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Proliferation and Growth Factor Expression in Abnormally Enlarged Placentas of Mouse Interspecific Hybrids

ULRICH ZECHNER,¹ MYRIAM HEMBERGER,¹ MIGUEL CONSTÂNCIA,² ANNIE ORTH,³
IOANNIS DRAGATSIS,⁴ ANGELA LÜTTGES,¹ HORST HAMEISTER,⁵ AND REINALD FUNDELE^{1*}

¹Max-Planck-Institut für Molekulare Genetik, Berlin, Germany

²Laboratory of Developmental Genetics and Imprinting, The Babraham Institute, Cambridge, United Kingdom

³Laboratoire Genome et Populations, Université de Montpellier, Montpellier, France

⁴Department of Genetics and Development, Columbia University, New York, New York

⁵Abteilung Humangenetik, Universität Ulm, Ulm, Germany

ABSTRACT It has been shown previously that abnormal placental growth occurs in crosses and backcrosses between different mouse (*Mus*) species. In such crosses, late gestation placentas may weigh between 13 and 848 mg compared with a mean placental weight of approximately 100 mg in late gestation *M. musculus* intraspecific crosses. A locus on the X-chromosome was shown to segregate with placental dysplasia. Thus in the (*M. musculus* × *M. spretus*)F1 × *M. musculus* backcross, placental hyperplasia cosegregates with a *M. spretus* derived X-chromosome. Here we have investigated whether increased cell proliferation and aberrant expression of two genes that are involved in placental growth control, *Igf2* and *Esx1*, may cause, or contribute to placental hyperplasia. Increased bromodeoxyuridine labeling of nuclei, reflecting enhanced proliferation, was indeed observed in hyperplastic placentas when compared with normal littermate placentas. Also, increased expression of *Igf2* was seen in giant cells and spongiotrophoblast. However, when *M. musculus* × *M. spretus* F1 females were backcrossed with males that were heterozygous for a targeted mutation of the *Igf2* gene, placentas that carried a *M. spretus* derived X-chromosome and were negative for a functional *Igf2* allele exhibited an intermediate placental phenotype. Furthermore, in early developmental stages of placental hyperplasia, we observed a decreased expression of the X-chromosomal *Esx1* gene. This finding suggests that abnormal expression of both *Igf2* and *Esx1* contributes to abnormal placental development in mouse interspecific hybrids. However, *Esx1* is not regulated by IGF2. © 2002 Wiley-Liss, Inc.

Key words: mouse; interspecific hybridization; placental dysplasia; proliferation; IGF2; ESX1

INTRODUCTION

Abnormal placental development was described in interspecific crosses of the genus *Mus* (Zechner et al.,

1996). Opposite effects on placental growth, i.e., placental hyperplasia and hypoplasia, were observed in reciprocal crosses and backcrosses. Hyperplastic placentas were seen in matings *M. spretus* (*Msp*) female × *M. musculus* (*Mmu*) male (SM) and in the SM × *Mmu* (SMM) and MS × *Mmu* (MSM) backcrosses (BCs; in all matings, the female is indicated first). Hypoplastic placentas appeared in the MS cross and in the MSS and SMS BCs (Zechner et al., 1996). Identical results were obtained when the *Msp* mice were substituted by mice of the two other European species *M. macedonicus* and *M. spicilegus* (Zechner et al., 1996). In all cases, the placental tissue that was mainly affected was the spongiotrophoblast, which was consistently enlarged in the SM, SMM, and MSM placentas. This enlargement always coincided with a significant increase of glycogen cell number (Zechner et al., 1996). In contrast, spongiotrophoblast was reduced in the reciprocal cross and BCs and in some cases it was missing completely. This phenotype consistently resulted in fetal growth retardation or death (Zechner et al., 1996), even though the role of placental dysplasia in fetal growth was not clear-cut (Kurz et al., 1999).

It was suggested that these phenotypes are caused by the aberrant interactions between loci or gene products derived from the genomes of the different species, which in the usual, intraspecific context interact normally (Zechner et al., 1996, 1997). Possible examples for such interactions could be envisaged as growth factor/growth factor receptor binding or heterodimerisation of transcription factors. Although a

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Dr. Zechner's present address is Abteilung Innere Medizin I, Universität Ulm, Ulm, Germany.

Dr. Hemberger's present address is Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Canada.

*Correspondence to: Reinald Fundele, Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, D-14195 Berlin-Dahlem, Germany. E-mail: fundele@molgen.mpg.de

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region on the X-chromosome designated *Ihp*d (interspecific hybrid placental dysplasia) was shown to be involved in the production of aberrant placental phenotypes (Zechner et al., 1996), neither the X-chromosomal nor the putative autosomal loci have been identified to date.

Expression pattern and function could implicate several X-chromosomal genes, such as *Pem* (Lin et al., 1994), *Plac1* (Cocchia et al., 2000), *Psx1* (Chun et al., 1999), or *Esx1* (Li et al., 1997), as candidate genes for interspecific hybrid placental dysplasia (IHPD). *Esx1*, a paired-like homeobox gene, is an especially interesting candidate. The gene maps to the distal region of the mouse X-chromosome (Li et al., 1997). During prenatal development, *Esx1* expression is restricted to extraembryonic tissues, including the chorionic ectoderm, visceral yolk sac endoderm, and the labyrinthine trophoblast (Li et al., 1997). Intriguingly, targeted mutation of the *Esx1* gene resulted in a placental phenotype, which exhibited a striking resemblance to the placental hyperplasia observed in the MSM BC (Li and Behringer, 1998). Thus *Esx1* mutant placentas were consistently larger than those of wild-type control fetuses, and both *Esx1* mutant and the hyperplastic MSM BC placentas exhibited a characteristic disruption of the spongiotrophoblast-labyrinth boundary with large wedges of spongiotrophoblast invading into the labyrinth. Excessive numbers of glycogen cells, a derivative of spongiotrophoblast were an additional shared trait. Furthermore, in interspecific hybrids within the rodent genus *Peromyscus* a similar placental phenotype (Rogers and Dawson, 1970) segregates with the X-chromosomal region that harbours the peromyscid *Esx1* orthologue (Vrana et al., 2000). Together, these findings made *Esx1* an obvious candidate gene for IHPD, even though no clear association between IHPD and *Esx1* could be seen in *Mus* (Hemberger et al., 1999).

No strong autosomal candidate genes for murine IHPD have been described to date. However, recently it was suggested that in the placental hybrid dysgenesis effects described in *Peromyscus*, autosomal imprinted genes may play a major role (Vrana et al., 1998, 2000). An autosomal imprinted gene which has been shown to be pivotal in control of placental growth is *Igf2* (Baker et al., 1993; Lopez et al., 1996). Interestingly, deletion of the functional paternal allele of *Igf2* causes reduced differentiation of glycogen cells, that is, a phenotype that is opposite to that of the *Esx1* mutation and the hyperplastic MSM placentas. Together, these findings suggested that both *Igf2* and *Esx1* could be involved in the generation of IHPD, possibly with opposite activity. To better understand the developmental processes that lead to IHPD, we have investigated growth of hybrid placentas and the expression of *Igf2* (Baker et al., 1993) and *Esx1* (Li et al., 1997; Li and Behringer, 1998).

RESULTS

Growth Characteristics of Interspecific Placentas

It was shown that in normal mouse development, placental weight reaches its maximum at embryonic day (E) 14 of gestation and then remains constant or decreases slightly toward the end of gestation (McLaren, 1965). To determine whether placentas from interspecific crosses exhibit a similar growth curve, pregnant F1 females were dissected between E12 and E19. Placental weights were determined at E12, E14, E16, E18, and E19, and fetuses were used for genotyping. In addition, fetuses were dissected at these stages from intraspecific *Mmu* × *Mmu* matings. This analysis showed that placentas from MSM matings that carry the *Msp* derived alleles on the X (X^{Msp}) chromosome continue to grow until the end of gestation. In contrast to this, X^{Msp} placentas and the intraspecific *Mmu* placentas did not grow significantly after E14 to E16 (Fig. 1A).

Proliferation in Interspecific Placentas

Placental cell proliferation was assessed in E12 and in E18 placentas by bromodeoxyuridine (BrdU) incorporation (Fig. 1B–I). At E12, a significant difference was visible between X^{Mmu} and X^{Msp} placentas. E12 X^{Msp} placentas contained numerous proliferating spongiotrophoblast cells and even secondary giant cells that had incorporated BrdU were consistently observed (Fig. 1E). In contrast to this, only very few proliferating cells were detected in the spongiotrophoblast of E12 X^{Mmu} placentas and no proliferating giant cells were detected at all in E18 X^{Mmu} placentas. In the E18 MSM placentas, significantly increased proliferation in X^{Msp} compared with X^{Mmu} placentas was still evident. This finding was most pronounced in the chorionic plate (Fig. 1F,G). In both X^{Msp} and X^{Mmu} placentas, endothelial cells lining the fetal blood vessels constituted the majority of proliferating cells (Fig. 1F,G). In the spongiotrophoblast, no proliferating cells were observed in 6 E18 X^{Mmu} placentas; however, cells that had incorporated BrdU were detected at low frequency in 6 X^{Msp} littermate placentas (Fig. 1I). Very rarely, BrdU-positive secondary giant cells were found in E18 X^{Msp} placentas (Fig. 1H).

Expression of *Igf2* and *Esx1* in MSM Placentas

To determine whether abnormal expression of *Igf2* or *Esx1* might contribute to the generation of IHPD, an *in situ* expression analysis of these genes was performed. Expression of *Igf2* was confined to cells derived from fetal portions of the placenta (Fig. 2A,B). In X^{Msp} placentas, the number of *Igf2*-expressing giant cells was markedly increased compared with the X^{Mmu} placentas. Giant cells of X^{Msp} placentas often displayed a strong labeling for *Igf2* mRNA, whereas giant cells of X^{Mmu} placentas were mostly unlabelled (Fig. 2C–G). As already described in detail in one of our previous stud-

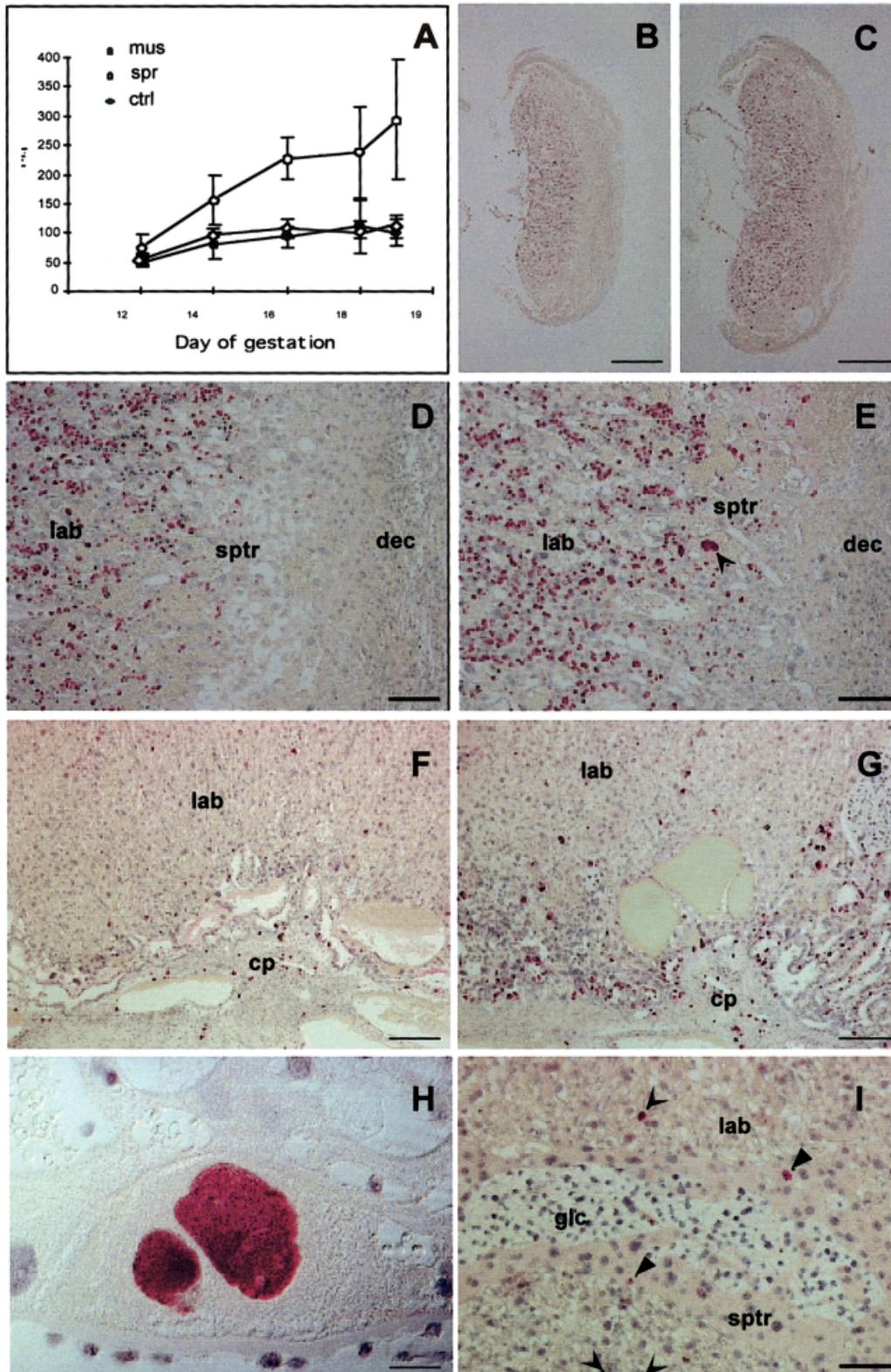


Fig. 1. **A:** Postmidgestation growth curves of placentas derived from MSM BC1 placentas with X^{Msp} or X^{Mmu} genotypes and intraspecific Mmu controls. Numbers of placentas analyzed were as follows. At embryonic day (E) 12: X^{Mmu} , 10; X^{Msp} , 6; Mmu , 48. E14: X^{Mmu} , 12; X^{Msp} , 12; Mmu , 25. E16: X^{Mmu} , 9; X^{Msp} , 18; Mmu , 115. E18: X^{Mmu} , 54; X^{Msp} , 41; Mmu , 46. E19: X^{Mmu} , 5; X^{Msp} , 5; Mmu , 13. **B,C:** Two littermate placentas derived from an E12 MSM BC after anti-bromodeoxyuridine immunohistochemistry. The placenta shown in B had an X^{Mmu} genotype and weighed 36.6 mg; the placenta shown in C had an X^{Msp} genotype and weighed 58.8 mg. **D:** Magnification of the X^{Mmu} placenta shown in B, proliferating cells are present mainly in the labyrinth. **E:** Magnification of the X^{Msp} placenta shown in C, proliferating cells are present in both the labyrinth and in the

spongiotrophoblast. A labeled giant cell nucleus (arrowhead) is also present. **F,G:** E18 littermate placentas from an MSM BC with X^{Mmu} (F) and X^{Msp} (G) genotypes and weights of 97.8 (F) and 305 (G) mg. Many more proliferating cells are visible in the chorionic plate of the X^{Msp} placenta than in that of the X^{Mmu} placenta. **H:** Higher magnification of a proliferating giant cell in an E18 X^{Msp} placenta. **I:** Higher magnification of proliferating spongiotrophoblast cells in an E18 X^{Msp} placenta. Solid arrowheads indicate BrdU-labelled nuclei situated in spongiotrophoblast; dented arrowheads indicate nuclei situated in the labyrinth. cp, chorionic plate; dec, decidua; glc, glycogen cells; lab, labyrinth; spr, spongiotrophoblast. Scale bars = 1 mm in B,C, 22.5 μ m in D,E, 35 μ m in F,G, 3.8 μ m in H, 15 μ m in I.

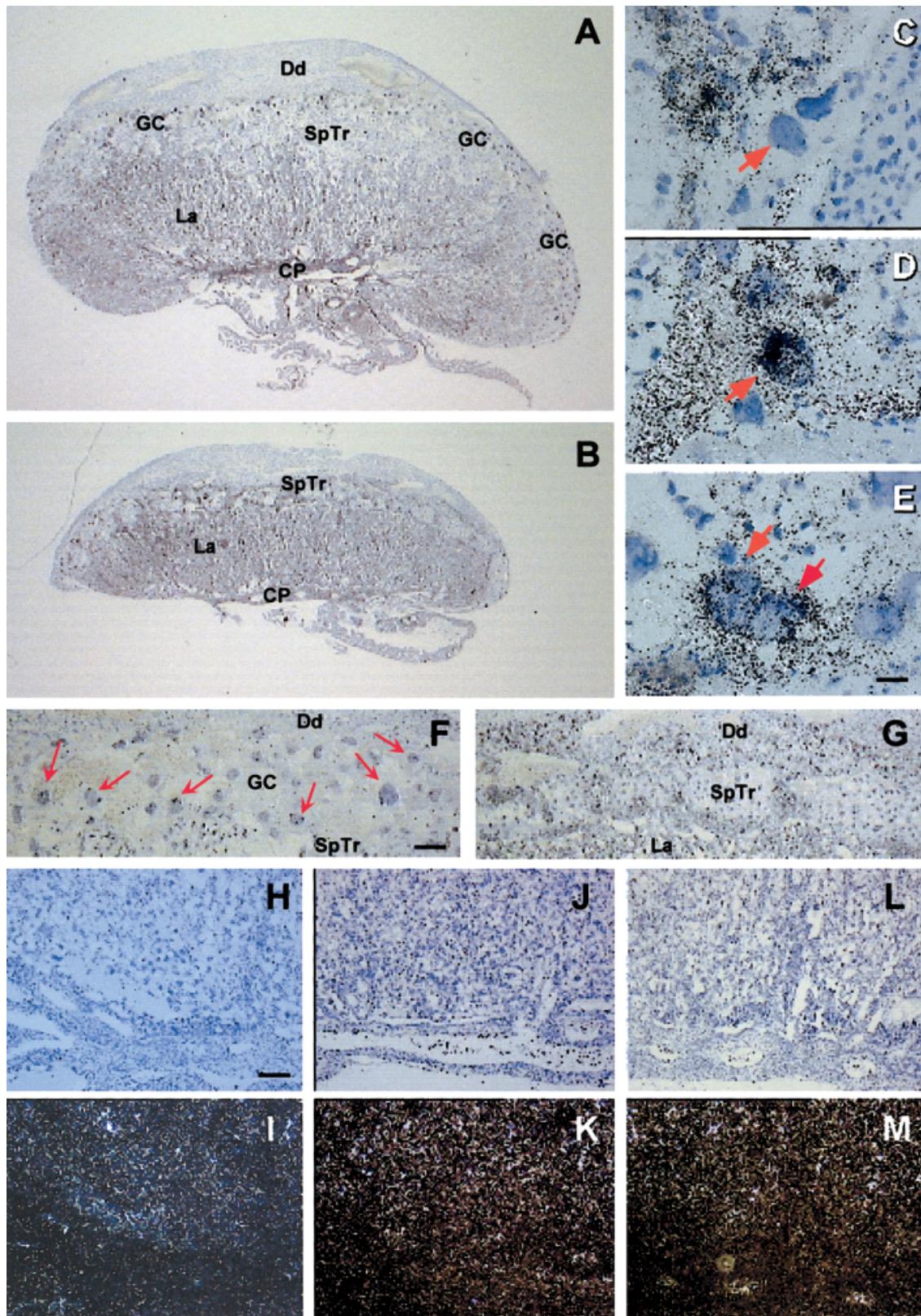


Fig. 2. Expression of *Igf2* and *Esx1* in embryonic day (E) 14 and E18 MSM BC placentas. **A,B:** RNA in situ hybridization analysis of *Igf2* in an E18 X^{Msp} (A) and an E18 X^{Mmu} (B) placenta. In both placentas, *Igf2* expression (grey staining) was observed in the chorionic plate (CP), the labyrinthine region (La), and subregions of the spongiotrophoblast (SpTr) representing glycogen cells. In the X^{Msp} placenta, expression was also often observed in giant cells (GC), whereas giant cells of the X^{Mmu} placenta were mostly unlabelled. Dd, decidua. **C:** Higher magnification of the border area between the fetal placental portion and maternal decidua (Dd) in the X^{Mmu} placenta shows the lack of *Igf2* expression. **D,E:** Higher magnification of giant cells (red arrows) derived from the X^{Mmu} placenta shows strong labelling for *Igf2* mRNA. **F,G:** Higher magnification of the border area between the fetal placental portion and maternal decidua (Dd) in the X^{Msp} (F) and the X^{Mmu} (G) placenta. Note the high amount of *Igf2* expressing giant cells (red arrows) in the X^{Msp} placenta compared with the X^{Mmu} placenta, which in this area is completely devoid of giant cells. **H–M:** *Esx1* expression in X^{Msp} (H,I) and X^{Mmu} (J–M) E14 littermate placentas. In all cases, chorionic plate and adjacent labyrinth are shown. No qualitative *Esx1* expression differences are visible between the hyperplastic (H,I, 287 mg) and the normal sized (J,K, 87.6 mg; L,M, 88.3 mg) E14 placentas. Scale bars = 7.6 μm in C–E, 15 μm in F,G, 20 μm in H–M.

ies (Kurz et al., 1999), strong hybridization signals were also observed in glycogen cells (data not shown). Thus the increased number of *Igf2*-expressing glycogen cells in X^{Msp} placentas (Zechner et al., 1996; Kurz et al., 1999) lead to an overall increase of *Igf2* levels compared with X^{Mmu} placentas. To investigate *Esx1* expression in interspecific placentas, 5 E14 placentas derived from 1 litter were hybridized. As described before (Li and Behringer, 1997), *Esx1* was expressed mainly in the labyrinthine trophoblast in both X^{Msp} and X^{Mmu} placentas (Fig. 2H–M). However, specific hybridization signals were also detected in a subset of spongiotrophoblast cells. No obvious qualitative differences in *Esx1* expression could be observed between X^{Msp} and X^{Mmu} E14 placentas.

Phenotype of MSM/*Igf2*[−] Placentas

Phenotypes of MSM placentas carrying a paternally inherited null allele of the *Igf2* gene were investigated at day 18 of gestation. The average weight of the *Igf2*^{−/−}/ X^{Mmu} MSM placentas was 60.5 ± 11.3 mg (N = 10), the average weight of the *Igf2*wt/ X^{Mmu} MSM placentas was 89.0 ± 19.7 mg (N = 8). *Igf2*^{−/−}/ X^{Msp} placentas weighed 138.0 ± 44.3 mg (N = 8), and *Igf2*wt/ X^{Msp} placentas weighed 253.5 ± 75.0 mg (N = 8). Histologic analysis of the placentas by hematoxylin and eosin (HE) staining and *in situ* hybridization (ISH) with the spongiotrophoblast specific marker gene 4311 revealed a strong reduction in the number of glycogen cells in null placentas compared with wt placentas (Fig. 3). This reduction occurred in both X^{Mmu} and X^{Msp} *Igf2*[−] placentas. In X^{Msp} null placentas, reduced glycogen cell differentiation coincided with a reduced invasive growth of the spongiotrophoblast into the labyrinthine region (Fig. 3B,H,C) compared with X^{Msp} wt placentas (Fig. 3A,G). Thus the hyperplastic IHPD phenotype was less pronounced in X^{Msp} null placentas. In X^{Mmu} null placentas, however, the absence of a functional *Igf2* allele led to an almost complete loss of glycogen cell differentiation and, therefore, to an increased penetrance of the normally moderate hypoplastic placental phenotype seen in X^{Mmu} wt placentas (Fig. 3E,F,J).

Quantitative *Esx1* Expression in MSM and MSS BC1 Placentas

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Esx1* expression was carried out in placentas derived from MSM and MSS BCs. A significant negative correlation was observed between placental weight and *Esx1* expression on fetal days 12 and 14 in the MSM BC1 (Fig. 4A,C), and on E12 in the MSS BC1 (Fig. 4B). In later developmental stages, no significant correlation could be observed (Fig. 4D–F).

Quantitative *Esx1* Expression in *Igf2*[−] Placentas

To determine whether expression of *Esx1* is influenced by *Igf2* expression levels, a Northern blot containing mRNAs isolated from 4 E18 *Igf2*[−] and 4 E18 wt

placentas was hybridized with an *Esx1* cDNA probe. Loss of *Igf2* expression did not cause any change in *Esx1* mRNA expression (Fig. 4G). Thus no correlation between *Igf2* expression and *Esx1* expression was observed.

DISCUSSION

We have pursued several experimental approaches designed to elucidate the molecular and genetic mechanisms that lead to the occurrence of abnormal placentas in mouse interspecific hybrids. Thus we could show that the growth characteristics of interspecific X^{Msp} placentas are entirely different from those described for a normal mouse placenta (McLaren, 1965). X^{Msp} placentas continue to grow until the end of gestation, whereas intraspecific Mmu placentas stop to grow between E14 and E16 (McLaren, 1965). The growth of interspecific X^{Mmu} placentas, however, closely resembles that of the normal Mmu placentas.

To explain these growth differences, we analysed the proliferation in X^{Msp} and X^{Mmu} placentas. Significant differences were observed at both E12 and E18 of gestation. The most striking observation was the markedly increased amount of proliferating cells in the spongiotrophoblast of E12 X^{Msp} placentas compared with E12 X^{Mmu} placentas. This result suggests that the dramatic placental weight increase of X^{Msp} placentas observed after E12 of gestation is mainly caused by the increased proliferation rate of cells located particularly in the spongiotrophoblast, which is also the region most strongly affected in placental hyperplasia. In addition, that few BrdU labeled spongiotrophoblast cells are still present in the E18 X^{Msp} placenta also correlates very well with the fact that X^{Msp} placentas still gain weight and grow between E18 and E19 of gestation. Our previous results had indicated that the placental tissue mainly affected in IHPD is the spongiotrophoblast (Zechner et al., 1996). Hence, it was surprising that two nonspongiotrophoblast cell types, i.e., secondary giant cells and endothelial cells, also exhibited increased proliferation in X^{Msp} placentas. This finding was especially surprising in the case of the secondary giant cells as these do not normally proliferate beyond E13 of gestation. However, these findings suggest that a diffusible growth factor produced by spongiotrophoblast is increased in X^{Msp} placentas. They also raise the possibility that this increased proliferation could cause abnormalities in the labyrinth that could lead to blood stasis or edema. This process could then act to further increase placental weights.

An important growth factor in placental development is IGF2 (Redline et al., 1993; Correia-da-Silva et al., 1999). Also, *Igf2* is an imprinted gene, and it has been shown recently that imprinting may be disrupted in interspecific *Peromyscus* hybrids (Vrana et al., 1998). When we compared *Igf2* expression in X^{Msp} and X^{Mmu} placentas, two differences were seen. First, elevated levels of *Igf2* were detected in giant cells of E12 X^{Msp} placentas. This finding suggests that the contin-

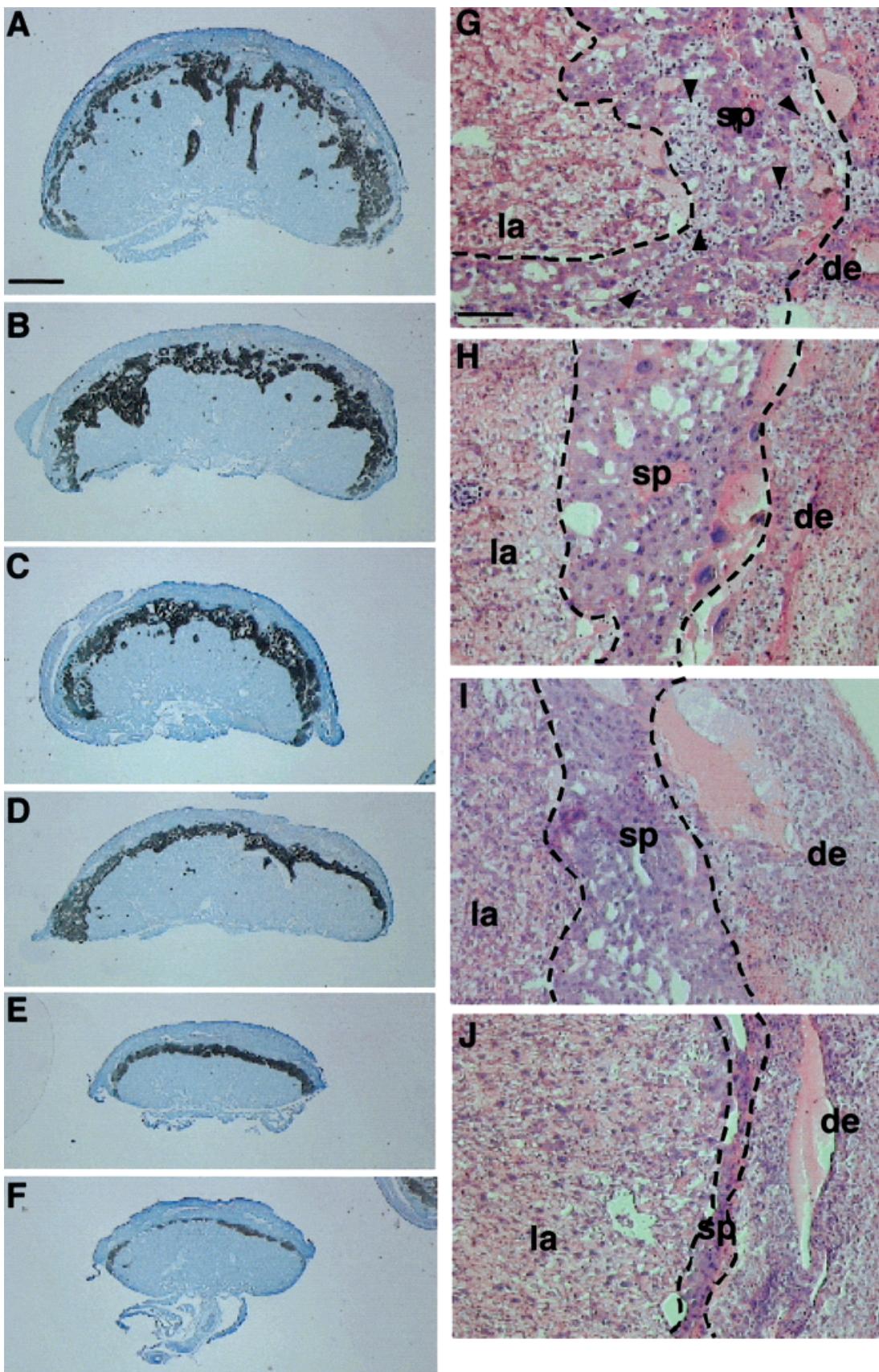


Fig. 3. A–J: Embryonic day (E) 18 placentas derived from ms F1 females mated with *Igf2wt*[−] males are shown after hybridization with a 4311-specific probe (A–F) and after staining with hematoxylin/eosin (G–J). **A,G:** *Igf2wt/X^{Msp}* placenta weighing 224 mg. **B,H:** *Igf2[−]/X^{Msp}* placenta weighing 179 mg. **C:** *Igf2[−]/X^{Msp}* weighing 117 mg. **D,I:** *Igf2wt/X^{Mmu}* with 113 mg. **E:** *Igf2[−]/X^{Mmu}* with 54 mg. **F,J:** *Igf2[−]/X^{Mmu}* weighing

42 mg. Borders between decidua (de), spongiotrophoblast (sp), and labyrinth (la) layers are indicated by the lines. Note that large glycogen cell areas (arrowheads) are present in the *Igf2wt/X^{Msp}* placenta (G) but absent in the *Igf2[−]/X^{Msp}* (H), *Igf2wt/X^{Mmu}* (I), and *Igf2[−]/X^{Mmu}* (J) placenta. Scale bars = 1 mm in A (applies to A–F), 22.5 μ m in G (applies to G–J).

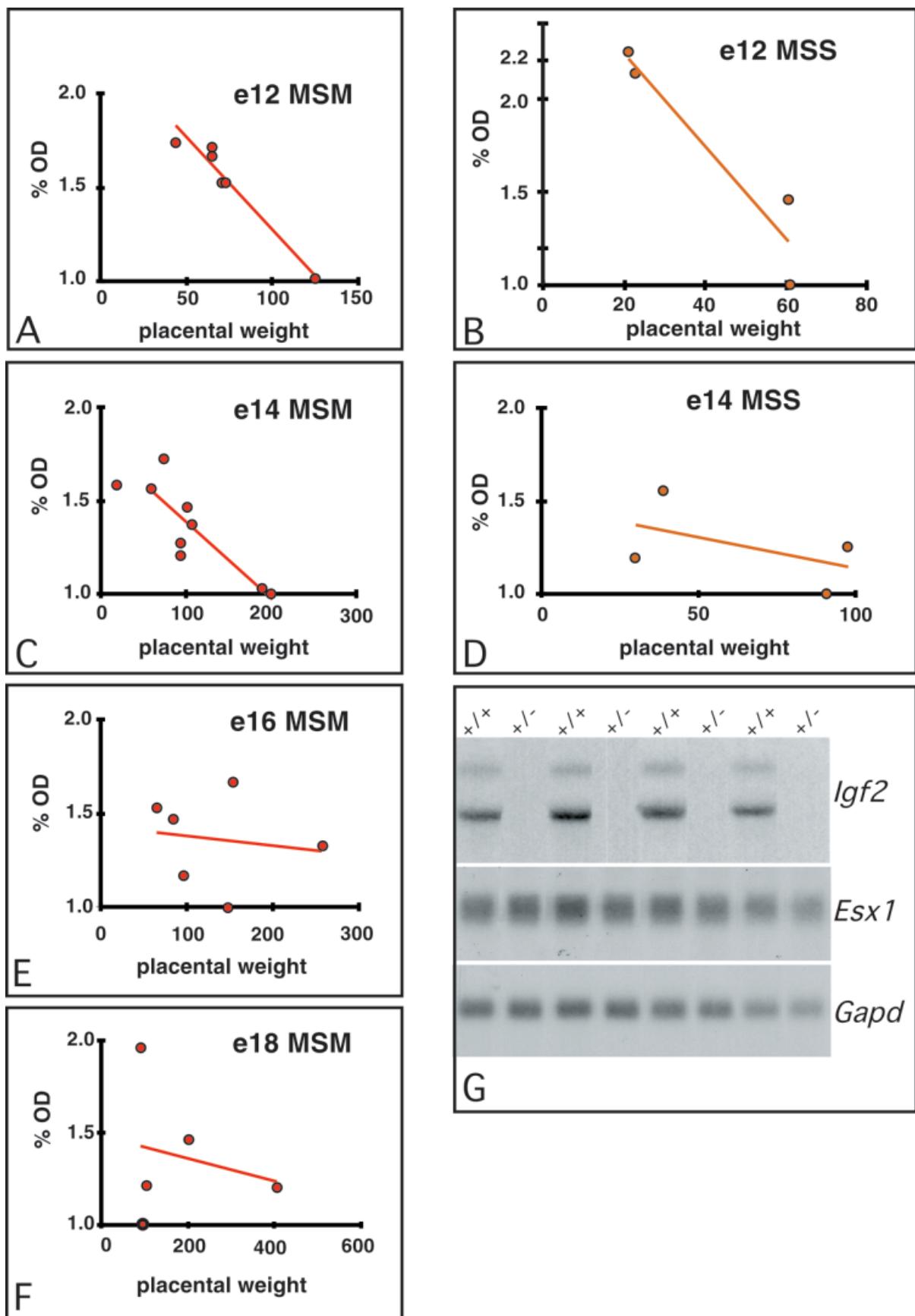


Fig. 4. **A–F:** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Esx1* expression levels of MSM (A,C,E,F) and mss (B,D) BC1 placentas. RT-PCR product intensities were densitometrically quantified and lowest values set as 1.0. Regression slope and *t*-test values areas follows. A: -0.01, $P < 0.001$; B: -0.025, $P = 0.05$; C: -0.004, $P <$

0.01; D: -0.003, $P = 0.49$; E: -0.0005, $P = 0.78$; F: -0.0006, $P = 0.72$. **G:** Northern hybridization analysis of *Igf2*, *Esx1*, and *Gapd* expression in four different embryonic day (E) 18 *Igf2*^{-/-} (+/−) placentas and four different E18 wt (+/+) placentas.

ued DNA synthesis observed in secondary giant cells of E12 X^{Msp} placentas is mediated by the growth promoting activities of *Igf2*. Second, the increased number of glycogen cells lead to an elevated expression level of *Igf2* in X^{Msp} placentas. In this context, it is important to note that in *Igf2* null mice a reduction in the number of glycogen cells has been observed (Lopez et al., 1996). Although these findings show that aberrant *Igf2* expression occurs in IHPD, they do not allow a conclusion as to whether this is a cause or a consequence of IHPD.

To more stringently address this question, we investigated the IHPD phenotype in the absence of IGF2. The phenotype of mice with a targeted disruption of the *Igf2* gene is growth reduction of both fetus and placenta during the last two thirds of gestation (DeChiara et al., 1990, 1991; Baker et al., 1993). Consequently, if IGF2 was the essential growth factor causing IHPD, we would have had to expect a complete absence of placental hyperplasia in *Igf2*^{-/-}/X^{Msp} mice. This was not observed. Both X^{Msp} and X^{Mmu} placentas with *Igf2*^{-/-} genotype showed decreased placental weights when compared with *Igf2* wild-type littermates; however, the *Igf2*^{-/-}/X^{Msp} placentas were still larger than the *Igf2*^{-/-}/X^{Mmu} placentas. Thus, we have to conclude that IHPD is primarily caused by the deregulation of factors other than IGF2, even though increased and prolonged expression of the *Igf2* gene probably contribute to the phenotype. There is some evidence that the gene product of the X-linked *Gpc3* gene, which is mutated in the Simpson-Golabi-Behmel syndrome (SGBS), interacts with IGF2 (Pilia et al., 1996). Patients with SGBS and mice with a targeted mutation of *Gpc3* suffer from overgrowth phenotype (Pilia et al., 1996; Cano-Gauci et al., 1999); however, placental growth has to the best of our knowledge never been described in SGBS. Still, it is possible that the increased *Igf2* expression levels in the MSM placentas are caused by abnormal expression of an X-linked gene like *Gpc3* whose product interacts with IGF2. *GPC3* is expressed in the syncytiotrophoblast of the human placenta (Khan et al., 2001).

An especially attractive candidate gene for IHPD is *Esx1*, which is located on the distal region of the X-chromosome at 57 cM (Li et al., 1997). This is just at the distal border of the chromosomal region that was initially identified as IHPD candidate region. However, this linkage analysis could have been influenced by the presence of multiple loci on the X chromosome that contribute to IHPD (Hemberger et al., 1999). This interferes with the identification of exact positions of individual loci (N. Schork, personal communication). Mice with a maternally inherited mutant allele of *Esx1* exhibit a placental phenotype that very closely resembles MSM IHPD placentas (Li and Behringer, 1998). This finding suggested that *Esx1* mRNA levels could be decreased in placental hyperplasia and increased in placental hypoplasia. This was indeed observed in the early developmental stages that were analyzed in the present study. As *in situ* hybridisation for *Esx1* showed no abnormal distribution of transcripts in the X^{Msp}

compared with the X^{Mmu} placentas, this reduction cannot be caused by a change in the number of labyrinthine cells that express *Esx1*. As we found no alterations of *Esx1* expression levels in *Igf2*^{-/-} placentas we can also exclude the possibility that deregulation of *Esx1* expression is a consequence of varying placental IGF2 levels. Deregulation of both *Esx1* and *Igf2* expression seem to be two independent events occurring during the development of IHPD. These findings suggest that *Esx1* contributes to IHPD, even though late gestation placentas no longer exhibit aberrant expression of *Esx1*. It could be envisaged that decreased expression of *Esx1* in early placentas is sufficient to induce a growth promoting process that supports increased proliferation of X^{Msp} placentas until term. More specifically, it could be argued that *Esx1* is important in the control of spongiotrophoblast growth and differentiation, as shown by the highly increased proportion of glycogen cells in both *Esx1* mutant and interspecific hyperplastic placentas. In a previous study that made use of congenic and subcongenic mouse strains, we have shown that IHPD cannot be caused by a single locus on the X-chromosome but rather by multiple loci that have to act synergistically (Hemberger et al., 1999). Considering our present results, it seems certain that *Esx1*, like *Igf2*, is one of these loci. Hence, deregulation of *Esx1* in interspecific hybrid placentas plays an important role in causing IHPD, independently from IGF2.

EXPERIMENTAL PROCEDURES

Mice

The following mouse strains were used in the present study: *Msp* strains SMZ, SEG, and SFM; and *Mmu* strain (C57BL/6 × C3H)F1 (B6C3F1). For the production of MSM BC1 mice, interspecific hybrid F1 females were mated with B6C3F1 males. For the production of MSM BC1 mice with a nonfunctional paternal *Igf2* allele, interspecific hybrid F1 females were mated with male mice carrying the inactivated *Igf2* gene (DeChiara et al., 1990). The day of the vaginal plug was designated day 1 (E1) of gestation.

Isolation of Tissues

Pregnant females were killed between E8 and E19 of gestation. At all developmental stages, placentas or implantation sites containing trophectoderm-derived cells were used for histology, whereas fetal tissues and/or yolk sac were taken for genotyping. For histology, placentas were fixed either in 4% paraformaldehyde or in Serras fixative at 0°C. After fixation, tissues were processed, embedded in paraffin, and sectioned at 5 to 7 µm.

Immunohistochemistry

For BrdU labelling of proliferating cells, E12 and E18 pregnant females were injected with 50 mg BrdU/kg body weight 10 and 60 min, respectively, before death. Placentas were fixed in Carnoy's fixative.

After processing and sectioning, detection of incorporated BrdU was performed essentially as described before (Jägerbauer et al., 1992) by using a monoclonal antibody (mAB) against BrdU (Boehringer Mannheim) and an alkaline phosphatase conjugated anti-mouse AB (Dako). Controls were performed by omission of the first antibody or immunohistochemistry on tissues of mice that had not been injected with BrdU.

RNA In Situ Hybridization, Northern Hybridization, and RT-PCR

For ISH and Northern hybridization, *Igf2* and *Esx1* probes provided by Drs. A. Surani and J. Cross were used. Radioactive ISH using 35 S-labeled probes was performed following a standard protocol (Adam et al., 1996). Spongiotrophoblast was identified by ISH with a 4311 probe provided by Dr. J. Rossant (Lescisin et al., 1988). For *Igf2*, placentas aged between E8 and E18, for *Esx1* 5 placentas from 1 E14 MSM litter were hybridized. For controls, sections were hybridized with the sense probes. Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Northern hybridization was performed as described previously (Krause et al., 1999). For RT, random hexanucleotide primers were used. Primers for *Esx1* were 5'-AGA AGG GCC AAG TGG AGACG and 5'-GAA AAG GTG GCA GAG GAG G; and for *Gapd* 5'-GAA GCT TGT CAT CAA CGG GAA GCC C and 5'-GCA TCG AAG GTG GAA GAG TGG GAG T. PCR conditions for simultaneous amplification of *Esx1* and *Gapd* were 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C for 18, 21, 24, 27, and 30 cycles. Primer concentrations were 1.7 pmol for both *Esx1* primers, and 0.5 pmol for both *Gapd* primers in a total volume of 50 μ l. After each round of cycles, 10 μ l were removed from the reaction. After the final cycle, all reaction products were applied to one gel. Densitometry was carried out by using the PC-BAS 2.0 program. *Esx1* values were normalized against *Gapd*.

Genotyping

For genotyping by PCR analysis, genomic DNA was prepared from fetuses by using the Kristal mammalian genomic DNA kit (Cambridge Molecular Technologies). To determine X-chromosomal genotypes, the following microsatellite markers from the Massachusetts Institute of Technology (MIT) were used: *DXMit54*, *DXMit86*, *DXMit8*, and *DXMit65*. For the detection of the *Igf2* mutation, we used the forward primer 5'-CTA GCT CAA AGC CTG CGT TTC TTT-3' corresponding to intron sequence located 30 bp upstream from the splice junction of the first *Igf2* exon and a *neo* reverse primer 5'-ATC CAT CTT GTT CAA TGG CCG ATC CC-3'. PCR cycling was for 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C for 35 cycles.

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