Identification and characterization of the t(w73) candidate gene Ortc3

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Mutations that affect the development and function of the extraembryonic membranes and placenta

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Intra-uterine development of the mammalian embryo undergoes four distinct phases of dependence on maternal nurturing (shown in Fig. 1). The first phase, that can also occur in vitro, is the preimplantation stage that is dependent on maternal resources deposited in the maturing oocyte. In this phase the fertilized single cell embryo (or zygote) increases in cell number to approximately 32 cells by reducing divisions that take place with no increase in mass. Cellular differentiation directed mainly by the relative position of cells, occurs to form a blastocyst that is competent to implant into a receptive uterine wall (Fig. 1A). At the site of implantation the uterine wall proliferates to produce a hyper-vascularized cell mass known as the decidua, that surrounds the embryo with large pools of maternal blood. Maternal nurturing at this second phase, from 4.5 to 8.5 days post coitum (dpc), occurs by diffusion of nutrients, gases, and waste products from the maternal blood pools and decidual cells across the developing membranes that enfold the implanting embryo and are known as the extraembryonic membranes (Fig. 1B). Once implantation occurs, development and growth of the embryo proceeds. The third phase of maternal nurturing of the embryo starts at gastrulation with the formation of the visceral yolk sac. This bi-layered membrane contains blood islands that produce blood cells and a network of blood vessels that are necessary, because of increased embryo size, to circulate nutrients and waste products between the maternal blood pools and the embryo (Fig. 1C). Finally, to meet the increased demands of the embryo during its progressive growth, in the fourth phase of maternal nurturing, the yolk sac is replaced by the chorioallantoic placenta as the primary source of nutrient, gas, and waste exchange (Fig. 1D). The placenta has numerous functions in addition to being the medium of maternal-fetal exchange. It allows anchorage of the fetus to the uterus, formation of vascular connections, endocrine regulation of fetal and maternal tissues, and immunological protection of the fetus from its maternal host.

A time table of the key steps in the development of the extraembryonic lineages and placenta is shown in Figure 2. All of these individual steps in the formation of the extraembryonic membranes and placenta are essential for fetal development. In contrast, severe abnormalities in the development of the embryo proper, including absence of the complete organ systems do not prevent the conceptus from being carried to term (1). For instance embryos with anencephaly resulting from neural tube closure defects in Tcfap2a (AP-2) mutants, or Foxd1 (BF-2) deficient embryos with severe kidney abnormalities, do not die until the perinatal period (2,3).
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Figure 1 Stages in development of the extraembryonic lineages. A, late blastocyst; B, egg cylinder; C, yolk sac; D, mature placenta. ICM, inner cell mass; PrE, primitive endoderm; TE, trophoderm; 1° GCS, primary giant cell; EPC, ectoplacental cone; 2° GCS, secondary giant cell; VE, visceral endoderm; EEE, extraembryonic ectoderm; E, epiblast; RM, Reichert’s membrane; PE, parietal endoderm; BI, blood island; VYS, visceral yolk sac; PYS, parietal yolk sac; CH, chorion; AL, allantois; AM, amnion; DB, decidua basalis; GC, giant cell; SP, spongiosotrophoblast layer; L, labyrinth; CP, chorionic plate; UC, umbilical cord; dpc, days post coitum.

The use of homologous recombination to generate null alleles in the mouse has, unexpectedly led to the identification of a large number of genes (169 are listed in Table 1) that affect the structure and function of the extraembryonic membranes and placenta. A molecular analysis of implantation and placentation in mouse models is likely to give information on disorders of human placentation despite the fact that mouse and human placentation have several differences (4). Humans show a wide range of placental anomalies such as miscarriage, preeclampsia, choriocarcinoma, and intrauterine growth retardation (5-7). As the molecular basis of these human pregnancy syndromes is little understood, it is likely that mouse mutants

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- implantation
- trophoblast invasion
- decidualization
- formation of VYS
- gastrulation
- chorioallantoic fusion
- formation of placenta

**Figure 2** Time table.
Time table representing the key steps in the development of the extraembryonic membranes and placenta in mouse.

will lead to identification of some of the genes involved. In addition to being of relevance in increased understanding of human placental disease, information on placental development might also increase our knowledge of tumor biology. Cellular mechanisms used by trophoblast cells during implantation and placentation, such as those involved in adhesion, invasion, angiogenesis, and immune surveillance are generally silent in the adult, but may be re-used by cancer cells as they metastasize within the body (reviewed in (8)).

As a preliminary to introducing the extraembryonic and placental mutations listed in Table 1, a short summary of normal mouse development is given below based on several recent reviews (9-13).

**Development of the extraembryonic membranes and placenta**

The first differentiation event during embryogenesis subsequently gives rise to the trophoblast lineage. It arise as a simple epithelium surrounding the inner cell mass (ICM) at the blastocyst stage, 3.5 days after fertilization, and mediates the process of implantation (Fig. 1A). Just before attachment of the blastocyst the hormones estrogen and progesterone prime the uterus to accept embryo attachment by inducing changes in vascular permeability of the uterine capillaries. The trophoblast cells subsequently attach to the uterine epithelium and the uterus clamps around the blastocyst (14). Transepithelial invasion of trophoblasts results in apoptosis of the uterine epithelium. Phagocytosis of these cells by trophoblast giant cells facilitates their access to maternal blood vessels. Epithelioid transition and proliferation of the underlying stroma results in a massive thickening of the endometrium, called the decidua (14). The decidua is thought to play a role in the restriction of trophoblast invasion by acting as a physical barrier as well as by the production of inhibitors of proteolytic enzymes. Furthermore, it may also contribute to the protection of the conceptus from the maternal immune system. In
addition, maternal blood pools in the decidua supply nutrition to the conceptus until formation of the placenta (15).

After implantation the trophoblast cells that overlie the ICM (polar trophectoderm) continue to proliferate and grow inwards to form the extraembryonic ectoderm, and outwards to form the ectoplacental cone (Fig. 1B). Signals stimulating trophoblast proliferation arise from the inner cell mass and its derivatives in the embryonic ectoderm and induce proliferation of trophoblast stem cells in the polar trophectoderm and later in the extraembryonic ectoderm of the chorion. The mural trophoblast cells furthest away from the influence of the ICM stop dividing, and ultimately differentiate into primary trophoblast giant cells. The outermost cells of the ectoplacental cone differentiate into secondary giant cells, which line the maternal blood spaces in the ectoplacental cone (16). Trophoblast giant cells are polyploid cells formed by endoreduplication (DNA replication in the absence of cell division). They have invasive capacity and likely mediate implantation into the uterus. Furthermore, they produce angiogenic factors and vasodilators to ensure maternal blood flow to the implantation site as well as hormones unique for gestation (12).

Whereas the trophoblast solely gives rise to extraembryonic structures, the ICM gives rise to the entire fetus as well as the extraembryonic mesoderm and primitive endoderm. The primitive endoderm migrates from the enlarging ICM (or epiblast) onto the basal surface of the trophoblast layer and differentiates into parietal endoderm (PE). It produces molecules such as metalloproteinases and their inhibitors that are supposed to be involved in tissue remodeling during implantation (17) and deposits, in rodents only, a thickened basement membrane called Reichert’s membrane. The trophoblast layer, Reichert’s membrane, and parietal endoderm compromise the outermost extraembryonic layer known as parietal yolk sac (PYS), a primitive diffusion barrier that is involved in the absorption of nutrients from the maternal blood surrounding the conceptus (Fig. 1B). The PYS, is only present until E13.5 and is then reabsorbed (reviewed in (10)). Visceral endoderm (VE) cells on the inner side of the PYS cover both the embryonic and extraembryonic region and secrete a wide spectrum of serum proteins as albumin, transferrin, and other molecules normally produced in the liver and the pancreas indicating that the VE is substituting for these fetal organs before they are formed (18). After gastrulation the newly formed extraembryonic mesoderm lines the inner layer of endoderm to form the visceral yolk sac (VYS). Blood islands form and contain hemangioblasts, the common precursor of endothelial and hematopoietic cells (Fig. 1C, (19)). Endothelial cells differentiate and form a rather homogeneous primary vascular plexus, the primitive capillary plexus. This process of vasculogenesis is followed by extending the vascular plexus through formation of new capillaries and remodeling into small and larger branched vessels, collectively called angiogenesis. Maturation of the developing vasculature involves recruitment of perivascular cells and deposition of a basement membrane leading to stabilization of the vascularization (20). Ultimately this leads to the formation of the primary circulatory system, the vitelline yolk sac circulation, a crucial component in transfer of gases and nutrient diffusing across the PYS from the maternal environment to the growing embryo. A third membrane directly surrounding the embryo is the amnion (Fig. 1D). It develops after gastrulation, when the extraembryonic mesoderm migrates and pushes both embryonic and extraembryonic ectoderm inside to form the amniotic folds. Upon fusion of the amniotic fold and rotation of the mouse embryo, the amnion and chorionic membranes are formed. The mature post-implantation embryo at 13.5 dpc is thus enfolded in three distinct extraembryonic membranes.

The basic body organs are established by day 10.5 post coitum (pc) in the mouse.
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From this time until birth, at 19–21 days (depending on the mouse strain), the embryo shows an approximately 50-fold increase in growth. At the time when metabolic requirements of the growing embryo approach the capacity of the yolk sac, the choioallantoic placenta starts to form. The mature placenta is composed of three distinctive trophoblast cell structures (Fig. 1D). First, the trophoblast giant cells that form the outer layer in contact with the maternal decidua. Second, the spongiotrophoblast that forms by expansion and flattening of the ectoplacental cone and is non-vascularized. Third, the formation of the labyrinthine layer is induced after fusion of the allantois to the trophoblast cells of the chorion. Fetal blood vessels grow out of the allantoic mesoderm, invade the chorionic plate, and instruct the trophoblast stem cells that reside in the chorionic plate to differentiate. Invading trophoblast cells form the (syncytiotrophoblast) barrier between maternal blood sinuses and fetal blood vessels (reviewed in (21)). Maternal blood sinuses enter the labyrinth and after folding and branching the labyrinth forms a huge surface area for nutrient and gas exchange between mother and fetus. By day 10 pc the mature placenta replaces the yolk sac as the primary means of nutrients, gas, and waste exchange and this stage is a critical point in development that can be arrested by mutations in many genes in diverse pathways (1). Growth of the labyrinth continues up to a few days before birth. The allantois contributes to the umbilical cord connecting the embryonic blood vessels to the choioallantoic placenta.

Phenotypes

A literature search (PubMed, http://www.ncbi.nlm.nih.gov) was made to identify all mouse mutants that have been reported to affect different stages of extraembryonic membrane and placental development or function. In Table 1, 169 mutations that have been reported as resulting in an extraembryonic phenotype are listed. They are arranged per stage of embryonic development and then classified according to their specific phenotype.

Uterus/decidua

Successful implantation in the uterus requires the synchronized hormone priming of the uterus to a receptive state and development of the embryo to the blastocyst stage. In Prlr (the prolactin receptor) mutants, these hormone signals are disturbed because the corpus luteum in the ovary does not receive prolactin support. This results in multiple reproductive abnormalities including an implantation failure (22). Among several mutations that directly affect the maternal contribution to implantation, those that affect Lif (cytokine), Hmx3, and Hoxall (homeodomain transcription factors) are the most severe. These mutant females show a complete failure of initiation of implantation, that coincides with an absence of Lif expression in the uterine endometrial gland that normally peaks preceding implantation of the blastocyst (23-25). Deficient development of stromal and glandular cells might explain the loss of Lif expression in Hoxall mutants (25), whereas Hmx3 may function upstream of Lif both resulting in a similar failure of implantation (24). In contrast, ablation of Hoxa10 (homeodomain transcription factor) does not disturb expression of Lif and results in a combination of implantation failure and defects in decidualization due to decreased vascular permeability and steroid-dependent stromal cell proliferation (26,27). Expression of Pigs2, better known as Cox2, an cyclooxygenase that catalyzes the rate-limiting step in prostaglandin synthesis, is down regulated in the stroma of Hoxa10 deficient mice (27). Ablation of Pigs2 results in similar defects in implantation and decidualization (28). Thus, the Lif and prostaglandin pathways
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appear to be distinct, but both are required for normal decidualization.

Reduced fertility is observed in Csf2 (also called GM-CSF) deficient females, but it affects development in a much later stage than those described above. Embryos in a homozygous mother show a reduced labyrinthine layer and glycogen cells in the placenta, which is most evident if the fetus is also Csf2 deficient (29). This indicates that fetal Csf2 can partly rescue the absence of maternal Csf2, which is required for optimal growth and survival.

Immune surveillance

As trophoblast cells express paternal surface antigens on their surface, both the mother as well as the embryo have generated mechanisms to escape immune attack of the trophoblast cells by the maternal immune system. Absence of Fga (Fas ligand) in the mother results in extensive leukocyte infiltrate and necrosis at the decidual–placental interface (30). Loss of Crry, a complement regulator, at the trophoblast surface results in complement deposition at the maternal-fetal interface and concomitant placenta inflammation leading to embryonic death (31). Similarly, absence of S100a8 (a calcium binding secreted protein) in fetal cells at the fetomaternal border results in infiltration with maternal immune cells (32). In contrast, ablation of the cytokine Csf1 in the mother results in reduced fertility due to unrestrained infection in the reproductive tract due to impaired recruitment of neutrophils (33).

Peri-implantation

The first lineage to differentiate in the mammalian embryo is the trophoblast lineage. This differentiation is perturbed in mutants for Cdhl or Catnal (E-cadherin or α-E-catenin), both components of adherens junctions, resulting in a defect in blastocyst formation (34,35). Subsequently, this early trophectoderm layer differentiates at the late blastocyst to form an invasive trophoblast layer that mediates implantation of the embryo into the uterine wall after hatching of the zona pellucida. Mutants of the Bcl2 family member Mcll fail to hatch from the zona pellucida and do not implant into the uterus (36). Embryos that are mutant for Bsg (Basigin, encoding a glycosylated membrane protein) show implantation at reduced frequency, whereas the few survivors are sterile due to a uterine implantation defect, suggesting an interaction between these molecules at the fetomaternal interface (37). A slightly later block in development was observed in the rtw73 (i.e. r73) mutant, for which the gene is not yet cloned, and the agouti A* mutants that lacks Raly, encoding a RNA binding protein (38). Both mutants initiate implantation, but fail to establish an intimate contact with the maternal decidua due to defective giant cell differentiation, and die around day 7.5 and 6.5 pc, respectively (39,40).

Trophoblast differentiation

The simple epithelium at the outside of the blastocyst can differentiate into many different cell types that all have their specific functions in extraembryonic development. Defects in trophoblast differentiation can therefore result in developmental blocks at different stage of development. Transcription factors such as Eomes, Esrrb (Err2), Ascl2 (Mash2), Ets2, Handl, Gcm1, Dlx3, and Esxl, that mediate these different stages of trophoblast differentiation, and their mutants have recently been reviewed (12).

Extraembryonic endoderm differentiation

Primitive endoderm cells that overly the ICM in the blastocyst (Fig. 1A) start
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migrating along the primary trophoblast giant cells, and differentiate into parietal endoderm. The endoderm that remains associated with the ICM as it develops into the epiblast, differentiates into visceral endoderm. A total block in primitive endoderm differentiation is observed in Grb2 (a growth factor receptor bound protein) deficient mice, which can be rescued by introducing a Grb2-Sos1 chimeric protein (41). However, Sos1 deficient mice do not reproduce this early phenotype suggesting functional redundancy at this level in the signaling pathway (42). A similar block in endoderm differentiation is observed in Fgf4 (a fibroblast growth factor) and Fgfr2 (Fgf receptor) mutants (43,44), suggesting that Grb2 might act downstream of Fgf signaling in this process. A more specific block towards the parietal endoderm lineage can be found in Lamp1 (Laminin γ1) deficient mice. Absence of this major component of most basement membranes results in an absence of parietal endoderm and the subsequent formation of the Reichert’s membrane, most likely because of the absence of a trophoderm basement membrane that acts as a scaffold for migrating endoderm cells (45). In contrast, Dag1 (Dystroglycan, a cellular receptor for laminin) ablation disrupts only the formation of the Reichert’s membrane but allows normal parietal endoderm differentiation (46).

Visceral endoderm differentiation is affected in Gata6 and Tcf2 (or vHnf1) mutants (47-49). Whereas Gata6 mutants show an absence of expression of early (Gata4) and late (Hnf4 and serum proteins) markers (47), Tcf2 mutants do form visceral endoderm and have normal expression of Gata4 and Gata6, but fail to differentiate further and do not show expression of late markers (48), although an independent targeting reported a phenotype very similar to Gata6 (49). This suggests that both Gata6 and Tcf2 act upstream of Hnf4, that subsequently directs expression of downstream endodermal genes as serum proteins (50). Although Gata4 expression is down regulated in Gata6 mutants, absence of Gata4 does not show a visceral endoderm defect (51,52). Because the visceral endoderm plays a crucial role in supporting the metabolism and growth of the early embryo prior to the onset of placental function, these visceral endoderm mutants often have severe defects in embryonic growth, which have been determined to be secondary to the defects in the VE by the use of chimeric aggregation experiments. In these experiments, aggregation of mutant embryos with wildtype tetraploid cells, that can only contribute to extraembryonic lineages, shows that the defect is only in the extraembryonic tissues and allows rescue of the embryo (53).

Egg cylinder

In the early post-implantation period the conceptus undergoes rapid proliferation to form an elongated egg cylinder (Fig. 1B). Madh4 (better known as Smad4) deficient mice exhibit a defect in elongation, especially in the extraembryonic part of the egg cylinder, as well as impaired visceral endoderm differentiation (54,55). Although the latter defect was not seen, the defect in the extraembryonic portion of the egg cylinder in Madh2 (or Smad2) mutants was an exact copy of that observed for Madh4 (56,57). This suggests that Smad2 and Smad4 function together in egg cylinder elongation. The Smad family members function as intracellular effector molecules for Tgfβ family receptors. These receptor activated Smads form heteromeric complexes with Smad4, are translocated to the nucleus, and function subsequently as transcriptional regulators via their interaction with DNA binding proteins. The more extended phenotype resulting from gene ablation of Madh4 can therefore be explained by inactivation of multiple pathways. In addition, Acrv1b (the activin receptor ActRIB) appears to transduce signals via Smad2, which is reflected by the similar phenotype seen in the Acrv1b mutant (58). A thus far unrelated gene Extl, involved in heparan sulphate synthesis and association of
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Hedgehog with the cell surface, is also required for elongation of the extraembryonic region (59).

**Visceral endoderm function**

Some mutations do not disturb visceral endoderm formation and differentiation, but specifically affect the transport function of the VE. Lipid transport is severely disturbed in Apob and Mttp (encoding the large subunit of Mtp) deficient mice (60,61), whereas vesicle transport is impaired after ablation of the mouse homologue of the Huntington disease gene Hdh (62). In addition to the metabolic function, the VE plays important roles establishing an anterior-posterior axis, in anterior patterning in the underlying epiblast, and in induction of gastrulation (63). Mutants that affect this patterning function of the visceral endoderm will not be further discussed here.

**Yolk sac vasculature**

After gastrulation, groups of mesodermal cells form the extraembryonic blood islands in the visceral yolk sac. Cells in the center become primitive blood cells, whereas cell at the periphery differentiate into endothelial precursors (mutants that specifically affect hematopoiesis were not included in this review). Upon endothelial differentiation a primary vascular plexus is formed (vasculogenesis), which subsequently is remodeled into a mature vascularization (angiogenesis). Multiple signaling pathways are required to fully execute endothelial differentiation and vascularization. The well described effects of Vegf and its receptors Kdr (Flk1) and Flt1 on endothelial differentiation and vasculogenesis as well as the involvement of Agpt, Tie1, and Tek (Tie2) of the Angiopoietin/Tie family in angiogenesis are reviewed by Yancopoulos et al. (64). Formation of a primary vascular plexus in the yolk sac is disturbed in Cd5 or Cd2 (VE-cadherin or N-cadherin) null mice, but not caused by apparent cell-adhesion defects (65,66). In 50% of the Tgfb1 (the transforming growth factor β1) mutants, angiogenesis of the yolk sac vasculature is disturbed, whereas an identical phenotypes with full penetrance is observed in mice deficient for Tgfb2 or Eng (type II Tgβ receptor or Endoglin), an ancillary Tgβ receptor (67,68). In contrast to mutations in Vegf and its receptors, only the extraembryonic and not the embryonic vasculature is affected in mutants of the Tgβ family, resulting in less and weaker blood vessels in the yolk sac. Furthermore, Madh5 (Smad5) and Acvrll (Alk1) mutants exhibit a very similar phenotype, suggesting a role for Alk1 as type I receptor and for Smad5 in downstream signaling in the Tgβ1 pathway (69,70). Defects in angiogenic remodeling of the yolk sac vasculature are observed after ablation of the genes Ephb2 (EphrinB2) and EphB4 and closely resemble the defects in Ang/Tie mutants. The bi-directional signaling between EphrinB2 expressed on arteries and EphB4 on vascular endothelial cells of veins is required for intercalating growth between arteries and veins in the yolk sac (71,72). Mutation in the hypoxia inducible factors Hif1α, Epas1 (Hif2α), and their dimerizing partner Arnt all result in impaired remodeling of the primary yolk sac plexus into larger and smaller vessels (73-75), suggesting that hypoxia induces maturation of the developing vasculature most likely via induction of Vegf.

Part of the blood coagulation system is adapted to perform a function in the yolk sac vasculature. Incomplete embryonic lethality is observed for deficiencies of the coagulation factors F2 (or Prothrombin), F5, F3 (Tissue Factor), and its inhibitor Tpdi due to defects in the integrity of the yolk sac vasculature (76-81). These factors are involved in a common mechanism to generate active Thrombin. Surprisingly, factor F7 deficiency, the only known Tissue
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Factor ligand, does not cause embryonic lethality, but can reverse the phenotype of the Tfpi mutants, suggesting that Tfpi modulates the activity of the F3/F7 complex (82,83). No embryonic lethality was observed in Fn1 (i.e. Fibrinogen) mutants, which may indicate that the embryonic defects are not caused by defective blood coagulation, but one of the other functions described to Thrombin (84).

An important role for the Mapk pathway in both yolk sac and placental angiogenesis appeared from several gene inactivations. Map3k3 (Mekk3) or Mapk14 (p38αMapk) deficiency results in defects in both yolk sac and placental angiogenesis (85,86), whereas Mef2c null mice show most severe defects in the yolk sac vasculature (87,88), suggesting a role for Mekk3 in activation of Mef2c through p38αMapk cascade in the yolk sac.

Chorioallantoic fusion

Mesenchyme derived from the posterior primitive streak at 7.5 dpc grows from the caudal region of the embryo across the exocoelomic cavity towards the chorionic plate. Subsequent chorioallantoic fusion is required for umbilical cord and placenta development and therefore prerequisite for continued development. A defect in chorioallantoic fusion can appear at several levels. Absent or abnormal allantois development is seen in mutants for the transcription factors Rbpsuh (or RBP-Jk), T, Lhx1 (or Lim1), the endosomal protein Hgs (or Hrs), the bone morphogenetic proteins Bmp4, Bmp5 and Bmp7 double mutants, and the proprotein convertase Pcsk3 (or Furin) deficient mice (89-95). Deficiency of Mrj-pending (co-chaperone) and Esr5b (transcription factor) however, results in abnormalities in the chorion (96, 97), whereas an adhesion defect is the cause of failure of chorioallantoic fusion in Vcam1 and Itga4 (α4 integrin) mutants (98-100). This defect of chorioallantoic fusion appears often with incomplete penetrance in a number of mutants, probably because several independent pathways exist to establish chorioallantoic fusion. A failure in fusion results in death around day 10 of gestation, the time when the embryo becomes critically dependent on a functional placenta (Fig. 2).

Placenta structure

Most mutants affecting placental morphology show labyrinth defects that directly affect the nutrition of the embryo. However, three mutants describe primary lesions in the spongiotrophoblast layer, Ascl2 (better known as Mash2), Egfr, and the heat shock transcriptional factor Hsf1(101-104). All of them also affect the labyrinth layer to a lesser extent.

Very early defects in the labyrinthine layer have been observed in Gcm1 and Hsp84-1 (previously called Hsp90β) deficient mice, that show no differentiation of labyrinthine trophoblast and no invasion or branching of fetal blood vessels (105-107). Whereas Gcm1 is expressed only in the chorionic trophoblast, the primary function of Hsp90β resides in the allantois, which normally induces differentiation of chorionic trophoblast upon fusion. Reduced numbers of labyrinthine trophoblast are the result of mutation of either the hepatocyte growth factor Hgf or its receptor Met (108,109). As it has been shown that Hgf is secreted by the allantois and Met is expressed on the labyrinthine trophoblast, it is very likely that this signaling is responsible for the allantois induction of trophoblast differentiation (108). Esx1 and Dlx3 mutants have specific defects in the vascularization, although only expressed in the labyrinthine trophoblast (110,111). This clearly demonstrates that the trophoblast cells guide the vascular development in the labyrinth.

Vascularization of the labyrinth is affected in AP-1 transcription factors Junb and Fosl1 deficient mice (112,113). Reduced interdigititation of maternal and fetal vessels in the
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labyrinth is seen in Map2k1 (Mek1) and Itgav (αv integrin) mutants (114,115). Mek1 deficient cells have reduced migration in response to fibronectin, which would fit with a role for Mek1 downstream of the αvβ3 integrin (115).

Relative late defects are observed upon ablation of the Wnt2, Lifr, and the retinoic acid receptor Rxra genes. They suffer from a disorganized labyrinthine layer, revealed in excessive maternal blood pooling, fibrin deposition, thickening of the trophoblast layer, decrease in the number of fetal capillaries, and edema (116-118). Combined deficiency of Rxra and Rxb however, results in a block of the labyrinth trophoblast differentiation combined with a failure of vascularization (119). A very similar phenotype was observed in mice deficient for Pparg, encoding the heterodimerizing partner for Rxr (120), suggesting an early role for Rxr/Pparg heterodimers in labyrinth formation.

Placenta function

Inactivation of some genes have identified important functions in placenta, although their phenotype is often not as striking as many genes that affect placental structure. Ablation of the transcription factors Gata2 or Gata3 results in severe reduction of placental lactogen I expression, without affecting the differentiation of the trophoblast giant cells (121). The expression of proliferin is also affected in both mutants, but this defect is more pronounced in Gata2 deficient mice resulting in a decreased neovascularization in the adjacent decidua (121). Inactivation of the Ada (adenosine deaminase, an enzyme in the purine catabolism pathway) gene results in accumulation of toxic Ada substrates in the embryo leading to liver damage and fetal death (122). This appears to be the result of lack of placental Ada that prevents the accumulation of toxic substrates in the embryo (123). Ablation of Gjb2, encoding the connexin 26 gap junctional channel, result in embryonic lethality without specific abnormalities. Upon specific transport studies, a role in glucose transport between mother and fetus was demonstrated (124).

Several genes play important roles in placenta, without being essential for survival under normal laboratory circumstances. Mice deficient for the multidrug resistant Pgp transporters Abcb4 and Abcb1 for instance can survive normal development (125). However, they appear to play important roles at the feto-maternal barrier, as deficient embryos show increased penetration of several harmful drugs into the embryo as a result of increased transport over their placentas (126,127). Similarly, absence of the M11 and Mt2 genes (Metallothionein I and II, involved in intracellular metal storage) results in increased cadmium accumulation in fetuses (128), suggesting a role for placental Mt in protecting the fetus from cadmium toxicity. The copper transporting ATPases Atp7a and Atp7b are involved in copper transport over the placenta, as their mutants show accumulation of copper in the placenta and copper deficiency in the embryo (129,130). Furthermore, the calcium sensing receptor Gprc2a exerts its affect on transplacental transport of calcium via Pthlh (or PTHrP), that by itself is required for calcium transport over the placenta without affecting normal development (131, 132). Finally, Slc22a3 (or Orc3) deficient mice have reduced transport of the prototypical uptake2 substrate MPP+ from the placenta to the fetus (133).

Conclusion

A large number of mutations generated by homologous recombination have been reported recently that affect the structure and function of the extraembryonic lineages and pla-
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centa (as shown by the 169 genes listed in Table 1). As the placenta and the extraembryonic membranes have a function in embryonic nutrition, it is not surprising that the mutants that affect formation of these structures result in lethality. What is surprising, is the identification of so many placental and extraembryonic mutants. This could be explained by the complexity of these extraembryonic organs. The placenta and the VYS are not only involved in exchange between mother and fetus, but have additional roles such as producing serum factors and hormones, and protecting the fetus from the maternal immune system as well as from toxic substances. As was shown in this review, all of these functions can be compromised by mutations in certain genes. For some of the genes inactivated by gene targeting, the placental function was rather unexpected, because they were initially identified as key players in other organ systems. An explanation for this could be re-utilization of already existing genetic pathways for the development of extraembryonic lineages that only appeared recently in evolution (10).

The strategy of gene targeting has unraveled some pathways involved in specific steps of extraembryonic development. For instance, the role of the Tgfβ pathway in vascular remodeling in the yolk sac and the Mapk pathway in yolk sac and placental angiogenesis. By classifying the mutants, interactions might be suggestive in those with very similar phenotypes. Studying these mutants in more detail, for instance by microarray technology, might unravel novel pathways involved in placental structure and function. The information retrieved can than be further used to investigate human pregnancy disorders and to test the proposal that common cellular mechanisms are involved in embryonic implantation and in metastatic spread of human tumors.

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<th>Table 1  Mutations that affect structure and function of placenta and extraembryonic membranes.</th>
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<td>Implantation</td>
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<td>Hoxa10 (26,27); Hoxa11 (25); Lif (23); Ptgs2 (28);Pr1r (22); Hmx3 (24)</td>
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<td>Fga (84); Csfl (33); Srd5a1 (135); Csfl2 (29); Tnfsf6 (30)</td>
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<td>Mcl1 (36); Bsg (37); Catenu (35); Cdh1 (34); Eomes (136); Raly (39); tclw73 (40)</td>
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<td>Bmp5 (and Nodal) (137)</td>
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Overview of all well described mutations with an extraembryonic phenotype arranged per stage of embryonic development and further classified according to their specific phenotype. Inactivations created by gene targeting are depicted in plain, mutations that are spontaneously arisen or induced by mutagenesis in bold, and transgene insertions are underlined. Official gene symbols approved by the nomenclature committee are given. The mutations were found by searching the PubMed database with combinations of the following keywords: placenta, ectoplacental cone, trophectoderm, trophoblast, implantation, chorion, allantois, amnion, yolk sac, vascularization, angiogenesis, extraembryonic, null, deficient, homozygous, knockout, and in references of published reports.

**Scope of this thesis**

The research described in this thesis uses positional cloning to find a key gene regulating the development and function of extraembryonic tissues. This approach was chosen because of the difficulty in identifying genes participating in extraembryonic development, based on their proposed function. As an example, some genes thought to play key roles in implantation and placentation based on their expression profile, such as the metalloprotease Mmp9 and the proteases in the Plasminogen/Plasmin system, turned out to be dispensable for embryonic development after gene targeting (205,206). In the complementary approach of positional cloning the starting point is a phenotype of relevance whose mapped position has been well characterized. Novel identified genes are tested for candidature using several criteria and good candidates are subsequently applied in a genetic experiment. We used the r<sup>73</sup> naturally occurring mouse mutant that has a specific defect in the trophoblast lineage during implantation and has been accurately mapped to a 500 kb region on mouse chromosome 17.

In Chapter 2, the positional cloning of the Slc22a3/Orc13 gene from the r<sup>73</sup> critical region and the cloning of its human homologue is described. The Slc22a3 gene encodes an organic cation transporter and has an appropriate expression profile since it is specifically expressed in the placenta during embryogenesis and is temporally regulated in this tissue (Chapter 2). Despite demonstrating expression in the early post-implantation period and
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despite identification of a r^73-specific polymorphism, genetic complementation showed that the Slc22a3 gene is not responsible for the r^73 phenotype (Chapter 3). Further expression studies revealed a specific expression of Slc22a3 in the labyrinthine layer of the placenta, the area of exchange between mother and fetus at this stage of development. Slc22a3 has been proposed to function in extraneuronal monoamine clearance (also known as uptake2), and this function is supported by the co-expression with the monoamine degrading enzyme Maaa (Chapter 4). Functional studies further support this by showing reduced transport of MPP^+, a known substrate of uptake2, into Oret3 deficient embryos. These studies identify the placenta as a novel uptake2 organ at the feto-placental border (Chapter 5).
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