Identification and characterization of the t(w73) candidate gene Ortc3
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Chapter 6

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The extraembryonic membranes and placenta are critical for embryonic development in mammals. Besides the exchange of nutrient, gas, and waste products, the extraembryonic lineages are involved in invasion, angiogenesis, in producing serum factors and hormones, and in protecting the embryo from the maternal immune system. Many genes have been identified to be critical for the development and function of the extraembryonic structures by the use of homologous recombination to inactivate genes (Chapter 1). The research described in this thesis aimed to provide more insight into the development and function of the placenta by the complementary approach of positional cloning. The \( rm^{73} \) naturally occurring mouse mutant has a specific defect in the extraembryonic lineage, most likely in the generation of the invasive trophoblast giant cells, which results in early post-implantation death (1,2). A 500 kb critical region containing \( rm^{73} \) was previously defined (3-5) and used to identify a novel candidate gene for this mutation (Chapter 2). This gene, encoding the organic cation transporter \( Orct3/Slc22a3 \), is specifically expressed in the placenta during embryogenesis and appears to be clustered with two family members \( Orct1/Slc22a1 \) and \( Orct2/Slc22a2 \) both in mouse and human. Genetic complementation of the \( rm^{73} \) mutant with an \( Orct3 \) null allele demonstrated that absence of \( Orct3 \) is not causing the \( rm^{73} \) phenotype, despite the presence of a \( rm^{73} \)-specific polymorphism within the \( Orct3 \) gene and expression of \( Orct3 \) in early post-implantation stages (Chapter 3). A YAC transgene covering 300 kb of the critical region, did not rescue the early lethality, excluding the closely linked \( Orct2 \) gene as a candidate gene and confirming the previous exclusion of the \( Igf2r, Air \), and \( Orct1 \). These experiments reduce the previous 500 kb critical region to a 150 kb region in the distal part and the 50 kb intergenic region between \( Orct3 \) and \( Orct2 \). In the proximal region, a second embryonic lethal was identified by an enhancer trap approach and mapped to intron1 of \( Orct2 \) without affecting its expression (6). Although the stage of lethality and the presence of transgene-induced rearrangements has not been determined, this suggests that there might reside another potential \( rm^{73} \) gene in this proximal region. In addition, sequencing of the syntenic region in humans (GenBank Accession No. Nt_007122) has revealed an expressed sequence tag in this region. Ongoing sequence analysis of the \( t \) complex might give some additional information (7), as many genes are arranged differently on a \( t \) allele, as was shown by duplication of \( Plg \) and \( Orct3 \) ((8) and Chapter 3).

In the second part of this thesis the function of \( Orct3 \) in placenta has been studied by expression analysis as well as functional studies in the \( Orct3 \) deficient mice. \textit{In vitro} transport studies performed with the rat and human \( Orct3 \) has previously showed their potency to transport monoamines as serotonin, the catecholamines noradrenaline and dopamine, and the neurotoxin MPP\(^+\) (9-11). Monoamines function as neurotransmitters in the nervous system and as hormones in the control of physiologic processes as blood pressure. Two uptake systems are involved in the clearance of extracellular monoamines. The neuronal uptake\(_1\) is located at presynaptic nerve endings and mediates re-uptake from released neurotransmitter via the noradrenaline transporter \textit{Net}, the dopamine transporter \textit{Dat}, and the serotonin transporter \textit{Sert} (reviewed in (12)). A second extraneuronal uptake system is involved in the transport of circulating monoamines in the plasma compartment (13). The uptake\(_2\) system has been identified in myocardial cells, smooth muscle cells of the uterus, vascular smooth muscle cells, and glandular and fat tissue (13). Both uptake systems are coupled to the intracellular enzymes that
mediate subsequent metabolism of the monoamines. The neuronal transport system is linked to the monoamine oxidases Maoa and Maob, of which Maoa is primarily responsible for metabolism of the three major physiological occurring monoamines, noradrenaline, dopamine and serotonin(14). Metabolism of the extraneuronal monoamines is mediated by both Catechol-O-methyltransferase (Comt) and the Mao enzymes (13).

Based on the kinetic parameters, inhibitor profile, and the widespread expression pattern, Orct3 was suggested to function as the extraneuronal monoamine transporter also known as uptake2(10, 11). To test the role of Orct3 as extraneuronal monoamine transporter in vivo, we performed transport experiment with radiolabeled MPP+, a non-degradable substrate for uptake2, in wildtype and Orct3 deficient adult mice. The only organ that showed a significant difference in MPP+ uptake was the heart, an organ previously indentified to have uptake2 activity (13). Almost fourfold less MPP+ accumulated in the heart of Orct3 deficient mice, suggesting an important role for Orct3 in heart (Chapter 5).

Most abundant expression for Orct3 was found in the placenta (19 and Chapter 2), an organ never tested for uptake2 activity. Surprisingly, expression of the human uptake1 transporters NET and SERT were also described in this non-innervated organ (15-17) as well as the metabolizing enzymes MAOA and COMT(18-20). To clarify the role of monoamine clearance in placenta, we performed detailed expression analysis of both uptake, and uptake, transporters as well as the intracellular enzymes in mouse placenta (Chapter 4). The expression data suggest a role for uptake1 transporters at the maternal side of the placenta, which provides support for the functional activities previously described for NET and SERT at the maternal side of the human placenta based on brush border studies (17, 21, 22). Our studies in mice placenta identify a second clearance pathway in the labyrinth layer, the exchange area between mother and fetus. This layer expresses both the uptake2, transporter Orct3 as well as the intracellular degrading enzyme Maoa in a similar pattern. To test the role of Orct3 as the uptake2, transporter in placenta, we performed transport experiments with radiolabeled MPP+ in pregnant mice (Chapter 5). Threefold less MPP+, was accumulated in the homozygous embryos as compared to wildtypes, but no differences were found in the placentas of both genotypes. These results pin-point Orct3 activity to the feto-placental interface, a novel location for uptake2.

Indications for the physiological function of uptake2 in placenta came from previous studies in sheep. In sheep embryos, circulating monoamine levels are low despite a high monoamine production rate. This can be explained by a high turnover (23, 24), which protects the fetus from detrimental effects in the fetal blood circulation caused by high circulating monoamine levels (25). The placenta accounts for 50% of the total intrauterine clearance (26), which is only partly inhibited by uptake, inhibitors (27). Similarly, in vitro perfusion experiments with human placentas showed uptake of fetal noradrenaline by the placenta, which was dependent on MAOA activity (28). The mechanism underlying the placental monoamine clearance from the fetal circulation was previously unidentified. Our expression and transport data indicate that Orct3 can mediate uptake of monoamines from the fetal circulation followed by intracellular degradation by Maoa.

Surprisingly, mice deficient for Orct3 show no overt defects that would indicate monoamine imbalance. In addition, we have been unable to identify significant differences in the steady-state levels of two tested monoamines between wildtype and knockout embryos and placentas. Similarly, absence of Sert, Net, or Maoa do not result in embryonic lethality either (29-31). Because Net and Sert are initially also expressed in the labyrinth layer, they might take over from Orct3. Alternatively, the embryos could adapt their catecholamine synthesis to pre-
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vent detrimental effects of monoamine imbalance. Thus, the physiological significance of these pathways in development is not yet clear. Further studies, using different combinations of these knockouts, might help to resolve these questions.
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References
