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Review

The role of megalin (LRP-2/Gp330) during development

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Abstract

Megalin (LRP-2/GP330), a member of the LDL receptor family, is an endocytic receptor expressed mainly in polarised epithelial cells. Identified as the pathogenic autoantigen of Heymann nephritis in rats, its functions have been studied in greatest detail in adult mammalian kidney, but there is increasing recognition of its involvement in embryonic development. The megalin homologue LRP-1 is essential for growth and development in *Caenorhabditis elegans* and megalin plays a role in CNS development in zebrafish. There is now also evidence for a homologue in *Drosophila*. However, most research concerns mammalian embryogenesis; it is widely accepted to be important during forebrain development and the developing renal proximal tubule. Megalin is also expressed in lung, eye, intestine, uterus, oviduct, and male reproductive tract. It is found in yolk sacs and the outer cells of pre-implantation mouse embryos, where interactions with cubilin result in nutrient endocytosis, and it may be important during implantation. Models for megalin interaction(s) with Sonic Hedgehog (Shh) have been proposed. The importance of Shh signalling during embryogenesis is well established; how and when megalin interacts with Shh is becoming a pertinent question in developmental biology.
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Introduction

The low-density lipoprotein (LDL) receptor gene family originated early in metazoan evolution (May and Herz, 2003). To date, proteins in this family have only been identified in multicellular organisms; genome analysis has revealed no homologues in unicellular eukaryotes, suggesting no role in the division and survival of these organisms (Herz and Bock, 2002). The mammalian LDL receptor family consists of seven core members (LDLR, VLDLR, ApoER2, MEGF7, LRP, LRP-1b, and megalin) and three distantly related receptors (LRP-5, LRP-6, and LR11/SorLA). Ligands have been identified for each of the family members; for review see Hussain et al. (1999). Orthologues of some members, almost identical in structure to their mammalian counterparts, have been identified in *Caenorhabditis elegans* and *Drosophila melanogaster* (Herz and Bock, 2002). The *Drosophila* gene CG12139 was identified as a megalin homologue in March 2006 (http://www.genecards.org).

LDL receptors are endocytic transmembrane glycoproteins characterised by a large extracellular domain, comparatively short intracellular domains (Hussain et al., 1999), and, for core members, a single membrane-spanning domain that anchors the protein to the plasma membrane (May and Herz, 2003). The cytoplasmic tail contains one or more NPxY motifs that act as docking sites for adaptor proteins containing phosphotyrosine-binding (PTB) domains and as signals for endocytosis. Core members are also characterised by the presence of cysteine-rich repeats of both ligand binding and EGF types and YWTD domains that form folds of β-pleated sheets (Herz and Bock, 2002). The more distantly related receptors share some but not all of the defining structural features of the core members of the family, along with additional structural or functional domains. For example, LRP-5 and LRP-6 do not contain the intracellular NpxY motif shared by the core members but have five copies of PPP(S/T)P motifs instead (He et al., 2004).

The work of Brown and Goldstein (1979) was ground breaking in terms of understanding receptor-mediated endocytosis,

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demonstrating that a receptor for LDL (LDLR) was essential for internalisation and intracellular processing. This work led to the establishment of a model for receptor-mediated endocytosis that has been applied successfully to more recently discovered LDLRs with specificities for different kinds of lipoproteins. As well as functioning in the cellular uptake of extracellular ligands, LDLRs regulate various other biological processes including lipid and vitamin metabolism. Some have a role in cellular signalling and others function during embryogenesis, and it has been established that all members of the LDLR family are present in the CNS.

Reviewing the structure and function of each of the LDLR family members is beyond the scope of this article. The mammalian LDLR family has been the subject of a comprehensive review (Hussain et al., 1999) and more specialised reviews concerning the role(s) of LDLRs in the CNS are available (Herz, 2001; Herz and Bock, 2002; May and Herz, 2003). This article will focus on one member of the family, megalin, emphasising its roles during homeostasis and development, and also its interactions with the developmental morphogen sonic hedgehog (Shh). The interactions between Shh and megalin will be examined and a brief outline of the Shh pathway will be presented; readers unfamiliar with the Shh signalling pathway are directed to the review by Cohen (2003).

Megalin, associated molecules and endocytosis

Like all members of the LDLR family, megalin is an endocytic transmembrane glycoprotein. It does not require associated molecules for endocytosis and degradation of ligands but binding to such ligands may be facilitated by proteoglycans (Marino et al., 2001). Receptor-associated protein (RAP), which is co-expressed with megalin in many developing tissues, has been utilised to study megalin–ligand trafficking and is involved in the biosynthesis of members of the LDL receptor gene family. The membrane receptor cubilin has many ligands in common with megalin. Although cubilin has no transmembrane domain and is therefore reliant on megalin for endocytosis of certain ligands (CC10 in kidney, for example) and both receptors interact during peri-implantation, interactions with cubilin are not necessary for megalin function.

Megalin

Megalin (gp330/LRP-2) was initially identified as the pathogenic autoantigen of Heymann nephritis (Kerjaschki and Farquhar, 1982). By 1996, the gene had been sequenced in rat and humans (Saito et al., 1994; Hjälm et al., 1996) and mapped to chromosome 2 (Xia et al., 1993). It is a 600-kDa integral membrane protein found in many absorptive epithelia (Raychowdhury et al., 1989) and is both structurally and functionally related to low-density lipoprotein receptor-related protein (LRP), another core member of the LDLR family. Both proteins are of similar size, bind many of the same ligands (Willnow et al., 1992; reviewed in Hussain et al., 1999), and are co-expressed in many epithelia during development (Kounnas et al., 1994). Megalin contains a single transmembrane domain (Xia et al., 1993), is known to act as an endocytic receptor and is expressed primarily on the apical surfaces of polarised epithelial cells (Kounnas et al., 1994). The extracellular region contains 17 EGF-like, 36 LDLR type A and 37 LDLR type B domains and YWTD domains (Springer, 1998). The cytoplasmic (C-terminal) region contains three NPxY motifs; residues 107–136, which include the second motif, are critical for apical sorting and targeting, whereas the regions containing the first and third NpxY motifs are needed for efficient endocytosis and signalling activities (Takeda et al., 2003). The extracellular YWTD domains are important in the pH-dependent dissociation of ligand complexes (Davis et al., 1987). A schematic representation of megalin protein and its binding domains is shown in Fig. 1A.

Expression of megalin has been reported in a wide variety of adult tissues including mammary epithelia, thyroid follicular cells and the ciliary body of the eye (Lundgren et al., 1997). It is found in the intestinal brush border (Yammani et al., 2001), the male reproductive tract (Van Praet et al., 2003), uterus and oviduct (Argraves and Morales, 2004), gallbladder epithelium (Erranz et al., 2004), type II pneumocytes (Chatelet et al., 1986; Lundgren et al., 1997) and kidney. It is also expressed in yolk sacs (Lundgren et al., 1997). Data from megalin-deficient mice imply developmental roles in the kidney along with lung and CNS (Willnow et al., 1996). The roles and functions of megalin during embryogenesis, and specifically organogenesis, will form the basis of the following section of this review.

Cubilin

The 460-kDa receptor protein cubilin (gp280) is a peripheral protein that complexes with megalin and is required for megalin-dependent endocytosis of many ligands in kidney tubules and other epithelia (Birn et al., 2000a; Hammad et al., 2000; Barth and Argraves, 2001; Moestrup and Verroust, 2001; Nykjaer et al., 2001; Christensen et al., 2003). For example, in the kidney CC10 binds to cubilin before undergoing megalin-dependent internalisation and degradation (Burmeister et al., 2001). Cubilin mediates the endocytosis of gastric intrinsic factor–cobalamin (vitamin B_{12}) complexes from the distal small intestine (Xu et al., 1999), as does the functionally related protein amnionless (AMN) (Strope et al., 2004), a 48-kDa apical membrane protein. Cubilin and AMN are subunits of a receptor complex called cubam (Fyfe et al., 2004); AMN is required for its membrane attachment and export from the endoplasmic reticulum, and extracellular cubilin is involved in apical sorting of the complex (Coudroy et al., 2005). AMN is also responsible for cubilin brush-border expression (He et al., 2005).

Cubilin lacks a membrane-spanning domain (Moestrup et al., 1998) and is attached to the extracellular surfaces of epithelial cells by its 110-residue N-terminal sequence (Bork and Beckmann, 1993). This N-terminal region contains a number of EGF-like, bone morphogenic protein (BMP)-like and complement (C1r/C1s)-like repeats (Kristiansen et al., 1999). Although the distribution of cubilin is more restricted than that

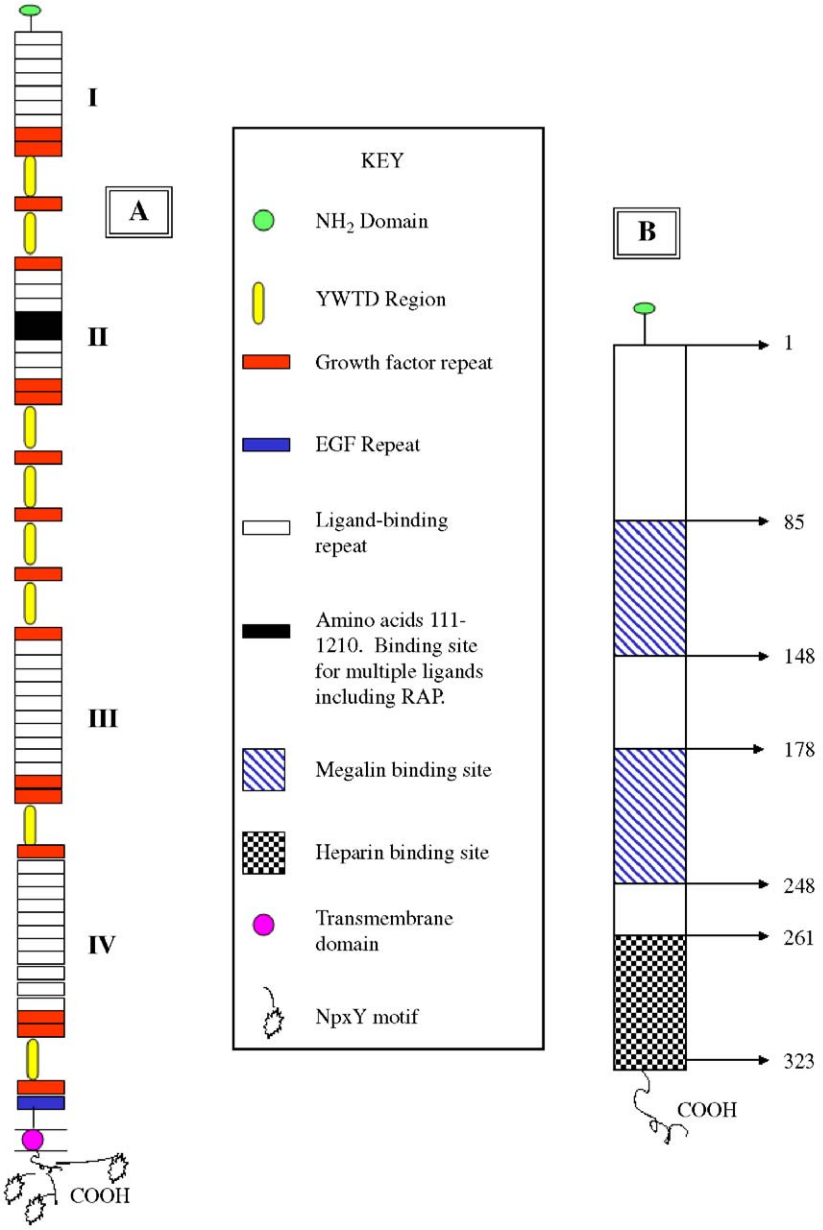

Fig. 1. Binding domains of megalin and RAP. (A) Schematic representation of megalin protein, highlighting the binding site for RAP (amino acids 1111–1210) within the second cluster of ligand-binding repeats. (B) Ligand-binding map of RAP, showing the two megalin-binding sites (between amino acids 85–148 and 178–248) and the binding site for heparin (amino acids 261–323). This figure was adapted from Orlando and Farquhar (1994), Saito et al. (1994), Orlando et al. (1997), and Herz (2001).

of megalin (for a review, see Christensen and Verroust, 2002), in both neonatal and adult mice the cubilin gene is transcribed in tissues that also express megalin (Hammad et al., 2000). This led to the proposal that it functions in endocytosis only as a complex with megalin. However, a study by Moestrup's group (Fyfe et al., 2004) utilising transfected cells in which megalin could not be detected demonstrated that the cubam receptor (mentioned above) can function independently of megalin and that AMN is responsible for the endocytosis of cubilin–ligand complexes (Fyfe et al., 2004). The authors do not exclude the possibility that megalin can/does interact with the cubilin/AMN complex and a functional relationship between cubilin, AMN and megalin has been suggested by Strope et al. (2004). However, it may be misleading to suggest an absolute requirement for megalin during cubilin–ligand endocytosis.

Receptor-associated protein (RAP)

Receptor-associated protein (RAP) is so-called because it co-purifies with both megalin (Kounnas et al., 1992; Orlando et al., 1992) and LRP (Strickland et al., 1990). In humans and mice, it is a 39-kDa protein (Kounnas et al., 1994); the rat homologue has a molecular mass of 44 kDa (Orlando et al., 1992). RAP is co-expressed with both LRP and megalin in various tissues

during development (Kounnas et al., 1994). In the early 1990s, RAP was shown to bind megalin, and it was proposed to assist in biosynthetic processing and/or trafficking of this receptor (Kounnas et al., 1992). RAP modulates ligand binding by members of the LDLR family (Battey et al., 1994), promotes the folding, trafficking and maturation of LDL (Li et al., 2002) and is crucial for the normal processing and function of megalin (Birn et al., 2000b). The proposed functions of RAP have led, rightly or wrongly, to it being referred to as a specialised molecular chaperone and/or escort protein (Bu et al., 1995; Willnow, 1998; Willnow et al., 1995; Birn et al., 2000b; Li et al., 2002). Irrespective of nomenclature, it is clear that RAP has important roles during biosynthesis of LDLR family members and endocytosis of megalin–ligand complexes (as discussed below). It binds to megalin with high affinity (K_d = 8 nM) (Kounnas et al., 1992) and this binding is calcium dependent (Christensen et al., 1992; Biemesderfer et al., 1993). Two primary megalin-binding sites have been identified, between residues 85 and 148 and between residues 178 and 248 (Orlando and Farquhar, 1994; see Fig. 1B).

It has been shown that residues 1111–1210 in megalin encompass a binding site for various ligands including RAP (Orlando et al., 1997). Although the authors of this study concluded that megalin contained one binding site that is common to several ligands, a more recent report suggests that megalin contains more than a single RAP-binding site (McCarthy et al., 2002). The most closely related member of the LDLR family, LRP, has multiple RAP-binding sites (Williams et al., 1992), so it is reasonable to suppose that the same applies to megalin. However, the details of megalin–RAP binding interactions remain elusive (Kounnas et al., 1992), although it is clear that accessory molecules such as proteoglycans can play a role.

Heparan sulphate proteoglycans (HSPGs)

Heparan sulphate proteoglycans (HSPGs) have been implicated in megalin function (McCarthy et al., 2002); however, megalin does not specifically bind heparin (Orlando and Farquhar, 1993) and the interaction is indirect. In the thyroid gland, HSPGs are involved in megalin binding to thyroglobulin (Tg). Marinò et al. (1999a) identified a 15 amino acid sequence between Arg^{689} and Lys^{703} in the *carboxyl*-terminal region of rat Tg that constitutes a heparin-binding region involved in megalin binding. HSPGs have been shown to bind to this heparin-binding sequence on Tg, facilitating binding of the prohormone to megalin and ultimately its transcytosis (Lisi et al., 2003). These studies demonstrate the importance of HSPGs for megalin to bind certain ligands.

In vitro studies demonstrate that RAP can bind directly to heparin; the heparin-binding site is between residues 261 and 323 (Orlando and Farquhar, 1994). The megalin- and heparin-binding sites in RAP are non-contiguous, implying that the glycosaminoglycan site may be physiologically exposed when RAP is bound to megalin (Orlando and Farquhar, 1994). This means that megalin requires heparin-binding ligands in order to interact with HSPGs for signalling and/or endocytosis. Therefore, HSPGs may interact with megalin in different ways depending on the tissue and ligand(s) involved.

Mechanisms of megalin action: endocytosis and signalling

After the initial discovery in the early 1980s that circulating autoantibodies bind to megalin causing Heymann nephritis, much research focused on the role of megalin in the renal system. Antibody–megalin complexes result in immune deposits in the glomerular basement membrane, which in turn lead to damage in the glomerular capillary wall and inevitably proteinuria (as reviewed by Kerjaschki and Neale, 1996; Christensen et al., 1998). Numerous studies including analysis of megalin-deficient mice and dogs with a functional defect in cubilin demonstrate that both proteins are key receptors in the endocytosis of various ligands in the kidney proximal tubule (Leheste et al., 1999; Birn et al., 2000a). They interact during early embryogenesis to provide nutrition to the developing embryo, although cubilin is not the only co-receptor involved in megalin function and endocytosis, and it is not essential for megalin to function.

Because megalin was originally recognised as an important mediator of kidney proximal tubule function, research has focused predominantly on its role in endocytosis. Megalin and its orthologues are proposed to endocytose dietary sterols such as cholesterol, and this function is not only important in many adult tissues but also during embryogenesis. Megalin is required in order that cholesterol can be taken up by the visceral yolk sac in early post-implantation mammalian blastocysts (Assémat et al., 2005a) and the neuroepithelium before neural tube closure (Willnow et al., 1996); in *C. elegans*, the orthologue of megalin is also thought to be essential for sterol uptake and therefore normal development (Yochem et al., 1999).

However, the interactions between megalin and scaffold proteins, which are usually mediated through cytosolic adaptors, imply a diversity of other functions in cellular communication and signal transduction (Gotthardt et al., 2000). Interactions involving megalin's cytoplasmic (C-terminal) moiety are responsible for its polarised distribution in epithelial cells (Marzolo et al., 2003), and several adaptor molecules that bind this moiety to various intracellular proteins have been identified. These include proteins with PTB and PDZ domains and proteins with ankyrin and tetratrico repeat elements (Gotthardt et al., 2000; Petersen et al., 2003; Rader et al., 2000). Another potential intracellular adaptor molecule, megalin-binding protein (MegBP), has been identified by Petersen and co-workers (2003). The binding site for MegBP was mapped to a proline-rich element of the N-terminal region of the megalin tail, and it is thought to bind megalin to a range of intracellular proteins. Proteins linked to megalin via MegBP include transcriptional regulators such as the SKI-interacting protein (SKIP), a co-activator of the vitamin D receptor (Petersen et al., 2003). The discovery that MegBP links SKIP to megalin suggests that megalin might participate in the regulation of transcription, and the recent observation that megalin can undergo proteolysis in situ in the membrane might suggest a role in cell signalling (Zou et al., 2004). In support of

this, it has been shown to associate with a member of the guanylate kinase family, PSD-95 (Larsson et al., 2003).

The adaptor protein disabled-2 attaches to FxNPxY sequences in the megalin tail (Gallagher et al., 2004), as does autosomal recessive hypercholesterolemia (ARH). ARH is a PTB protein (Garcia et al., 2001); it binds to the first of the two FxNPxY motifs (Nagai et al., 2003). It is first seen in association with internalised megalin in clathrin-coated pits, then in early endosomes, followed by recycling endosomes and finally at the cell surface again (Nagai et al., 2003). ARH appears to facilitate megalin-related endocytosis and possibly acts as a chaperone during the process.

Studies in which cells are given exogenous RAP have been utilised to study megalin–ligand trafficking and the distribution of both RAP and megalin during endocytosis. They do not refer to endogenous RAP, which is not secreted extracellularly. They reveal that although trafficking through early endosomes is characteristic of ligand-bound megalin, in the absence of ligands this process involves RAP (Bu et al., 1995; Willnow et al., 1995). Like ARH, RAP appears to be necessary during endocytosis for normal processing and subcellular distribution of megalin. It has been demonstrated to bind to megalin shortly after its synthesis in the rough endoplasmic reticulum (Biemesderfer et al., 1993). During endocytosis, megalin–RAP complexes appear to cycle through late endosomes. From these, megalin is returned to the cell surface whereas RAP undergoes lysosomal degradation (Czekay et al., 1997). Levels of megalin in kidney proximal tubule cells fall significantly in the absence of RAP. Concomitantly, its distribution changes; less is detected on the brush-border membrane and relatively more is found on the rough endoplasmic reticulum (Birn et al., 2000b).

Megalin and homeostasis

Megalin is detected on embryonic structures that give rise to homeostatic organs such as the thyroid, parathyroid (Assémat et al., 2005b) and kidney (Sahali et al., 1993; Assémat et al., 2005b). With the exception of the metanephros, there is little information concerning the expression and/or function(s) of megalin during the development of organs primarily involved with body homeostasis. However, as outlined below, it is clear that megalin is important in controlling body homeostasis in adult mammals. This implies that proper expression and function of megalin during development of these organs may be prerequisites for normal maturation and survival.

Thyroid

A negative-feedback pathway involving thyroid stimulating hormone (TSH) prevents over- and under-secretion of the thyroid hormones. The functional unit of the thyroid, the follicle, is composed of a layer of thyrocytes (epithelial cells) surrounding a lumen containing colloid, of which a major constituent is the prohormone thyroglobulin (Tg). Tg is synthesised and then released into the lumen of the follicle by thyrocytes (Ericson, 1981). The release of thyroid hormones requires the iodination of tyrosyl residues on Tg (Taurog et al., 1996), its uptake by thyrocytes and transportation to lysosomes, where proteolytic cleavage results in hormone release (as reviewed by Dunn et al., 1992). Megalin has a key role in thyroid hormone secretion and therefore in the control of body homeostasis.

Megalin is expressed in a TSH-dependent manner on the apical surface of thyrocytes, binds to Tg with high affinity and mediates its endocytosis (Marinò et al., 1999b, 2000); the role of HSPGs in this process was mentioned earlier. Most Tg endocytosed by this mechanism is low hormonogenic; it undergoes apical–basolateral transcytosis and is released into the blood stream (Marinò et al., 2000; Lisi et al., 2003). This is thought to divert low-hormonogenic Tg from the lysosomes, allowing mainly high-hormonogenic Tg to undergo proteolytic cleavage and consequent release of hormones, thereby favouring more effective hormone secretion (Lisi et al., 2003). Cell culture studies incorporating megalin blocking experiments and immunoprecipitation data suggest that lack of functional megalin on thyrocytes impairs the transcytosis of low-hormone Tg, which therefore competes with high-hormonogenic Tg for the lysosomal pathway; this should result in hypothyroidism (Lisi et al., 2003). Ninety-eight percent of megalin−/− mice die perinatally (Willnow et al., 1996), but for unknown reasons some do survive to adulthood. A study comparing adult mice heterozygous for the disrupted megalin gene with megalin−/− mice revealed hypothyroidism in the KO animals, accompanied by low serum levels of Tg and high levels of TSH (Lisi et al., 2005).

Kidney and parathyroid

The functions of megalin have been studied in greatest detail in the renal proximal tubule, where it is associated with the sodium-potassium exchanger (Biemesderfer et al., 1999). It is necessary for the reabsorption of retinol (Christensen et al., 1999), vitamin D (Hilpert et al., 2002), vitamin B_{12} (Birn et al., 2002) and calcium (Frick and Bushinsky, 2003) and for angiotensin II uptake (Gonzalez-Villalobos et al., 2004). Megalin is strongly expressed in proximal tubule (PT) cells in adult rodent kidneys (Birn et al., 2000b; Biemesderfer et al., 1992), primarily in coated pits along the apical domain (Biemesderfer et al., 1992) and in microvilli of the brush border (Kerjaschki and Farquhar, 1983; Christensen et al., 1995). It is also expressed in endocytic vesicles, membrane recycling compartment dense apical tubules (DAT) and some lysosomes (Christensen et al., 1995). Megalin is expressed at low levels in glomerular podocytes of Lewis rats (Assémat et al., 2005b). No other rat strain or species, including humans (Ronco and Debiec, 2005), has been shown to express megalin in these cells.

Vitamin A (VA) derived metabolites (retinoids) increase embryonic viability and positively impact embryonic development (Eberhardt et al., 1999). Plasma retinol (ROH) is transported by retinol-binding protein (RBP). Although the liver is the main site of RBP synthesis, and hepatic VA storage and mobilisation are important for homeostatic regulation of retinol, approximately 50% of the circulating ROH pool

originates in the kidneys. In 1999, a study by Christensen et al. demonstrated a crucial role for megalin in VA homeostasis and a further study in 2005 utilising mice with a kidney-specific defect in megalin gave insight as to the intrinsic nature of that role. The latter study demonstrated that megalin is essential for ROH homeostasis by virtue of its role in ROH-RBP reabsorption in kidney PT, and also that kidney-specific megalin inactivation affects hepatic VA storage; rapid hepatic VA mobilisation ensues, probably in response to uncontrolled urinary ROH excretion (Raila et al., 2005). Although many questions remain regarding regulation of VA metabolism, megalin has a crucial homeostatic role.

There is significant functional interplay between kidney and parathyroid gland in respect of calcium, phosphate, vitamin D and parathyroid hormone (PTH) metabolism. Within minutes of reduction in extracellular [Ca^{2+}], PTH, produced and secreted by parathyroid chief cells, is released into the circulation. It mobilises calcium by osteoclastic breakdown of bone, increases renal reabsorption and stimulates renal conversion of 25-OH vitamin D_3 to 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], which in turn increases intestinal absorption of dietary calcium (Hilpert et al., 1999).

The unique ability of the parathyroid gland to respond to changes in extracellular [Ca^{2+}] is due to Ca^{2+}-sensing molecules on parathyroid cell surfaces, thought to be involved in regulating cytoplasmic [Ca^{2+}] and PTH release (Nemeth and Scarpa, 1987). One such Ca^{2+}-sensing molecule, later identified as megalin, was discovered by Juhlin and co-workers (1987, 1988, 1990). It contains Ca^{2+}binding sites on its extracellular domain (Christensen et al., 1992; Lundgren et al., 1994) and is found in several rat cell types with presumed or demonstrated Ca^{2+}-sensing ability; many of these also express parathyroid hormone-related protein (PTHrP) (Lundgren et al., 1997). Antibodies binding to megalin inhibit the effect of increased external [Ca^{2+}] on PTH secretion (Juhlin et al., 1987).

PTH is predominantly cleared from the circulation by glomerular filtration. Renal PTH catabolism is impaired in megalin KO mice, resulting in a 4-fold increase in levels of amino-terminal PTH fragments in urine compared to wild-type litter mates (Hilpert et al., 1999). However, the total amount and subcellular localisation of PTH/PTHrP receptor in kidney PT cells is unaffected (Hilpert et al., 1999). RAP-deficient mice have decreased renal megalin expression and disturbed endocytosis in the PT (Birn et al., 2000b). It is thought that lysosomal degradation of PTH is mediated through a RAP-sensitive receptor (Hilpert et al., 1999), and RAP-deficient mice have impaired PTH-induced endocytosis (Bacic et al., 2003). It is unclear whether reduced megalin levels are solely responsible for this or if other factors are also affected by RAP deficiency, but PTH receptor expression in kidney PT cells is not affected in RAP KO mice (Bacic et al., 2003).

Dietary inorganic phosphate (P_i) intake and PTH regulate P_i, which is reabsorbed in the renal PT via Na^+/P_i cotransporter type IIa (NaP_i-IIa). Megalin is involved in regulating NaP_i-IIa, and PTH-induced internalisation of NaP_i-IIa in kidney is delayed in RAP-deficient mice although its distribution and abundance remain unaltered (Bacic et al., 2003). Residual megalin expression may be sufficient for endocytosis, or other proteins may partially compensate for RAP. Alternatively, RAP might alter the expression or function of other proteins important for NaP_i-IIa function/retrieval (Bacic et al., 2003).

Circulating levels of $1,25(OH)_2D_3$ depend mainly on the renal cytochrome P450 enzyme 25-hydroxyvitamin D_3-α-hydroxylase (1α-hydroxylase), which is potentially induced by PTH. The function of 1α-hydroxylase is not fully defined but activity/expression is seen in extra-renal sites expressing megalin, where local synthesis of $1,25(OH)_2D_3$ appears to fulfil autocrine or paracrine functions (Hewison et al., 2000). Parathyroid gland chief cells express 1α-hydroxylase mRNA and protein (Segersten et al., 2002). Megalin is also expressed in these cells (Lundgren et al., 1997), consistent with its putative capacity for local synthesis of $1,25(OH)_2D_3$, which contributes to feedback regulation of parathyroid function by suppressing PTH gene transcription and inhibiting PTH secretion and cell proliferation (Hellman et al., 1999; Silver et al., 1999).

Decreased renal $1,25(OH)_2D_3$ production and high phosphate levels may be principal causes of parathyroid glandular enlargement in hyperparathyroidism (Slatopolsky et al., 1999). Although work concerning the role(s) of 1α-hydroxylase, PTH and Ca^{2+}-sensing receptors in hyperparathyroidism (HPT) and adenomas has been carried out in the last decade (Lundgren et al., 1997; Farnebo et al., 1998; Knutson et al., 2000; Segersten et al., 2002), no clear correlation between the levels of these various molecules and disease severity has been elucidated.

Megalin and development

Work concerning the expression and possible functions of megalin has centred on the renal system, owing to its discovery as the Heymann nephritis autoantigen, and on the CNS, because of the phenotypic characteristics of megalin−/− mice. However, there is a growing body of evidence for megalin functions in the male and female reproductive tracts and the thyroid gland, and it is known to be expressed in several other organs, even though definitive functions have not been investigated. This section outlines what is known about the role of megalin in thyroid, the reproductive system, and the developing CNS and renal system and identifies other organs in which it is expressed. Current knowledge of megalin function in the nematode *C. elegans* and zebrafish is reviewed briefly before the expression and possible functions of the protein during mammalian organogenesis are discussed.

C. elegans

The nematode *C. elegans* comprises approximately 1000 cells. Several genes in this organism share conserved domains with members of the mammalian LDLR family, notably those encoding LRP-1 and LRP-1b. These high molecular mass proteins were originally named to reflect their close structural similarities with mammalian LRP (Yochem and Greenwald, 1993), but subsequent studies have shown that LRP-1 is more closely related to megalin (Saito et al., 1994). The role of LRP-1b in *C. elegans* remains unclear (Herz and Bock, 2002), but

there is now considerable evidence that LRP-1 is essential for growth and development. Mutations prevent shedding and degradation of old cuticle during larval moults and usually cause growth arrest at the moult between the third and fourth larval stages (Yochem et al., 1999). These effects impair the larva's ability to feed (Herz and Bock, 2002).

The components of the cuticle, a collagen-rich exoskeleton and environmental barrier, are secreted from the hyp7 syncytium. This structure makes up most of the epidermis (hypodermis) of *C. elegans* larva; its apical plasma membrane is the outermost membrane for most of the body length and LRP-1 is present on the apical surface. LRP-1 might regulate extracellular proteases; thus, the inability of mutant larvae to degrade the cuticle could be the result of inadequate proteolysis of the exoskeleton (Yochem et al., 1999). The proposal is based on two observations. First, the extracellular moiety of LRP-1 must be close to or in contact with the overlying cuticle if shedding is to occur. Second, the mammalian homologue of LRP-1, megalin, is believed to regulate extracellular proteases; this may partly account for its role in cholesterol metabolism and lung abnormalities found in null mice (see below) (Willnow et al., 1996).

The phenotypic effect of sterol starvation in *C. elegans* and other nematodes, incomplete moults, is indistinguishable from that of LRP-1 mutation (Bottjer et al., 1984; Coggins et al., 1985). This observation has led to an alternative proposal. Megalin endocytoses dietary sterols such as cholesterol from extracellular fluids that are in contact with polarised epithelia. LRP-1 is also endocytic, as evidenced by the presence of large multivesicular bodies in the syncytial epidermis (Yochem et al., 1999). Because nematodes absorb sterols mainly through the epidermis rather than the intestine (Fleming and Fetterer, 1984) and LRP-1 is present on the apical surface of hyp7 syncytium, it is suggested that LRP-1, like megalin, is required for sterol uptake.

This proposal remains speculative because it is not clear why sterols are essential for nematodes (Chitwood, 1992). However, Ce-imp-2, a critical regulator of development, potentially interacts with lipid-lipoprotein receptor-mediated pathways (Grigorenko et al., 2004). Knockdown of Ce-imp-2 leads to embryonic death and an abnormal moulting phenotype in *C. elegans* (Grigorenko et al., 2004); this protein is required for proper development and complete shedding of old cuticle in all four larval moults. Loss of function in several other genes including daf-9 and let-512, which are involved in lipid and steroid homeostasis or signalling, results in abnormalities that include moulting defects (Jia et al., 2002; Roggo et al., 2002). The loss of *Ce-imp-2* function was mimicked by cholesterol depletion and partly suppressed by expression of a Ce-lrp-1 derivative; Grigorenko et al. (2004) conclude that Ce-imp-2 may promote trafficking of Ce-lrp-1 to the cell surface. The cholesterol depletion effect suggests that Ce-imp-2 acts upstream of LRP-1 in *C. elegans* and promotes cholesterol biosynthesis.

Zebrafish

Analysis of megalin expression during CNS development has been carried out in rodents and zebrafish (Willnow et al., 1996; McCarthy et al., 2002; Assémat et al., 2005b). In zebrafish embryos, megalin is prominent in the floor plate of the neural tube by 16 h post-fertilisation and 8 h later is expressed in cells of the ventral floor plate and the apical surfaces of cells lining the neural tube lumen. Later (33 h) it is detected in cells of the luminal surface of both the forebrain and midbrain, with strong expression at the midbrain–hindbrain border. There is no megalin expression in the notochord (McCarthy et al., 2002).

Mammalian development: peri-implantation

Megalin is expressed in the mammalian embryo long before development of the foetal shape, let alone specific organs. Following fertilisation, rounds of cell division result in the formation of a blastocyst, the endpoint of pre-implantation development; this comprises the inner cell mass (ICM) and, surrounding the ICM, the trophectoderm (TE). Cells of the ICM are predominantly apolar and undifferentiated, whereas the TE is a functional epithelium with outward-facing apical cell surfaces. It contributes to blastocoele expansion at the 32-cell stage (Wiley, 1984), is important for implantation, gives rise to extra-embryonic tissues but does not contribute to the formation of embryonic tissues (Rossant, 1986).

Gueth-Hallonet et al. (1994) reported that megalin is first synthesised in 16-cell stage embryos and is found in coated pits between the microvilli of TE cells undergoing epithelial differentiation. Secondary lysosomes form during the 16-cell stage (Fleming and Pickering, 1985; Fleming et al., 1986). This led Gueth-Hallonet et al. (1994) to conclude that megalin first appears during final maturation of the endocytic system. However, Assémat et al. (2005a) showed that megalin is first expressed in 8-cell stage embryos when segregation of TE and ICM is initiated. Cubilin is also expressed in 8-cell stage embryos and has a very similar expression pattern to megalin in pre-implantation and early post-implantation embryos; formation of megalin–cubilin complexes allows uptake of apolipoprotein A-I (apoA-I) by TE cells in the blastocyst (Assémat et al., 2005a). Megalin appears in the embryo concurrently with the development of the endocytic system; it complexes with cubilin in pre-implantation embryos in order to provide nutrition to the developing foetus.

Shortly before implantation occurs (5 days post-fertilisation in mouse), ICM cells differentiate to form the primitive endoderm (Gardner, 1985), which expresses high levels of both cubilin and megalin in membrane-coated pits and endosomes. This primitive endoderm then differentiates into parietal and visceral endoderm (VE), which express megalin, cubilin and AMN (Sahali et al., 1988; Kalantry et al., 2001). AMN and cubilin form the subunits of the cubam receptor, and both molecules may be required for endocytosis and/or transcytosis of ligands in the VE during gastrulation for proper growth of the embryo (Strope et al., 2004). After implantation, VE differentiates into the visceral yolk sac (VYS), which until the establishment of the allantoic placenta is the only interface between the foetus and mother. A role for megalin during implantation is suggested by its restricted expression in primitive endoderm.

Also, megalin binds plasminogen activator (PA)/inhibitor (PAI) complexes (Willnow et al., 1992), which are produced in vitro by mouse blastocysts cultured at the time of implantation (Strickland et al., 1976; Sappino et al., 1989). Megalin and LRP show significant homology and bind many of the same ligands, including PA–PAI complexes (Bu et al., 1992; Christensen et al., 1992; Kounnas et al., 1992, 1993; Willnow et al., 1992). Both these LDLR family members are present in pre-implantation blastocysts, suggesting that both are involved in implantation.

The VYS is crucial for survival and normal growth in rodents and the endodermal cells of this structure express megalin, cubilin and amnionless (Sahali et al., 1988; Kalantry et al., 2001). Despite the appearance of amnionless in the primitive endoderm after implantation (Kalantry et al., 2001), cholesterol uptake by VYS early post-implantation appears to be mediated by megalin/cubilin complexes (Assémat et al., 2005a). AMN is required for murine gastrulation but is not essential for human gastrulation (Tanner et al., 2003) and this probably reflects, at least in part, differing maternal-embryonic exchange mechanisms. A study by Carney and co-workers (2004) highlighted the fact that rats, rabbits and humans utilise fundamentally different strategies for waste/nutrient exchange. Whereas rodents exclusively rely on the VYS, rabbit embryos are not completely enclosed by this structure until gestational day 13, and in humans the chorioallantoic placenta is the important exchange structure with the VYS possibly fulfilling a supplemental role (Carney et al., 2004). The varying role of the VYS possibly also explains why cubilin is apparently not fundamental to human development.

In summary, megalin is involved in nutrition of the pre-implantation and the early post-implanted blastocyst, is required (in conjunction with cubilin and AMN) for the normal growth and nutrition of the peri-implantation embryo and may have a role in the implantation process itself.

Kidney

Kidney development in mammals involves the formation of a pronephros, mesonephros (the functional kidney in fish and amphibians) and finally the metanephric kidney (comprehensively reviewed in Kuure et al., 2000). The collecting duct system of the metanephros derives from an initially unbranched outgrowth of the Wolffian duct, the ureteric bud (UB) (Davies and Davey, 1999), both of which express megalin (Sahali et al., 1993; Assémat et al., 2005b). Glial cell line-derived neurotrophic factor (GDNF) released by surrounding mesenchyme is essential for bud initiation and subsequent development (Pichel et al., 1996; Sainio et al., 1997). It acts upstream of the MAP kinase pathway (Fisher et al., 2001) and is reliant on HSPGs for signalling (Barnett et al., 2002).

By E11.5 in mouse, the UB has branched once, and by E12.5 nephrogenesis has begun; later nephrons do not appear until after birth. Biemesderfer et al. (1992) described four stages of nephron development on the basis of ultrastructural studies (Larsson, 1975; Larsson and Maunsbach, 1975). Stage I (renal vesicle) is characterised by the appearance of the first epithelial features, including the zona occludens separating the apical and basolateral domains. However, the glomerular and PT epithelia do not become distinguishable until stage II (S-shaped body). By stage III, capillaries invade the forming glomeruli, and microvilli are detectable on PT cells. PT epithelia and glomeruli are fully differentiated by the end of stage IV (Biemesderfer et al., 1992). Kidney formation is an example of morphogenesis reliant on branching epithelium, resulting in a so-called ‘epithelial tree’. Nephrons found in the centre of this tree are formed early in development, with newer nephrons at the periphery of the organ.

During nephrogenesis, Biemesderfer et al. (1992) reported that megalin is not expressed until stage III of nephrogenesis and that expression is restricted from the outset to apical clathrin-coated membrane domains and endosomes. However, a later study demonstrated megalin expression in S-shaped bodies during stage II, and in the perinuclear envelope and cytoplasmic vesicles as well as at the apical surface (Abbate et al., 1994). By stage III, co-localisation of RAP and cytoplasmic megalin was evident in the perinuclear envelopes and RER cisternae of immature PT cells; RAP was not detected at the cell surface (Abbate et al., 1994). By stage IV, apical staining in PT appears to be restricted to the coated pit region of the brush border (Biemesderfer et al., 1992) but it is heterogeneous; not all the microvilli express megalin (Abbate et al., 1994). RAP forms a heterodimer with megalin in RER within 30 min of megalin synthesis (Biemesderfer et al., 1993). Taken together with the co-localisation data presented above, this is consistent with the hypothesis that RAP assists in the folding and assembly of megalin within the ER, and in trafficking of newly synthesised megalin to the cell surface.

Clathrin distribution appears to be random during early stages of nephrogenesis but by stage IV it is almost exclusively expressed in the brush border of the PT, paralleling megalin expression (Biemesderfer et al., 1992). Exogenous horseradish peroxidase (HRP) uptake has been used to demonstrate that nephrons are functional (Rodman et al., 1986). During the latter stages of nephrogenesis, megalin and HRP were found to localise in the same PT cells, often in the same endosomes, and in general HRP was not endocytosed if megalin was absent (Biemesderfer et al., 1992). As nephrons mature, megalin becomes progressively concentrated in clathrin-coated pits on the apical domain of PT cells. This coincides with vascularisation of the glomerulus and initiation of glomerular filtration, and therefore the ability of the PT to reabsorb protein coincides with megalin expression (Biemesderfer et al., 1992; Christensen and Verroust, 2002) and the apical distribution of clathrin at the brush border. This is consistent with the endocytic role of megalin in the adult kidney. Although newborn megalin−/− kidney PT cells have smaller and less apical vesicles compared to wild-type cells, megalin is not required for kidney development (Willnow et al., 1996) but it is necessary for proper renal function.

CNS

Megalin may contribute to neurological conditions such as Alzheimer’s disease by virtue of its ability to endocytose the

apolipoproteins apoJ, apoE and apoJ-apoE particles (Chun et al., 1999), suggesting a role in the adult brain. Its importance in the developing brain was highlighted by the introduction of megalin knockout (KO) mice (Willnow et al., 1996). Although they also show pronounced lung and kidney defects, these mice are mainly characterised by an abnormal head shape. Generally, newborn megalin−/− mice have a shortened nose, flattened forehead, often a characteristic protrusion in the midline of the crown and in some cases small eyes or no eyes at all. The olfactory bulbs are almost always absent and the forebrain hemispheres are fused (Willnow et al., 1996).

The expression patterns of megalin and cubilin during CNS development in the rat have recently been documented (Assémat et al., 2005b). Early in development (E11), megalin is detected in the neuroepithelium of the neural tube and the notochord, but notochordal expression is lost by E13. Both receptors are detected in ventral regions by E13: in the optic recesses, the ventral diencephalon, the ventral spinal cord, the newly formed choroid plexus and also in the primordium of the pineal gland, the hippocampal ventricular zone and strongly in the cortical hem (Assémat et al., 2005b). After E15, expression of both receptors is progressively restricted, becoming limited to the choroid plexus, the ventricular zone of the ventral lateral vesicle and cortical hem, spinal cord and ventricular regions of the third and fourth ventricles (Assémat et al., 2005b).

During normal murine development, megalin expression is restricted to the apical surface of the neuroepithelium at E9.5, and in megalin−/− embryos this expression is lost (Willnow et al., 1996). The apical surface of the neuroepithelium is exposed to amniotic fluid before neural tube closure, so in wild-type animals megalin is ideally situated for endocytosis of molecules from this medium. Failure to endocytose essential nutrients for growth and development of the neuroepithelia may contribute to the defects seen in megalin−/− mice. This is akin to the proposed role of megalin in the peri-implantation embryo discussed above.

The striking features of the megalin−/− mouse brain are a markedly reduced telencephalon (TEC) and holoprosencephaly (HP) (Willnow et al., 1996; Spoelgen et al., 2005), but the aetiology of this holoprosencephalic syndrome is unclear. One contributing factor may be disruption of nutrient uptake, specifically cholesterol-rich lipoproteins, before neural tube closure. Impaired cholesterol biosynthesis is also likely to play a part, as is the effect of megalin gene disruption on other important mediators of CNS development. The brain synthesises cholesterol (Dietschy et al., 1983); preventing cholesterol synthesis results in HP, which supports the hypothesis that megalin is required for normal cholesterol transport in the embryo (Willnow et al., 1996). The drug AY9944 inhibits 7-dehydrocholesterol-Δ^7-reductase, a late enzyme in the cholesterol biosynthetic pathway; when administered to rats it produces a holoprosencephalic syndrome similar to that seen in megalin KO mice (Roux et al., 1979). However, there is also evidence that disruption of pathways involved in the specification of the dorsoventral axis of the rostral neural tube contributes to HP. Components of these pathways include BMPs (Golden et al., 1999) and Shh (Chiang et al., 1996).

The earliest phenotypic characteristic of megalin−/− embryos is the reduced size of the TEC (Willnow et al., 1996), and this is accompanied by a decrease in neuroepithelial wall thickness, most noticeable in the entopeduncular area (AEP) (Spoelgen et al., 2005). In wild-type mice, megalin and Shh are co-expressed in the AEP, but Shh expression is lost by E10.5 in megalin KOs. It is therefore likely that loss of Shh signalling in the AEP of mutant mice is responsible for the small size of the TEC and the loss of ventral cell fate in this structure (Spoelgen et al., 2005). Concordant with this loss of signalling, megalin−/− embryos present with impaired establishment of neuroepithelial progenitors and considerably reduced oligodendroglial and interneuronal populations in the ventral forebrain, as found in mice with perturbed Shh signalling (Fuccillo et al., 2004). Shh does not function in isolation; unless Fgf 8 and BMP4 act in synergy with it, proper proencephalic development does not ensue. These components play key roles in dorsoventral patterning of the rostral head (Ohkubo et al., 2002). In E9.5 megalin−/− embryos, BMP4 (a potent antagonist of Shh signalling) is overexpressed, resulting in enhanced and ventrally expanded BMP4 signalling in the proencephalon (Spoelgen et al., 2005). BMP4 binds strongly to megalin and can be endocytosed by this receptor in BN16 cells, raising the possibility that megalin is a receptor for BMP4 in the early forebrain (Spoelgen et al., 2005). It is conceivable that megalin is a negative regulator of BMP4 in dorsal neuroepithelium, and that in mutant mice a loss of megalin results in an increase in dorsal BMP4 signals, suppressing Shh and causing HP and impairment of ventral cell fates (Spoelgen et al., 2005). Although the precise details have not yet been elucidated, it is clear that a lack of megalin results in defects in Shh and BMP4 signalling pathways and in impaired establishment of ventral cell fates in the developing CNS. Interactions between the Shh signalling pathway and megalin have been hypothesised, particularly with regard to the CNS, and possible modes of interaction are discussed later.

Reproductive tracts

Megalin has important roles during development of reproductive organs, primarily through its ability to internalise sex steroids bound to carriers such as sex hormone-binding globulin (SHBG) and specifically deliver them to target tissues (Hammes et al., 2005). In adults, expression of cubilin, megalin and apoJ in the reproductive tract epithelia of both sexes appears to be required for the functional maturation of spermatozoa, probably through direct interaction.

Spermatozoa released into seminiferous tubules are functionally immature. To become capable of fertilisation, they must undergo structural and biochemical changes, including remodelling of the plasma membrane to reduce phospholipid content and alter the cholesterol composition, as they travel through the efferent ducts, epididymis and vas deferens (reviewed by Hermo et al., 1994; Cooper, 1998; Jones, 1998). In adult males, megalin is found in epithelia of the efferent ducts, epididymis and vas deferens, and it mediates the endocytosis of apoJ (Andonian and Hermo, 1999; Morales et al., 1996). Cubilin is

co-expressed with megalin in these tissues; it may be important in spermatozoa maturation through plasma membrane interactions and must bind to megalin in order to be endocytosed (Van Praet et al., 2003).

In addition to these effects on the maturing sperm plasma membrane, megalin is expressed by epithelia lining the ductal region and ampulla of the rat seminal vesicle and binds to seminal vesicle secretory protein II (SVS-II) with high affinity. It can mediate SVS-II endocytosis leading to lysosomal degradation, and this is inhibited by RAP (Ranganathan et al., 1999). After fertilisation, both megalin and cubilin appear to have roles during blastocyst formation and embryo implantation and also in providing essential nutrients to the developing foetus (see above).

Megalin is strongly expressed in the efferent ducts throughout postnatal development in male rats, but not until postnatal day 21 is it expressed in any part of the epididymis (Hermo et al., 1999). Epididymal expression is transient in the distal segment and the distal caput. Megalin expression is maintained throughout postnatal development in all other areas of the epididymis except the proximal initial segment, which does not express the protein (Hermo et al., 1999). This expression pattern correlates with apoJ, a secretory product of Sertoli cells involved in remodelling the spermatozoon plasma membrane (Law and Griswold, 1994) and therefore probably in the maturation of spermatozoa.

Notwithstanding the maturation processes within the male reproductive tract, ejaculated spermatozoa are unable to fertilise an ovum; capacitation is only acquired after residence in the female reproductive tract (Chang, 1951; Austin, 1952). Apical expressions of megalin and cubilin in uterine epithelium and the oviduct are evident throughout the oestrous cycle, with maximal expression during the oestrous and metestrous stages; apoJ and apoA-I are also expressed in these epithelia (Argraves and Morales, 2004). Uterine glands may synthesise both these proteins (Argraves and Morales, 2004), which potentially accept sperm cholesterol as they do during maturation in the male reproductive tract. Spermatozoa do not express megalin or cubilin (Morales et al., 1996; Van Praet et al., 2003). They are therefore reliant on epithelia expressing these receptors so that ligands such as apoJ and apoA-I, the main constituent of HDL, can remodel the plasma membrane to establish acrosomal responsiveness and capacitation. It is likely that maturation of spermatozoa and fertilisation capacity are coupled processes, requiring removal of cholesterol from the plasma membrane, initially in the male reproductive tract and then in the female (Argraves and Morales, 2004).

Megalin KO mice that survive to adulthood present with testicular maldescent and vaginal obstruction, phenotypes that can be induced by anti-oestrogens (Chadwick et al., 1988) and anti-androgens (Van der Schoot and Elger, 1992). Megalin−/− females possess a closed vaginal cavity, resulting in gross inflation of the uterus owing to fluid accumulation (Hammes et al., 2005); in wild-types, the vaginal cavity opens 4–5 weeks after birth (Rodriguez et al., 1997). The vaginal tissue of megalin KO mice is reminiscent of prepubertal tissue of wild-types (Hammes et al., 2005). Its histological appearance, together with the fact that anti-oestrogens delay vaginal opening (Chadwick et al., 1988; Ashby et al., 2002), suggests that defective oestrogen signalling is responsible for this malformation.

In megalin−/− males, the left testis remains in the body cavity rather than the scrotum owing to failure of the inguinoscrotal phase (postnatal) of testes descent. This cryptorchid testis is very small and poorly developed, with degenerated germinal epithelium of the seminiferous tubules, an absence of germ cells, and an epididymis devoid of sperm (Hammes et al., 2005). In addition, the cranial suspensory ligament (CSL), one of two tissues controlling the position of the gonads in both sexes (Zimmermann et al., 1999), was still present in new-born KO mice, tethering the testes and epididymides to the abdominal body wall dorsal to the kidney. Normally, it regresses during development and is absent in newborns. Nevertheless, the descended testis in male KO mice appeared to be normal (Hammes et al., 2005). This study suggests that megalin deficiency impairs spatially and temporarily restricted activities of sex steroids, in line with distinct expression pattern of the receptor during genital development (Hammes et al., 2005). Although the right testis of megalin−/− male mice appear to function normally, it is not clear whether spermatozoa produced by these mice are capable of fertilisation.

The role of megalin in other organs

During respiratory tract development in rats, cubilin and megalin are expressed in the epithelium of the tracheal diverticulum at E11. In subsequent stages of development, both proteins are found in the bronchial lumen of developing lung buds and the apical regions of epithelial cells lining the trachea. By the end of gestation, few bronchi remain positive (Assémat et al., 2005b). Most megalin−/− mice die perinatally from respiratory insufficiency. Lungs of newborn KO mice are characterised by emphysematous and atelectic areas, and it is thought that impaired pulmonary inflation and alveolar expansion are the cause of death (Willnow et al., 1996). Megalin expression has been reported in adult rat type II pneumocytes (Chatelet et al., 1986; Zheng et al., 1994) and human type II lung epithelial cells (Lundgren et al., 1997), and it has been proposed that in adult lung megalin may be important in supplying vitamin E to such cells (Kolleck et al., 2002). Immortalised foetal rat alveolar pre-type II cells express high levels of megalin, which can participate in regulating proteinase activity (Stefansson et al., 1995). This might be relevant to lung physiology and pathology; Willnow et al. (1996) proposed that an imbalance in proteolysis may be responsible for the lung abnormalities in megalin-deficient mice. There is supporting evidence from literature concerning α_1-antiproteinase deficiency and quartz-induced emphysema (Zay et al., 1995), and transgenic mice overexpressing collagenase (D'Armiento et al., 1992), in which increased extracellular proteolytic activity causes analogous structural changes.

Developing sensory organs express megalin and cubilin. In the adult eye, megalin has been detected in the retina and ciliary

body (Zheng et al., 1994; Lundgren et al., 1997) and it is important, although probably not essential, for normal eye development. It is expressed in the optic vesicle, which gives rise to the optic cup, at E11 in rats; but by E18, the only megalin-expressing areas are the outermost layers of the neural retina and the presumptive ciliary body. No expression is evident in the lens vesicle, corneal ectoderm or the perioptic mesenchyme (Assémat et al., 2005b). The role(s) of megalin in this organ is unclear but, as noted above, many megalin−/− newborn mice present with small eyes, or in some cases no eyes at all (Willnow et al., 1996). Pax-6 is a master control gene for eye morphogenesis (as reviewed by Gehring, 1999). Gene disruption is characterised by a small eye phenotype in heterozygous mice and in humans; homozygous pax-6 mutation is lethal to murine embryos. Not only do they lack eyes but the nose is also absent, and they have some brain damage.

In adult rat ear, megalin is expressed in the cochlear duct, specifically on the apical surfaces of marginal cells of the stria vascularis, epithelial cells of spiral prominence and Reissner's membrane (Mizuta et al., 1999). During development, both cubilin and megalin are expressed in various presumptive structures. After E14, the sensory epithelia of the vestibular (utricle, saccule) and cochlear components, as well as the semicircular ducts, express both molecules; by E18 both are expressed in vestibular and cochlear hair cells (Assémat et al., 2005b). Malformations in the ears of megalin KO mice have not been reported and it is unclear what role(s) megalin fulfils in this organ. The role of megalin in the nose is also unknown. At E14 in rats, the olfactory epithelium and forming vomeronasal organ strongly express megalin. However, the basal cells of the olfactory epithelium, the developing snout and whiskers do not (Assémat et al., 2005b).

The lumen of the developing foregut expresses megalin and cubilin, as do some cells of the intestinal tube and epithelial cells lining the stomach. There is transient expression in the oesophagus (E14–E17 in rats), and although the dorsal pancreas shows low levels of megalin expression early in development, none is apparent by E14 (Assémat et al., 2005b). In adults, megalin is expressed in the intestinal brush border (Yammani et al., 2001), and throughout development it is expressed in the epithelium of the small intestine (Assémat et al., 2005b). It is thought to complex with cubilin in the gastrointestinal uptake and transport of vitamin B_{12} (Yammani et al., 2001), a process that also requires AMN, most likely in a complex with cubilin (He et al., 2005). There is also recent evidence to support a role for megalin in delivering folate to suckling animals. Folate binds to high affinity folate-binding proteins (FBPs); megalin has been shown to bind bovine and human milk FBPs and mediate cellular uptake of FBP in vivo and in vitro (Birn et al., 2005). Adult human and mouse gallbladder epithelia express megalin, and it has been proposed that megalin has a role in gallstone pathogenesis through disruption of cholesterol adsorption by the gallbladder epithelium (Erranz et al., 2004). To date, there is no information on the expression or role of megalin during gallbladder development.

Megalin is present in adult mammary epithelia (Lundgren et al., 1997) but as yet no role during mammary gland development has been reported. It probably has a role in the developing heart; mesothelial cells of the pericardium express both megalin and cubilin (Assémat et al., 2005b). It appears that there is no megalin expression in the developing liver, spleen or musculoskeletal system (Assémat et al., 2005b).

Megalin expression, transient or otherwise, during organogenesis of many structures and systems indicates that it is an important mediator during development. The perinatal death of megalin-deficient mice supports this inference.

Interactions with other developmental pathways

Sonic hedgehog (Shh)

There is now in vitro evidence that Shh is included among the multitude of molecules with which megalin is known to interact functionally (McCarthy et al., 2002). Shh is a developmental morphogen with important roles during CNS formation, development of limb buds, the pulmonary system, the reproductive tract and the eye as well as other organs (reviewed in Fisher and Howie, 2005). Work by Ingham and colleagues (1991) led to the articulation of the now well-established model of Hedgehog (Hh) signalling in *Drosophila* and a parallel Shh pathway in vertebrates. Many of the detailed interactions have been elucidated, but some aspects of the pathway remain speculative. In-depth discussions of both the Hh pathway in *Drosophila* and its vertebrate counterpart are available (Cohen, 2003; Fisher and Howie, 2005). Only a general overview will be presented here.

Biologically active Shh (N-Shh) is formed by autocatalytic cleavage of the precursor protein, resulting in a 19-kDa ligand with a covalently linked cholesterol moiety at the C-terminus. It has been proposed that the addition of cholesterol allows N-Shh to carry out long-range signalling (Lewis et al., 2001), as does the molecule Dispatched (Disp). In resting cells, the receptor for Hh, patched (Ptch), inhibits signalling by the transmembrane molecule smoothened (smo). This eventually results in cleavage of the molecule cubitis interruptis (Ci) and the formation of CiR, a truncated form that acts as a transcriptional repressor, preventing the transcription of Ci target genes. The mammalian homologues of Ci are the Gli transcription factors. When Hh binds to Ptch (possibly with the aid of HSPGs), the inhibition of smo is repressed, allowing downstream signalling. Cos-2 tethers a group of segment polarity proteins to microtubules, and full-length Ci is bound to these. Recruitment of cos-2 to smo causes Ci to dissociate from the cytoskeleton, preventing its cleavage to CiR. Full-length Ci is translocated to the nucleus, where it acts as a transcription factor for target genes containing Ci-binding sites (see Fig. 2).

The importance of Hh/Shh in developing organisms cannot be overestimated; its roles are many and varied, encompassing both early and late stages of embryogenesis. Like Shh, megalin also has roles in early development as evidenced by its presence in TE cells (Gueth-Hallonet et al., 1994) and the VYS (Sahali et al., 1988). Its expression during organogenesis, particularly of the renal proximal tubule (Christensen and

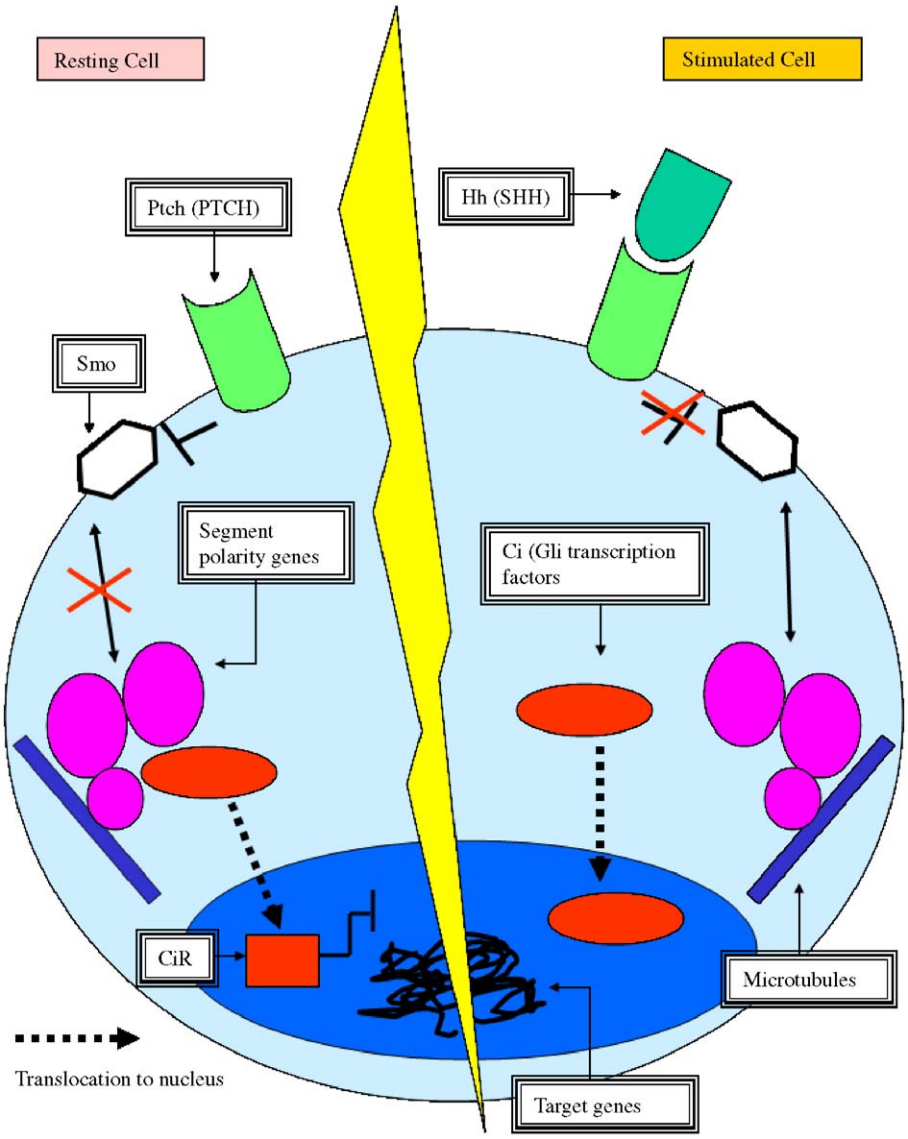

Fig. 2. Simplified overview of the hedgehog signalling pathway. When Hh binds to Ptch, the inhibition of smo is repressed and full-length Ci can translocate to the nucleus and activate target genes. When Ptch remains unbound it inhibits signal transduction by smo, and this ultimately results in the formation of Ci^{75} (CiR), which represses the transcription of target genes. This diagram provides a basic schematic of the Hedgehog pathway in *Drosophila*. Mammalian homologue genes are in parentheses where applicable.

Verroust, 2002) and during forebrain development (Willnow et al., 1996), is well documented.

Megalin–Shh interactions

Many developmental abnormalities in mice that lack components of the Shh pathway are akin to those found in megalin−/− mice. The most noticeable abnormalities in megalin-deficient embryos involve the CNS (Willnow et al., 1996). Shh−/− and smo−/− mice, as well as mice lacking dispatched (Disp), which is critical for the secretion/long-range signalling of N-Shh, display neurodevelopmental abnormalities. This is also the case in smo−/− zebrafish embryos and partially rescued Ptc−/− embryos (McCarthy and Argraves, 2003). Such striking similarities have led to the suggestion that megalin is a regulatory component of the Shh signalling pathway (Herz and Bock, 2002).

Megalin has been shown to bind Shh in a radiolabelled ligand-binding assay, by ELISA and surface plasmon resonance (SPR) measurements (McCarthy et al., 2002). A recombinant fusion protein of N-Shh with glutathione-*S*-transferase (GST-N-Shh) was able to bind megalin with a dissociation constant (K_d) of 21 nM in the presence of calcium, and it has also been suggested that Shh and megalin are co-expressed early in the development of the nervous system (McCarthy et al., 2002). Interactions among megalin, RAP and Shh have been investigated. Megalin–Shh complexes are endocytosed by BN cells (rat yolk sac cell line) (McCarthy et al., 2002), and it has been argued that megalin-mediated endocytosis leads to lysosomal degradation,

evidenced by the presence of TCA-soluble proteolytic fragments of ligands in media after in vitro culture (Stefansson et al., 1995). However, megalin–N-Shh complexes appear resistant to dissociation at pH 4.5; the mechanism(s) by which some ligands bypass lysosomal degradation is unclear.

The available in vitro data, together with the similarities found in null mutant embryos, have led to the proposal of possible models for the role of megalin in the neurodevelopmental biology of Shh (McCarthy and Argraves, 2003), which Cohen (2003) has discussed (inter alia). Three possible models based on the commentary of McCarthy and Argraves (2003) are outlined below and discussed in the light of available evidence. However, it must be stressed at the outset that it is not yet clear whether megalin constitutes a component of the Shh pathway/network, or if it merely binds Shh and internalises it in the relevant compartments of the target cell.

The first model proposes that N-Shh signals directly via megalin (Fig. 3A). As mentioned previously, HSPGs in the thyroid aid the binding of megalin to its ligands (Lisi et al., 2003). It may be the case that in cells that co-express Shh and megalin, possibly during early development of the CNS as proposed by McCarthy et al. (2002), Shh binds to HSPGs, which then facilitate binding to megalin, thereby allowing these proteins to interact, after which signalling ensues. This suggestion is not implausible and has at least some circumstantial support. For instance, HSPGs synthesised by the enzymatic action of tout velu, an integral membrane protein of the EXT gene family, regulate Shh movement (Bellaiche et al., 1998). A very similar model for Shh binding to Ptch on cell surfaces has been advanced (Cohen, 2003).

A second proposal is that cells internalise Shh by megalin-mediated endocytosis, a process that is reliant on RAP (Fig. 3B). The observation that Shh can be internalised by megalin-containing cells during development of the CNS and also by BN cells (McCarthy et al., 2002) lends credence to this proposal. Megalin-dependent endocytosis of Shh may be required to regulate its availability to Ptch, which is one target of Shh. Alternatively, endocytosis may deliver Shh to vesicular pools of Ptch (Cohen, 2003), a plausible reason for its apparent ability to bypass lysosomal degradation.

A third model of Shh–megalin interaction is that Shh undergoes transcytosis whereas megalin internalises Ptch and smo (Fig. 3C). Again, this idea is consistent with the apparent resistance of Shh to lysosomal degradation. There is no evidence to date that megalin and Ptch can interact (McCarthy and Argraves, 2003); it may be the case that megalin requires the aid of HSPGs and/or RAP in order to internalise Ptch–smo complexes. However, a somewhat more plausible idea is that Shh acts as a ligand bridge for internalisation of Ptch and smo. Support for this comes from the observation that in kidney epithelial cells, Ptch–smo complexes are internalised after Shh binding (Incardona et al., 2002). This third model implies that megalin is either a component of the Shh pathway or that it can interact with various components thereof.

Conclusions: questions and reflection

Megalin has roles in mammalian development that range from maturation of spermatozoa, through provision of nutrition to the early embryo around the time of implantation, to involvement and/or expression during the normal development of a wide range of organs (lung, kidney, gastrointestinal tract, endocrine glands, CNS, special sense organs). Before its involvement in development was suspected, megalin was best-known as a widely distributed endocytic receptor on apical membranes of polarised epithelia. Although it associates with a wide variety of ligands, especially when acting in conjunction with cubilin, its primary role is in the uptake of lipoprotein components, notably cholesterol. Primarily, megalin is a device for moving apolar substrates into or across epithelial cells.

Is this property relevant to its role in development? The evidence for megalin having a role in sterol uptake in *C. elegans* is consistent with this possibility, as is the cholesterol-transporting function in sperm maturation, CNS development and possibly the peri-implantation mammalian embryo. Because N-Shh undergoes a lipophilic modification, having a cholesterol moiety covalently linked to the C-terminal in order to interact with its receptor Ptc and for long-range signalling (Lewis et al., 2001), this role could also be relevant to the proposed involvement of megalin in the Shh signalling pathway.

As discussed in the previous section, megalin and Shh clearly interact. However, there are arguments against megalin being a component of the Shh signalling pathway. First, megalin KO mice have very different (and much less dramatic) lung phenotypes from Shh KOs. Lack of Shh is always an embryonic lethal phenotype; 98% of megalin-deficient mice die perinatally. This suggests that either megalin and Shh do not interact during lung development or such interaction is not essential for development of the pulmonary system so long as Ptc is present and functional. The fact that 2% of megalin-deficient mice survive to adulthood with no obvious sign of respiratory insufficiency lends credence to the latter view. Second, RAP KO mice are indistinguishable from wild types though megalin expression is only about 23% of normal (Birn et al., 2000b). Therefore, a 4-fold reduction in megalin expression is compatible with life; this is not true of Shh expression.

In the 98% of megalin KO mice that die perinatally, the lungs are not inflated and are characterised by thickened alveolar walls; this is thought to be the cause of death (Willnow et al., 1996). Megalin is expressed in adult type II cells of rodents and humans (Chatelet et al., 1986; Zheng et al., 1994; Lundgren et al., 1997), the principal producers of lung surfactant, suggesting that what matters for proper lung inflation and surfactant production at birth is a “megalin pathway”. That is, megalin must normally endocytose a particular ligand (or must be internalised by these cells) at an appropriate stage of development, otherwise insufficient surfactant is produced and the lungs, though more or less phenotypically normal, are not fully functional. Clara cells also produce surfactant components, though not surfactant protein C (SP-C) (Horowitz et al., 1991), which is important in the spreading of the surface

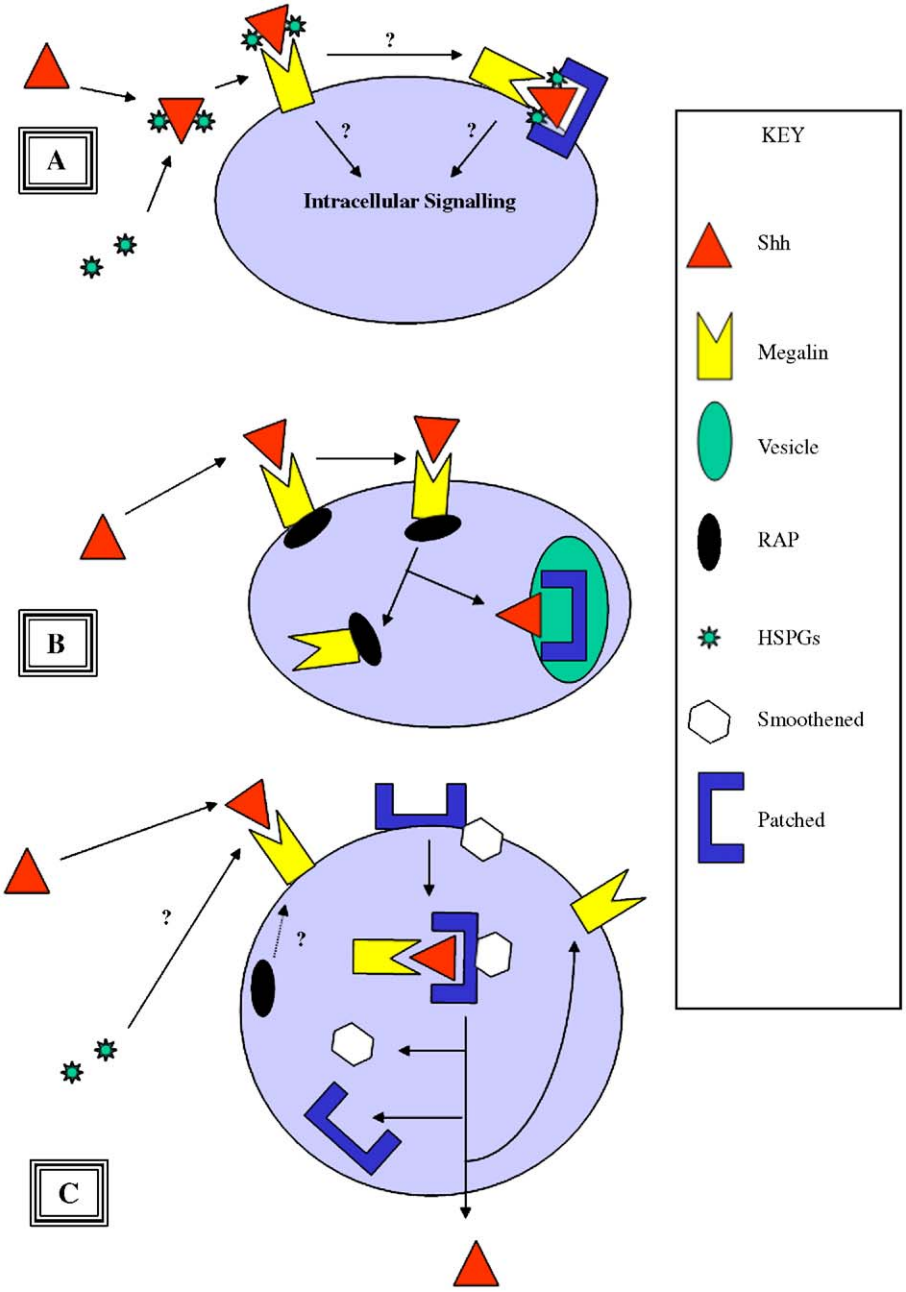

Fig. 3. Megalin–Shh interactions. (A) Shh is known to bind to megalin with high affinity and this may be aided by interaction with HSPGs. Once bound, the Shh–megalin complex may result in intracellular signalling via a 'megalin' pathway. An alternative hypothesis is that the Shh–megalin complex interacts with Ptch on the cell surface and then signalling ensues. (B) Shh binds to megalin on the cell surface and undergoes endocytosis, possibly delivering Shh to vesicular pools of Ptch. The process requires interaction with RAP. (C) Shh undergoes transcytosis whereas megalin internalises Ptch and smo. Shh may act as a ligand bridge for a complex that includes megalin, Shh, Ptch and smo, and it may be that RAP and/or HSPGs are involved. Ptch and smo are internalised whereas megalin delivers Shh to the extracellular surface of the cell, and then is recycled back to the cell membrane.

film and preventing alveolar collapse. If megalin is not expressed on Clara cells, then any surfactant produced by megalin KO mice would presumably lack SP-C. The fact that 2% of such mice survive to adulthood argues that megalin does not act in isolation in this process.

Analogous "megalin pathways" might also operate in other organs. For example, although there is considerable evidence that megalin is involved in the Shh pathway in CNS development (see above), is it possible that some of the effects seen in megalin−/− mice are attributable to abolition of a "megalin pathway" rather than dysfunctional megalin–Shh interactions? Megalin is not present in neurons in the adult brain but it plays an important role in transport of ligands across the blood–brain and blood–cerebrospinal fluid barriers; in particular, it appears to be the only receptor for ApoJ, ApoE and ApoJ-ApoE particles, all of which accumulate in the brain in Alzheimer's disease (AD) (Chun et al., 1999). It would be interesting to know whether the brain tissue of megalin-

deficient mice that survive to adulthood demonstrates accumulation of these particular ligands. If so, this would support the idea of a "megalin pathway" that is distinct from the Shh signalling network, at least in the adult brain if not in the developing CNS.

The point of these speculations is to suggest that megalin might have a more crucial role in adult, as well as in developing organs, than is widely suspected at present. Megalin was only identified a little over two decades ago and its role in development has been investigated for less than a decade. Although its expression patterns in most adult and embryonic tissues have been described, many of megalin's functions and interactions with other signalling pathways remain to be elucidated. It is hoped that this insight into megalin and its roles in development will encourage research into the many questions that are now open.

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