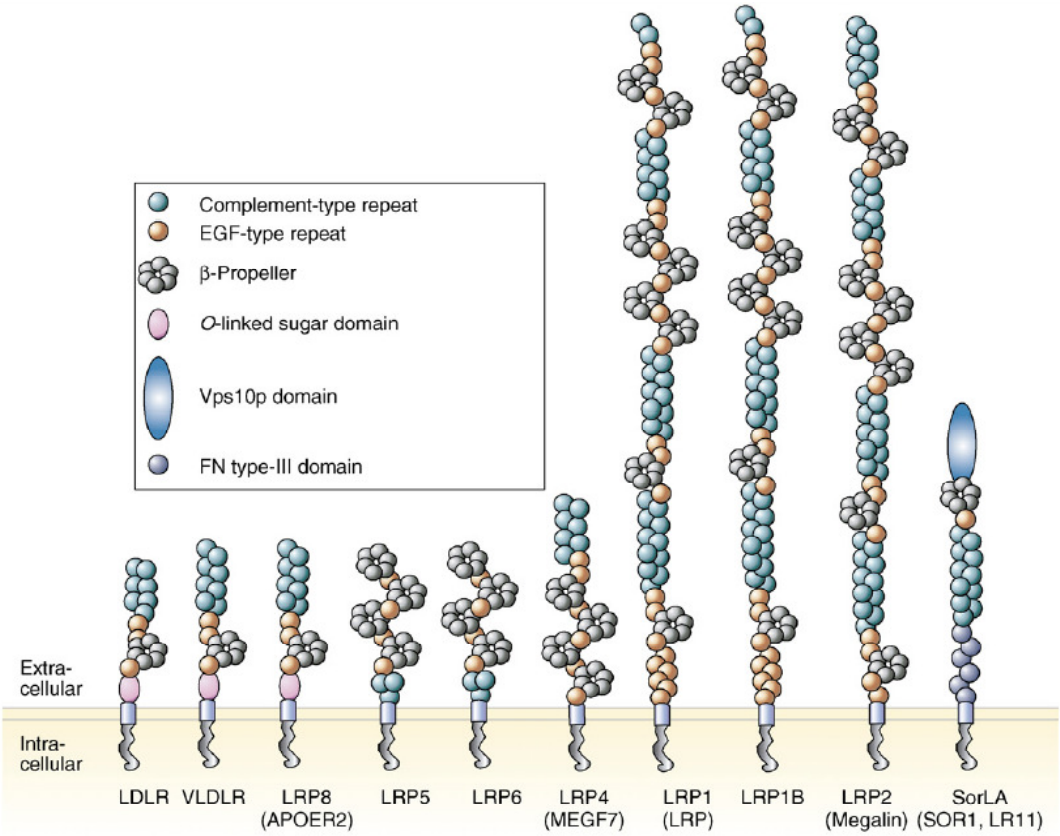

Figure 1.1. Structure of the LDL receptor family members. Most receptors on the picture constitute core members of the protein family. More distantly related members include LRP-5, LRP-6, and SorLA, which are characterized by unique combinations of motifs. See text for more details [Willnow et al., 2007]. (source: Andersen and Willnow, 2006)

There are five characteristic building blocks of LDL receptor family members, which constitute these proteins in various compositions. The extracellular part of the receptors contains ligand-binding domains, formed by cysteine-rich complement-type repeats. The epidermal growth factor (EGF) precursor homology domain, containing cysteine-rich growth factor repeats and YWTD (Tyr-Trp-Thr-Asp) sequences, is essential for the pH-dependent release of the ligands in the endosomes. O-linked

sugar domains constitute serine and threonine-rich stretches on the extracellular part of the receptor.

The trans-membrane spanning domain anchors the receptor to the membrane. All cytoplasmic domains have unique recognition sites for adaptor proteins suggesting different fates for ligands internalized by individual receptors. At least one NPXY (Asn-Pro-Xxx-Tyr; Xxx stands for any amino acid) sequence can be found in the intracellular domain, which mediates the clustering of LDL receptor family members into clathrin-coated pits [Schneider and Nimpf, 2003; Willnow et al., 2007].

1.1.3 Functional Features of Prominent LDL Receptor Family Members

LDL Receptor

The first member of the LDL receptor family discovered was the LDL receptor (LDLR), a 160kDa protein controlling plasma cholesterol levels. The LDLR binds lipoproteins by the recognition of apolipoprotein E (apoE) and apolipoprotein B-100 (apoB-100), which can be found on the surface of certain lipoprotein particles. Hence, receptor-mediated endocytosis is a main regulatory mechanism for cholesterol homeostasis. The founding member of the LDL receptor family seems to be the only one exclusively involved in lipid metabolism [Hussain, 2001].

Genetic defects in the LDL receptor result in the inability to clear lipoproteins from the bloodstream, which leads to the accumulation of LDL particles in the circulation [Nykjaer and Willnow, 2002]. Autosomal, dominantly inherited familial hypercholesterolaemia (FH) is the disease caused by mutations in the LDLR gene, which comes along with early atherosclerosis and premature cardiovascular disease (CVD). Hundreds of mutations can be attributed to five classes of disease-causing mutations at the LDLR gene locus [Brorholt-Petersen et al., 2002].

The chicken LDLR has been conserved throughout evolution, sharing characteristic features with the mammalian receptor; it supplies lipoprotein-derived cholesterol for steroid production in ovarian follicular cells [Schneider, 2007].

LDLR-related Protein

The LDLR-related protein (LRP-1) is a 600kDa multifunctional cargo receptor that removes its ligands from the surface of various types of cells. LRP-1 is ubiquitously expressed and can mostly be found in vascular smooth muscle cells (SMCs), hepatocytes, and neurons. The functions of the receptor range from lipoprotein transport to regulation of cell surface protease activity, control of tissue invasion, and protection against atherosclerosis. Recent studies showed a crucial role of LRP-1 in signaling functions including cell migration and proliferation, and the regulation of vascular permeability [May et al., 2007].

The chicken protein is conserved, showing 83% overall identity with human LRP-1. After post-translational cleavage, a 515kDa extracellular subunit is produced. Chicken LRP-1 binds Ca^{2+}, α_2-macroglobulin, and vitellogenin. The receptor is not expressed in oocytes, but coevolved with genes playing a role in the reproduction of avian species [Schneider, 2007].

VLDL Receptor, LR8

The very low-density lipoprotein (VLDL) receptor, a member of the LDL receptor gene family with eight ligand-binding repeats, is predominantly expressed in mammalian heart, skeletal muscle, and adipose tissue [Nimpf and Schneider, 1998]. This highly conserved receptor (95kDa) is similar to the LDLR, just containing one additional complement-type repeat in the ligand-binding domain. This protein is involved in the extrahepatic metabolism of triglyceride-rich lipoproteins, having an affinity for apoE. Moreover, the VLDLR binds lipoprotein-unrelated ligands including urokinase-type plasminogen activator and plasminogen activator inhibitor complexes, thrombospondin, and reelin [Schneider and Nimpf, 2003].

In egg laying species, the VLDLR homologue is also termed LR8, due to its eight ligand-binding repeats. This endocytic receptor is crucial for reproduction by mediating specific uptake of VLDL and vitellogenin (VTG) into growing oocytes. Furthermore, LR8 is also expressed on the surface of endodermal endothelial cells (EECs) of the extraembryonic yolk sac, where it mediates the uptake of VLDL from the yolk and subsequent transport into the embryo [Hermann et al., 2000].

A point mutation at the *lr8* locus can be observed in a mutant chicken strain, called "restricted ovulator" (R/O). These hens are not able to lay eggs due to the inability of the oocytes to enter the rapid growth phase. As a result of sustained VLDL and VTG synthesis in the liver, R/O females develop severe hyperlipidemia and atherosclerotic features [Schneider and Nimpf, 2003].

1.2 The Role of LRP-2/Megalin/gp330

1.2.1 Overview

LRP-2 is a very large, multiligand member of the LDL receptor gene family. Initially termed gp330, the receptor was originally identified as the pathogenic antigen in Heymann nephritis (HN), an autoimmune disease in rats. This rat kidney disease presents an excellent animal model for studying human membranous glomerulonephritis.

After purification from HN rat glomeruli, the antigen was named gp330 due to the molecular weight estimated by its mobility during gel elecrophoresis. First, the receptor was found in the renal proximal tubule and in glomeruli, but subsequently additional epithelia expressing LRP-2 were identified. Many ligands were determined, including the receptor-associated protein (RAP), lipoproteins, and enzymes and their inhibitors [Christensen and Birn, 2001; Orlando et al., 1992].

When in 1994 cDNA cloning and sequencing was completed by Saito et al., the name megalin was suggested due to its large size. The 4660 amino acid (aa) sequence was expected to constitute a mature glycoprotein of 516.715kDa [Saito et al., 1994]. The generation of a megalin-deficient mouse was another step to gain further information about the role of this receptor. Megalin knockout mice manifest abnormalities in epithelial tissue including lung and kidney, and die perinatally from respiratory insuffiency. In the brain, holoprosencephaly is caused by impaired development of the neuroepithelium [Willnow et al., 1996].

1.2.2 Structural Features of LRP-2

LRP-2, a 517kDa single-chain receptor, has a large amino-terminal extracellular domain, one transmembrane domain, and a short carboxy-terminal cytoplasmic tail. It is the largest known mammalian single-chain receptor. The human gene, located on chromosome 2, shows 77% homology to the rat DNA sequence. The non-glycosylated form has a molecular weight of 517kDa, but after posttranslational modification the receptor becomes heterogenously glycosylated with various forms of N-glycans and oligo/poly-α2,8-deaminoneuraminic acid is added, to result in a molecule with an approximate molecular mass of 600kDa. A 25 amino acid sequence, adjacent to the amino-terminus, represents a potential signal peptide sequence [Christensen and Birn, 2002].

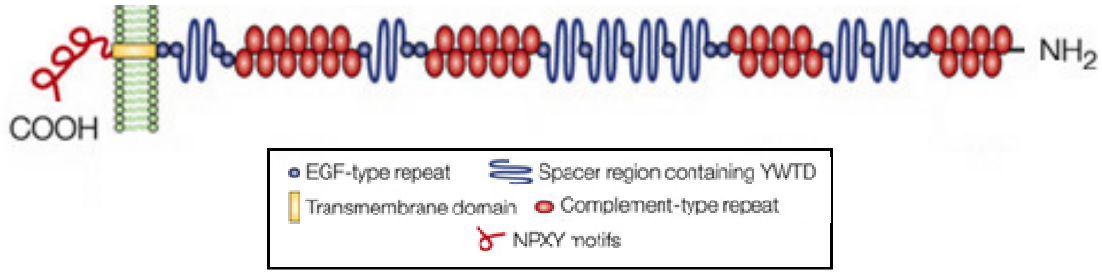

Figure 1.2. Schematic diagram depicting the modular structure of LRP-2. (Adapted from Christensen and Birn, 2002)

Four clusters of cysteine-rich complement-type repeats constitute the ligand-binding regions, which are separated by a total of 17 EGF-like repeats. Eight cysteine-poor spacer regions contain YWTD motifs. The single transmembrane domain is connected to the cytoplasmic tail, which varies between different gene family members. Three NPXY motifs can be found on the carboxy-terminus, responsible for clustering the receptor into coated pits. Additionally three conserved Src-homology binding regions, three proteinase kinase C phosphorylation sites, and seven casein kinase II sites mediate the binding to cytoplasmic adapter proteins [Christensen and Birn, 2001; Moestrup and Verroust, 2001].

1.2.3 Tissue Distribution of LRP-2

LRP-2, an integral membrane protein, is expressed on the apical surface of polarized epithelial cells. The receptor can be found in various adult tissues including the intestinal brush border, gallbladder epithelium, thyroid follicular cells, and type II pneumocytes of the lung and in the kidney. LRP-2 is also expressed in the mammary epithelium, uterus and oviduct, and in the male reproductive tract [Fisher and Howie, 2006].

Since LRP-2 operates in concert with cubilin, which is a peripheral membrane glycoprotein, a co-localization can be observed in various tissues [Christensen and Birn, 2002].

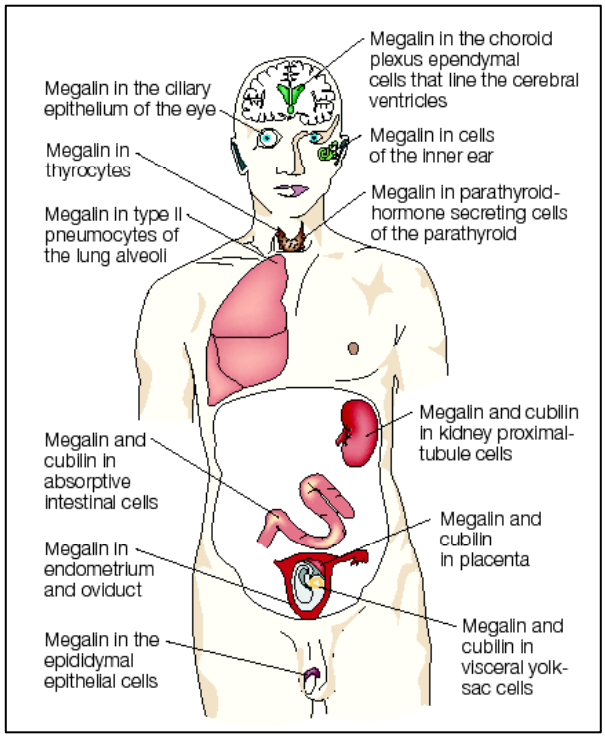

Figure 1.3. Organs expressing LRP-2 and cubilin. (source: Christensen and Birn, 2002)

The role of this receptor during embryonic development is pinpointed by its wide distribution in embryonic and extraembryonic tissues. After fertilization, rapid cell division results in the formation of a blastocyst, which marks the endpoint of pre-implantation development [Fisher and Howie, 2006]. LRP-2 can be detected in rat and mouse embryonic day 4 trophoectodermic cells, which constitute the outer wall of

the blastocyst and finally form the fetal part of the placenta. During development the primitive endoderm cells differentiate into the visceral endoderm (VE) of the yolk sac, in which the receptor is also present. LRP-2 is highly expressed in the neuroepithelium, the ventricular zone of the developing brain ventricles and the spinal cord, the neural retina, the sensory epithelia of the vestibular and cochlear organs, and the olfactory epithelium, demonstrating its crucial role in brain development [Christensen and Birn, 2002; Kozyraki and Gofflot, 2007].

In the early stages of renal development, LRP-2 can be detected in the mesonephric tubules and the early metanephric renal vesicles, but later on expression becomes restricted to glomerular and proximal tubule cells [Christensen and Birn, 2002].

1.2.4 LRP-2 is a Multifunctional Receptor

LRP-2 is a multifunctional receptor, which binds and internalizes various kinds of ligands including lipoproteins, protease-protease inhibitor complexes, vitamin-vitamin binding protein complexes, and hormones [May et al., 2007]. A summary of the so far identified ligands is given in Table 1.1. The promiscuity of the receptor can be partly explained by its 36 complement-type repeats, encoding many possible recognition sites, but the number of repeats cannot fully explain so many structurally unrelated ligands. The cooperation with co-receptors like cubilin could be a strategy to broaden its ligand spectrum [Nykjaer and Willnow, 2002]. Plasma proteins like apoE, lipoprotein lipase, lactoferrin, and aprotinin bind to the second ligand-binding region. The intracellular chaperone, receptor associated protein (RAP), is able to bind to several binding sites, thereby inhibiting the binding of most ligands. Ligand binding of LRP-2 depends on the presence of calcium, which stabilizes binding sites [Kozyraki and Gofflot, 2007].

Although ligands vary in structure and function, various groups can be distinguished. Proteins with a carrier function for vitamins, lipids, hormones, and minerals constitute a group of nutritionally relevant ligands. The nutritional aspect of protein uptake by LRP-2 applies especially to its function in the yolk sac [Moestrup and Verroust, 2001].

Vitamin-binding proteins Transcobalamin–vitamin B_{12} Vitamin-D-binding protein Retinol-binding protein Folate-binding protein	***Lipoproteins*** Apolipoprotein B Apolipoprotein E Apolipoprotein J/clusterin Apolipoprotein H Apolipoprotein M	***Immune- and stress-response-related proteins*** Immunoglobulin light chains PAP-1 β_2-microglobulin
Steroid hormone binding proteins Sex hormone-binding protein-estrogens Sex hormone-binding protein-androgens	***Hormones and hormone precursors*** Parathyroid hormone Insulin Epidermal growth factor Prolactin Thyroglobulin Leptin	***Enzymes and enzyme inhibitors*** PAI-1 PAI-1–urokinase PAI-1–tPA Pro-urokinase Lipoprotein lipase Plasminogen β-amylase β_1-microglobulin Lysozyme
Other carrier proteins Albumin Lactoferrin Hemoglobin Myoglobin Odorant-binding protein Transthyretin Metallothionein	***Drugs and toxins*** Aminoglycosides Polymyxin B Aprotinin Trichosanthin Somatostatin analogues	***Others*** RAP Ca^{2+} Cytochrome *c* Bone morphogenic protein 4 Sonic hedgehog

Table 1.1. Ligands of LRP-2. (adapted from: http://www.recepticon.com/introduction.htm; Kozyraki and Gofflot, 2007)

Furthermore, binding of proteins like albumin or immunoglobulin G light chains suggests a protein-rescuing function of the receptor. Another ligand group including enzymes, enzyme-protein complexes, and toxins, is cleared by LRP-2, indirectly regulating the toxicity of substances in tissue fluids [Moestrup and Verroust, 2001].
Latest findings have revealed that the endocytic uptake by the receptor is not only involved in cargo transport, but also mediates superordinate regulatory processes. LRP-2 is able to regulate the availability of signaling molecules by limiting their activity or by delivering signaling molecules to their target cells [May et al., 2007].

Endocytosis and the Kidney

Because LRP-2 was initially identified as a receptor important for renal proximal tubule function, research primarily focused on its role in endocytosis, binding and internalizing dietary sterols [Fisher and Howie, 2006].
The renal proximal tubules are important for the recycling of various solutes after the filtration through the glomerulus. Low molecular weight metabolites are bound and internalized, which otherwise would be lost in the urine. In addition to water, other blood components including ions, glucose, and amino acids are absorbed in the

proximal tubule. Plasma proteins, like plasma carrier proteins, peptide hormones, and lysozyme, are also filtered through the glomerulus and subsequently reabsorbed. The epithelial cells of the proximal convoluted tubule (PCT) are the cells responsible for the uptake of macromolecules. Tissues involved in endocytic activities often increase their surface by the formation of microvilli, thus creating an apical brush border. Furthermore, clathrin-coated pits and members of the endocytic apparatus like endosomes, lysosomes, and dense apical tubules facilitate rapid uptake of ligands [Leheste et al., 1999].

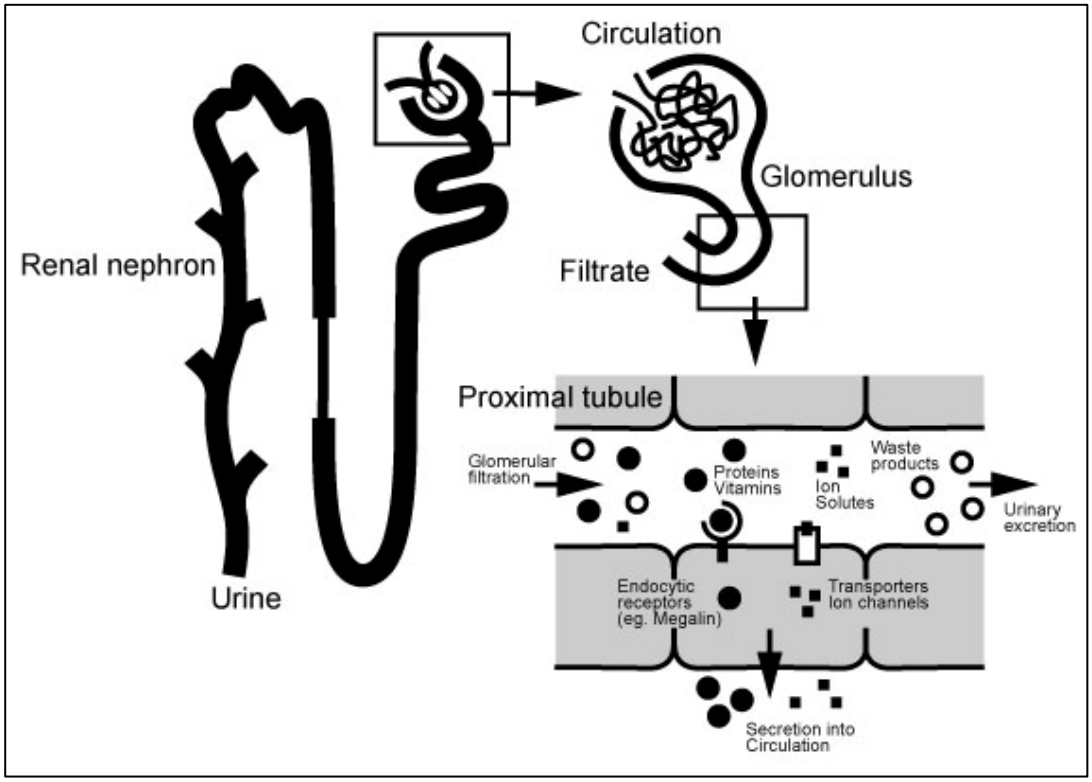

Figure 1.4. A schematic illustration of the kidney and receptor-mediated endocytosis in the renal proximal tubule. (source: http://www.recepticon.com/megalinreceptor.htm)

LRP-2 is a key receptor for the uptake of proteins in the PCT. Several grams of protein are internalized and degraded each day, but the rescue mechanism for protein-bound components may be physiologically even more important [Moestrup and Verroust, 2001].

LRP-2 and Embryonic Development

In mammals, LRP-2 is expressed prior to implantation (the attachment to the wall of the uterus) of the embryo. The blastocyst, composed of the inner cell mass (ICM) and the trophoectoderm (TE), is formed after several cell divisions. In early post-implantation blastocysts, the receptor mediates transport of dietary sterols particularly into the neuroepithelium [Fisher and Howie, 2006].

Yolk sacs can be found in all species, displaying different structures and functions. However, in the nematode *Caenorhabditis elegans* (*C. elegans*) for instance a LRP-2 homologue plays an important role in growth and development, mediating sterol uptake. In other non-mammalian model organisms including zebrafish and fruitflies, LRP-2 expression is important for embryonic development. In rodents, endodermal cells migrate from the ICM to the blastocoelic cavity finally forming the yolk sac structure around embryonic day 7. After nine days it completely surrounds the embryo and the absorbtive surface is directed towards the maternal circulation. Until embryonic day 10, this remains the only interface between the mother and the embryo. Then, an allantoic membrane is generated [Moestrup and Verroust, 2001]. To conclude, LRP-2, expressed in the endodermal cells of the yolk sac, is needed for normal growth, nutrition, and development from pre-implantation to blastocyst implantation in rodents [Fisher and Howie, 2006].

The receptor is crucial for development not only at an early embryonic stage, it is also involved in proper organogenesis. During formation of the brain, the central nervous system (CNS), and sensory organs, LRP-2 is a crucial regulator of development. In epithelia of the kidney, respiratory tract, foregut, stomach, and small intestine the receptor is also involved in organogenesis [Fisher and Howie, 2006].

Recent evidence showed that LRP-2 has a biological function as a signal transducer. The interaction of the receptor with bone morphogenic factor 4 (BMP4) demonstrates an involvement in the sonic hedgehog (Shh) signaling pathway. BMP4, a negative regulator of Shh, accumulates in LRP-2 deficient animals, thereby reducing Shh signaling and causing impaired forebrain development [May et al., 2007].

Another mechanism involves receptor action in the kidney. In the course of a Notch-like pathway, the cleaved intracellular domain of LRP-2 may be involved in signaling processes, which have to be elucidated in the future [May et al., 2007].

Vitamin Metabolism

Three vitamin-binding proteins are ligands for LRP-2. These vitamin-carriers include retinol-binding protein (RBP), vitamin D-binding protein (DBP), and transcobalamin-transporting vitamin B_{12} [Christensen and Birn, 2002]. The carrier, bearing vitamin, is taken up and degraded, whereas the vitamin is internalized and recycled back to circulation [Moestrup and Verroust, 2001]. The vitamin in complex with its binding protein is reabsorbed in the renal proximal tubule during the filtration, which is important for the re-uptake of vital substances reducing their loss in the urine. During embryonic development a similar mechanism is supposed to mediate the vitamin transport in the yolk sac and the placenta [Christensen and Birn, 2002].

In the circulation, vitamin D is generally bound to DBP, a steroid-binding protein with high affinity for LRP-2. The receptor, in cooperation with cubilin, mediates DBP uptake in the kidney. This mechanism not only prevents protein excretion, but also leads to 25-(OH) vitamin D_3 activation by the hydroxylation to 1,25-$(OH)_2$ vitamin D_3, thus regulating calcium homeostasis [Christensen and Birn, 2002].

In LRP-2 deficient mice, the loss of the receptor results in the elevated excretion of 25-(OH) vitamin D_3 and DBP in the urine. The lack of vitamin D corresponds with bone mineralization defects [May et al., 2007].

LRP-2 reabsorbs vitamin A and vitamin B_{12} and their binding proteins in a similar way, thereby contributing to vitamin homeostasis [May et al., 2007].

Steroid Hormone Uptake

Steroid hormones are regulators of vertebrate metabolism, reproduction, and embryonic development. These signaling molecules bind to target cells, enter them, and interact with transcription factors, thereby regulating gene expression. Steroids are bound to carrier proteins in the circulation. After reaching their target, these hormones are released and transverse the plasma membrane by free diffusion due to their small size and lipophilic nature. The classical free hormone hypothesis states that only unbound hormones are biologically active. However, latest research indicates the importance of specific mechanisms for hormone uptake [Lin and Scanlan, 2005].

Since, LRP-2 is expressed in steroid-responsive organs of male and female reproductive tracts, it seems to be important for the active transport of steroid hormones. The receptor is responsible for the uptake of androgens and estrogens in complex with the sex hormone binding globulin (SHBG). Because of the lack of the receptor, LRP-2 knockout mice show impaired male and female reproductive tract development beside other defects [Hammes et al., 2005].

Even though there are normal levels of hormones circulating in the blood, LRP-2 deficient embryos fail to acquire enough sex steroids for reproductive tract maturation [Willnow et al., 2007].

To conclude, LRP-2 appears to be crucial for the functionality of tissues, which depend on high levels of steroid hormones [Lin and Scanlan, 2005].

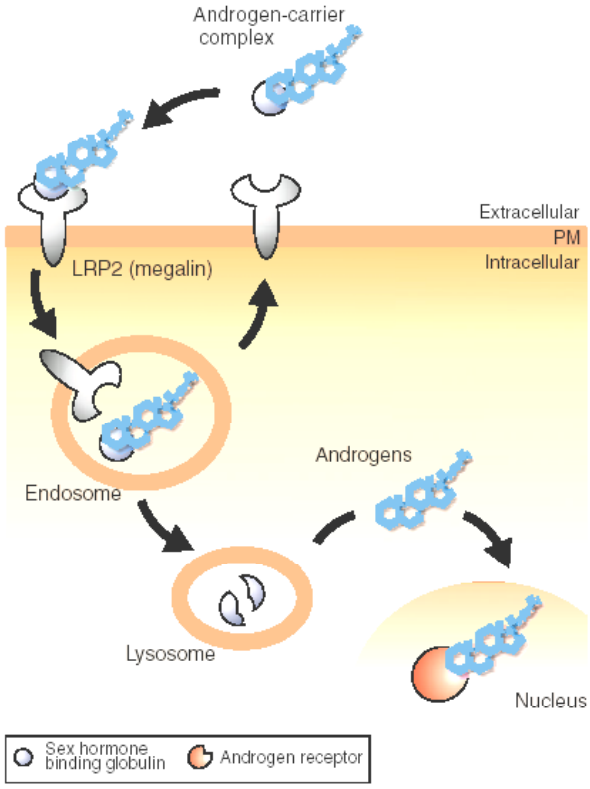

Figure 1.5. LRP-2 mediates the uptake of sex hormones bound to carrier proteins. Androgens are solubilized by the sex hormone-binding globulin (SHBG). The uptake of SHBG-bound androgens requires LRP-2 binding to SHBG, which is subsequently internalized by receptor-mediated endocytosis. SHBG is degraded in lysosomes, while the hormone enters the cytoplasm to regulate gene expression [Willnow et al., 2007]. (source: Willnow et al., 2007)

1.3 The Chicken, a Powerful Model Organism to study Lipid Metabolism

1.3.1 Introduction

The chicken, *Gallus gallus*, is a model organism with a long history. Initially, it has been used for developmental biology studies, but it has also contributed new insights into immunology, genetics, virology, cancer, and cell biology [Stern, 2005].
In regards to lipid-related events, the laying hen as well as the developing embryo provide excellent systems for research on lipid metabolism [Speake et al., 1998].

1.3.2 Lipid Metabolism in the Laying Hen

The development and growth of female germ cells in oviparous (egg-laying) species, called oogenesis, involves extensive lipid transport, mediated by receptor-mediated endocytosis [Schneider, 2007].
Under the control of estrogen, yolk precursors are synthesized in the maternal liver [Speake et al., 1998]. Receptor-mediated endocytosis is responsible for the uptake of precursors, including VLDL and vitellogenin (VTG), by growing oocytes. A repertoire of gradually sized oocytes undergoing rapid growth is generated in the stroma. The largest follicle, designated F1, contains the next oocyte to ovulate. The five largest follicles are termed F1 to F5, a discrimination of smaller ones is difficult. After entering the oviduct, fertilization of the oocyte occurs if the hen has been inseminated. Structural components develop around the oocyte during the passage through the oviduct [Schneider, 2007].
The oviduct is a long and flexible tube, which develops from the left ovary. In contrast the right ovary degenerates during embryonic development and is rudimentary present until the first day of hatching. The oviduct can be subdivided into five sections (see Figure 1.6), namely infundibulum, magnum, isthmus, uterus, and vagina [Schwarze and Schröder, 1985].

While the oocyte traverses the oviduct for 25 hours, structural components, egg white proteins, water, egg membranes, and the shell are generated to result in a laid egg [Schneider, 2007].

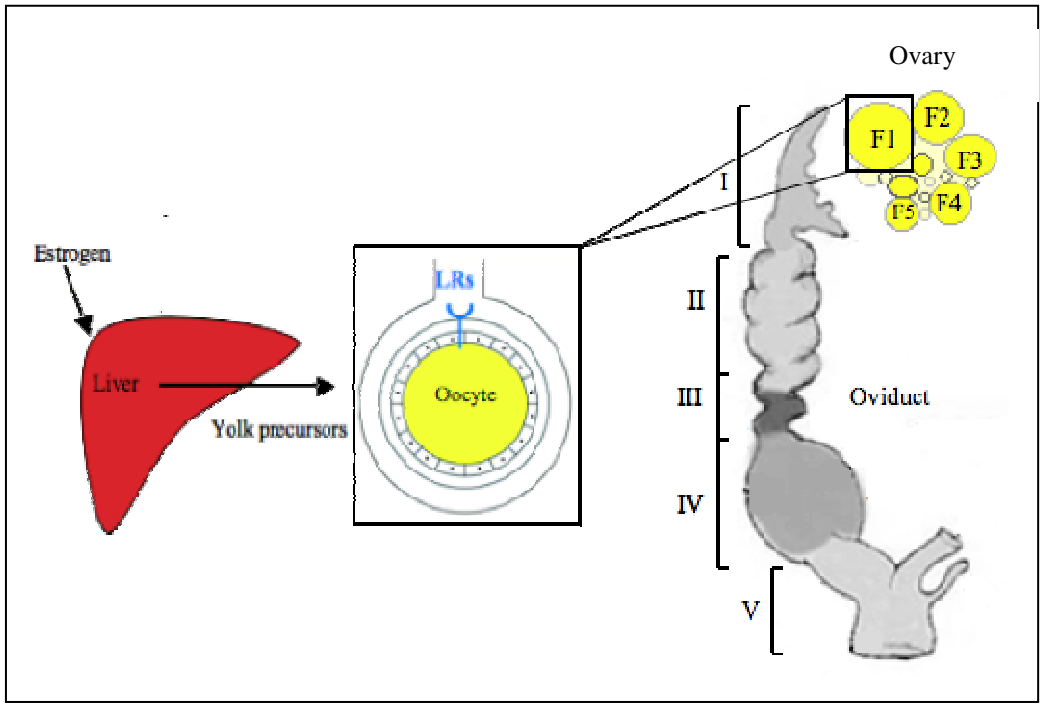

Figure 1.6. Schematic illustration of the oocyte development. Receptor mediated endocytosis is responsible for the uptake of yolk precursors, produced by the maternal liver under the influence of estrogen. The steadily growing follicles are designated in a size-dependent manner, F1 to F5. The biggest follicle (F1) contains the oocyte, which is the next to ovulate. After ovulation and possible fertilization, the oocyte travels through the oviduct to finally result in the laid egg. The five sections of the oviduct indicated in the figure are infundibulum (I), magnum (II), isthmus (III), uterus (IV), and vagina (V).
(adapted from: http://people.eku.edu/ritchisong/avianreproduction.html)

1.3.3 Embryonic Development of the Chicken

The development of the chicken embryo constitutes an outstanding model for lipid and lipoprotein transport mechanisms. The yolk is the exclusive source of nutrients for the embryo including lipids, carbohydrates, and protein [Hermann et al., 2000].
In the early stages of development, extraembryonic structures including amnion, allantois, and yolk sac are formed. The embryo floats inside the liquid-filled amniotic cavity. The allantois, adjunctive to the hindgut of the embryo, quickly increases in

size until the amnion and the yolk sac are enclosed [Schwarze and Schröder, 1985]. In the lumen of the allantois excretion products are stored. On the outside, the allantois fuses with the chorion to give rise to the chorioallantois. This membrane is the site for oxygen and carbon dioxide exchange through the pores of the eggshell [Bellairs and Osmond, 2005]

The avian yolk sac is in many respects analogous to the mammalian placenta, which also provides nutrients for the embryo. The membrane of the yolk sac is responsible for the uptake of yolk constituents and their transfer into the embryonic circulation [Speake et al., 1998].

During the first two weeks of development very little lipid uptake occurs. The last week of embryogenesis is characterized by a massive rate of yolk lipid transport via the yolk sac membrane [Hermann et al., 2000]. The progressive withdrawl of the yolk sac into the abdominal cavity starts around day 19 and is completed before hatching. The yolk sac remains the only nutrient supply for several days after hatching, mediating the uptake of about 5 grams of yolk [Speake et al., 1998].

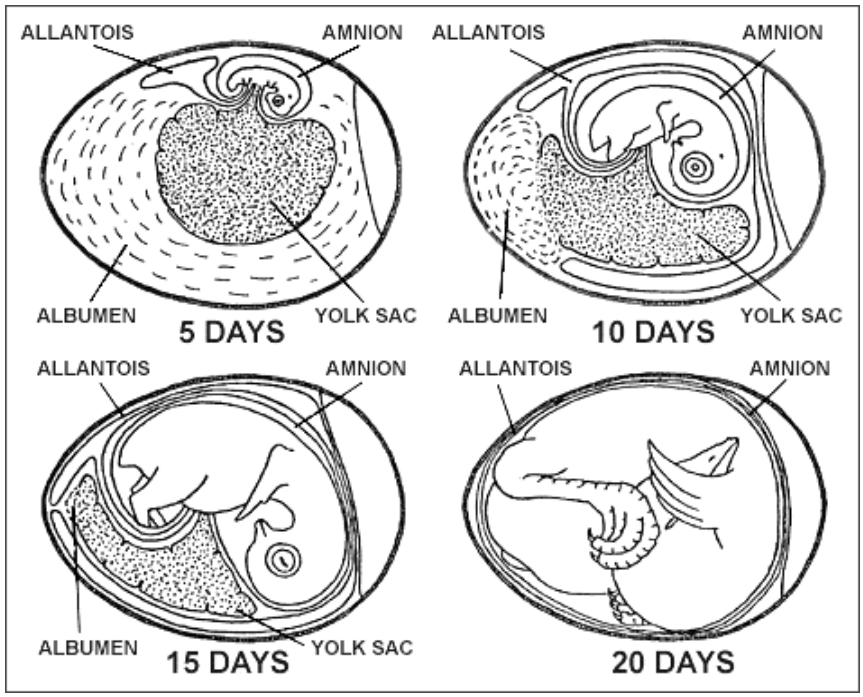

Figure 1.7. Illustration of the progressive growth and development of the chicken embryo and the extraembryonic membranes. The position of the three most important membranes including yolk sac, allantois and amnion are indicated in the picture.

(source: http://chickscope.beckman.uiuc.edu/resources/egg_to_chick/development.html)

The avian yolk sac is composed of two layers, an outer layer of mesenchymal cells (mesoderm) pervaded by fetal blood vessels, and an inner single layer of large, yolk-laden endodermal cells. These endodermal endothelial cells (EEC), facing the yolk compartment, express lipoprotein receptors [Hermann et al., 2000].

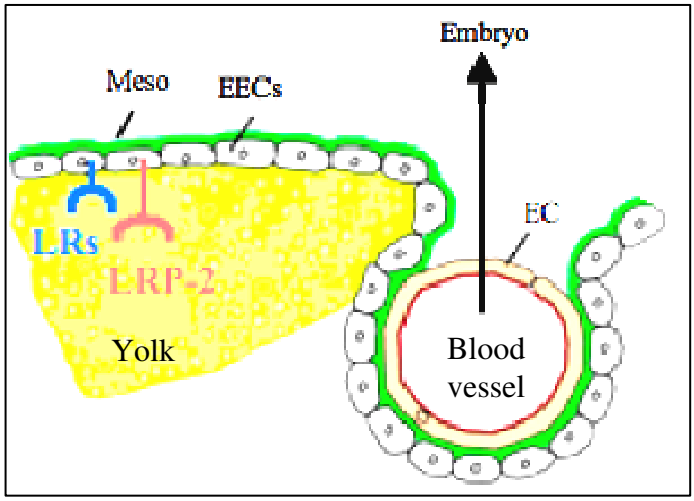

Figure 1.8. During the development of extraembryonic tissues, the yolk sac grows to finally enclose the yolk surface. Endothel cells (EC) line the interior surface of the blood vessels, which are embedded in the yolk sac membrane. Two layers constitute the yolk sac, an inner single layer of endodermal endothelial cells (EEC) and an outer layer of mesenchymal cells (Meso). LRs expressed on the surface of EECs are responsible for the uptake of nutrients from the yolk and their secretion into the embryonic circulation.

Chapter 2

Materials and Methods

2.1 Chemicals and Enzymes

Chemicals obtained from Amresco, AppliChem, BioRad, Fluka, Merck, Pierce, Riedel-de Haën, Roche, Roth, Sigma-Aldrich, and Starlab were used for the production of buffers, solutions, and media. Restriction enzymes were purchased from Roche.

Diverse polymerases were used:

- DyNAzyme™ EXT DNA Polymerase from FINNZYMES
- High Fidelity PCR Enzyme Mix from Fermentas
- Klenow Polymerase from Amersham Biosciences
- SUPERScript™ Rnase H⁻ Reverse Transcriptase from Invitrogen

2.2 Bacterial Strains and Vector Systems

Strain	One Shot TOP10 Chemically Competent E.coli
Genotype	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
Reference/Source	Invitrogen

Table 2.1. Properties of E.coli TOP10

Vector	pCR2.1-TOPO
Size	3931 basepairs
Genotype characteristics	LacZα fragment, M13 reverse priming site, MCS, T7 promoter/priming site, M13 forward priming site, f1 origin, kanamycin resistance, ampicillin resistance, pUC origin
Reference/Source	Invitrogen, TA cloning kit

Table 2.2. Properties of T/A cloning vector

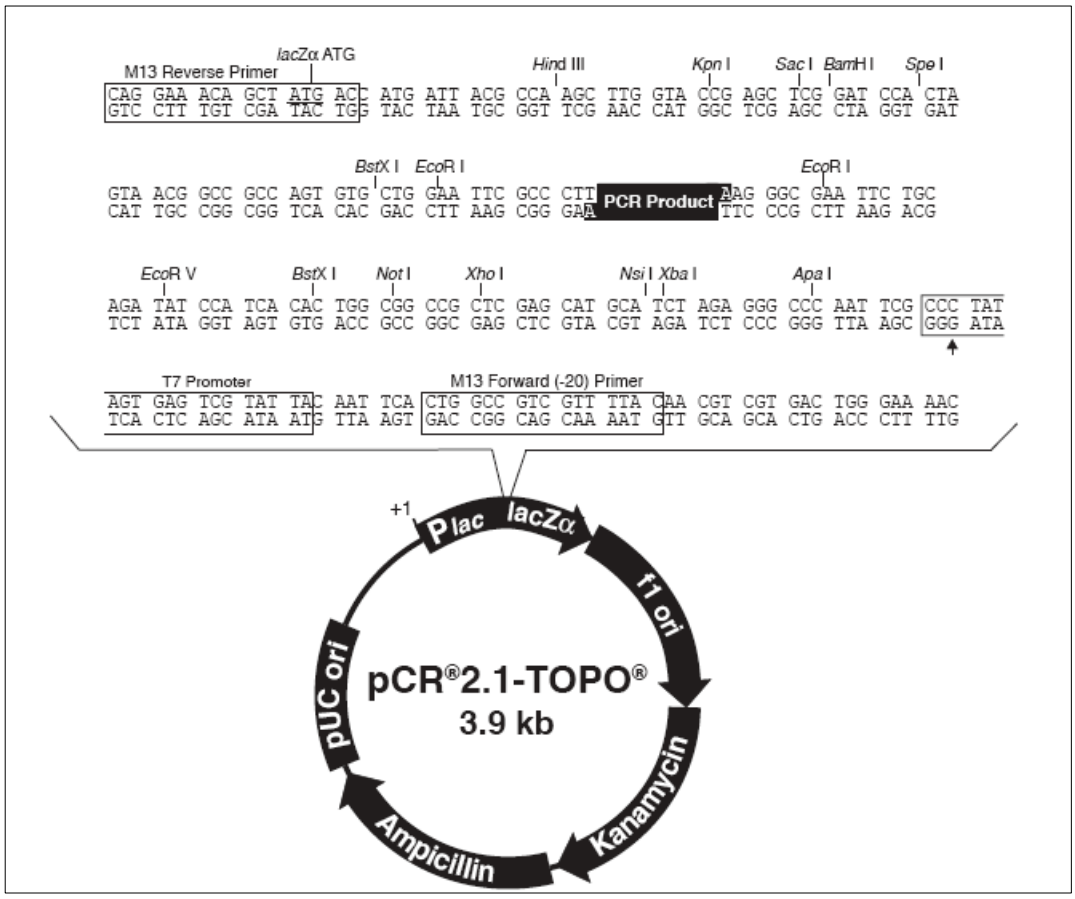

Figure 2.1. Genetic map of the pCR2.1-TOPO vector (http://www.invitrogen.com/)

2.3 Animals

Derco brown laying hens and roosters (10-40 weeks old) were purchased from Heindl Co. (Vienna, Austria) and maintained on layer's mash with free access to water and feed under a daily light period of 16 hours. Furthermore, a R/O (restricted ovulator) breeding colony was kept under the same conditions. Fertilized eggs were incubated at 37.5°C and 55% relative humidity for a chosen time period or until hatching.

2.4 Oligonucleotide Primers

The designed oligonucleotides were synthesized by MWG-Biotech AG, Ebersberg, Germany.

Name	Sequence	T_m (°C)
T/A cloning vector		
M13fw	5'-G TAA AAC GAC GGC CAG-3'	51.7
M13rv	5'-CAG GAA ACA GCT ATG AC-3'	50.4
Chicken LRP-2		
Fwd.Megalin_1	5'-ATGGGAACTCGGCAGCAGACG-3'	63.7
Rev.Megalin_2	5'-TGACAGCGATACTGGCAGCTC-3'	61.8
Fwd.Megalin_3	5'-GGAGTGTTAGCGATTGGAGGC-3'	61.8
Rev.Megalin_4	5'-CCTCTTTAACAAGATTGGCGG-3'	57.9
Rev.Megalin_5	5'-CCACACTACCAGCTCCTGTTA-3'	59.8
Chicken LRP-380		
5gg.ooc.LRP	5'-TGT CCC GGG ACC CAC CAC TGC-3'	67.6
3gg.ooc.LRP	5'-CTG GCA GCC CGG TCA CGA TGG-3'	67.6
Standard Primers		
Oligo (dT)	5'-TTT $(TTT)_{2-4}$TTT-3'	

Table 2.3. Primers used for PCR

2.5 Antibodies

Name	Epitope	Source
Anti-chicken LRP-2	Peptide	Rabbit
Anti-chicken LR-8	Peptide	Rabbit
Anti-rabbit IgG peroxidase conjugated (Sigma)	Rabbit IgG	Goat
Anti-rabbit IgG biotin conjugated (Sigma)	Rabbit IgG	Goat
Alexa Fluor® 488 anti-rabbit (Invitrogen)	Rabbit IgG	Goat

Table 2.4. Antibodies

2.6 Molecular Biological Methods: DNA

2.6.1 cDNA Synthesis

SUPERScript™ RNase H- Reverse Transcriptase from Invitrogen was used to perform cDNA synthesis. After total RNA isolation from chicken tissue (see 2.7.1) the following components were mixed together in a sterile Eppendorf tube:

10µl Total RNA
1µl dNTPs (10mM each)
1µl Oligo (dT) (500µg/ml)

The mixture was heated to 65°C for 5 minutes and then cooled on ice. The following components were added:

4µl First Strand buffer
2µl 0.1M DTT

After incubation at 42°C for 2 minutes, 1µl SUPERScript™ II reverse transcriptase (200U/µl) was added and mixed by pipetting up and down. The cDNA synthesis reaction was performed at 42°C for 50 minutes and finally stopped by heating at 70°C for 15 minutes.

2.6.2 Polymerase Chain Reaction (PCR)

A 50µl or 25µl reaction volume was used for Reverse Transcriptase PCR (RT-PCR). The components were kept on ice and mixed in a sterile PCR tube. PCR conditions depended on the primer pair used (see Tab. 2.3). A touch-down thermocycling program was used to optimize PCR conditions (see Tab. 2.6). All PCR products were analyzed by agarose gel electrophoresis.

High Fidelity PCR Enzyme Mix from Fermentas:

5µl 10x High Fidelity PCR Buffer with 15 mM $MgCl_2$
2.5µl DMSO
1µl dNTP mix (10mM each dNTP)
1µl Forward primer (20pM)
1µl Reverse primer (20pM)
1µl DNA template
0.5µl High Fidelity PCR Enzyme Mix (5U/µl)
xµl sterile H_2Odd to a final volume of 50µl

Table 2.5. PCR setup using the High Fidelity PCR Enzyme Mix

	Number of cycles	Temperature	Time
Lid temperature		99°C	
Initial denaturation		94°C	5min
Denaturation	3	94°C	1min
Annealing		x°C + 6°C	1min
Elongation		72°C	xmin
Denaturation	3	94°C	1min
Annealing		x°C + 3°C	1min
Elongation		72°C	xmin
Denaturation	33	94°C	1min
Annealing		x°C	1min
Elongation		72°C	xmin
Final elongation		72°C	6min
Pause		4°C	∞

Table 2.6. Touch-down PCR conditions

2.6.3 Quantitative Real Time PCR

Quantitative Real Time PCR (qPCR) was performed using the LightCycler® 480 system (Roche), requiring the LightCycler® FastStart DNA Master SYBR Green I kit (Roche). After the cDNA was prepared from embryonic and adult tissues, the samples were diluted with water (1/20). A freshly prepared 10^{-1} to 10^{-9} serial dilution of the target PCR product was prepared starting from a 1/20 dilution. Every sample was measured twice and compared to the serial dilution, which served as an internal standard.

A master-mix was set up in a sterile Eppendorf tube. 14µl of the mixture were pipetted into a LightCycler® 480 Multiwell Plate 96. The diluted sample was added and the 96 well plate was analyzed in the LightCycler® 480.

4.5µl Sterile H_2Odd
1µl Forward primer
1µl Reverse primer
7.5µl SYBR Green Mix
add 1.5µl diluted template

Table 2.7. qPCR setup using a LightCycler® FastStart DNA Master SYBR Green I kit

	Number of cycles	Temperature	Time
Denaturation	45	95°C	15sec
Annealing		60°C	15sec
Elongation		72°C	16sec
Melting curve	1	95°C	10sec
		75°C	20sec
		98°C	
Cooling		50°C	∞

Table 2.8. qPCR conditions using a LightCycler®480

2.6.4 DNA Gel Extraction

The QIAquick Gel Extraction kit from QIAgen® was used for the elution of DNA fragments from agarose gels. This protocol allowed to extract and purify DNA of 70bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer. All centrifugation steps were carried out in a table-top microcentrifuge at 13000 rpm at room temprature. The desired DNA band was excised from the agarose gel with a sterile scalpel and transferred into an Eppendorf tube. Three volumes of buffer QG were added to 1 volume of gel (100mg gel weight = 1 gel volume = 100µl buffer QG). The gel was dissolved completely by incubation at 50°C for 10 minutes and repeated vortexing every 2-3 minutes. In case of a desired DNA fragment size of >500bp or <4kb, 1 gel volume of isopropanol was added to the mixture and mixed well. The sample was loaded onto a QIAquick column and centrifuged for 1 minute. To wash, 500µl of buffer QG were added to the column, followed by centrifugation. Additionally, bound DNA was washed with 750µl buffer PE. After centrifugation for

1 minute the flow-through was discarded and the column was centrifuged for an additional minute to remove residual ethanol from the column. The DNA was eluted by adding 50µl of buffer EB, incubating for 1 minute, and finally centrifuging for 1 minute.

EB buffer
10mM Tris-HCl pH 8.5

2.6.5 T/A-Cloning

Taq-polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosin (A) to the 3' ends of PCR products. The linearized pCR2.1-TOPO vector has a single, overhanging 3' deoxythymidine (T) residue.
Topoisomerase I is covalently bound to the vector. It allows PCR inserts to ligate with the vector, without the need of adding DNA ligase to the reaction. The following ingredients were mixed:

4µl PCR product (purified by gel extraction)
1µl Salt solution (1.2M NaCl, 0.06M $MgCl_2$)
1µl TOPO vector (pCR2.1)

The reaction was performed for 5 minutes at RT. 2µl of the reaction were used for transformation.

2.6.6 Transformation of Competent *Escherichia coli* (*E. coli*)

One aliquot (50µl/tube) of chemically competent *E. coli* cells was thawed on ice. 2µl of the ligation mixture were directly pipetted into the vial of competent cells and mixed by tapping gently. The reaction was incubated on ice for 30 minutes. Afterwards, the cells were heated for 30 seconds at 42°C and cooled on ice for 2 minutes. For regeneration, 250µl of provided S.O.C. medium were added and the cells were incubated for 1 hour at 37°C with vigorous shaking. 50µl and 150µl of the transformation mixture were plated onto LB-Amp/X-Gal plates and incubated o/n at

37°C. White colonies were checked for containing the correct plasmid by preparation of the plasmid DNA mini scale, followed by restriction enzyme digestion and agarose gel electrophoresis.

LB-Amp plates
10g Peptone from Casein
5g Yeast Extract
10g NaCl
12g Agar-Agar
H_2Odd ad 1000ml
100µg/ml Ampicillin

LB-Amp plates containing X-Gal
50µl of X-Gal solution (50 mg/ml) were plated on LB-Amp plates.

2.6.7 Mini-Preparation of Plasmid DNA

Preparation of plasmid DNA was carried out using the FastPlasmid™ Mini kit from Eppendorf. This protocol yields high purity plasmid DNA isolated from 1.5ml of *E. coli* bacterial cultures. Only a single solution for cell resuspension, lysis, and DNA binding is needed. All centrifugation steps were carried out in a table-top centrifuge at maximum speed (13000 rpm) and RT. To get an overnight-culture, a single white bacterial colony from a LB plate was transferred into 5ml of LB medium containing the appropriate antibiotic in a loosely capped eprouvette. After vigorously shaking o/n at 37°C, 1.5ml of the bacterial culture were transferred into an Eppendorf tube and harvested by centrifugation for 1 minute.

The medium was removed by decanting and the cell pellet was resuspended in 400µl of ice-cold complete lysis solution by vigorously vortexing for a full 30 seconds. After the cell lysate was incubated at RT for 3 minutes, it was transferred to a Spin Column Assembly by pipetting and centrifuged for 1 minute. The filtrate was discarded. Bound DNA was washed by adding 400µl of Wash buffer followed by centrifugation for 1 minute. The filtrate was discarded again and the Spin Column was

dried by an additional centrifugation step. DNA was eluted by adding 50µl of Elution buffer and spinning for 1 minute.

LB medium
10g Trypton
5g Yeast Extract
10g NaCl
H_2Odd ad 1000 ml

LB-Amp
LB medium
100µg/ml Ampicillin

2.6.8 Restriction Enzyme Digestion

For digestion of Mini-Preps the following components were mixed and incubated for one hour at 37°C:

4µl Mini-Prep DNA
x units Restriction enzyme
1.5µl Appropriate 10x restriction buffer
xµl H_2Odd to a final volume of 15µl

The appropriate restriction buffer was chosen according to the recommendations of the producer. The restriction digest was checked by agarose gel electrophoresis.

2.6.9 DNA Gel Electrophoresis

Separation of DNA fragments was performed by gel electrophoresis using 1% (w/v) agarose gels with a constant voltage of 100V for approximately 30 minutes. DNA samples were mixed with 5x DNA loading buffer containing Bromophenol Blue or Xylene Cyanol FF serving as front marker and glycerol to increase the density of the

sample, ensuring that DNA was evenly loaded. 1x TAE was used as electrophoresis running buffer. The 1kb Plus DNA Ladder from Invitrogen was used as a size marker.

To visualize DNA fragments under ultraviolet light (366nm), the agarose gel contained the fluorescent intercalating dye ethidium bromide (40µg/100ml).

50x TAE, pH 8.0
2M Tris-HCl
1M Acetic acid
0.1M EDTA

Ethidium bromide stock solution
10mg/ml in H_2Odd

5x DNA loading buffer
5ml 100% Glycerin
2ml 0.5M EDTA
12ml 50x TAE
3ml H_2Odd
Bromophenol blue or Xylene Cyanol FF

2.6.10 Radiolabelling of DNA Fragments

For the labelling of DNA fragments the Amersham Megaprime™ DNA labelling kit was used. The following components were mixed in an Eppendorf screwcap tube:

4µl Purified PCR fragment
24µl DEPC treated H_2Odd
5µl Primer (random hexamers provided by the kit)

The mixture was heated to 95°C for 5 minutes and cooled on ice. The content was collected by short centrifugation. 10µl labelling buffer, 2µl Klenow Polymerase (5U/µl) and finally 5µl α-^{32}P dCTP (10µCi/µl; Hartmann Analytics) were added. The

mixture was spun and incubated at 37°C for 1 hour. The reaction was stopped by heating up to 95°C for 5 minutes. Then the ^{32}P-labelled DNA was used as a probe for Northern blot analysis.

2.6.11 DNA Sequencing

For sequence analysis Mini-preparations of plasmid DNA were sent to VBC-Genomics Bioscience Research GmbH, Vienna, Austria.

2.7 Molecular Biological Methods: RNA

2.7.1 Isolation of Total RNA

Two different methods for total RNA isolation were used, including the NucleoSpin® RNA II kit from Macherey-Nagel and TRI Reagent from Molecular Research Center, Inc.

RNA Isolation with NucleoSpin® RNA II kit from Macherey-Nagel

To achieve a high purity of RNA for production of cDNA, the NucleoSpin® RNA II kit was used for isolation. The kit allowed the purification of up to 70µg of total RNA from up to 50mg of tissue. All centrifugation steps were carried out in a table-top centrifuge at RT.

- Homogenization: 30-50mg frozen tissue supplied with 350µl buffer RA1 were homogenized in a sterile glass potter.

- Cell lysis and filtration of the lysate: The homogenized tissue was lysed by addition of 3.5µl β-mercaptoethanol, followed by vigorous vortexing. A NucleoSpin® filter unit was placed in a collection tube, the lysate was added and centrifuged at 11000x g for 1 minute. The filter unit was discarded and 350µl of 70% ethanol were added to the lysate to adjust the RNA binding conditions.

- Bind RNA: The lysate was loaded onto A NucleoSpin® RNA II column. The column was centrifuged at 8000x g for 30 seconds and then placed in a new collection tube. 350µl Membrane Desalting buffer (MDB) were added to the column, before centrifugation at 11000x g for 1 minute in order to desalt.

- Digest DNA: DNase reaction mixture was prepared by adding 10µl DNase I to 90µl DNase reaction buffer and mixed by flicking the tube. 100µl of this mixture were pipetted onto the center of the silica membrane of the column and incubated at RT for 15 minutes.

- Wash and dry silica membrane: For the first wash step 200µl buffer RA2 were added to the column, followed by centrifugation at 8000x g for 30 seconds. The flow-through was discarded and the column was placed into a new collection tube. The second wash step was performed by addition of 600µl buffer RA3 to the column and centrifugation at 8000x g for 1 minute. Again the flow-through was discarded and the column was put back into the same collection tube. In the third and final wash step, 250µl buffer RA3 were added and the column was centrifuged at 11000x g for 2 minutes to dry the membrane completely. The column was subsequently placed into a nuclease-free 1.5ml Eppendorf tube.

- Elution of pure RNA: To elute bound RNA, 40µl of RNase-free H_2O were added to the column and centrifuged at 11000x g for 1 minute. The concentration of eluted RNA was measured (see 2.7.3) and the samples were stored at -80°C.

RNA Isolation: TRI Reagent from Molecular Research Center, Inc.

- Homogenization: 50-500mg tissue (frozen in liquid N_2 and stored at -80°C) were homogenized in TRI reagent (1ml per 100mg tissue) in a sterile polypropylene Falcon tube using an Ultra-Turrax-T25 homogenizer.

- Phase separation: The homogenate was incubated for 5 minutes at RT to allow complete dissociation of nucleoprotein complexes. After the addition of 0.2ml chloroform per 1ml of TRI Reagent the homogenate was covered tightly and vigorously shaken for 15 seconds. The mixture was incubated at RT for 15 minutes and centrifuged at 12000x g for 15 minutes at 4°C.

- RNA precipitation: The aqueous phase was transferred carefully into a sterile Eppendorf tube. RNA was precipitated by addition of 0.5ml isopropanol per 1ml TRI reagent. The sample was vortexed and incubated at RT for 10 minutes followed by centrifugation at 12000x g for 30 minutes at 4°C. The RNA pellet was washed with 75% ethanol and centrifuged at 12000x g for 5 minutes at 4°C. The ethanol was removed and the RNA pellet was briefly air-dried for 3-5 minutes. RNA was dissolved at RT in an appropriate quantity of 0.1% DEPC treated H_2Odd. After determination of RNA concentration (see 2.7.3), samples were stored at -80°C.

DEPC treated H_2Odd
0.1% Diethyl Pyrocarbonate (DEPC)
mixed with H_2Odd and autoclaved.

2.7.2 Purification of poly(A) RNA

To purify poly(A) RNA the NucleoTrap® mRNA kit was used. The kit allows the isolation of poly(A) RNA from a total RNA preparation via latex beads covalently modified with oligo(dT) residues. Poly(A) RNA binds to these latex beads under high-salt conditions and can be eluted with water or low salt buffer.

250µg of total RNA, in a volume of 200-500µl DEPC treated H_2Odd, were supplemented with the same volume of RM0 binding buffer. 15µl thoroughly resuspended oligo(dT) latex beads per 100µg total RNA were added. After mixing, the sample was heated at 68°C for 5 minutes followed by incubation at RT for 10 minutes with 2 minute intervals of inverting the tubes. The mixture was centrifuged at 2000x g for 15 seconds and at 11000x g for 2 minutes. All centrifugation steps were carried out in a table-top centrifuge at RT. The supernatant was discarded and the pellet dissolved in 600µl washing buffer RM2. The oligo(dT) latex bead suspension was transferred onto the NucleoTrap® microfilter and centrifuged at 2000x g for 15 seconds and at 11000x g for 2 minutes. After discarding the flow-through, the pellet was washed with 500µl washing buffer RM3 by directly resuspending on the

microfilter until the suspension became milky. The tube was centrifuged at 2000x g for 15 seconds and at 11000x g for 2 minutes and the flow-through was discarded. The washing step with washing buffer RM3 was repeated and finally the NucleoTrap® microfilter was dried by centrifugation at 11000x g for 1 minute. The pure poly(A) RNA was eluted by the addition of 20µl prewarmed (68°C) RNase-free H_2O per 10µl oligo(dT) latex beads. The resuspended bead suspension was incubated at 68°C for 7 minutes. The eluate was collected by centrifugation at 11000x g for 1 minute. The elution step was repeated in order to increase the yield. After determination of RNA concentration (see 2.7.3), samples were stored at -80°C.

2.7.3 Determination of RNA Concentration

For determination of RNA concentration the NanoDrop® ND-1000 Spectrophotometer was used. Nucleic acid samples were checked for concentration and quality. The sample concentration in ng/µl based on absorbance at 260nm and the selected analysis constant was determined.

The sampling arm was opened to pipette 2µl of the blank or of the sample onto the lower measurement pedestal. The sampling arm was closed and the spectral measurement initiated using the operating software on the PC. The liquid column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement made. The NanoDrop® ND-1000 Spectrophotometer was cleaned by wiping the sample from both the upper and lower pedestals with a soft laboratory wipe.

2.7.4 Northern Blot Analysis

Northern blotting was carried out using the glyoxal gel electrophoresis method and the urea-PAGE method with minor changes. All solutions were prepared with RNA grade chemicals. H_2Odd was treated with 0.1% DEPC.

Glyoxal gel electrophoresis method

- Sample preparation: The following components were put on ice in a fresh sterile Eppendorf tube:

  25µg Total RNA in 6µl H_2Odd
  11.8µl DMSO
  2.4µl 0.1M $NaHPO_4$ buffer
  3.5µl 40% Glyoxal

  The samples were incubated at 50°C for 1 hour and subsequently put on ice and 6.3µl glyoxal loading buffer were added. For marker preparation 3µl 0.5-10Kb RNA ladder (Invitrogen) were mixed with 11.8µl DMSO, 2.4µl 0.1M $NaHPO_4$ buffer, 3.5µl 40% Glyoxal, and 6.5µl Glyoxal loading buffer and incubated at 65°C for 10 minutes.

- Gel electrophoresis: 1.2% agarose gel was prepared with 10mM $NaHPO_4$ buffer, which was also used as Running buffer. The gel was run at 80V for 90 minutes and the direction of the gel was changed every 30 minutes to avoid a pH and salt gradient.

- Capillary transfer: After gel electrophoresis the gel was equilibrated in Equilibration buffer I for 20 minutes, in Equilibration buffer II for 20 minutes, and in 10x SSC for 30 minutes at RT shaking. To transfer the RNA onto the membrane a blot was prepared as followed: A glassplate was covered with 6 glassplate-sized Whatman papers soaked in 20x SSC, the gel was applied upside down, followed by a nylon membrane (Hybond™-N for nucleic acid transfer from Amersham Biosciences) and 6 dry membrane-sized Whatman papers, one package of green towels and 0.5kg weight. Blotting o/n at RT.

- RNA staining: After marking the slots, the membrane was rinsed in 2x SSC, placed on a Whatman paper and auto-crosslinked in a Stratagene UV crosslinker. RNA was stained with Methylenblue for 5 minutes and destained with DEPC treated H_2Odd. The stained membrane was photocopied and the replica served as size marker for 28S and 18S rRNA, which represent a loading control.

- Hybridization: Prehybridization (to block the free positions on the membrane) was performed in a glass roller-bottle filled with 10ml of prewarmed hybridization solution for 3 hours at 65°C. The radiolabelled probe was added to a final concentration of 1-2x 106cpm/ml. The membrane was hybridized o/n at 65°C.

- Washing: Washing buffers were prewarmed at 65°C. The membrane was washed twice with washing buffer A for 10 minutes and once with washing buffer B for 5 minutes. Finally, the membrane was fixed in a film cassette and exposed to a film (GE Healthcare, Amersham Hyperfilm™ MP) at -80°C for several days depending on the intensity of the signal.

1M Na-Phosphate Buffer
462mM Na_2HPO_4
469mM NaH_2PO_4
pH 6.8
add DEPC treated H_2Odd up to 200ml

Glyoxal loading Buffer
50% Glycerol
10mM Na-Phosphate Buffer
0.4% Bromophenolblue
0.25% Xylene Cyanol FF

Methylenblue Staining Reagent
0.04% Methylenblue
0.5M NaOAc pH 5.2

Equilibration Buffer I
50mM NaOH
150mM NaCl
add DEPC treated H_2Odd up to 250ml

Equilibration Buffer II
100mM Tris-HCl buffer pH 7.5
150mM NaCl
add DEPC treated H_2Odd up to 150ml

Strip Buffer
0.1x SSC
0.1% SDS

20x SSC
3M NaCl
0.3M Na-Citrat
pH 7.2
add DEPC treated H_2Odd up to 250ml

Hybridization Solution
1% BSA Fraction V
40% DEPC treated H_2Odd
7% SDS
500mM Na-Phosphate Buffer pH 6.8
1mM EDTA
mixed in order shown above,
final volume should be 10ml

Washing Buffer A
0.5% BSA
5% SDS
40mM Na-Phosphate buffer
1mM EDTA
add DEPC treated H_2Odd up to 250ml

Washing Buffer B
1% SDS
40mM Na-Phosphate buffer
1mM EDTA
add DEPC treated H_2Odd up to 500ml

Urea-PAGE method

- Sample preparation: 10µg total RNA in 10 µl H_2Odd were mixed with 2x RNA loading buffer and heated to 65°C for 10 minutes and subsequently put on ice. For marker preparation 5µl 0.5-10Kb RNA ladder (Invitrogen) were mixed with 2x RNA loading buffer, heated to 65°C for 10 minutes, and put on ice.

- Gel electrophoresis: Bio-Rad Mini-PROTEAN and Hoefer miniVE systems were used for gel elecrophoresis. The components of the gel were mixed (see Tab. 2.8), the gel was poured and the comb inserted. After polymerization, the slots of the gel were rinsed with 1x TBE, which was also used as running buffer. The gel was run at 200V for 1 hour and 30 minutes.

- Transfer: For transfer a peQlab PerfectBlue 'Semi-Dry' Electro blotter was used. Transfer of RNA from Urea-gels to nitrocellulose membranes (Hybond™-N Extra for optimized nucleic acid transfer, Amersham Bioscience) was performed in a peQlab PerfectBlue 'Semi-Dry' Electro Blotter. The blot was prepared as followed: 3 Whatman papers soaked in 1x TBE were applied to the blotting unit, followed by the nitrocellulose membrane, the gel, and finally 3 more wet Whatman papers. To avoid air bubbles a falcon tube was rolled over the blot. The blot was covered with the lid of the blotting unit. Blotting was performed at 400mA per gel for 1 hour at RT.

- RNA staining: After the slots were marked, the membrane was crosslinked in a Stratagene UV crosslinker, rinsed with 6x SSC for 10 minutes and crosslinked again.

- Hybridization: Prehybridization (to block the free positions on the membrane) was performed in a glass roller-bottle filled with 10ml of prewarmed hybridization solution for 3 hours at 65°C. The radiolabelled probe was added to a final concentration of 1-2x 106cpm/ml. The membrane was hybridized o/n at 65°C.

- Washing: The membrane was washed 3x for 30 minutes, successively using washing buffer I, II and III. the membrane was fixed in a film cassette and exposed to a film (GE Healthcare, Amersham Hyperfilm™ MP) at -80°C for several days depending on the intensity of the signal.

	4% gel	6% gel
25% AA/BisAA (1:19) – Urea 8M	2.4ml	3.6ml
10x TBE	1.5ml	1.5ml
8M Urea	11.1ml	9.9ml
10% APS	110µl	110µl
TEMED	15µl	15µl

Table 2.8. Composition of gels used for Urea-PAGE

25% Acrylamide/Bisacrylamide Solution (1:19) – Urea 8 M

625ml Acrylamide/Bisacrylamide solution 40 % (1:19)
8M Urea
add DEPC treated H_2Odd up to 1l

2x RNA Loading Buffer

95% Formamide
0.025% Xylene cyanol FF
0.025% Bromophenol blue
12.5mM EDTA
0.5% SDS
add DEPC treated H_2Odd up to 10ml

10x TBE

440mM Tris-base
440mM Boric acid
10mM EDTA
add DEPC treated H_2Odd up to 1l

Methylenblue Staining Reagent

0.04% Methylenblue
0.5M NaOAc pH 5.2

Washing Buffer I

25ml 20x SSC
adjusted to 1l with DEPC H_2Odd

Washing Buffer II

50ml 20x SSC
6.25ml 20% SDS
adjusted to 1l with DEPC H_2Odd

Washing Buffer III

25ml 20x SSC
6.25ml 20% SDS
adjusted to 1l with DEPC H_2Odd

2.8 Molecular Biochemical Methods: PROTEIN

2.8.1 Preparation of Triton® X-100 Total Extracts

Freshly obtained chicken tissue was placed in ice-cold homogenization buffer (4ml/g wet weight) and homogenized with an Ultra-Turrax T25 homogenizer 3x for 20 seconds each. The homogenates were centrifuged for 10 minutes at 620x g at 4°C. The supernatant was transferred to a fresh tube and 1/20 volume of 20% Triton® X-100 was added. Then the mixture was incubated at 4°C for 30 minutes and finally centrifuged at 300000x g for 1 hour at 4°C. The supernatant was aliquoted and stored at -20°C. Protein concentration was determined by the method of Bradford using Assay Dye Reagent Concentrate (BioRad).

Homogenization Buffer
20mM Hepes pH 7.4
300mM Sucrose
150mM NaCl
As protease inhibitor Complete EDTA-free tablets from Roche were added.

2.8.2 Preparation of Membrane Extracts

For preparation of membrane extracts the use of Triton® X-100 as detergent is recommended. Buffer A (5ml/g wet weight) was added to the tissue samples, followed by homogenization using an Ultra Turrax T25 homogenizer, 3x 30 seconds at setting 8. If necessary, homogenization was extended. The samples were centrifuged at 2000x g for 10 minutes at 4°C in a SS34 rotor and the supernatant was poured over 4 layers of cheesecloth into a fresh beaker on ice. The filtrate was centrifuged at 100000x g for 1 hour at 4°C in a TLA 100.3 Rotor (Beckmann). The supernatant was discarded and the walls of the centrifugation tube were washed with H_2Odd to remove lipids. The pellet was washed by resuspending in 3ml buffer A using a 19G needle followed by a 23G needle. The sample was centrifuged at 100000x g for 1 hour at 4°C. The supernatant was discarded and the pellet was resuspended in 0.625ml buffer B, using

again a 19G needle and subsequently a 23G needle. Afterwards, 4% of the total volume of 4M NaCl were added and the sample was sonicated for 30 seconds at setting 6 using a Bandelin sonicator (Bandelin, Berlin, Germany). 26% of the total volume of sterile H_2Odd and 20% of the total volume of 5% Triton® X-100 were added and the sample was vortexed. Finally, the sample was centrifuged at 100000x g for 1 hour at 4°C and the supernatant was aliquoted and stored at -20°C. Protein concentration was determined using the method of Bradford.

Buffer A
20mM Tris-HCl, pH 8
1mM $CaCl_2$
150mM NaCl

Buffer B
250mM Tris-Maleate, pH 6
2mM $CaCl_2$
As protease inhibitor Complete EDTA-free tablets (Roche) were added.

2.8.3 Determination of Protein Concentration

The protein concentration was measured with the method of Bradford using Assay Dye Reagent Concentrate (BioRad). 1µl of protein-extract was incubated with 1ml of Bradford reagent for 5 minutes and then analyzed in an UV spectrometer at 595nm. Protein concentration was calculated as followed:

$$\frac{\mu g \text{ standard}}{OD \text{ standard}} \times \frac{OD \text{ sample}}{\mu l \text{ sample}} = \mu g/\mu l \text{ protein}$$

2.8.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis: Bio-Rad Mini-PROTEAN and Hoefer miniVE systems were used for gel electrophoresis. The components of the separating gel were mixed (see Tab. 2.9), the gel was poured and the comb inserted. After the gel was overlaid with isopropanol the components of the stacking gel were mixed and poured onto the separating gel after complete removal of the isopropanol. The comb was inserted and after polymerization the slots of the gel were rinsed with H_2Odd. The gels were put into the buffer chamber filled with electrophoresis buffer. The protein samples were mixed with Laemmli buffer (reducing or non-reducing). In case of reducing conditions the samples were incubated for 5 minutes at 95°C and loaded onto the gel. 5µl of Kaleidoskop Prestained Protein Standard (Bio-Rad) served as a length marker. The gel was run at 180V until the Bromophenolblue front reached the bottom of the gel. The gels were used for transfer to a nitrocellulose membrane (Western Blot Analysis) or staining with Coomassie Blue.

Components (1 gel)	Stacking gel 4%	Separating gel 6%
H_2Odd	1.525ml	2.675ml
Tris-HCl, pH 8.8	-	1.25ml
Tris-HCl, pH 6.8	0.625ml	-
30% Polyacrylamide	0.325ml	1ml
10% SDS	0.025ml	0.05ml
10% APS	0.0125ml	0.025ml
TEMED	0.005ml	0.005ml

Table 2.9. Composition of gels used for SDS-PAGE

<u>30% Polyacrylamide</u>

29.2% Acrylamide

0.8% N,N'-Methylenebisacrylamide

4x Laemmli Buffer (non-reducing)
31.2% Glycerol
6% SDS
20mM Tris-HCl, pH 7.5
Bromophenolblue
add H_2Odd to 20ml

1x Electrophoresis Buffer
250mM Tris-HCl
192mM Glycine
0.1% SDS

0.5M Tris-HCl Buffer
0.5M Tris-HCl
pH 6.8

2.8.5 Coomassie Stain

The SDS-gel was stained by shaking in Coomassie Brilliant Blue solution for 1 hour. The gel was destained for a few hours at RT or o/n shaking at 4°C in destain solution before drying in a gel vacuum dryer. Alternatively, the gel was washed 15 minutes with H_2Odd and stained by shaking in GelCode R Blue Stain reagent from Pierce for 1 hour. The gel was destained in H_2Odd for several hours or o/n at 4°C.

Coomassie Solution
10% Acetic Acid
25% Isopropanol
0.862g Coomassie Blue R250
1950ml H_2Odd

Destain Solution
30% Ethanol
10% Acetic Acid

2.8.6 Western Blot Analysis

Wet blotting: The proteins were transferred from SDS-gels to nitrocellulose membranes (Hybond™-C Extra for optimized protein transfer, Amersham Bioscience) by using a Bio-Rad Mini Trans-Blot cell. The blot was prepared as followed: 1 fiber pad and 3 Whatman papers soaked in 1x Transfer buffer were applied to the transparent side of the blotting cassette, followed by the nitrocellulose membrane, the gel and finally 3 more wet Whatman papers and 1 fiber pad. To avoid air bubbles a falcon tube was rolled over the blot. The blotting cassette was put into the electrode assembly, inserted in the tank and covered with the lid of the blotting unit. Blotting was performed at 100V for 1.5 hours on ice. To check the transfer efficiency, the membrane was stained with Ponceau S. Blotted proteins and the standard became visible by destaining with H_2Odd. The lanes of the loaded samples and the standard were marked and excess parts of the membrane were cut off.

Immunoblot: The membrane was blocked for 1 hour at RT with 5% skimmed milk powder in 1x TBS-T (Tris-Buffered-Saline) solution. After removing the blocking solution, the primary antibody was diluted in 5% skimmed milk powder in 1x TBS-T and the membrane was incubated o/n at 4°C with gentle agitation. The membrane was washed 3x 10 minutes in 1x TBS-T, followed by incubation with an HRP-conjugated secondary antibody diluted in 1x TBS-T. After 1.5 hours of incubation at RT with gentle agitation the solution was discarded and the membrane was washed another three times with 1x TBS-T for 10 minutes.

Detection: Enhanced chemiluminescence (ECL) from Pierce was used to visualize proteins on immunoblots. ECL is based on enzyme-catalyzed production of light (horse radish peroxidase conjugated to the secondary antibody, catalyzing the oxidation of luminol in the presence of H_2O_2). Finally, the membrane was incubated with a 1:1 mixture of ECL solution I and II for 3 minutes at RT and put into an exposure cassette. Films (Pierce, CL-XPosure™Film) were exposed in the dark room for a few seconds up to 20 minutes, depending on the intensity of the signal and background.

1x Transfer Buffer

25mM Tris-HCl

192mM Glycine

Ponceau S

0.2% Ponceau S

3% Trichloracetic Acid

dilute with 1x TBS

10x TBS

1.37M NaCl

0.027M KCl

0.25M Tris-HCl

with HCl to pH 7.4

1x TBS-T

0.1% Tween

1x TBS

pH 7.4

2.9 Histological Methods

2.9.1 Immunohistochemistry

Freshly isolated tissues were fixed in Tissue-Tek® O.C.T. Compound at -24°C. Sections of 5-10µm were cut on a Microm HM500 OM Cryostat, fixed on SuperFrost® slides (Menzel-Glaeser) and stored at -20°C. The slides were incubated in 1:1 Acetone/Methanol by gentle shaking for 5 minutes and subsequently washed 3x 5 minutes with 1x PBS. By using the DakoCytomation Pen a barrier to liquids were drawn. The incubation steps were done in a humid chamber. The sections were incubated o/n at 4°C with the appropriate antibodies in 1x PBS.

- Fluorescence

  The sections were rinsed 3x 5 minutes in PBS and incubated with goat α-rabbit fluorescence-labeled secondary antibodies (Alexa Fluor-488 conjugated, 1:1000 dilution, Molecular Probes) for 1 hour. Counterstaining of cell nuclei was performed with DAPI (1:1000, Molecular Probes). Specimens were mounted in fluorescent mounting media (DAKO) and analyzed by fluorescence microscopy (Axiovert 135, Zeiss)

- Biotin

  The sections were rinsed 3x 5 minutes in PBS and incubated with biotinylated goat α-rabbit secondary antibody (1:250 dilution, Sigma) for 1 hour. To get a higher specificity the slides were incubated with Streptavidin Peroxidase Polymer (1:250, Sigma) for 20 minutes. To get the color reaction the sections were incubated with AEC+ Substrate-Chromogen (ready-to-use solution, DAKO). The specimens were mounted in Glycergel® Mounting Medium (DAKO) and analyzed by light microscopy (Axiovert 10, Zeiss).

Chapter 3

Results

3.1 Characterization of Chicken LRP-2

3.1.1 Current State of Research

A chicken-specific LRP-2 antibody has already been generated in the course of previous studies. After cloning of a chicken LRP-2 fragment into a pGEX-5X-1 vector, the construct was expressed in *E. coli*. The GST fusion protein was purified by affinity chromatography and subsequently injected into New Zealand white rabbits for antibody production.

The resulting polyclonal antibody is directed against the carboxy-terminus of LRP-2, containing three NPXY motifs. The protein sequence recognized by the chicken LRP-2 antibody (ggGSTMegHis) is shown in Figure 3.1.

```
MPPACRCMYGGTCYIDESGLPKCKCSYGYTGDYCEIGLSKGVPPGTTAVAVLLTVILIII 4424

IGVLAVGGFFNYRRTGSILPALPKLPSLSSLVKSAENGNGVTFRSGADVSMDIGGSVFEG 4484

DSAIDRAMQMNENFAVESGKQPITFENPMYATRDSGAGGTSEVTVIQPTQVTGAGSVENE 4544

NFENPVYASEVSPGPKEPPPRTDTPQESKWSFFKRKMKQNTNFENPIYAEMEKEQQQDTE 4604

NVPPSSPSLPPKITLKRDQPLAYTATEDTFKDTANLVKEDSVV----------------- 4647
```

Figure 3.1. This figure shows a part of the LRP-2 protein sequence (ENSGALT17651). The sequence used as antigen is indicated in grey. The three NPXY (Asn-Pro-Xxx-Tyr; Xxx stands for any amino acid) motifs are highlighted in green.

3.1.2 Cloning of Chicken LRP-2 cDNA

In the chicken, LRP-2 has not been clearly identified. A transcript with the length of 13935bp in the Ensembl chicken genome database (ENSGALT00000017651) is designated as LRP-2 [Schneider, 2007]. A 15165bp long transcript, with the accession number XM_422014 can be found in NCBI Blast.

To gain further knowledge about the amino-terminus and the potential start-codon of the sequence, total RNA was isolated from chicken kidney and reverse transcribed into cDNA using Oligo (dt) primers. The primerset Fwd.Megalin_1 and Rev.Megalin_2 was used to obtain a 971bp PCR fragment. This fragment was cloned into the pCR2.1-TOPO vector, which was subsequently transformed into *E. coli* Top10 cells. The plasmids were isolated using mini preparation, followed by restriction enzyme digestion with *EcoRI*. The fragments were analyzed on an agarose gel and further subjected to DNA sequencing. Afterwards, the cloned sequence was aligned with the existing sequences (see Figure 3.2), described above.

```
ENSGALT17651      ----------------------GGTTCCTGTAGTACATCTGCTCTTCTGAGCTCTGTACTG 39
XM422014          ------------------------------------------------------ATGG 4
Cloned            ATGGGAACTCGGCAGCAGACGGGTGGAAAAGCAGCAACTGCCTGCGGAGTGCTCCTGTTG 60
                                                                           *

ENSGALT17651      CTAAC---CTGCTTGTTACTTTGTTTTACTACAGGCTGTGGAGGTGGACAGTTTCGCTGT 96
XM422014          AGGACT--CTGCATATGGACGTGGT--ACTCCAGGCTGTGGAGGTGGACAGTTTCGCTGT 60
Cloned            CTCGCTGCGTGCATCCTGCGGGGCAGTTGCCAAGGCTGTGGAGGTGGACAGTTTCGCTGT 120
                      *    *** *        *         ****************************

ENSGALT17651      GGAAATGGTCGCTGTATTCCTGCAGACTGGAGATGTGACGGGGCTAGAGATTGCTCAGAT 156
XM422014          GGAAATGGTCGCTGTATTCCTGCAGACTGGAGATGTGACGGGGCTAGAGATTGCTCAGAT 120
Cloned            GGAAATGGTCGCTGTATTCCTGCAGACTGGAGATGTGACGGGGCTAGAGATTGCTCAGAT 180
                  ************************************************************

ENSGALT17651      GATACAGATGAAGCTGGTTGTCCTCAACCTACTTGTGAGGGAAATCAGTTTCAGTGTCAG 216
XM422014          GATACAGATGAAGCTGGTTGTCCTCAACCTACTTGTGAGGGAAATCAGTTTCAGTGTCAG 180
Cloned            GATACAGATGAAGCTGGTTGTCCTCAACCTACTTGTGAGGGAAATCAGTTTCAGTGTCAG 240
                  ************************************************************

ENSGALT17651      ACTGATGGTGAATGTATCCCACATTCATGGGTGTGTGATGATGAGGAAGATTGTGAAGAT 276
XM422014          ACTGATGGTGAATGTATCCCACATTCATGGGTGTGTGATGATGAGGAAGATTGTGAAGAT 240
Cloned            ACTGATGGTGAATGTATCCCACATTCATGGGTGTGTGATGATGAGGAAGATTGTGAAGAT 300
                  ************************************************************

ENSGALT17651      GGATCAGATGAACATCAACAGTGTCCGGGAAGGACGTGCTCAAGTCAGCAGTTTACATGC 336
XM422014          GGATCAGATGAACATCAACAGTGTCCGGGAAGGACGTGCTCAAGTCAGCAGTTTACATGC 300
Cloned            GGATCAGATGAACATCAACAGTGTCCGGGAAGGACGTGCTCAAGTCAGCAGTTTACATGC 360
                  ************************************************************

ENSGALT17651      TCAAATGGACAGTGTATCCCAAGTGCATACAGGTGTGATCGAGTAAAGGACTGCACTGAT 396
XM422014          TCAAATGGACAGTGTATCCCAAGTGCATACAGGTGTGATCGAGTAAAGGACTGCACTGAT 360
Cloned            TCAAATGGACAGTGTATCCCAAGTGCATACAGGTGTGATCGAGTAAAGGGCTGCACTGAT 420
                  ********************************************** **********

ENSGALT17651      GGAACAGATGAAAGAGACTGCCGCTACCCAAGATGTGAACAGTTATCCTGTGCAAACGGA 456
XM422014          GGAACAGATGAAAGAGACTGCCGCTACCCAAGATGTGAACAGTTATCCTGTGCAAACGGA 420
Cloned            GGAACAGATGAAAGAGACTGCCGCTACCCAAGATGTGAACAGTTATCCTGTGCAAACGGA 480
                  ************************************************************

ENSGALT17651      GCATGTTTTAATGCAAGCCAACGATGTGATGGCAAAGTTGATTGCAGAGATACTTCTGAT 516
XM422014          GCATGTTTTAATGCAAGCCAACGATGTGATGGCAAAGTTGATTGCAGAGATACTTCTGAT 480
Cloned            GCATGTTTTAATGCAAGCCAACGATGTGATGGCAAAGTTGATTGCAGAGATACTTCTGAT 540
                  ************************************************************

ENSGALT17651      GAAGCTAATTGCACACGTGGATGTGCCAGTACGCAGTTTCAGTGTGCTAATGGAGAATGT 576
XM422014          GAAGCTAATTGCACACGTGGATGTGCCAGTACGCAGTTTCAGTGTGCTAATGGAGAATGT 540
Cloned            GAAGCTAATTGCACACGTCCATGTGCCAGTACGCAGTTTCAGTGTGCTAATGGAGAATGT 600
                  ******************  ***************************************

ENSGALT17651      ATCCCACAGGCCTTTATGTGTGACCATGATGATGACTGTGGAGACAGGAGTGATGAAAAC 636
XM422014          ATCCCACAGGCCTTTATGTGTGACCATGATGATGACTGTGGAGACAGGAGTGATGAAAAC 600
Cloned            ATCCCACAGGCCTTTATGTGTGACCATGATGATGACTGTGGAGACAGGAGTGATGAAAAC 660
                  ************************************************************
```

```
ENSGALT17651      TCTTGCACTTATGCAACCTGTAGAGGCAACTTCTTCACTTGTCCAAGTGGCCGCTGCATT 696
XM422014          TCTTGCACTTATGCAACCTGTAGAGGCAACTTCTTCACTTGTCCAAGTGGCCGCTGCATT 660
Cloned            TCTTGCACTTATGCAACCTGTAGAGGCAACTTCTTCACTTGTCCAAGTGGCCGCTGCATT 720
                  ************************************************************

ENSGALT17651      CATCAGAGCTGGATATGTGATGGTGATGATGACTGTGAAGATAATGAAGATGAAAGAGGA 756
XM422014          CATCAGAGCTGGATATGTGATGGTGATGATGACTGTGAAGATAATGAAGATGAAAGAGGA 720
Cloned            CATCAGAGCTGGATATGTGATGGTGATGATGACTGTGAAGATAATGAAGATGAAAGAGGA 780
                  ************************************************************

ENSGALT17651      TGTGAAAGTAGTTATCATGAATGCTACCCGGGAGAGTGGGCCTGCCCTGAGTCAGGGCAT 816
XM422014          TGTGAAAGTAGTTATCATGAATGCTACCCGGGAGAGTGGGCCTGCCCTGAGTCAGGGCAT 780
Cloned            TGTGAAAGTAGTTATCATGAATGCTACCCAGGAGAGTGGGCCTGCCCTGAGTCAGGGCAT 840
                  ***************************** ******************************

ENSGALT17651      TGTATTCCAATTGGGAAAGTGTGCGATGGAGCTGCAGACTGCCCTGCAGGAGAAGATGAA 876
XM422014          TGTATTCCAATTGGGAAAGTGTGCGATGGAGCTGCAGACTGCCCTGCAGGAGAAGATGAA 840
Cloned            TGTATTCCAATTGGGAAAGTGTGCGATGGAGCTGCAGACTGCCCTGCAGGAGAAGATGAA 900
                  ************************************************************

ENSGALT17651      ACGAACATCACGGCAGGCAGACATTGCAACATATCACACTGTGCTGCTCTGAGCTGCCAG 936
XM422014          ACGAACATCACGGCAGGCAGACATTGCAACATATCACACTGTGCTGCTCTGAGCTGCCAG 900
Cloned            ACGAACATCACGGCAGGCAGACATTGCAACATATCACACTGTGCTGCTCTGAGCTGCCAG 960
                  ************************************************************

ENSGALT17651      TATCGCTGTCATTCTTCTCCTTCAGGAGGGATGTGTTATTGCCCTTCGGGATATACAATA 996
XM422014          TATCGCTGTCATTCTTCTCCTTCAGGAGGGATGTGTTATTGCCCTTCGGGATATACAATA 960
Cloned            TATCGCTGTCA---------------------------------------------------- 971
                  ***********
```

Figure 3.2. Nucleotide sequence alignment of two different LRP-2 sequences and the cloned fragment. The primers are indicated in green. The most probable start codon in frame is highlighted in yellow, resulting in the longest open reading frame (ORF).

3.1.3 Tissue Distribution of LRP-2 Transcripts

To determine the tissue distribution of chicken LRP-2 mRNA, Northern blot experiments were performed. RNA was prepared from different laying hen (LH) and embryonic tissues and subsequently used for Northern blot analysis. PCR with the primers Fwd.Megalin_3 and Rev.Megalin_4 generated a 655bp LRP-2 fragment, which was ^{32}P radiolabeled, was used as probe.

Hybridization signals showed high expression levels of LRP-2 mRNA in the laying hen kidney. Ovarian stroma was used as a negative control. The 15kb transcript was also present in the yolk sac and its EEC layer, but not in its mesoderm.

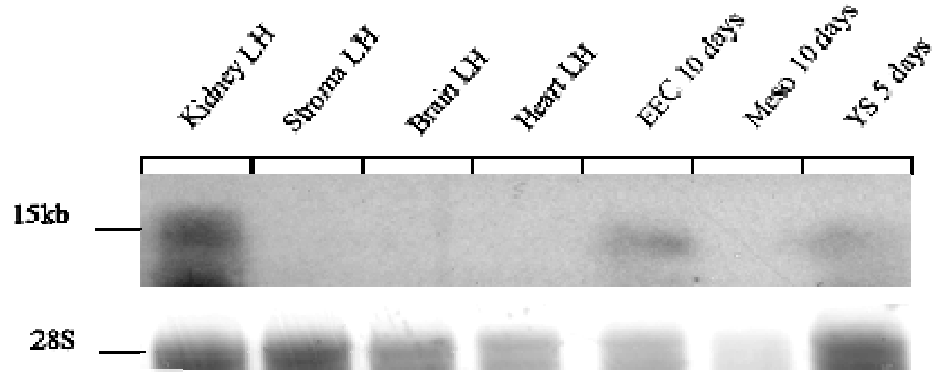

Figure 3.3. Northern blotting shows expression of transcripts in various laying hen and extraembryonic tissues as indicated. 15µg of total RNA extracts were electrophoretically separated. The blot was hybridized with a ^{32}P labeled 655bp LRP-2 PCR fragment. Methylene blue staining of the 28S rRNA is shown for loading control.

3.1.4 PCR Analysis of LRP-2 Tissue Distribution

RT-PCR was used to further analyze LRP-2 tissue distribution. A touch-down PCR program was used to generate a 655bp fragment. The primer pair Fwd.Megalin_3 and Rev.Megalin_4 was used.

As shown in Figure 3.4, the strong bands can be observed in the kidney, brain, and section V of the oviduct (see Figure 1.6) of laying hens and in all embryonic tissues tested. The data obtained from RT-PCR only partially correspond to the results obtained by Northern blot analysis.

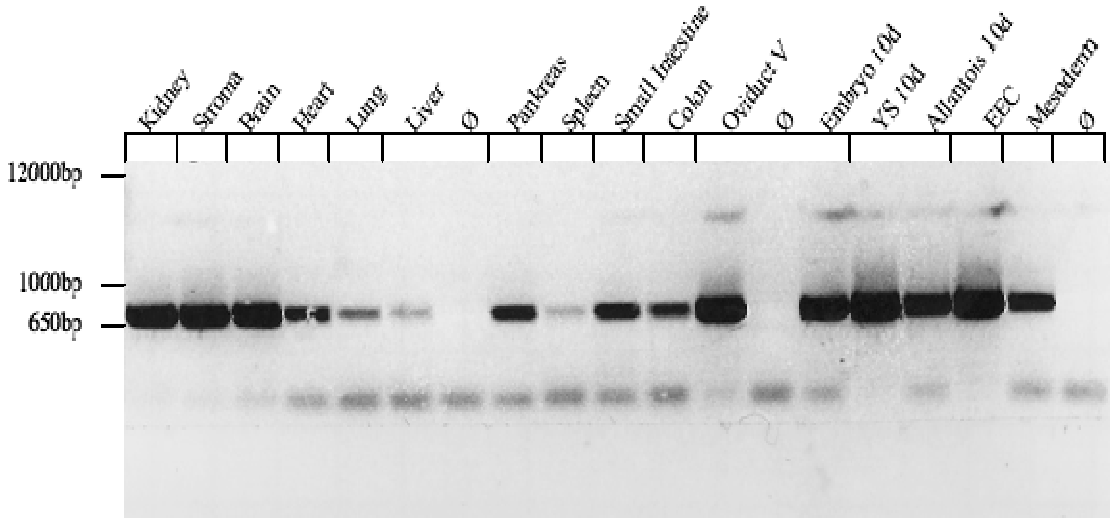

Figure 3.4. RT-PCR analysis of chicken LRP-2 using a touch-down program (see Materials and Methods). Expression can be observed in all LH and embryonic tissues tested. Lanes labeled with the symbol ∅ contain PCR products obtained without cDNA.

The PCR shown in Figure 3.4 indicates very high LRP-2 expression in the section V of the oviduct. To further investigate expression patterns of the female reproductive tract, cDNA of all sections was subjected to PCR analysis. Figure 3.5 shows that LRP-2 expression levels in the five segments of the laying hen oviduct differ significantly.

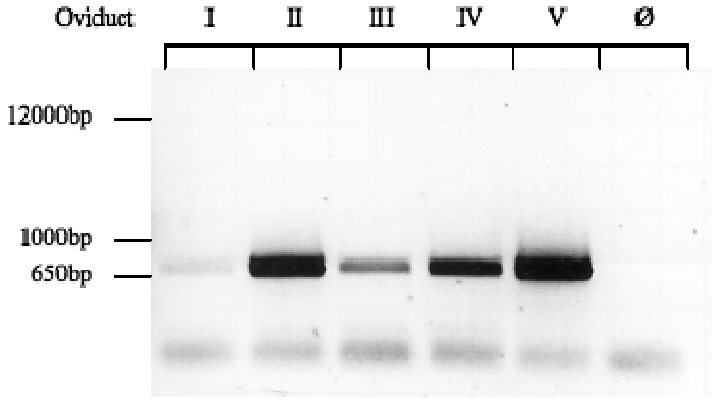

Figure 3.5. RT-PCR analysis of chicken LRP-2 in the five different segments of the LH oviduct. The primers Fwd.Megalin_3 and Rev.Megalin_4 were used to generate a 655bp fragment. Segments II and V show the strongest bands. Lanes labeled with the symbol ∅ contain PCR products obtained without cDNA.

3.1.5 Quantitative Analysis of mRNA Levels

Finally, a quantitative method was used to determine mRNA levels in different chicken tissues. Quantitative real time PCR analysis was performed with the primer pair Fwd.Megalin_3 and Rev.Megalin_5 using SYBR Green I. 45 cycles were performed in the LightCycler® 480. The melting curve was monitored to measure the melting temperature.

The efficiencies with values ranging from 1.913 to 1.932 were calculated from the internal standard curve. To further calculate the concentrations and standard deviations of the measurements, the LightCycler® Software (version 1.2) was used. The absolute quantification can be used to compare mRNA levels in different tissues.

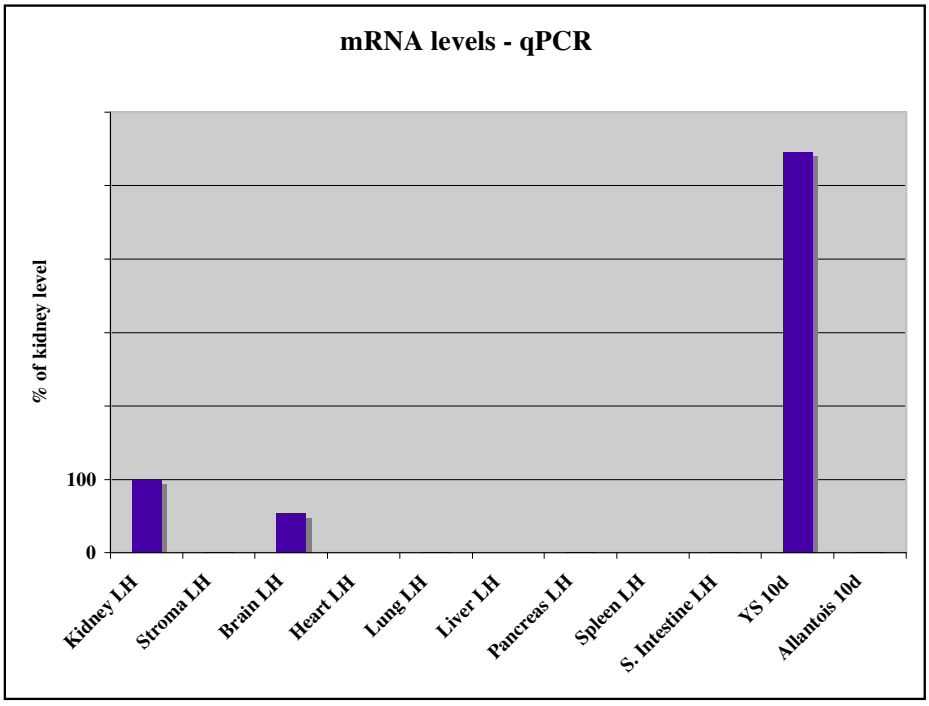

Figure 3.6. Quantitative PCR analysis of chicken LRP-2 mRNA in various LH and extraembryonic tissues. High expression levels can be observed in adult kidney and brain. In the yolk sac of 10 day old embryos, the transcript level is 5.5 times higher than in the kidney. LRP-2 mRNA can also be found in other tissues, but only at marginal levels.

The mRNA level in the laying hen kidney was arbitrarily set to 100%. Figure 3.6 shows that a huge amount of mRNA is present in the yolk sac of a 10-day-old embryo. LRP-2 mRNA expression can also be detected in the brain. Very low expression levels were observed in all other tissues tested.
The quantitative PCR analysis of transcript levels largely correlates with the results of the Northern blotting experiments.

3.2 Analysis of LRP-2 Protein Distribution

To investigate the LRP-2 protein distribution, membrane extracts were prepared since LRP-2 is a transmembrane receptor. The polyclonal anti-chicken LRP-2 antibody described above was used for detection of the protein in various tissues.
In laying hen tissues including heart, lung, liver, pancreas, spleen, oviduct, stroma, and colon receptor expression could not be detected (results not shown). In the adult animal, the kidney is the only organ in which LRP-2 can be found, thus serving as a positive control (Figure 3.7).

3.2.1 LRP-2 Distribution in Chicken Embryonic Tissues

The aim of these experiments was to find specific expression patterns during embryonic development in various tissues. First of all, we wanted to determine the localization of LRP-2 in the yolk sac. Therefore, the yolk sac was washed in a high saline buffer, and the two different layers (EECs and mesodermal cells) were torn apart with watchmaker forceps. Membrane protein extracts were subjected to gel electrophoresis and further analyzed by Western blotting.

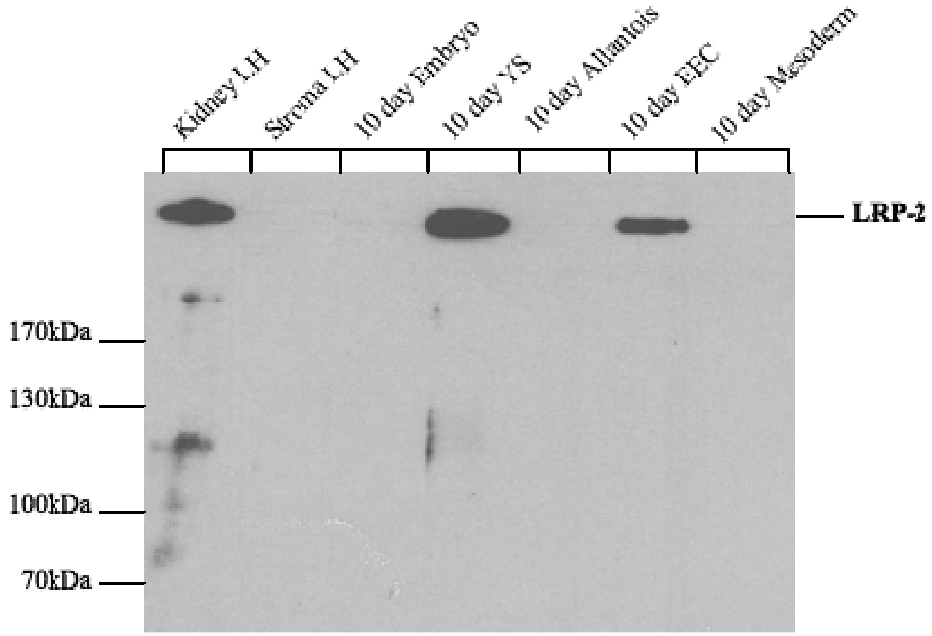

Figure 3.7. For Western blotting, 50µg of chicken membrane protein were separated on a 6% SDS-polyacrylamide gel under non-reducing conditions. The analyzed tissues are indicated on the picture. The anti-chicken LRP-2 antiserum was used at 1:1000 dilution. HRP-conjugated goat-anti-rabbit secondary antibody was diluted 1:50000. The chicken kidney serves as a positive control, the stroma represents the negative control.

Figure 3.7. demonstrates the expression of LRP-2 in the yolk sac and in the endodermal endothelial cell layer, but not in the mesoderm. These data indicate that the receptor may be exclusively localized to the EECs.

To analyze whether there is time-dependent LRP-2 expression in the yolk sac during embryonic development, tissue samples of various developmental stages were investigated. Yolk sacs from 5, 10, 15, and 20 day-old embryos represent a wide choice of developmental time points. Also yolk sacs of 1- and 3 day-old chicks were included in the study.

The results of Western blot analysis (see Figure 3.8) indicate that protein expression starts around day 5 and gradually increases to finally peak in the last trimester of embryonic development. Even three days after hatching a signal can be observed.

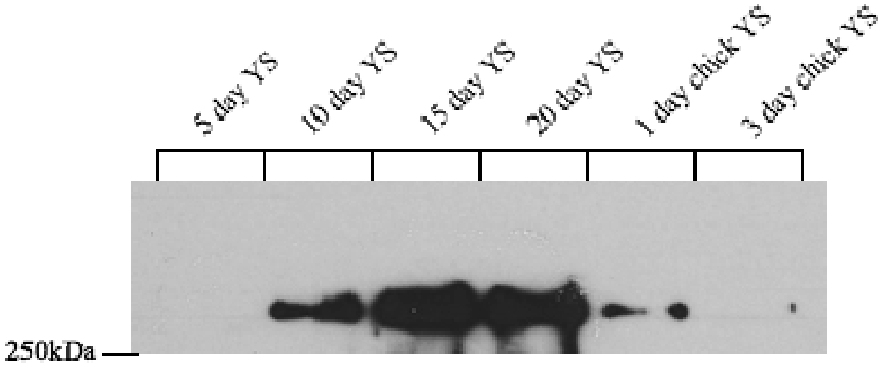

Figure 3.8. Western blotting of LRP-2. 50µg of chicken yolk sac membrane protein extracts were separated by SDS-PAGE using a 6% gel (non-reducing conditions). The antiserum to chicken LRP-2 was diluted 1:1000. HRP-conjugated goat-anti-rabbit antibody was used as secondary antibody (1:50000).

Several embryonic tissues were also tested for the presence of LRP-2 protein including brain, heart, liver, and kidney of 15 and 20 day-old embryos and of 1 day-old chicks (data not shown). LRP-2 expression could only be detected in the embryonic kidney, which led to further investigations.

A time-dependent expression pattern of LRP-2 was observed in the developing kidney (Figure 3.9). The receptor expression dramatically increases during progressive development.

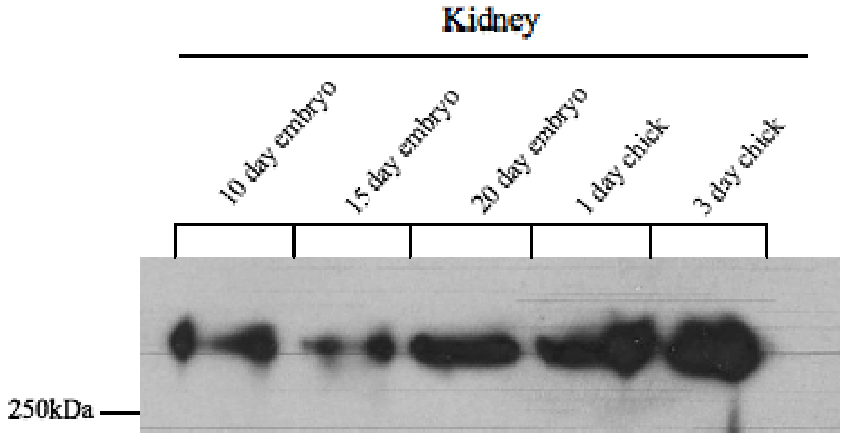

Figure 3.9. Western blot experiment using 30µg of chicken kidney membrane protein extracts separated on SDS-polyacrylamide 6% gels under non-reducing conditions. Anti-chicken LRP-2 antiserum was used. HRP-conjugated goat-anti-rabbit secondary antibody was diluted 1:50000.

3.2.2 Sex Dependent LRP-2 Expression in the Chicken Kidney

To determine LRP-2 protein levels in the adult chicken, Western blot experiments were performed. At the protein level, LRP-2 could only be detected in the kidney (data not shown). To further analyze receptor expression in the kidney, hen and rooster kidneys of wildtype and R/O animals were used. Additionally, roosters were treated with estrogen in order to simulate the hormonal status of the laying hen. 10mg estrogen/kg body weight were administered to adult roosters. After 48 or 72 hours, the animals were sacrificed and tissues were used for further investigation. As Figure 3.10 shows, a significant difference between LRP-2 levels in laying hen and rooster can be observed. The receptor levels are higher in the kidney of female animals (see lane 2 and 3). In contrast, R/O roosters have higher LRP-2 levels than wildtype males (lane 3 and 6). Thus, estrogen causes an increase of receptor expression for 48 hours of estrogen treatment in male kidneys (lane 4). If males are sacrificed 72 hours after estrogen administration, LRP-2 levels almost returned to normal due to the hormone degradation (lane 5).

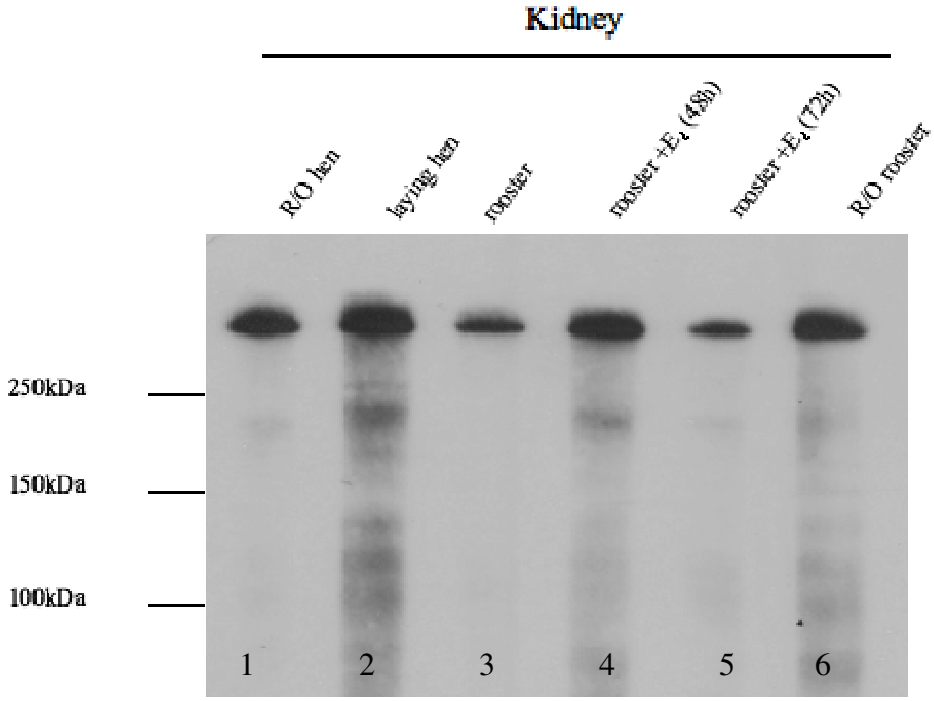

Figure 3.10. Determination of LRP-2 expression in adult chicken kidneys. 20µg of membrane protein extracts were subjected to Western blot analysis under non-reducing conditions. The anti-chicken LRP-2 antiserum was used at 1:1000 dilution. HRP-conjugated goat-anti-rabbit secondary antibody was diluted 1:50000.

3.3 Immunolocalization of Chicken LRP-2 in Yolk Sac Sections

To confirm the results obtained by Western blot analysis, immunolocalization of LRP-2 was performed using yolk sac sections. The yolk sac of 10 day old embryos, fixed in Tissue-Tek® O.C.T. Compound, was sectioned (5-10µm thickness), probed with anti-chicken LRP-2 antiserum (1:100 dilution) and stained using two different methods. To visualize LRP-2, either biotinylated goat anti-rabbit secondary antibodies and Streptavidin-linked peroxidase for color development, or fluorescence-labeled secondary antibodies were used.

Figure 3.11 shows the analysis under the light microscope. LRP-2 is indeed expressed in the EEC layer of the yolk sac. After fluorescence staining, shown in Figure 3.12, the receptor can be localized on the apical surface of the EECs.

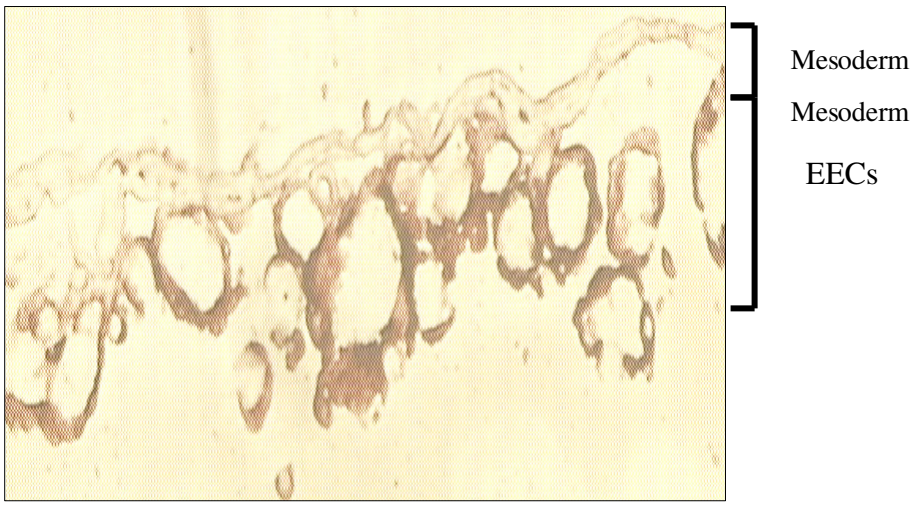

Figure 3.11. The image shows a section through the yolk sac of an 11 day-old embryo stained with anti-chicken LRP-2 antibody (1:100 dilution). Secondary antibody was used according to the protocol (see 2.9).

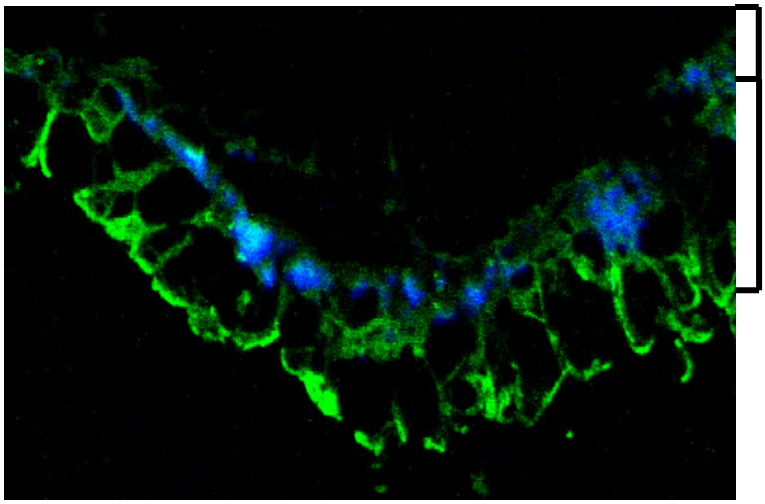

Figure 3.12. An 11 day-old chicken embryo yolk sac section was stained with anti-chicken LRP-2 antiserum (1:100 dilution). Goat anti-rabbit IgG fluorescence-labeled secondary antibodies (Alexa Fluor-488 conjugated) were diluted 1:1000. Counterstaining of cell nuclei was performed with DAPI (1:1000 dilution), which appear blue. LRP-2 expression is indicated in green.

After separating the two layers of the yolk sac, including EECs and mesoderm, the individual layers were fixed in Tissue-Tek® O.C.T. Compound and stained using biotinylated goat anti-rabbit secondary antibodies and Streptavidin-linked peroxidase for development of the colored product. The result further confirms the localization of LRP-2 on the surface of the EECs (see Figure 3.13A).

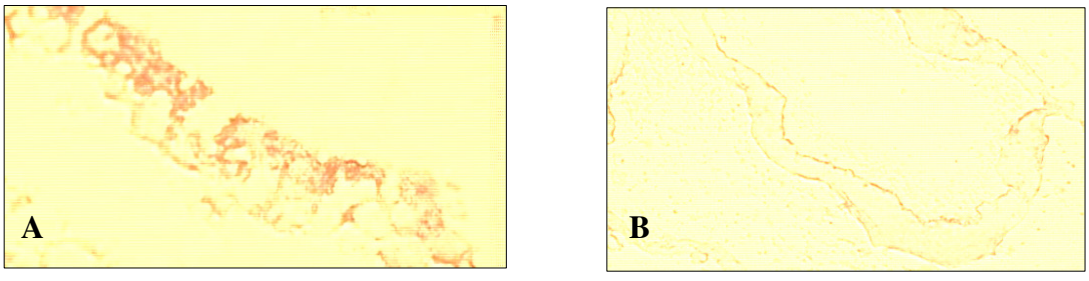

Figure 3.13. After separating the two layers of the yolk sac of 11 day-old embryos, membrane sections were stained with anti-chicken LRP-2 antiserum (1:250 dilution). Secondary antibodies were used according to the protocol (see 2.9). A: EECs are stained. B: In the mesoderm layer of the yolk sac no immunostaining was observed.

Chapter 4

Discussion

In the course of this study, the avian homologue to mammalian LRP-2 was characterized. The transcript ENSGALT00000017651, found in the Ensembl database is the designated chicken LRP-2 sequence [Schneider, 2007]. The mRNA sequence of the chicken receptor can be received under the accession number XM_422014 in NCBI Blast. These two sequences were aligned. Cloning of the N-terminus revealed the most probable start codon in frame by comparing it with the two different sequences.

Further insights into LRP-2 expression patterns should help to elucidate the receptor function in the chicken. The main expression sites for LRP-2 in mammals include kidney, small intestine, lung, thyroid, thymus, oviduct and uterus, and the yolk sac [Moestrup and Verroust, 2001]. In order to get an overview of expression sites and levels of the receptor in the chicken, Northern blotting experiments, RT-PCR, and qPCR analysis were performed. Northern blotting showed rather unspecific results. Due to the fact that the transcript comprises about 15kb, mRNA becomes easily degraded, resulting in a smear. After testing different protocols, specific results, showing a single band with the expected size, proved LRP-2 expression in the adult kidney and the yolk sac. To gain more specific information about RNA expression levels, RT-PCR was performed. By using this method, the receptor was shown to be

expressed in all of the tested adult and embryonic tissues indicated in Figure 3.4. Hence, LRP-2 expression sites in chicken widely correspond to mammalian ones. For quantitative analysis qPCR was used. Very basal levels of mRNA could be detected in most of the tissues including laying hen stroma, heart, lung, liver, pancreas, spleen, small intestine, and some extraembryonic tissues. The outstanding exceptions were the adult kidney and brain and the yolk sac of 10 day-old embryos, showing high LRP-2 expression.

LRP-2 is known to be essential for brain development, highlighted by receptor deficient mice, which show impaired forebrain development. Because LRP-2 is able to endocytose various apolipoproteins, specific roles in the adult brain were suggested, maybe explaining the high expression level found in the chicken brain [Fisher and Howie, 2006]. LRP-2 is present in endothelial cells of the brain, pointing at the possibility that the receptor mediates transport across the blood-brain barrier [Zlokovic et al., 1996]. The data obtained from qPCR experiments showed that LRP-2 is present in the chicken brain, indicating roles in the adult animal.

Furthermore, the receptor, which is expressed on the brush border surface of the renal proximal tubule, binds and reabsorbs several ligands [Leheste et al., 1999]. All collected data proved that LRP-2 is abundantly expressed in the adult chicken kidney. Therefore, LRP-2 expression in the kidney of adult animals seems to mediate equal recycling mechanisms.

In mice, nephrogenesis, which can be divided into four stages, starts by day 12.5 of embryonic development. LRP-2 is not expressed until stage II, where it can be located in S-shaped bodies, the perinuclear envelope, cytoplasmic vesicles, and the apical surface. With the initiation of glomerular filtration the receptor is concentrated in clathrin-coated pits, indicating that LRP-2 is required for proper renal function. However, studies with LRP-2 deficient animals showed that the receptor is not required for kidney development [Fisher and Howie, 2006]. In the chicken, the glomerular anlagen of the mesonephros start to develop in the 2.5 day-old embryo [Schwarze and Schröder, 1985]. We are able to isolate the kidney of 5 day old chicken embryos. Beginning at that time point, LRP-2 expression can be detected in the kidney of the developing chicken. During renal organogenesis the receptor expression steadily increases to finally reach the highest levels after hatching. This expression pattern indicates that LRP-2 may not be essential for renal development but crucial for initiation of filtration and proper kidney function, similar to the

situation observed in mammals [Fisher and Howie, 2006].

The avian yolk sac resembles the mammalian placenta in many aspects, providing nutrients for the embryo and mediating the transfer of yolk components to the embryonic circulation [Speake et al., 1998]. The yolk sac can be found in all species, however displaying different structures and functions. In rodents, the yolk sac completely surrounds the embryo and the absorptive epithelia face the maternal circulation. This interface, expressing LRP-2, remains functional during the whole pregnancy, but around embryonic day ten, the placenta is established, taking over nutrient supply [Moestrup and Verroust, 2001]. The chicken yolk sac is composed of two membranous layers, one is composed of loosely associated mesenchymal cells connected to a network of blood vessels, the other contains large EECs [Hermann et al., 2000]. The determination of the exact localization of the receptor contributes to the understanding of its actions. Immunohistochemical studies of yolk sac sections showed that LRP-2 is expressed on the surface of the EECs facing the yolk compartment. This finding was confirmed by Western blotting results, showing that after the separation of the two yolk sac layers, the anti-chicken LRP-2 antibody exclusively recognizes the receptor in the EECs. LRP-2 expression on the apical surface of chicken yolk sac epithelial cells is consistent with its localization found in other tissues.

The first two weeks of development mostly carbohydrates and proteins are taken up from the yolk, to be later replaced by yolk lipids [Kanai et al., 1996]. During the second half of embryogenesis, lipids are intensively transported across the yolk sac membrane, indicating the rapid growth phase. In the last days before hatching, the yolk sac is withdrawn into the abdominal cavity. The nutrient supply, consisting of lipid reserves, is maintained until the third day after hatching [Speake et al., 1998]. The expression pattern of LRP-2 in the yolk sac during chicken development should also give detailed information about the endocytic processes mediating nutrient supply. Western blot analysis showed that LRP-2 expression starts very early in the yolk sac and gradually increases to reach a plateau in the third week of embryogenesis. Thereupon, the protein expression slowly decreases, but can even be detected after hatching. All these developmental processes are reflected by the LRP-2 expression pattern. This data leads to the suggestion that LRP-2 is an essential receptor for lipoprotein uptake of yolk components via endocytosis, especially during the growth period in the last trimester of development.

Yolk precursor production means a massive metabolic effort for the laying hen, leading to lipid synthesis from carbohydrates in the liver. The lipid secretion into the circulation results in 20-fold plasma VLDL concentration and in elevated levels of vitellogenin and yolk-specific proteins, e.g. vitamin-binding proteins. The production of yolk precursors in the maternal liver is under the strict control of estrogen [Speake et al., 1998]. In mammals, VLDLR (termed LR-8 in the chicken) can be detected in various tissues including heart, muscle, brain, and adipose tissue. In chicken, LR8 is the major oocyte receptor, but very basal levels can be found in some tissues, resembling mammalian expression patterns. In R/O animals the receptor is not functional due to a point mutation at the *lr8* locus [Schneider et al., 1998]. R/O hens are not able to lay eggs and develop severe hyperlipidemia and atherosclerotic features. R/O roosters are not affected due to the fact that LR8 is mostly located to oocytes [Schneider, 2007].

The data obtained from Western blot experiments show a significant difference between LRP-2 protein levels in laying hen and rooster kidneys. The highest LRP-2 expression level can be observed in the laying hen kidney. There is an obvious sex-dependent discrepancy of LRP-2 expression levels in the kidney. Due to the fact that reproductive processes are under the control of estrogen in the laying hen, LRP-2 expression seems to be regulated by steroid hormones. This can be supported by the results gained from estrogen treatment of roosters, which show increased receptor expression after 48 hours past a single estrogen administration comparable to laying hen. When estrogen treated roosters are sacrificed 72 hours after the procedure, the receptor levels have already decreased. This observation can be explained by hormone degradation processes. High plasma concentrations of estrogen regulate the hepatic expression of genes coding for vitellogenin, apoVLDL-II, vitamin-binding proteins, and enzymes in laying hens and in estrogen-treated roosters [Speake et al., 1998]. Hence, LRP-2 expression could be controlled by the same mechanism. The receptor upregulation in the kidney may be important for recycling processes in the renal proximal tubule involved in reproduction.

On the other hand, in R/O animals another situation can be observed. There is hardly any difference between receptor expression levels in the laying hen and R/O females, but there is a big gap between LRP-2 expression observed in wildtype and R/O roosters. R/O males have an increased receptor expression level. Increased LRP-2

levels in R/O rooster kidneys and high receptor levels in R/O hen kidneys may be a result of LR-8 mutation, compensating the loss of functional receptors.

Further investigation in sex differences of LRP-2 expression should give an insight in regulatory mechanisms of estrogen. The comparison of wildtype and R/O embryonic development may be able to elucidate possible rescue processes compensating for the loss of functional LR-8.

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Abbreviations

A	adenine
aa	amino acid
Amp	ampicillin
APS	ammonium persulfate
BM	basement membrane
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	Celsius
Ca	calcium
cDNA	complementary DNA
Cl	chloride
cpm	counts per minute
C-terminal	carboxy-terminal
Da	dalton
DAPI	4’,6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DNA	desoxyribonucleic acid
dNTP	desoxynucleotidetriphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FH	familial hypercholesterolemia
Fwd	forward

G	guanine
g	gram or gravity
gg	Gallus gallus
GST	glutathione-S-transferase
h	hour
HDL	high-density lipoprotein
HRP	horseradish peroxidase
kb	kilobases
kDa	kilodalton
l	liter
LA-repeat	LDL receptor type A repeat
LB	luria bertani
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LH	laying hen
LRP	low-density lipoprotein receptor-related protein
M	molar
µg	microgram
mg	milligram
min	minute
µl	microliter
ml	milliliter
mM	millimolar
mRNA	messenger RNA
nm	nanometer
nt	nucleotides
N-terminal	amino-terminal
o/n	over night

OD	optical density
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered-saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
qPCR	quantitative PCR
Rev	reverse
R/O	restricted ovulator
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase-PCR
s	second
SDS	sodium dodecyl sulfate
T	thymine
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TBS	tris-buffered-saline
TEMED	N,N,N´,N´tetramethylethylendiamine
T_m	melting temperature
U	unit
UV	ultraviolet
V	volt
VLDLR	very low-density lipoprotein receptor
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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09/1994 – 06/2002	BG/BRG Perau (Highschool), Peraustraße, Villach, Austria, with the focus on natural sciences

Practical Experience

07/2006	Practical training in the Laboratory Dr. Holzweber, Medical specialist for med. a. chem. laboratory diagnostics

09/2005	Laboratory for clinical microbiology, LKH Villach (Villach Regional Hospital), Institute for Pathology, Chief physician Dr. Günter Alpi
07/2005	Practical training in the Laboratory Dr. Holzweber, Medical specialist for med. a. chem. laboratory diagnostics

Language Skills

German, native language
English, fluent
Italian, basic knowledge

Computer Skills

Touch typing
MS Office
Adobe Photoshop
Online research databases
Solid knowledge of Apple and Microsoft operating systems

Oral Presentations

J. A. Plieschnig, T. M. Bajari, W. J. Schneider, M. Hermann. The role of LRP-2 during embryonic development. Austrian Atherosclerosis Society (AAS) annual conference, St. Gilgen, 2008