New Functions for Old Genes in the Mouse Placenta

UMASHANKAR SINGH
Dissertation presented at Uppsala University to be publicly examined in Lindahlsalen, EBC, Norbyvägen 18A, Uppsala, Tuesday, May 30, 2006 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Different species are separated by pre-zygotic reproductive barriers which impede gene flow between them. Rarely, when pre-zygotic barriers break down, interspecific hybrids are produced that display abnormal phenotypes, collectively called hybrid dysgenesis effects. Interspecies hybrid placental dysplasia (IHPD) in the genus Mus is a very consistent X-linked hybrid dysgenesis effect. Reproductive cloning and mutation of the gene Exs1 lead to placental hyperplasias with phenotypic similarities to IHPD. Comparative gene expression analysis of these three different models of placental hyperplasia showed that different mechanisms underlie these placental hyperplasias. We also identified several genes for which roles in placentation had not been studied earlier. We screened five of these genes, Car2, Ncam1, Phnl, Cacnb3 and Cpe for their functions in placentation. Analysis of the spatio-temporal expression patterns of these genes during mouse placental development showed that they are ectopically expressed in IHPD placentas. Placental phenotype and gene expression was then studied in mice mutant for these genes. Our results show that complicated by the expression of functional counterparts, deletion of these genes failed to produce any consistent phenotype. Incompletely penetrant phenotypes were found in Cacnb3 and Cpe mutants. The Cpe mutant placentas recapitulated some IHPD phenotypes, despite co-expression of Cpd, a functionally redundant gene. Deregulated expression of Cpe and Cpd prior to manifestation of IHPD phenotype indicated that these are causally involved in IHPD and might be speciation genes in the genus Mus. We found that AT24 placentas also exhibit deregulated expression of these genes and could be used as a model to study IHPD. We tried rescuing the AT24 placental phenotype, by decreasing the expression of the over expressed genes. Normalization of transcript levels of these genes did not rescue the AT24 phenotype, thus indicating that up-regulation of these genes is a down-stream event in the generation of IHPD.

Keywords: hybrid dysgenesis effects, placental hyperplasia, functional screening, giant cell, glycogen cell

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### Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>ASMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cM</td>
<td>Centimorgan</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DXMit</td>
<td>DNA segment, X chromosome, Massachusetts Institute of Technology</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day post coitum</td>
</tr>
<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>F1</td>
<td>First filial generation</td>
</tr>
<tr>
<td>IAP</td>
<td>Intracisternal A particle</td>
</tr>
<tr>
<td>IHPD</td>
<td>Interspecies hybrid placental dysplasia</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intra-uterine growth restriction</td>
</tr>
<tr>
<td>LINE</td>
<td>Long interspersed elements</td>
</tr>
<tr>
<td>MMA</td>
<td><em>Mus macedonicus</em></td>
</tr>
<tr>
<td>MMU</td>
<td><em>Mus musculus</em></td>
</tr>
<tr>
<td>MSP</td>
<td><em>Mus spretus</em></td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>MMU x MSP (female always shown first in all crosses)</td>
</tr>
<tr>
<td>MSM</td>
<td>MS x MMU backcross 1</td>
</tr>
<tr>
<td>MSS</td>
<td>MS x MSP</td>
</tr>
<tr>
<td>MX</td>
<td>MMU x MMA</td>
</tr>
<tr>
<td>MXM</td>
<td>MX x MMU</td>
</tr>
<tr>
<td>MXX</td>
<td>MX x MMA</td>
</tr>
<tr>
<td>MXMM</td>
<td>MXM backcross 2</td>
</tr>
<tr>
<td>N</td>
<td>Genomic copy number</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PL1</td>
<td>Placental lactogen 1</td>
</tr>
<tr>
<td>PL2</td>
<td>Placental lactogen 2</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription PCR</td>
</tr>
<tr>
<td>SM</td>
<td>MSP x MMU</td>
</tr>
<tr>
<td>SMS</td>
<td>SM x MSP</td>
</tr>
<tr>
<td>XM</td>
<td>MMA x MMU</td>
</tr>
</tbody>
</table>
Introduction

The concept of Species

Since the time of Linnaeus, the term “species” has been used to mean the basic taxonomic unit into which living organisms can be classified. For various purposes, different definitions of species are used and defining species has largely become a context specific practice.

**Morphological species concept**: The morphological species concept suggests that a species is a group of individuals with fundamental resemblance in morphology and anatomy. This concept is widely employed in taxonomic classification of living organisms on the basis of external features. For classification of asexually reproducing and extinct organisms, this is the only concept of species that can be used (Linnaeus, 1735). Darwin also dwelled on the idea that species are no more than just well marked varieties (Darwin, 1858).

**Evolutionary species concept**: Evolutionary species concept defines species as a single lineage of ancestor-descendant population which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate (Simpson, 1944).

**Ecological species concept**: According to this concept, all organisms of a species share same ecological resources and compete for them. They tend to be on the same trophic level, at same nodes in the food web and compete for same habitats (Mayr, 1942; Dobzhansky, 1951; Rice & Hostert, 1993; Schluter, 1996; Schluter, 2000). This concept however does not include the fact that the individuals of a species also compete with each other for mates.

**Biological species concept**: The biological species concept dwells on reproductive isolation as the main feature of species (Dobzhansky, 1951; Mayr, 1963). According to this concept, a species is a group of actually or potentially interbreeding populations of organisms which are reproductively incompatible with other such groups. While the biological species concept is widely accepted, it also has certain drawbacks, which in my eyes are minor. This concept does not explain asexually reproducing species and plants evolving by polyploidy. Biological species concept can not be applied to extinct organisms as the reproductive incompatibilities of extinct organisms can not be established.

The biological species concept is applied throughout this thesis.
Speciation and reproductive barriers

From the biological species concept, it follows that there is no or very little gene flow across different species under natural circumstances. The gene flow between distinct species is prevented by numerous mechanisms collectively termed as reproductive barriers, which act as boundaries between species by inducing reproductive isolation (Grether, 2005; West-Eberhard, 2005). Species are constantly evolving entities and so are the barriers to gene flow between them (Grether, 2005; West-Eberhard, 2005). Evolution of novel species from the existing ones is called speciation. Speciation takes place by a variety of mechanisms, each involving roles of different types of reproductive barriers.

Geographical isolation and allopatric speciation: The term allopatry means living in different geographical areas. If a population of a parent species is split into two or more subpopulations by geographical barriers that do not allow gene flow between these subpopulations, then these subpopulations become allopatric to each other. Subpopulations occupying different geographical niches are amenable to different selection pressures. Adaptation in different environment, differential natural selection as well as genetic drift can eventually give rise to differences in gene pools, morphology and behavioral patterns. Thus, the allopatric subpopulations diverge from each other and from the parental species. Over a period of time the different subpopulations may evolve into different subspecies and slowly into distinct species. Since this process of speciation takes place in allopatric subpopulations of a parent species, it is called allopatric speciation (Barton and Hewitt, 1989; Liou and Price, 1994; Coyne and Orr, 2004).

To resolve the status of various allopatric subpopulations as true species, their reproductive incompatibility needs to be ascertained. If allopatric speciation has occurred successfully, then upon reunion such subpopulations will exhibit reproductive incompatibility and nullified gene flow between them.

Sympatric speciation: The word sympatric refers to organisms living in same geographical region. Sympatric speciation is the process of formation of new species from the parental species in the absence of geographical barriers (Dieckmann and Doebeli, 1999). Since all the subpopulations occupy same habitat (at least initially) they are sympatric with each other and yet they evolve into distinct reproductive groups. Being sympatric, it is likely that random interbreeding will very efficiently eliminate any genetic differences that might arise in a population. However, very strong competition can cause disruptive selection, in which the extreme phenotypes are selected at the cost of individuals with intermediate phenotypes. Consequently, assortative mating takes place, in which individuals with extreme opposite phenotypes do not prefer to mate with each other (Kondrashov and Shpak, 1998). Gradually, such subpopulations with extreme phenotypes will develop com-
plete reproductive isolation and will become distinct biological species. Hence, intense competition is required to drive sympatric speciation (Polechova and Barton, 2005).

**Parapatric speciation**: In the context of speciation, the term “parapatric” refers to populations spread over a vast area which are geographically contiguous along an environmental gradient. In such populations, even in the absence of any geographical barrier, random dispersal and matings do not take place. This can be due to (1) the area being too large for random dispersal, or, (2) different environmental conditions in different regions of the vast geographical expanse. Random interbreeding is required to eliminate genetic differences that arise in a population. However, for parapatric populations of such species, localized interbreeding can give rise to genetic differences between subpopulations. These subpopulations will experience different selection pressures due to differences in environmental conditions of the habitats they occupy in the large area. Thus new species can be formed in long run. Such a process of speciation is called parapatric speciation (Odeen and Florin, 2000; Garvilets, 2003).

**Speciation by interspecies hybridization**: Hybrids between two different species can give rise to new species if they are reproductively compatible amongst each other, but not with either of the parental species. The hybrids may also adapt to and occupy a habitat different from that of the parental species. This will exclude any competition from the parental species and the hybrids will continue to evolve into a new species (Dowling and Secor, 1997; Salzburger et al., 2002; Dietmar et al., 2005). Both, habitat specific adaptive superiority of hybrids over the parental species (extrinsic selection) and outcome of new gene-networks in the hybrid genome (intrinsic selection) drive speciation by interspecies hybridization (Arnold, 2004; Hegarty and Hiscock, 2005). In addition, hybridization between two sexual species of lizards *Cnemidophorus inornatus* and *Cnemidophorus tigris* is also known to have given rise to a distinct parthenogenetic species, *Cnemidophorus neomexicanus*.

Reproductive barriers are key elements in initiation and progression of speciation. They can be classified into two categories, pre-zygotic and post-zygotic.

**Pre-zygotic reproductive barriers**

Pre-zygotic barriers are those factors which prevent successful fertilization between gametes derived from two different species. By preventing formation of zygotes, these factors establish reproductive isolation of different species. Owing to various pre-zygotic barriers, mates from different species may not meet, do not exhibit appropriate reproductive behavior or that due to biochemical incompatibility, successful fertilization does not occur. Hence, pre-zygotic reproductive barriers are classified into different categories.
Niche incompatibility of potential mates: Potential mates may be reproductively isolated because they occupy reproductively incompatible ecological niches (Schluter, 1996, 2000; Coyne and Orr, 2004). There may be geographical barriers between habitats of potential mates, restricting populations/individuals from one habitat migrating and searching for suitable mates in another habitat. There can also be temporal isolation, such that a nocturnal species may not be able to meet its potential mate from a diurnal species.

Behavioral incompatibility of potential mates: Eliciting of specific mating behavior is an essential prerequisite for successful mating in many taxa. Differences in mating behavior can act as a pre-zygotic reproductive barrier. Behavioral isolation is the strongest barrier to gene flow between closely related species (Sorenson et al., 2003).

Biochemical incompatibility of gametes: External fertilization is common in several species of aquatic organisms. Between such species, behavioral isolation may not come into play. However, it is known that the sperm even from closely related but different species are not recognized by the species specific proteins present on the zona pellucida or on the oocyte surface. Hence, successful fertilization does not take place between gametes from different species, even if they are in contact with each other (Glabe and Vacquier, 1977, 1979).

Post-zygotic reproductive barriers
Pre-zygotic barriers to reproduction may break down leading to interspecific hybridization. In nascent species (stages of transition from a sub-population to species in sympatric or parapetetic speciation) this may happen naturally. Then, mechanisms of reproductive isolation that act downstream to successful interspecific fertilization, called post-zygotic reproductive barriers frequently manifest themselves. As a result of the post-zygotic reproductive barriers, many abnormal phenotypes are manifested on the hybrid progenies, which greatly compromise their reproductive success. The zygote may be unable to develop to term or the resulting hybrid progeny may be inviable and die at pre-reproductive age. Infertility of the hybrids is perhaps the best known post-zygotic reproductive barrier (Haldane, 1922; Wu and Palopoli, 1994) and it affects the heterogametic sex more than the homogametic sex (Haldane, 1922). Hybrids may also be reproductively incompatible as they may fail to attract mates due to different appearance and behavior.

Interspecific hybrids and hybrid dysgenesis effects
Interspecies hybridization does not occur in nature commonly. Nevertheless, the prezygotic reproductive barriers sometimes break down and this results in interspecies hybridization. This is often brought about under laboratory
conditions, however, natural interspecific hybridization has been reported between the rodent species MMU and MSP (Orth et al., 2002) as well as between different species of fruit flies (Dietmar et al., 2005). Some other interspecific hybrids are known between closely related species in various mammalian families (Table 1 and Figure 1).

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>Hybrid</th>
</tr>
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<tbody>
<tr>
<td>Equus caballus</td>
<td>Equus asinus</td>
<td>“Mule”</td>
</tr>
<tr>
<td>Equus asinus</td>
<td>Equus caballus</td>
<td>“Hinny”</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>Bos gruniens</td>
<td>“Yakow”</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>Bos bison</td>
<td>“Beefalo”</td>
</tr>
<tr>
<td>Panthera leo</td>
<td>Panthera tigris</td>
<td>“Liger”</td>
</tr>
<tr>
<td>Panthera tigris</td>
<td>Panthera leo</td>
<td>“Tigon”</td>
</tr>
</tbody>
</table>

**Table 1.** Examples of mammalian interspecies hybrids; the names of the hybrids mentioned here may not be standard.

These interspecies hybrids, however, have various abnormalities that can be manifested at physiological, behavioral, biochemical and molecular levels. The abnormalities associated with interspecific hybrids are collectively called hybrid dysgenesis effects. Post-zygotic reproductive barriers are very important factors affecting interspecific hybridization-mediated speciation.
Depending on the intensity, hybrid dysgenesis effects may act as postzygotic reproductive barriers. Interspecies hybrids have been created for investigating the mechanisms that are adversely affected when the alleles from two different species are paired in one genome. These studies not only tell us about the molecular basis of speciation, but they also provide us with new models to study the disease phenotypes represented by the hybrid dysgenesis effects.

Hybrid dysgenesis effects: causes

There are two different theories to explain why hybrid dysgenesis takes place. These are the models developed by (1) Fisher and (2) Bateson, Dobzhansky and Muller.

**Fisherian viewpoint of hybrid dysgenesis**

In the early 1930s, R. A. Fisher argued that single genes and not gene-networks are independent units on which natural selection acts in any lineage at a time. This favors the selection of alleles that elevate fitness in that genetic background and environment. Such alleles become fixed in different species irrespective of their compatibility or incompatibility with alleles becoming fixed in other species. Interspecific hybridization brings all such alleles present genome-wide in one species to interact with those of other species in the hybrid genome, creating a combination of the alleles which has never been subjected to natural selection before. As an outcome of the interactions of alleles from different lineages, negative epistasis often takes place, which is the primary cause of dysgenesis observed in hybrids (Fisher, 1930). Hence, the hybrid genotypes are less adapted to their environment compared to their parents and natural selection acts against them.

**Bateson-Dobzhansky-Muller model**

According to this viewpoint, natural selection tends to retain and propagate combinations of favorably and efficiently interacting groups of genes called coadapted gene complexes; for example those genes responsible for a certain pathway (Dobzhansky, 1951; Mayr, 1963). Let us assume a small coadapted gene complex comprising genes “A” and “B” in an ancestral species, such that the genotype of organisms in this population is “AA/BB” at these loci. If this population gets split up into two reproductively isolated subpopulations, then the genotype “AA/BB” will be subjected to different selection pressures in the two subpopulations. Over a period of time, in one subpopulation the allele “A” might evolve into “a” and become fixed. Similarly, in the other subpopulation, the allele “B” might evolve into “b” and become fixed (Figure 2).
Obviously, in these subpopulations, “a” is compatible with “B” and “b” is compatible with “A”. In an event of hybrids being produced between these two species (parental genotypes being “aa/BB” and “AA/bb”), “a” will have to interact with “b”. These interactions have never been subjected to natural selection before and there is a great likelihood that aberrant interaction between independently evolved alleles will cause breakdown of the processes downstream to the interactions of “a” and “b”, thereby causing a plethora of hybrid dysgenesis effects (Figure 2).

The house mouse and the genus Mus

The genus Mus (kingdom animalia, phylum chordata, class mammalia, order rodentia, family muridae, subfamily murinae) is approximately 6 million years old and was established after evolving as a separate lineage from the rats about 10 million years ago (Jaeger et al., 1986). By approximately 10,000 years ago, ancestors of the common house mouse had already evolved into at least four different populations; Mus musculus musculus, Mus musculus domesticus, Mus musculus castaneus and Mus musculus bactrianus (Auffray et al., 1991; Horiuchi et al., 1992; Boursot et al., 1993). Each of these different populations occupied non-overlapping ranges in and around the Indian sub-continent. It is believed that Mus musculus domesticus was present in the mountainous regions of present Pakistan, Mus musculus musculus was spread through the northern parts of present India, Mus musculus bactrianus, the founder population, was present in the central and
southern parts of present India, while *Mus musculus castaneus* was restricted to the present extreme north-east India and Bangladesh. With migrating human populations, these populations of mice spread round the globe (Figure 3). *Mus musculus domesticus* spread towards the Middle-East, occupying the Persian Gulf and west Asia, *Mus musculus musculus* dispersed northwards and spread over China and Asian part of Russia, while *Mus musculus bactrianus* and *Mus musculus castaneus* moved towards the south-east Asia. Eventually, *Mus musculus domesticus* inhabited the northern Africa, southern and western Europe, while *Mus musculus musculus* made its way to eastern Asia.
Europe through north-west Russia. The spread of *Mus musculus domesticus* to sub-Saharan Africa, Australia and the Americas is not more than 1000 years old (Lee, 1995). At present, these four populations are not sympatric with others in any known region. However, a hybrid zone that exists between *Mus musculus domesticus* and *Mus musculus musculus* in the middle of Europe has not expanded in the past 3000 – 4000 years (Sage et al., 1986 and Figure 4). At present, these four populations are classified as different subspecies of MMU. There are no pre-zygotic reproductive barriers between these subspecies; however, some mild hybrid dysgenesis effects have been reported (Moulia et al., 1991). Interestingly, interbreeding of *Mus musculus musculus* and *Mus musculus castaneus* has resulted in a new subspecies called *Mus musculus molossinus* (Yonekawa., 1988).

![Figure 5](http://www.informatics.jax.org)

**Figure 5.** Phylogenetic tree showing evolution of different species and subspecies of the genus Mus. Figure adapted from Mouse Genetics: concepts and applications by Lee M. Silver available at Mouse Genome Informatics http://www.informatics.jax.org.

In the genus Mus, the species MSP, MMA and *Mus spicilegus*, are very closely related to MMU (Figure 5) and have been studied earlier (Zechner et al., 1996). MSP is found in south-west Europe and the northern parts of Africa (Bonhomme and Guenett, 1989), MMA is found in the eastern Mediterranean region (Bonhomme, 1984), while *Mus spicilegus* is found to the north and west of the Black sea (Bonhomme et al., 1978, 1983; Sage, 1981, Figure 5). With the exception of rare cases of hybrids between MMU and MSP (Orth et al., 2002), these three species do not produce any hybrids in nature, even if they are sympatric. This confirms their status as different species.
However, under laboratory conditions, hybrids between MMU and any of these three species have been generated.

**Hybrid dysgenesis effects in the genus Mus**

In the genus Mus, interspecific hybrids have been created under laboratory conditions mainly between MMU and MSP or MMA (Bonhomme et al., 1978; 1984; Zechner et al., 1996). Hybrid dysgenesis effects have been documented in the F1 crosses MS, SM, MX, and MMU x *M. spicilegus*, in the backcrosses MSM, MSS, SMS and MXM and in further backcrosses (Matsuda et al., 1991; Pilder et al., 1991; Forejt, 1996; Zechner et al., 1996; Elliott et al., 2001; Shi et al., 2004). As described for hybrids between species of other genera, Mus hybrids also have defects in placental growth (hyperplasia or hypoplasia) (Zechner et al., 1996), growth (Shi et al., 2004), fertility, behavior (Shi, 2005) and lipid homeostasis (Singh et al., unpublished observations). To what extent they act as post-zygotic reproductive barriers in natural environment is not known. Nevertheless, these dysgenesis effects do affect the reproductive success of the hybrids. Infertility of the males is the most significant barrier to gene flow, and only females can be used to propagate the F1 hybrid genomes (Bonhomme et al., 1978; Zechner et al., 1996). Mentioned below are some general features of the hybrid dysgenesis effects observed in genus Mus, though they may not limited to Mus hybrids only.

1. **Reciprocal phenotypes are produced in reciprocal crosses:** This feature applies to the placental growth and post-natal growth of the hybrid progenies (Zechner et al., 1996; Shi et al., 2004). MS progeny have hypoplastic placentas whereas SM progeny have hyperplastic placentas (Zechner et al., 1996). The same holds true for crosses in which MSP is replaced by MMA (Zechner et al., 1996). Also, generally SM mice are larger than MS mice at adulthood (Shi et al., 2004).

2. **The heterogametic sex is affected more than the homogametic sex (Haldane’s rule):** In the genus Mus, infertility in F1 hybrids is observed usually in the males (XY) and only rarely in females (XX). On the other hand, in F1 hybrids between some species of birds, females (WZ), and not the males (ZZ) are infertile (Sætre et al., 1999). In addition to other factors, hybrid dysgenesis effects are caused by recessive alleles which are present genome-wide and interact epistatically with alleles present elsewhere in the genome. Such alleles, located on sex chromosomes with dose differences between sexes, become epistatically active in F1 hybrids with very high frequency, compared to other such alleles present on autosomes (Orr, 1993; Turelli and Orr, 1995; Orr and Turelli,
Thus, sex chromosomes appear to be more important in hybrid dysgenesis effects than autosomes.

Wide variations in the severity of phenotypes are observed amongst genetically identical F1 hybrids: Even when F1 hybrids are produced from parents belonging to inbred strains (>95% genetically homogenous) of different parental species, intriguing differences in certain hybrid dysgenesis phenotypes are observed. In many instances, littermate F1 hybrids can exhibit extreme phenotypes of a hybrid dysgenesis effect. Two very prominent examples include maternal or infanticidal behavior of females (Shi, 2005) and obesity or leanness in both males and females derived from SM and MS F1 crosses (Singh et al., unpublished observations). Maternal care is a behavior exhibited by females of several taxa, especially mammals. Normally, even virgin female mice take care of foreign pups and exhibit maternal behavior by collecting the pups, crouching and licking them and nest building, if they are in diestrous. In our laboratory, it was observed that a subset of genetically identical virgin MS F1 females in diestrous exhibited abnormal maternal behavior. The abnormality ranged from delayed response to the pups and lack of interest in some females to immediate infanticide in others (Shi et al., unpublished observations; Shi, 2005). Infanticidal behavior is known in feral mice, however, such strikingly different behavioral pattern in genetically identical animals, to the best of my knowledge, has never been described before. Similarly, we also found that only some of the genetically identical F1 hybrid mice develop large accumulation of visceral white adipose tissue, may weigh more than 40 g and exhibit several features of obesity and dyslipidemia starting at 4-5 months of age. This phenotype was accompanied by increased axial growth and was observed in both MS and SM crosses. While some of these mice become obese, others, littermates usually weigh less than 20 g and remain so lean that isolating any visceral adipose becomes difficult (Singh et al., unpublished observations). The fact that the extreme phenotypes of these hybrid dysgenesis effects are differentially manifested in mice which are largely genetically identical, indicates that at least in part, such hybrid dysgenesis effects are determined by non-genetic factors. Nevertheless, the manifestation of such hybrid dysgenesis effects is affected by the parental strains used to generate the hybrids.

The hybrid dysgenesis effect which results in placental overgrowth in the genus Mus is the focus of work presented in this thesis. In the following sections, I will discuss various forms of placenta, normal placental development in the mouse and placental overgrowth in detail.
The placenta

The placenta is a highly vascular organ, responsible for the sustenance of the developing fetus in utero. It is the site for exchange of nutrients, respiratory gases, hormones and fetal excreta between the maternal and fetal circulations, which are in close proximity to each other in this organ (Georgiades et al., 2002). It is a feto-maternal organ formed by interaction of extraembryonic membranes with the uterine blood supply. By definition, a true placenta is characteristic of mammals only; however, functionally similar organs have been also described for other taxa; for example, some sharks, snakes and lizards (Enders and Carter, 2006).

Four extraembryonic membranes, yolk sac, allantois, chorion and amnion are characteristic of all species of the classes, reptilia, aves and mammalia. Reptiles, birds and monotreme mammals lay shelled eggs, in which these extraembryonic membranes have specialized functions. The yolk sac contains mainly lipoproteins, which serve as nutritional reserve. The allantois serves to collect nitrogenous wastes, and the amnion filled with amniotic fluid provides a cushion to the growing embryo. The chorion forms the outermost extraembryonic membrane which is in close proximity with the porous egg shell and is involved in respiratory gas exchange. In the marsupials, the egg is not shelled and becomes embedded in the uterine wall. The yolk sac then establishes a close connection with the maternal blood supply through which nutrient exchange takes place. However, this association between the fetal and maternal tissues does not develop further proximity and complexity and is often called a yolk sac placenta (Enders and Carter, 2006).

In the eutherian mammals, the allantois and the chorion fuse with each other and form a ramified meshwork of highly vascularized fetal tissue which is in close proximity with maternal blood. Such a combination of the maternal and fetal tissues called a chorioallantoic placenta enables very efficient biochemical exchange across the two circulations. Hereafter, the term placenta will be used to imply a chorioallantoic placenta, unless specified otherwise. The efficiency of placent al exchange depends on various factors like area of contact, proximity between the between fetal and maternal circulations, and the direction of blood flow of the two circulations. Based on these parameters, the placenta can be classified into different types (Georgiades et al., 2002; Enders and Carter, 2004).

Classification of the placenta based on its shape

Depending on the shape of placentas and the area of contact between the fetal and maternal tissues, placentas have been categorized into different kinds. In horses and pigs, almost the entire surface of the allantochorion is involved in formation of the placenta. This is called diffuse placenta (Figure 6). In the cotyledonary placenta, multiple, discrete areas of attachment called cotyledons are formed by interaction of patches of allantochorion with en-
dometrium. The fetal portions of this type of placenta are called cotyledons; the maternal contact sites are called caruncles, and the cotyledon-caruncle complex a placentome (Figure 6). This type of placentation is observed in ruminants. In carnivores, elephants and seals, the placenta takes the form of a complete or incomplete band of tissue surrounding the fetus, called a zonary placenta (Figure 6). In rodents and primates, the placenta takes a compact disc-like shape and is called a discoidal placenta (Figure 6) (Balinsky, 1975).

**Figure 6.** Classification of the placenta depending on its shape. A, diffuse placenta of the horse, B, cotyledonary placenta of the cow, C, zonary placenta of the cat and D, discoidal placenta of the mouse. Figures adapted from http://www.vivo.colostate.edu/hbooks/pathphys/reprod/placenta/structure.html

**Classification of the placenta based on maternal-fetal proximity**

Just prior to formation of the placenta, there are a total of six layers of tissue separating maternal and fetal blood. There are three layers of fetal tissues, the endothelium lining allantoic capillaries, connective tissue of the chorioallantois and chorionic epithelium. All of these are components of a mature placenta as well. The three layers of maternal tissues are endothelium lining endometrial blood vessels, connective tissue of the endometrium and endometrial epithelial cells. The number of these layers which are retained - that is, not destroyed in the process of placentation - varies greatly among species. Horses, pigs and ruminants have epitheliochorial placenta in which all these three layers are retained (Figure 7). In the endotheliochorial placentas, present in the carnivores, only the uterine endothelium is retained (Figure 7). Furthermore proximity between the maternal and fetal sides is achieved in the hemochorial placentas of rodents and primates, in which the fetal allan-
tochorion bathes directly in the maternal blood (Figure 7) (Balinsky, 1975; Enders and Carter, 2004).

The development and functioning of the mouse placenta

In mice, like most other mammals, the ovulated oocyte, in which the second meiotic division is sustained, is ready for fertilization. Following successful fertilization the second polar body is released and meiosis is completed. After 30-35 hours post coitum, the first cleavage takes place. Around E2, further cleavage leads to 4 celled embryo, called an early morula. By E3.5, a 16 celled compact morula is formed. Going through these various stages, the embryo moves through the fallopian tubes towards the uterus and is still covered with the zona pellucida. Around E4, the compact morula, which consists of nearly 40 cells, starts to develop a fluid filled cavity inside, which is asymmetrically placed. This cavity called blastocoel is fully developed towards the end of E4 and the embryo at this stage is called blastocyst (Figure 8). The blastocoel displaces majority of the cells in the morula towards one side and remains covered on the other side by a single layer of cells. This asymmetrically placed clump of cells is called the inner cell mass and contains the embryonic stem cells. The single layer of cells that covers the blastocoel on one side actually is continuous over the inner cell mass and thus covers the entire blastocyst. The cells of this layer are morphologically distinct from the cells of the inner cell mass in that they are relatively larger.
and are flat in appearance. The cellular membrane thus formed is called the trophectoderm. At this stage, the blastocyst has reached the uterine lumen and is ready to hatch. Between E4.5-E5, the blastocyst hatches from the zona pellucida and is ready for implantation (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).

The inner cell mass at this stage divides rapidly. The monolayer of cells surrounding the blastocoel is called mural trophectoderm, and the trophectoderm overlying the inner cell mass is called polar trophectoderm. The rapidly proliferating polar trophectoderm consists of trophoblast stem cells. Before the end of E5, the blastocyst implants into the uterine wall. At this stage, the inner cell mass has started to differentiate into an epiblast, which lines the polar trophectoderm, and, a hypoblast, which underlies the epiblast and overlies the blastocoel. Shortly after implantation, the cells of mural trophectoderm stop dividing rapidly and subsequently exit mitosis, however, the DNA replication continues. Repeated endo-reduplication thus leads to a tremendous increase of N in the nuclei of these cells as they differentiate into primary giant trophoblast cells. The function of these cells is to invade into the maternal tissue of the uterus. The polar trophectoderm on the other hand proliferates rapidly and differentiates into a distal ectoplacental cone and a proximal extraembryonic ectoderm (Figure 8). The ectoplacental cone consists of secondary giant cells whereas the extraembryonic ectoderm retains the trophoblast stem cells. There is however no discrete boundary between the ectoplacental cone and the extraembryonic ectoderm and the two morphologically different tissue types are continuous with each other (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).

The inner cell mass continues to proliferate rapidly and the embryo becomes cylindrical in shape shortly after implantation. By E6, implantation sites measuring about 4-6 mm² can be seen in the uterine horns. The secondary giant cells extensively invade the uterine tissue and the rapidly growing ectoplacental cone starts to come in contact with maternal blood vessels. The inner cell mass further differentiates into extraembryonic and embryonic regions. The primary endoderm completely lines the mural trophectoderm and the parietal and visceral yolk sacs become clearly distinguished at this stage (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).

Between E6.5-7.5, the embryo attains the anterior-posterior axis. The primitive streak starts to be seen and the process of gastrulation begins for the first time. While at this stage, the ectoplacental cone continues to grow and establish proximity with maternal vasculature, the process of gastrulation gives rise to the mesodermal mesenchyme. This mesodermal mesen-
chyme gradually makes its way between the extraembryonic ectoderm and endoderm. The expanding mesoderm gradually forces the extraembryonic ectoderm to fold over the growing embryo, eventually splitting it into a distal layer of ecto-mesoderm called chorion and a proximal layer of ecto-mesoderm called amnion. The chorionic ectoderm is a flat structure, contains the trophoblast stem cells and is called chorionic plate. At the posterior end of the developing embryo, the expanding extraembryonic mesoderm creates a space within the mesodermal mesenchyme called extraembryonic coelom. By late E7.5, an allantoic bud starts to grow at this region of the embryo (Figure 8). This is an endo-mesodermal out pouching that evades into extraembryonic coelom. Unlike in reptiles and birds, in mammals the allantois does not grow to become functional. However, the mesodermal part of the allantoic bud continues to grow in the form of multiple fingers-like projections until it obliterates the extraembryonic coelom and further continues to grow and push against the mesodermal side of the chorion. This process of fusion of allantois and chorion, called chorio-allantoic fusion is a very important step in placental development of the mouse and a properly fused allanto-chorion is visible by E8-9. The chorionic trophoblasts continue to grow distally and develop primary villi which branch out to form complex meshwork of secondary villi. This complex meshwork of chorionic trophoblast is underlined by allantoic mesoderm. Meanwhile, the secondary giant cells enter into the decidua, and migrate some distance. Some of the secondary giant cells invade into the maternal tissue either in close association with the maternal artery or by migrating into the spiral maternal artery which brings maternal blood to the implantation site. These are called endovascular giant cells. They are atypical giant cells in that they are not as large as the primary giant cells. The interaction of the endovascular trophoblast with maternal artery leads to thinning of the arterial wall. While the very invasive giant cells arise out of the very distal end of the ectoplacental cone, the adjacent proximal part of the ectoplacental cone gives rise to a rather slowly dividing layer of cells. These cells also exit mitosis and undergo some rounds of endo-reduplication like giant cells, but do not develop such huge nuclei. This layer of cells is poorly vascularized and appears spongy. It is called spongiotrophoblast and is supposed to inhibit the growth of maternal vasculature into the chorio-allantoic part of the developing placenta. So, the maternal blood is drained in maternal endothelia only up to the spongiotrophoblast, after which, the chorionic trophoblasts are in direct contact with the maternal blood (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).
By E11, due to the concerted action of giant cell layer, spongiotrophoblast layer and underlying chorio-allantoic layer, the chorionic trophoblasts start to bathe directly in the maternal blood and haemotrophic nutrition is established for the developing embryo (Figure 8). The complex network of chorionic trophoblasts underlined by allantoic mesodermal blood vessels bathed by the maternal blood collectively making up the site for exchange between the maternal and fetal circulations and is called a labyrinth (Figure 8). The labyrinth consists of several different types of trophoblasts including several clusters of trophoblast stem cells. The chorionic trophoblasts become organized as a trilayer of cells. The largest of the chorionic trophoblasts, the mononuclear trophoblasts, are the ones which directly line the maternal blood lacunae and constitute the first layer, the most distal to the fetal side. These cells are distinctly seen in the labyrinth as cells with a prominent nucleus. The mononuclear trophoblasts do not have any microvillus structure. Unlike the true cellular nature of mononuclear trophoblasts, the two additional layers of chorionic trophoblasts are organized as syncytiotrophoblasts. The second and the middle layer of syncytiotrophoblasts, is also in touch with the maternal blood and has communication with the mononuclear trophoblasts through various fenestrations. The third layer of syncytiotrophoblasts is the

Figure 8. Different stages development of mouse placenta and schematic representation of labyrinthine organization. The allantois grows and pushes against chorion and forms the labyrinth. Figure based on and modified from Rossant and Cross, 2001.
only layer which is not in contact with the maternal blood. However, this layer of syncytiotrophoblasts shares a common basement membrane with the underlying allantoic blood vessels (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).

In addition, there are other cells like the pericytes and the macrophages which are usually located very close to the basement membrane separating the third layer of syncytiotrophoblast and the endothelia of the fetal capillaries. Interestingly, the pericytes are supposed to act as anchors which hold the various branches of fetal capillaries in position at one place, thereby making the labyrinthine structure more complex and increasing the surface area of exchange. By E11.5, all these structures are clearly organized and a functional placenta comes into being as a distinct organ (Figure 8). At E12, a new variety of cells start to differentiate from the spongiotrophoblast. These cells are small with a dense nucleus and glycogen-laden cytoplasm which is only weakly stained with eosin. These cells, called glycogen cells, are of unknown function, but are the only cell types which continue to invade into the decidua even in the very late stages of gestation (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).

As the embryonic development proceeds, the allantoic blood vessels, two arteries and one vein, become elongated and spirally inter-wound around each other and constitute the umbilical cord. The umbilical cord connects the fetus (at the site of allantoic bud formation) with the placenta (at the chorionic plate). The major fetal blood vessels can be seen branching out into the labyrinth at the chorionic plate. It follows that in a cross section, following different regions of a mature and functional placenta can be seen: from the maternal end towards the fetal end, the decidua (containing invasive glycogen cells of fetal origin), secondary giant cells, the junctional zone comprising of spongiotrophoblast, the labyrinth and the chorionic plate continuing into an umbilical cord. The placenta continues to grow in size and gains weight till E14.5 after which, the rate of placental weight gain plateaus. Typically, a normal placenta weighs around 100mg towards the end of gestation and supports the fetus which weighs around ten times the placenta itself. This indicates the efficiency of exchange occurring through this organ, which is the lifeline for the developing fetus (Balinsky, 1975; Kaufmann, 1982; Kaufman and Bard, 1999; Georgiades et al., 2002; Enders and Carter, 2004; Watson and Cross, 2005; EMAP Edinburgh Mouse Atlas Project).

The exchange between the fetal and maternal circulations is not only a passive process, rather a subset of the exchange is also active and is regulated by expression of specific molecules in the chorionic trophoblasts, for example certain ion channels and glucose transporter are actively involved in regulating the exchange of ions and glucose respectively. In addition to func-
tioning as an organ for feto-maternal exchange, the placenta also has several endocrine functions. The murine placenta synthesizes and secretes several peptide hormones which are structurally related to the pituitary hormones prolactin and growth hormone (Krieger, 1982; Gootwine, 2004; Dotsch et al., 1989). The two most prominent of these hormones are PL1 and PL2 (Gootwine, 2004). PL1 takes over the functions of pituitary prolactin just after the ectoplacental cone is formed. However, in more than second half of the pregnancy, PL2 is the major lactogenic hormone. PL1 followed by PL2 also maintain the corpus luteum in the ovary throughout the pregnancy and maintains the production of progesterone (Gootwine, 2004). In addition to PL1 and PL2, a third hormone called proliferin is also secreted by the giant cells (Gootwine, 2004). Proliferin has no functional similarities to the pituitary prolactin. Instead, it is a cellular proliferation and growth associated hormone. It is needed for the growth of uterine epithelium during gestation, growth of blood vessels at the site of implantation and has angiogenic activities (Corbacho et al., 2002). It is interesting to note that proliferin mediated effects involve signaling through insulin-like growth factor 2 receptor (Volpert et al., 1996; Jackson and Linzer, 1997). Another protein, proliferin-related peptide has effects opposite to those of proliferin and is anti-angiogenic (Jackson et al., 1994). Proliferin related peptide is necessary for the functions of the junctional zone and is secreted from both, the giant cells as well as the spongiotrophoblast.

Factors responsible for trophoblast differentiation and placental growth

The formation of the placenta is apparently a very precisely orchestrated process. Various molecules function in executing these processes precisely. Though our knowledge about the molecular control of placental development is far from complete, several genes are known to play key roles in specific processes and transgenic mice have been very valuable tools in acquiring this knowledge (Cross et al., 1994; Rinkenberger et al., 1997; Cross, 2000; Hembeger and Cross, 2001; Rossant and Cross, 2001; Cross, 2005; Watson and Cross, 2005; Simmons and Cross, 2005). The maintenance of the trophoblast stem cell phenotype is maintained by the signaling of FGF4 through the FGFR2 (Tanaka et al., 1998; Hughes et al., 2004). Another important player in this pathway is Nodal (Ma et al., 2001; Ghuzman-Ayala et al., 2004) which maintains the trophoblast stem cell phenotype by regulating the expression of genes like Err2 (Pettersson et al., 1996; Luo et al., 1997; Tremblay et al., 2001), Eomes (Ciruna and Rossant, 1999; Russ et al., 2000), Cdx2 (Chawengsaksophak et al., 1997), and Mash2 (Ghuzman-Ayala et al., 2004), and Ap2g (Werling and Schorle, 2002). Mash2 is responsible for differentiation of spongiotrophoblast out of the cells of the ectoplacental cone, which are otherwise destined to differentiate into secondary giant cells only (Guillemot et al., 1995; Tanaka et al., 1997) and there are genes known
which counteract the effects of Mash2 in the trophoblast (Kraut et al., 1998). Stra13 and Hand1 promote giant cell differentiation (Cross et al., 1995; Hughes et al., 2004) and Socs3 regulated Lif1 signalling is responsible for restricting giant cell growth for labyrinthine growth (Takahashi et al., 2003; Roberts et al., 2001). Gcm1 (Schreiber et al., 2000), Arnt (Kozak et al., 1997), Mdfi (Kraut et al., 1998), are some examples of those genes which are responsible for formation of labyrinth. Many such genes, which when mutated cause defects in chorio-allantoic fusion, branching morphogenesis and establishment of a functional labyrinth. The literature on such genes has been extensively reviewed (Cross et al., 1994; Rinkenberger et al., 1997; Cross, 2000; Hemberger and Cross, 2001; Rossant and Cross, 2001; Kunath et al., 2004; Cross, 2005; Watson and Cross, 2005; Simmons and Cross, 2005).

Faithful maintenance of a process called genomic imprinting is very important for maintaining normal placental growth and function. The majority of genes in our genome are known to be expressed from both, maternal and paternal alleles. However, there is a subset of genes which are normally expressed only monoallelically in a parent of origin-dependent manner (Reik and Walter, 2001; da Rocha and Ferguson-Smith, 2004). It has been hypothesized that in the placental mammals, there is a competition between the maternal and the paternal genomes in the fetus, for the available nutritional resources during gestation. The paternally expressed genes extract nutrition and promote growth of the placenta and hence the fetus. The maternally expressed genes on the other hand conserve nutritional resources for other offspring and hence antagonize the growth promoting effects of the paternally expressed genes (Reik and Walter, 2001). Placenta, through which the nutritional exchange during gestation takes place, is also the primary site of this parental conflict. Remarkably, most of the 77 imprinted genes known so far in the mouse are expressed and imprinted in placenta (Coan et al., 2005). This parent conflict hypothesis is not universally valid for all imprinted genes. It has been observed that biallelic expression, changes in expression or ablation of many imprinted genes actually affect placental growth and some of them do so in a manner that agrees with this hypothesis. Some prominent examples include Igf2 (Baker et al., 1993; Constancia et al., 2002), Mest (Lefebvre et al., 1998), Ipl (Frank et al., 2002), Grb10 (Charalambous et al., 2003), an imprinted locus on mouse chromosome 12 (Georgiades et al., 2000) and Cdkn1c (Zhang et al., 1998; Caspary et al., 1999; Takahashi et al., 2000). On the other hand phenotypes of Mash2 and deficient mice do not fit into the paradigm of parent conflict hypothesis (Guillemot et al., 1995; Tanaka et al., 1997) (reviewed by Coan et al., 2004).
Cell type-specific marker genes

There are several genes which are known to be expressed in certain cell types specifically, and mRNA expression of these genes is used as marker for the identification of those specific cell types. Tphpa and Flt1 are expressed only in the ectoplacental cone during early development; however, later these genes are expressed exclusively in the spongiotrophoblast (Lescisin et al., 1988; He et al., 1999). Plf is expressed only by giant cells at all stages (Lee et al., 1988). Pl1 and Pl2 expression are widely used as markers for early and late giant cells respectively (Hall and Talamantes, 1984; Faria et al, 1990; Faria et al., 1991). Transcription factor Eomes is known to be expressed only in small clusters of trophoblast stem cells present in the labyrinth and has been used as trophoblast stem cell marker (Russ et al., 2000; Ciruna and Rossant, 1999). Mest is exclusively expressed by the fetal capillaries in the labyrinth (Mayer et al., 2000). CDKN1C, a protein expressed in the nucleus of many different cell types in a mature placenta, is strongly expressed by glycogen cells (Takahashi et al., 2000; Georgiades et al., 2002). Similarly, the protein α-smooth muscle actin is specifically expressed by pericytes (Ohlsson et al., 1999). In addition, the presence of various cell surface carbohydrates can be probed using certain specific lectins. Though several lectins are available for cell type-specific staining, only few are used commonly. For example, isolectin B4 stains syncytiotrophoblasts, fetal capillaries as well as many cells in the decidua (Bulmer and Peel, 1996; Hemberger et al., 1999). Thus, isolectin B4 has been extensively used for morphometric demarcation of spongiotrophoblast (not stained) against labyrinth and decidua (stained).

Mouse models of placental dysplasia

Like many other biological processes, placental development is also affected by several factors and studies on the mouse models of placental dysplasia have added to our knowledge about the various factors affecting placental growth. Based on the etiology, placental growth defects can be broadly classified into three categories: entirely genetic, entirely non-genetic and mixed. The entirely genetic models are those, in which a defect in placental growth is manifested as a result of heritable mutation of any specific gene or a chromosomal segment. The non-genetic models include placental growth defects associated with gestational diabetes and reproductive cloning. In such cases the placental phenotype is not determined by any genotype and hence is not genetically inherited by successive generations. Hybridization between different closely related species, in this context between MMU and MSP or MMA, however causes an intriguing placental phenotype which has mixed etiologies. While the placental phenotype of interspecies hybrids is
primarily determined by the genotype, it has some other features which show that some non-genetic mechanisms also underlie this interesting model of placental dysplasia.

Genetic models of placental overgrowth

Targeted deletions of several genes are known to cause placental dysplasia and imprinted genes are particularly interesting in this context. Some common examples have been mentioned above. Mutations of a large number of non-imprinted genes also affect mouse placental development. However in these cases, either the placental weight is not the main parameter affected, or it is reduced because there is an overall defect in placenta formation (Cross et al., 1994; Rinkenberger et al., 1997; Cross, 2000; Hemberger and Cross, 2001; Rossant and Cross, 2001; Kunath et al., 2004; Cross, 2005; Watson and Cross, 2005; Simmons and Cross, 2005).

Esx1 is known to be involved in the regulation of placental growth. The gene Esx1, located on the distal X chromosome codes for a homeobox transcription factor (Li et al., 1997; Fohn and Behringer, 2001). Esx1 is expressed widely in the extraembryonic compartments and at adulthood, the expression is limited to a subset of cells in male germ line only (Li et al., 1997; Fohn and Behringer, 2001). Since in the extraembryonic lineage the paternally inherited X chromosome is preferentially silenced (Takagi and Sasaki, 1975; Takagi et al., 1978; West et al., 1981), Esx1, like most other X-linked genes, is a maternally expressed in placenta (Li et al., 1997). The phenotypes caused by Esx1 deficiency are hence manifested only if the mutation is inherited from the maternal allele, while the conceptuses inheriting the mutation from the paternal allele have phenotypes identical to the wild type conceptuses (Li and Behringer, 1998).

Targeted deletion of Esx1 leads to IUGR resulting in 20% reduction in perinatal bodyweight of the maternal mutants (Li and Behringer, 1998). This difference in body weight is abolished at maturity indicating that the placental function is significantly compromised in these mutants. Despite an in-
crease in placental weight of the mutants, the embryos are smaller at birth because the labyrinthine structure is disrupted in the mutants leading to reduction in trans-placental exchange (Li and Behringer, 1998). The increase in placental size and weight (about 51% at E14.5 and 27% at E18.5) is mostly due to expansion of spongiotrophoblast into labyrinth (Figure 9), increased proliferation of glycogen cells and presence of edema like fluid-filled cysts in the spongiotrophoblast (Li and Behringer, 1998). The first signs of labyrinthine defects are visible at E12, when large aggregates of trophoblasts are seen in the labyrinth (Li and Behringer, 1998). At later stages of gestation, there are two defects manifested in the labyrinth; (1) the absence of laminated trophoblasts which give rise to the layer of syncytiotrophoblasts that lies adjacent to the fetal capillaries and (2) presence of vacuoles in innermost layer of syncytiotrophoblast indicating a defect in transport (Li and Behringer, 1998). Put together, these results indicated that Esx1 is important for proper growth of placenta, especially the basal layer and for normal development and functioning of the labyrinthine trophoblasts.

**Placental overgrowth in cloned mice**

Reproductive cloning is a process by which genetically identical copies of an organism can be made bypassing the normal process of fertilization of an oocyte with a sperm (Wakayama and Yanagimachi, 1999). Experimentally, the feasibility of this approach was first shown in the frog (Gurdon, 1962, 1986). The first confirmed mammalian clones were sheep (Kampbell et al., 1996), after which reproductive cloning has been successfully performed in various other mammals including the mouse (Wakayama and Yanagimachi, 1999; Wilmut et al., 1997; Wakayama et al., 1998, 1999; Kato et al., 1998; Galli et al., 1999; Wells et al., 1999; Kubota et al., 2000; Polejaeva et al., 2000). In this process, nuclei from terminally differentiated somatic cells are transferred into enucleated oocytes, obtained from suitable donors. The cytoplasmic environment of the oocyte reprograms the chromatin of the differentiated somatic cell in such a way that successful development may ensue (Rideout et al., 2001; Dean et al., 2003; Shi et al., 2003; Jaenisch et al., 2005). However, the probability of this process happening successfully is very low, around 2% (Tamashiro et al., 2003). Since such a reprogramming of the somatic cell nucleus does not change the base sequence of DNA, it is implied that it is not a genetic, but rather an epigenetic process. This epigenetic reprogramming redirects the somatic-like gene expression patterns to zygote-like gene expression patterns; a process achieved by rearrangement of epigenetic marks genome-wide. The very low success rate of reproductive cloning is attributed to aberrant epigenetic reprogramming (Rideout et al., 2001; Dean et al., 2003; Shi et al., 2003; Jaenisch et al., 2005). CpG methylation, a very well studied epigenetic mark is frequently disturbed even in
cloned mice (Inoue et al., 2002; Ogawa et al., 2003) that are born and survive and is frequently associated with deregulated expression of several genes (Humpherys et al., 2001; Mann et al., 2003; Suemizu et al., 2003). As allele specific CpG methylation patterns are keys to the maintenance of monoallelic expression of imprinted genes, deregulated and biallelic expression of imprinted genes is frequently seen in cloned mice (Humpherys et al., 2001; Ogawa et al., 2003; Mann et al., 2003; Suemizu et al., 2003). Aberrant epigenetic marks laid down during the process of cloning, cause abnormal phenotypes of placental overgrowth and obesity in cloned mice. However, ES cells from somatic cell nuclear transfer-derived blastocysts do not retain the aberrations in epigenetic marks and show remarkable transcriptional similarity to ES cells from normal blastocysts derived by fertilization (Brambrink et al., 2006).

The hyperplastic placentas of cloned conceptuses, hereafter referred to as cloned placentas, are on an average 3 times the weight of a normal mouse placenta at birth (Figure 10) and are observed in both male and female conceptuses (Tanaka et al., 2002). The histological features of cloned placentas include overgrowth of spongiotrophoblast compartment, increased numbers of glycogen cells and atypically round giant cells at the end of gestation (Tanaka et al., 2002). In addition the size of cells in the spongiotrophoblast is also generally larger than normal and the labyrinth contains fluid filled cysts (Tanaka et al., 2002). The cloned placentas exhibit deregulated expression of imprinted genes Igf2r, Cdkn1c, Mest, Grb10 and Mash2 (Tanaka et al., 2002). The change in expression has been attributed to changes in sizes of tissue compartments that express these genes as the expression of the non imprinted gene Tphpa is increased in cloned placentas (Tanaka et al., 2002). This implies that the deregulated expression of imprinted genes is not a cause but a consequence of placental hyperplasia; however, this has never been tested functionally. Since cloned conceptuses are produced in very small litter sizes, usually just 1 or 2 conceptuses per litter, an influence of systemic effects adds to the placental overgrowth (Tanaka et al., 2002). While the epigenetic lesions in cloned placentas have not been studied in
detail, it has been reported that in these placentas, the *Sall3* locus is an epigenetic hotspot of aberrant CpG methylation (Ohgane et al., 2004).

### Placental overgrowth in mouse interspecies hybrids

As I have mentioned earlier, placental growth defects are observed in several mammalian interspecies hybrids. Interspecies hybrid placental dysplasia (IHPD) in the closely related species of the genus *Mus* was reported by our laboratory almost a decade ago (Zechner et al., 1996). This first study used F1 and backcross hybrids mostly between MMU and MSP, and, MMU and MMA. Backcross hybrids MSM, MSS and MXM have also been used in the work presented in this thesis. Of all the mouse models of placental growth defects known so far, IHPD is peculiar for its several features discussed below.

#### Reciprocal phenotypes in reciprocal hybrids

IHPD is the only model which by similar etiology provides contrasting phenotypes in the placenta. Depending on the direction of crosses, determined by parent-species combination used to generate the hybrids, the placentas are either hyperplastic or hypoplastic (Figure 11). Placentas of the conceptuses derived from the cross MS and MX are hypoplastic (Zechner et al., 1996) whereas the reverse crosses SM and XM give hyperplastic placentas (Zechner et al., 1996). However, SM litters are difficult to obtain and so far, only one conceptus with an 848 mg placenta has been obtained (Zechner et al., 1996). In the backcrosses, MSM and MXM conceptuses have hyperplastic placentas whereas MSS and MXX conceptuses have hypoplastic placentas (Zechner et al., 1996). As discussed in proceeding sections, not only the placental dysplasia but the histo-morphological features are also reversed in reciprocal crosses. Since different reciprocal crosses with contrasting phenotypes are produced by using the mother of same genotype (MS F1 female in the case of MSM and MSS back crosses), the reciprocation of phenotypes can not be attributed to a difference in the intra-uterine environment.

#### A range of phenotypes with different intensities

In the genetic models of placental dysplasia and in the cloned placentas, the hyperplasia or hypoplasia occurs according to an all-or-none law. In such models, there is a clear distinction between normal and abnormal and a very narrow range of abnormal phenotypes exist. Only IHPD provides us with a model in which different ranges of placental phenotypes are seen, even amongst littermates (Zechner et al., 1996). Depending on the cross, the wet weights of IHPD placentas may vary from as low as 12 mg to 848 mg (Zechner et al., 1996). The hyperplastic phenotype is more pronounced in the males compared to female littermates (Zechner et al., 1996). The severe hypoplasia in the MSS and MXX backcrosses are frequently associated with
fetal death. The hybrids between MMU and MMA produce more severe placental phenotypes compared to the hybrids between MMU and MSP (Zechner et al., 1996). In addition to the weight, histo-morphological features also present as a gradient in such placentas.

IHPD is X-linked

IHPD has been found to segregate with alleles on the X chromosome, in MSM, MXM, MSS and MXX matings and further backcrosses (Zechner et al., 1996). For example, the MSP alleles on the X chromosome interact with MMU alleles elsewhere in the genome and cause hyperplasia in MSM backcross; the same holds true for MMA alleles in MXM backcross (Zechner et al., 1996, 1997). On the other hand MMU alleles on the X chromosome interact with MSP and MMA alleles causing hypoplasia in MSS and MXX backcrosses respectively (Zechner et al., 1996, 1997). The linkage to the X chromosome is spread along a large region in the proximal and central regions (Zechner et al., 1996). The highest score for linkage was found for the region of X chromosome harboring the marker DXMit8 and proximal to it (Zechner et al., 1996). This locus is also called IHPD locus. Several loci on the X chromosome give rise to placental hyperplasia in the MSM backcross as MMU mice congenic for MSP alleles on different parts of the X chromosome present with significantly increased placental weight (Hemberger et al., 1999). However, to what extent the MMU alleles on autosomes are involved in the placentas phenotypes of MMU backcross remain unknown. The homozygous congenic strain AT24, having MSP alleles from 12 cM to 31 cM

Figure 11. IHPD phenotype in E16 MSM littermate conceptuses. A, normal weight (MSM)M placenta, B, hyperplastic placenta, littermate of A. C, hypoplastic placenta from MXX backcross. Subphenotypes of hyperplastic IHPD placentas can be seen in AT24 placenta in D. Figures adapted from Zechner et al., 1996 and Hemberger et al., 1999.
on X chromosome in MMU background gives rise to moderately hyperplastic placentas which are histo-morphologically comparable to hyperplastic IHPD placentas (Figure 11) and sub-phenotypes of hyperplastic IHPD placentas are manifested in AT24 placentas consistently (Hemberger et al., 1999). Nevertheless, the occurrence of X linked placental hyperplasia could not be attributed to any single gene locus on the X chromosome as MMU mice, homozygous congenic for regions smaller than 20 cM (between 12 cM and 31 cM) did not produce any hyperplasia (Hemberger et al., 1999). This indicates that though multiple regions on X chromosome can determine placental weight independently, there is a threshold for the minimum amount of introgression needed to initiate placental dysplasia. However, this has been tested only for the AT24 region on the X chromosome and not for the other regions for which, only heterozygous congenic mice could be made owing to male sterility (Hemberger et al., 1999). The AT24 mice provide us with a very useful tool to study placental hyperplasia caused by MSP alleles on X chromosome in an otherwise homogenous genetic background.

While the different extents of overgrowth or undergrowth of placentas is primarily dependent on the genotype (Zechner et al., 1996), manifestation of opposite phenotypes in reverse crosses shows that there are parent of origin-dependent determinants of IHPD as well. Imprinted genes are expressed in a parent of origin-dependent manner and might be responsible for the reversal of phenotypes in reverse crosses. Very intriguingly, we found that many genes, both imprinted and non-imprinted ones are deregulated in opposite directions in MSM and MSS placentas (Singh and Fundele, unpublished results). One very interesting aspect of the linkage of IHPD to alleles on X chromosome is that the X chromosomes are also expressed in a parent of origin-dependent manner in trophoblast. Owing to preferential paternal inactivation in the mouse trophoblast, X chromosomes are of paramount epigenetic interest (Reik and Ferguson-Smith, 2005). It has been shown that under the experimentally induced conditions, where the hybrid progeny inherits only one X chromosome (no maternal X chromosome), MSP or MMA alleles inherited through paternal X chromosome can cause IHPD phenotypes similar to that of MSM or MXM placentas (Zechner et al., 1997). However, whether abnormalities in preferential paternal X inactivation also underlie IHPD, has not been tested. Nevertheless, there is preliminary evidence that disturbance in paternal X inactivation is not a frequent phenomenon in the IHPD (Hemberger et al., 2001). However, through genetic studies, it is indicated that complex genetic interactions in-trans and associated epigenetic changes on the X chromosome underlie IHPD (Zechner et al., 1997).

DNA methylation is an important epigenetic mark. Analysis of CpG methylation for several candidate genes and LINE-1 and IAP repeat elements genome-wide revealed no discernible differences between hyperplastic and normal weight IHPD placentas (Schütt et al., 2003). Sall3, a gene which is hypermethylated and deregulated in cloned hyperplastic placentas (Ohgane
et al., 2004) is also under expressed in MSM hyperplastic placentas compared to normal weight littermates (Singh and Fundele, unpublished observations). This might indicate some similarities between the epigenetic mechanisms underlying placental hyperplasia in cloned and IHPD placentas, however this remains elusive.

Histo-morphological features of IHPD placentas
Like in the *Esx1* mutant and cloned placentas, spongiotrophoblast is the tissue compartment most affected in IHPD placentas (Zechner et al., 1996; Hemberger et al., 1999). In the hyperplastic placentas, spongiotrophoblast overgrows into the labyrinth and a boundary between the basal layer and labyrinthine layer is lost (Zechner et al., 1996; Hemberger et al., 1999). In the hypoplastic placentas, the spongiotrophoblast is poorly grown and is very rudimentary (Zechner et al., 1996). The differentiation of spongiotrophoblast into glycogen trophoblasts is increased and pegs of glycogen trophoblasts can be seen in hyperplastic placentas, whereas in the hypoplastic placentas, this cell type is almost non existent (Zechner et al., 1996). While no clear cut function of this cell type is known, it has been shown that there is a correlation between fetal weight and increased differentiation of glycogen cells in IHPD placentas (Kurz et al., 1999). The number of giant trophoblasts is also significantly increased in the hyperplastic placentas. It is noteworthy that the IHPD placentas have aberrant expression of at least two genes responsible for placental growth; *Igf2* and *Esx1* (Zechner et al., 2002). The IHPD placentas and cloned placentas of comparable weight range resemble each other very much. The features of edematic cysts in the cloned placentas are also seen in extremely hyperplastic IHPD placentas (Zechner et al., 1996). Unlike the normal placentas, the hyperplastic IHPD placentas continue to gain weight even after E15 (Zechner et al., 1996). The labyrinthine structure and functioning is apparently normal in the hyperplastic IHPD placentas and there are no indications of compromised placental function. Despite the striking overgrowth of IHPD placentas, the size of the litters from MSM or MXM matings are not very small and frequently between 6 to 10 conceptuses are found in such matings. Hence the systemic effect adding to placental overgrowth is not as strong a confounding factor in IHPD as it is in the case of cloned placentas.

Despite widely different etiologies, *Esx1* mutants, cloned placentas and hyperplastic IHPD placentas have striking resemblance in phenotypes. While our knowledge about molecular mechanisms underlying placental development and function are not very well understood, these various models provide us with useful tools to explore into such mechanisms.
Aims

Paper I

IHPD, cloning by nuclear transfer and mutation of the gene *Esx1* represent three different models of placental hyperplasia with widely different etiologies. These models of placental hyperplasia share many histological features; for example increased size of the spongiotrophoblast, increased number of giant and glycogen cells. On the other hand, defects in labyrinthine structure and a consequent reduction in fetal weight is a feature of the *Esx1* mutant placentas only. Prior to this study, independent gene expression profiling was done on cloned placenta by various groups, whereas no such study was done on the IHPD and *Esx1* mutant placentas. It was also not clear from those studies if similar or exclusively different pathways were involved in placental overgrowth in these models. This study was undertaken to determine the molecular pathways underlying these different models of placental hyperplasia. We performed comparative large-scale mRNA expression profiling of the three models by using cDNA microarrays to find out how similar are the molecular mechanisms underlying them. In addition we also aimed at studying spatial expression patterns of selected genes in IHPD placentas. Since it was known for cloned placentas that changes in gene expression reflect overgrowth of the tissue compartments expressing them, we also tested this possibility in IHPD placentas.

Paper II

In the first paper we had reported a set of genes which were aberrantly expressed in different placental overgrowth models and had thus identified genes potentially important in murine placental developmental. It was a major challenge to identify functions of these genes in murine placentation, as for many of these, no role in placental development had been known or even expected. The aim of the next course of studies was to rapidly screen some of these genes for their roles in placental development. Opportunistically, we chose to analyze those genes for which mutant mice were already available at this time. In this study, which was the first one in this series of rapid functional screening, we focused on two genes, carbonic anhydrase 2 (*Car2*) and neural cell adhesion molecule 1 (*Ncam1*), which were both over expressed in
IHPD placentas. Our objectives were (1) to determine spatio-temporal expression profiles of these genes during mouse extraembryonic development, (2) to analyze placental phenotypes in mice homozygous mutant or wild type for Car2 or Ncam1, (3) and to see the effect of normalization of expression of these genes independently on the placental phenotypes of the AT24 mice. This was also the first study where we validated by gene expression profiling that the AT24 congenic strain could be used as a model to study IHPD. Finally, we also tested the possibility if co-expression of other members of the same gene families could confound our observations.

Paper III

In paper I, we had reported genes with at least 2-folds of change in the microarray hybridizations. This masked several genes with very consistent yet marginally less than 2-folds of change in expression. Fibulin1 was one such gene, whose splice variant D (Fbln1D) was over expressed in IHPD placentas by 1.8 folds. This gene codes for a protein known to be responsible for integrity of peripheral vasculature which could indicate its importance in labyrinthine function. In this paper we aimed at (1) ascertaining deregulation of expression of Fbln1D in IHPD placenta, (2) finding out spatio-temporal expression pattern of Fbln1D during mouse placental development, (3) assessing placental phenotype of Fbln1 mutant mice, (4) trying to rescue the AT24 placental phenotypes by normalization of Fbln1 expression and (5) checking the expression of genes which could provide functional redundancy.

Paper IV

In continuation with our pursuit to address functions of different genes in placentation, we next focused on the gene which codes for the beta 3 subunit of the voltage-dependent Ca^2+ channel (Cacnb3). This gene was also over expressed in IHPD placentas. As Ca^2+ homeostasis in important for several cellular functions, we wanted to explore the effects of over expression of this gene in placenta. For the functional analysis of Cacnb3 also, we followed our methodology of spatio-temporal expression profiling, phenotypic analysis of mutant placentas, rescuing placental phenotypes of AT24 mice and asking if functional counterparts of Cacnb3 are expressed in placenta.
Paper V

Of all the genes chosen for the functional studies, Cpe was different in two respects in that it was deregulated in IHPD as well as cloned placentas and it was under-, not over expressed. Our primary objectives in this paper were to identify spatio-temporal expression pattern of Cpe and to analyze placental phenotypes of Cpe null placentas. We then wanted to find genes whose expression is regulated by Cpe in order to understand how Cpe exerts its effects on placentation. We also went on to determine spatio-temporal expression pattern of Cpd, a proven functional counterpart of Cpe. To see if deregulated expression of Cpe (and Cpd) is a cause or a consequence of IHPD, we performed expression analysis for these two genes prior to the manifestation of dysplastic phenotype. Finally, we wanted to know if the decrease in Cpe expression is IHPD placentas is associated with changes in DNA methylation at the Cpe transcription start site.
Results and discussion

Paper I
Through comparative gene expression profiling, we found that different molecular mechanisms are involved in the hyperplastic phenotypes of IHPD, cloned and Esx1 mutant placentas. Such an expression analysis revealed a set of genes which were deregulated in IHPD irrespective of the species used to generate hybrids and the extent of hyperplasia, as we had used placental pools of different weight ranges (similar X chromosomal genotypes represented in each pool) from MSM and MXM backcrosses. In this study, we arbitrarily chose a threshold of 2-folds of change in expression and reported deregulated genes accordingly. There was only one gene with known function, Ramp2 and another EST representing a UniGene cluster of unknown function, found commonly deregulated in all the three models. Ppp2r4 and Ncam1 were the only genes commonly deregulated between cloned and Esx1 mutants, and, IHPD and Esx1 mutants respectively. However, Ncam1 expression was increased in IHPD whereas it was decreased in Esx1 mutant placentas. There were many genes, which were deregulated in only one of the three models investigated. There were more genes commonly deregulated between cloned and IHPD placentas compared to cloned and Esx1 mutants or IHPD and Esx1 mutants. These included Cpe and imprinted genes Dcn and Gatm. At a threshold of 2-folds, the genes Car2, Ncam1, Cacnb3 and Lhx3 were over expressed in IHPD placentas only. Some of the genes identified by us were also found similarly deregulated in cloned placentas by Humpherys et al., (2001) and Suemizu et al., (2003). When we looked at the expression of imprinted genes present on the microarrays, we found that there was very high variation in the expression. With exceptions of Gatm and Dcn, all other imprinted genes present on the microarrays, which included Dlk, Igf2, Snrpn, Tapal, Mest and Meg3, exhibited inconsistent and mild deregulation. From these results it was clear that though there are some similarities between IHPD and cloned hyperplastic placentas at transcriptional level, largely different mechanisms underlie these three models of placental hyperplasia.

We then performed mRNA in situ hybridizations to find out the spatial expression patterns of some of these deregulated genes. Interestingly, we found that the genes over expressed in hyperplastic IHPD placentas, for example Lhx3, were expressed in many parts of spongiotrophoblast and several
foci in the labyrinth. On the other hand, the genes under-expressed in IHPD placentas were expressed mostly in the decidua and some places in chorionic plate. However, the expansion of spongiotrophoblast in hyperplastic placentas was not responsible for the over expression of genes, as Tphpa, a gene specifically expressed in this tissue was not found over expressed in hyperplastic placentas.

Paper II

Car2 and Ncam1 had been found to be over expressed in IHPD hyperplastic placentas. We started our analysis with these two genes by studying their expression through different stages of placental development. Car2 was found to be expressed in decidua at E8. At E10 the expression was very strong in the ectoplacental cone and parietal yolk sac. From E12 till E18, the expression was primarily in spongiotrophoblast and some parts of labyrinth and in glycogen cells. Towards the end of gestation, the expression declined and at E18, very weak expression could be seen. For Ncam1, the expression at E8 and E10 was in decidual tissue and not in the ectoplacental cone. From E12 onwards, a weak and diffuse expression could be seen throughout the placenta. However, there was an induction of expression at E14 in the spongiotrophoblast and peaking at E16 in this tissue. At E18 most of Ncam1 expression was in the decidua. Glycogen cells also expressed Ncam1 very strongly at E14 and E16. Ectopic expression of both the genes was found in E18 IHPD and cloned placentas.

Homozygous mutation of neither Car2, nor Ncam1 had any significant effect on placental phenotype or fetal weight. However, we found that Car3 and Ncam2, redundant functional counterparts of Car2 and Ncam1 respectively were expressed in spongiotrophoblast and could possibly mask the effects of mutation of these genes.

Before using AT24 mice for rescue-based functional studies, we assayed the mRNA expression profile of AT24 placentas by cDNA microarrays as well as qRT-PCR analysis. We found that AT24 placentas had similarly deregulated expression of several genes previously found deregulated in IHPD placentas and this included Car2 and Ncam1. Chromosomal localization analysis showed that there was a disproportionately high number of deregulated genes in the MSP derived region of the AT24 X chromosome compared to the rest of the genome. Decrease in CAR2 and Ncam1 expression levels in AT24/+;Car2+/- and AT24/+;Ncam1+/- placentas respectively did not produce any phenotypic rescue. This showed that Car2 and Ncam1 are not upstream determinants of placental phenotypes seen in AT24 mice, and probably in IHPD placentas as well.
Paper III

Before starting our functional analysis of Fbln1 in placenta, we confirmed deregulation of Fbln1 expression in IHPD placentas. Northern blot hybridization on IHPD placentas showed that Fbln1D was indeed over expressed in hyperplastic placentas compared to normally weighing littermate placentas. In the wild type placentas, Fbln1D could be detected starting from E12 onwards. The bulk of expression was in the spongiotrophoblast, where it congruently overlapped with that of Tphpa. Glycogen cells in the decidua and giant cells did not express Fbln1. Some expression was also seen in the walls of blood vessels emanating out of the chorionic plate into the labyrinth. In the IHPD placentas however, the expression was ectopic. It was noteworthy that the three tissues most affected in IHPD, spongiotrophoblast, giant cells and glycogen cells, expressed Fbln1D and the expression in giant cells was heterogeneous. This ectopic expression was confirmed at the protein level.

Mutation of Fbln1 gene did not have any statistically significant effect on the placental weight, though there was an apparent tendency of the Fbln1 null placentas to be smaller than their wild type littermates. Interestingly, not only the other splice variant of Fbln1, Fbln1C, but also other members of the gene family, Fbln2 and Fbln5 were found to be expressed in placentas, raising the possibility that perhaps there is a functional redundancy for the Fbln1 gene in the placenta. Though the AT24 placentas had an increased expression of Fbln1D, the heterozygous mutation of Fbln1 in heterozygous AT24 background did not produce any significant effect on placental weight and morphology. Hence, it was confirmed that Fbln1 is not causally deregulated in IHPD.

Paper IV

As no previous reports were available that described the expression pattern of Cacnb3 in the mouse placenta, we started with mRNA in situ hybridization for this gene in placentas of different stages. Cacnb3 mRNA in situ hybridizations showed that this gene is expressed in ectoplacental cone at early stages. From E12 onwards, widespread expression in the spongiotrophoblast and several foci in the labyrinth could be seen. The glycogen cells, other than some of those in the decidua, were devoid of expression. The E18 IHPD placentas displayed a temporally delayed expression, resembling E16 wild type placentas. However, in spatial terms the expression was not ectopic and only few glycogen cells in the decidua expressed Cacnb3.

Mutation of the Cacnb3 gene did not have any significant effect on placental or fetal weight. Nevertheless, there was a regionalized labyrinthine defect observed in only approximately 20% of the homozygous mutants. The mononuclear trophoblasts in these defective regions of certain phenotypic
placentas were disorganized and were fewer in number compared to wild type littermates and normal appearing regions of the same placenta. Staining with isolectin B4 showed that the fetal vasculature was also distorted in the same regions and the area of contact between maternal and fetal tissues appeared to be reduced. A comparable but much more pronounced phenotype has been reported for Pdgfb and Pdgfrb mutants both and as pericytes, the cells anchoring fetal blood capillaries in labyrinth, are disorganized in these mutants, we wanted to see if pericytes are affected in Cacnb3 mutants as well. Indeed we found by ASMA staining that specifically in the regions affected, the pericytes were fewer in number and had disorganized presence. None of the heterozygous or wild type placentas showed this phenotype. We found that other members of the Cacnb3 gene family, Cacnb2 and Cacnb4 were expressed in placenta at mRNA and protein level both and can potentially take over the function of Cacnb3. Intriguingly, while Cacnb1 mRNA levels were detected at levels comparable to those of Cacnb2 and Cacnb4, we could not detect CACNB1 expression by western blot indicating that this gene is not translated in placenta.

Despite over expression of Cacnb3 in AT24 placentas and reduced Cacnb3 expression in heterozygous mutants, in the rescue experiments using AT24 mice, heterozygous Cacnb3 mutation failed to affect AT24 placental phenotype significantly. This showed that even though Cacnb mediated Ca^{2+} homeostasis is important for maintenance of proper labyrinthine structure, the function of Cacnb3 is not causative to the development of AT24, and reasonably, for IHPD placental phenotypes.

**Paper V**

By mRNA in situ hybridizations, Cpe expression was found in decidual tissue at E8 and E10 of gestation. From E12 till E16, expression was prominent in decidua and a diffuse but weak expression was visible in labyrinth as well. Towards the end of gestation, by E18, the expression in decidua and labyrinth declined, however, strong expression was seen in the chorionic plate at E18. No specific expression was seen in glycogen cells or giant cells. In IHPD placentas, the expression at E18 was temporally delayed and mimicked that of E16 wild type placentas.

Mutation of Cpe, which causes obesity and insulin insufficiency, did not affect placental weight significantly. In some litters, however, it was associated with IUGR. While overall morphology of mutant placentas was not affected, there was a phenotype of giant cells and/or glycogen cells seen only in the mutant placentas occasionally. The giant cells were increased in number and in some cases had nuclei larger than normal. The giant cell phenotype was accompanied in all cases by an increase in the population of glycogen cells as well. There was however one Cpe mutant placenta which had
only the phenotype of increased population of glycogen cells, without any detectable giant cell abnormality. BrdU labeling and in situ expression analysis of giant cell marker genes Pl2 and proliferin revealed no abnormalities with these two cell types. cDNA microarrays based expression profiling of Cpe mutant placentas using wild type littermates as controls showed that while there was little transcriptional disturbance and only 7 genes with known functions were found deregulated, yet Dtprp, a decidua and trophoblast-specific protein coding gene was found to be consistently over expressed in Cpe mutant placentas. Intrigued by the inconsistency of the phenotype, we assessed the expression of Cpd, a gene known to rescue loss of Cpe activity in Cpe mutants. mRNA expression profiles showed that spatio-temporal expression profiles for Cpe and Cpd were congruent from E12 onwards. The overlapping expression pattern of these two genes was also confirmed by immunohistochemical studies. When expression of Cpe and Cpd was assayed in E12 IHPD placentas, a stage when hyperplasia is not yet manifested, a very strong reduction in expression consistently correlated with fetal genotypes indicative of hyperplastic placentas by E18. This confirmed that decrease of Cpe and Cpd expression is an upstream phenomenon in IHPD and hence is very likely causally involved in IHPD. Interestingly, these genes were over expressed in E18 hypoplastic mss placentas compared to normal weight littermate placentas. Finally, we wanted to find what causes a decrease of Cpe transcript levels in IHPD placentas. We assayed CpG methylation in a 139 bp region, present upstream of the Cpe transcription start site. This region was chosen because it is CpG rich, is conserved between mouse, rat and human and is close to Cpe transcription start site. Sequences of PCR products of this region, amplified from sodium bisulfite treated genomic DNA showed that this region was unmethylated in wild type as well as IHPD placentas of different weights.
Conclusions

Though many genes are differently deregulated in IHPD and cloned hyperplastic placentas, they also share some similarities in gene expression patterns. The fact that there are two imprinted genes amongst the few genes commonly deregulated between these two different models indicates that at least in part there is a certain overlap between mechanisms underlying IHPD and cloned placentas. However, unlike cloned placentas, changes in gene expression in IHPD placentas are not due to changes in the tissue type expressing them.

Placental phenotype is not significantly affected by loss of function of Car2, Ncam1 and Fbln1 genes and this can be attributed to co-expression of counterparts which are functionally redundant to these genes. Cacnb3 and Cpe mutations cause abnormal placental phenotypes, though these are not completely penetrant for both these genes. This might again be due to differential expression of and rescue provided by the various functional counterparts. In the case of Cpe, this is reinforced by almost identical expression behavior of Cpe and Cpd. Of all the genes screened for their role in placenta in this study, Cpe is the only one, whose mutation recapitulates to some extent the phenotypes of IHPD and cloned placentas. This, combined with the finding that decreased Cpe and Cpd expression precedes placental hyperplasia, reveals Cpe and Cpd as candidate causative genes in IHPD and hence potential speciation genes.

The genome has been shown to be hypomethylated in the trophoblast lineage and methylation-independent expression regulation has been shown for imprinting cluster 2 in placenta. Our results of no methylation at the Cpe promoter locus might be a testimony to the fact that this methylation independent expression regulation in placenta applies to non-imprinted genes as well.

To confirm the possibilities of functional redundancies, simultaneous mutations of functional counterpart genes need to be created, which is a challenging task. It appears from these results that placenta expresses several genes as standby which come up to rescue the placental function in case any specific gene function is affected. This might be an evolutionary advantage for the successful functioning and resistance to somatic mutations, of the placenta.
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