Spongiotrophoblast Development

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The *Mash2* gene, which encodes a basic helix-loop-helix transcription factor, is one of the mammalian homologues of the *Drosophila achaete-scute* genes. It is strongly expressed in diploid trophoblast cells of the postimplantation mouse embryo. Targeted mutagenesis of *Mash2* revealed that loss of function results in embryonic lethality at midgestation, due to placental failure associated with a lack of spongiotrophoblast and reduced labyrinthine trophoblast layers. For the further study of Mash2 function in development of the trophoblast cell lineage, we have performed chimeric analysis combining *Mash2* mutant and wild-type embryos. We have addressed the question of whether the phenotype of the *Mash2* mutant embryo, which affects all of the three trophoblast cell layers, is caused by a cell autonomous or non-autonomous defect and whether Mash2 is required in both spongiotrophoblast and labyrinthine trophoblast development. Our results showed no contribution of *Mash2* mutant cells to the spongiotrophoblast layer in chimeric placentae at 10.5 and 12.5 days postcoitum, suggesting that the product of the *Mash2* gene is required cell autonomously during the development of the spongiotrophoblast. However, it seems that Mash2 is not required for development of labyrinthine trophoblast or giant cells, since high contributions of *Mash2* mutant cells were observed in those trophoblast cell layers in the chimeric placentae analyzed. We can therefore conclude that the primary and cell-autonomous function of Mash2 appears to be an involvement in the development of diploid trophoblast cells in the ectoplacental cone to form the spongiotrophoblast cell layer of the mature chorioallantoic placenta.   © 1997 Academic Press

INTRODUCTION

During mammalian development, the trophoblast is the first cell lineage to differentiate and gives rise to most of the extraembryonic tissues which are required for implantation and further development of the embryo proper within the uterine environment (Cross *et al.,* 1994; Rossant, 1986). Later in the development of the mouse embryo, the chorioallantoic placenta is the major site of exchange between the maternal and fetal blood circulations, and contains three trophoblast layers, namely, the labyrinthine trophoblast, spongiotrophoblast, and giant cell layers, which are each morphologically distinct (Rossant, 1995; Rossant and Croy, 1985). Failure of proper formation of the chorioallantoic placenta results in embryonic

lethality at mid gestation (Copp, 1995; Cross *et al.,* 1994). Recent genetic approaches have begun to unravel the genetic pathways regulating the development of this cell lineage (Copp, 1995; Cross *et al.,* 1994).

The *Mash2* gene, in particular, has been shown to encode a trophoblast-specific regulatory protein required for development of a subset of trophoblast cells (Guillemot *et al.,* 1994). *Mash2* (Johnson *et al.,* 1990), one of the mammalian homologues of the *Drosophila achaete-scute* genes (Campuzano and Modolell, 1992; Ghysen and Dambly-Chaudiere, 1988; Villares and Cabrera, 1987), encodes a basic helix-loop-helix transcription factor which is strongly expressed in the diploid trophoblast cell lineage during early mouse development (Guillemot *et al.,* 1994). *Mash2* transcripts are first detected in preimplantation stage embryos and become restricted to diploid trophoblast cells around implantation (Rossant *et al.,* in preparation). By 8.5 days postcoitum (d.p.c.), *Mash2* transcripts are observed in diploid trophoblast cells of the ectoplacental cone (EPC) and chorionic

ectoderm (Guillemot *et al.,* 1994). At 9.5–10.5 d.p.c., *Mash2* transcripts are still detectable in both labyrinthine trophoblast and spongiotrophoblast layer of the chorioallantoic placenta (Guillemot *et al.,* 1995), but a patchy pattern starts to be seen by 12.5 d.p.c. indicating a gradual decline in the level of transcripts within these regions (Scott and Cross, 1996; Rossant *et al.,* in preparation). Interestingly, *Mash2* transcripts are never detected in giant cells (Guillemot *et al.,* 1994).

Targeted mutagenesis of *Mash2* revealed that loss of Mash2 function results in embryonic lethality at midgestation, due to a placental failure associated with a lack of spongiotrophoblast, an improperly formed labyrinthine layer and thickened giant cell layer (Guillemot *et al.,* 1994). Embryos lacking Mash2 could be rescued to term and beyond by aggregation with tetraploid embryos (Guillemot *et al.,* 1994), suggesting that Mash2 does not play an essential role in development of the embryo proper, thereby implying a requirement for Mash2 solely within the trophoblast cell lineage.

Here we use chimeric analysis of *Mash2* mutant embryos to address the question of whether the observed developmental defect within the trophoblast cell lineages in *Mash2* mutant placentae is cell autonomous or non-cell autonomous. Our results show that, even though *Mash2* expression is detected in both the labyrinthine trophoblast and spongiotrophoblast cell lineages, Mash2 function is required cell autonomously for development of the spongiotrophoblast, but not for the development of labyrinthine trophoblast.

MATERIALS AND METHODS

ES Cell ↔ Tetraploid Embryo Aggregation

Tetraploid embryos were produced by electrofusion of 2-cell stage embryos (Kubiak and Tarkowski, 1985; Nagy and Rossant, 1993) and aggregated with small clumps of 10–15 ES cells in microdepressions as described previously (Nagy and Rossant, 1993). After overnight culture, aggregates that had successfully formed blastocysts were transferred to the uteri of 2.5-d.p.c. pseudopregnant CD-1 females. Embryos were then dissected at 10.5 and 12.5 d.p.c. to recover chimeric placentae for lacZ staining. The ROSA26 gene trap insertion line (Friedrich and Soriano, 1991), which expresses the β-galactosidase gene product ubiquitously and thus provides an *in situ* lineage marker for chimera analysis, was used to distinguish the origin of either the ES cells or the tetraploid embryos. The C16 ES cell line (Ciruna *et al.,* 1997), which is *Fgfr1*+/−; ROSA26/+ (hemizygous for the ROSA26 transgene), was used as a lacZ-positive ES cell control and aggregated with wild-type CD-1 tetraploid embryos. Previous analysis has shown that heterozygosity at *Fgfr1* locus has no effect on normal development (Yamaguchi *et al.,* 1994; Ciruna *et al.,* 1997). ROSA26/+ CD-1 embryos were also used to produce tetraploid embryos and aggregated with wild-type R1 ES cells (Nagy *et al.,* 1993).

Generation of Mash2 Mutant ↔ Wild-Type Chimeras

Mash2 heterozygous females carrying a maternally inherited wild-type allele and a paternally inherited null allele (Guillemot *et al.,* 1995) (*Mash2*+/−) were superovulated and crossed with males homozygous for the ROSA26 transgene. Embryos from this cross fall in two classes: *Mash2*−/+ (maternal inheritance of a *Mash2* null allele); ROSA26/+, and *Mash2*+/+; ROSA26/+ (Fig. 1). Since the *Mash2* gene is subject to genomic imprinting (Guillemot *et al.,* 1995), with the paternally inherited allele being functionally inactive, the class of embryos carrying a maternal null allele and a paternal wild-type allele (*Mash2*−/+) behave like homozygous null mutants (Guillemot *et al.,* 1995) (therefore, the term ''*Mash2* mutant'' is used hereafter for these embryos), and the second class act as controls. Eight cell embryos from this cross were aggregated with 8-cell wild-type CD-1 embryos as described (Wood *et al.,* 1993) and transferred to pseudopregnant females.

Pregnant females were sacrificed at 10.5 and 12.5 d.p.c. of gestation, and the placentae dissected away from the embryos and reserved for lacZ staining. A part of the yolk sacs was used for genotyping embryos by PCR using an assay that distinguished wildtype and null *Mash2* alleles (Guillemot *et al.,* 1995). Conceptuses that showed both blue and white staining cells and were positive by PCR for the null *Mash2* allele defined the experimental class of chimeras (*Mash2*−/+; ROSA26/+ ↔ *Mash2*+/+), while those that showed mixed blue and white cells but only the wild-type PCR band defined control chimeras (*Mash2*+/+; ROSA26/+ ↔ *Mash2*+/+) (Fig. 1). We found that all chimeric conceptuses analyzed had lacZ-positive cell contribution in the yolk sac (data not shown).

Whole Mount β-Galactosidase (lacZ) Staining of Placenta

Placentae were dissected in PBS, bisected with a scalpel blade, and fixed in 0.2% glutaraldehyde, 2 m*M* MgCl$_2$, 5 m*M* EGTA in PBS at 4°C for 30 min. They were then washed in three changes of PBS at 4°C for 5 min each, and one of the two halves of each placenta was subsequently stained in 1 mg/ml X-gal, 5 m*M* K$_3$Fe(CN)$_6$, 5 m*M* K$_4$Fe(CN)$_6$, 0.02% NP-40, 0.01% deoxycholate, 2 m*M* MgCl$_2$, 100 m*M* sodium phosphate, pH 7.3, at 37°C overnight. Stained placentae were then washed in PBS, refixed in 4% formaldehyde in PBS, and kept at 4°C prior to photographing. Embryos including the yolk sacs were also stained for lacZ to assess general levels of chimerism (data not shown).

Cryosections

The second half of each placenta was processed for cryosectioning after the first fixation and washing. Processing was carried out through a graded series of sucrose concentrations from 15 to 30% in PBS at 4°C for 5–12 hr for each step, washed in O.C.T. (Tissue-Tek) at 4°C for 5–12 hr, and then embedded in O.C.T. and frozen on dry ice. Sections were then cut at 10 μm, mounted on slides, washed in PBS for 5 min, and subsequently stained in X-gal solution, as described above, at 37°C overnight. Sections were counterstained with Nuclear Fast Red.
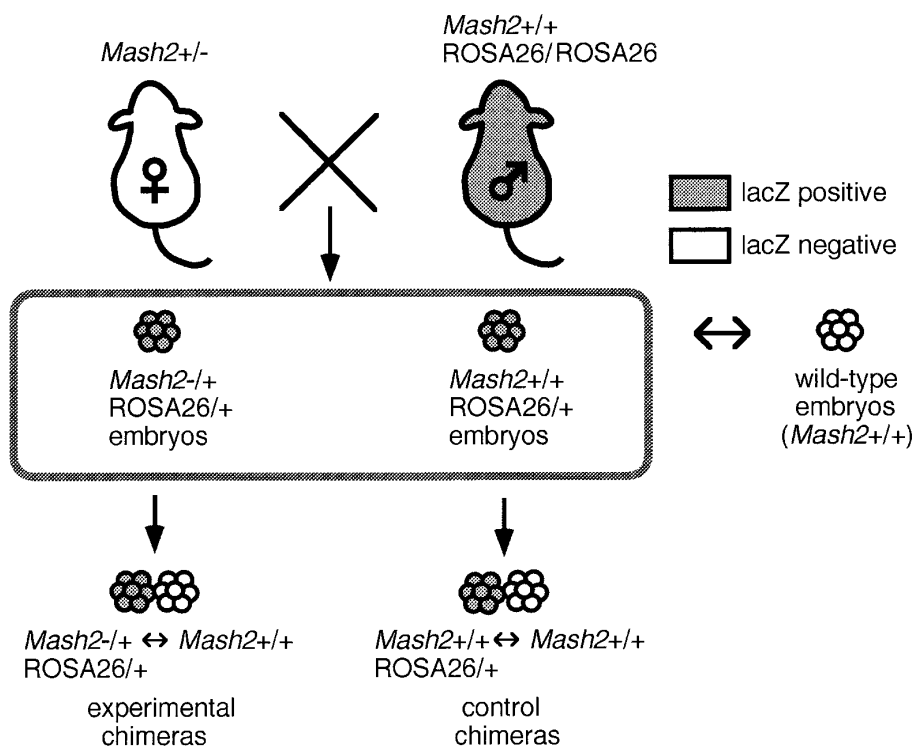
FIG. 1. The experimental strategy for generation of chimeras.

RESULTS

Spatial Distribution of Trophoblast and Nontrophoblast Cells in the Placenta

Before analyzing the chimeric contribution to the various layers of placenta in the *Mash2* experimental series, it was important to be able to distinguish trophoblast cells from fetally derived nontrophoblast cells and maternal cells (Rossant and Croy, 1985). This is particularly true in the labyrinthine layer, where fetally derived endothelial and mesenchyme cells are intimately interspersed with the labyrinthine trophoblast cells (Rossant and Croy, 1985). Previous studies of reconstituted blastocysts using GPI isozyme variants as markers have estimated that about 70% of the 13- to 15-d.p.c. placenta is trophoblast, 30% is maternal in origin, and 4% develops from the inner cell mass (ICM) (Rossant and Croy, 1985). However, this study could not determine the spatial distribution of the trophoblast cells. To assess this, we generated ES cell ↔ tetraploid embryo chimeras in which either component was marked with ROSA26 transgene (Fig. 2). It has been shown in such chimeras that the tetraploid cells and ES cells show complementary distributions, with the tetraploid cells forming the trophoblast and primitive endoderm lineages and the ES cells making the primitive ectoderm-derived components (Nagy *et al.,*

1990, 1993). Thus in placentae from such chimeras, ES cells will contribute to the nontrophoblast tissues such as endothelial and mesenchyme cells in the labyrinthine layer, and tetraploid cells will form the trophoblast cells.

Before analyzing these chimeras, control ROSA26/+ placentae were stained with X-gal to ensure that all cells of the placenta expressed the marker gene. This was observed to be the case, although expression was weaker in the spongiotrophoblast and giant cells than in the labyrinthine layer of the placenta (Figs. 3A and 4A). Control CD-1 placentae showed minimal background staining (Fig. 3B).

In placentae from R1 ES cell ↔ ROSA26/+ tetraploid embryo aggregates at 12.5 d.p.c. (Figs. 2A and 2C), contribution of blue cells was observed in all trophoblast layers but not in primitive ectoderm-derived tissues, consistent with previous reports (Nagy *et al.,* 1990, 1993). On the other hand, in the labyrinth of *Fgfr1*+/−; ROSA26/+ ES cell ↔ CD-1 tetraploid embryo chimeric placenta at 12.5 d.p.c. (Figs. 2B and 2D), the contribution of blue cells was only observed in allantois, fetal blood vessels, and mesenchyme cells, showing a complementary pattern to Figs. 2A and 2C. Heterozygosity for the *Fgfr1* mutation has no effect on development (Yamaguchi *et al.,* 1994; Ciruna *et al.,* 1997). These results revealed the spatial distribution of trophoblast and nontrophoblast cells in mature placenta at 12.5 d.p.c.

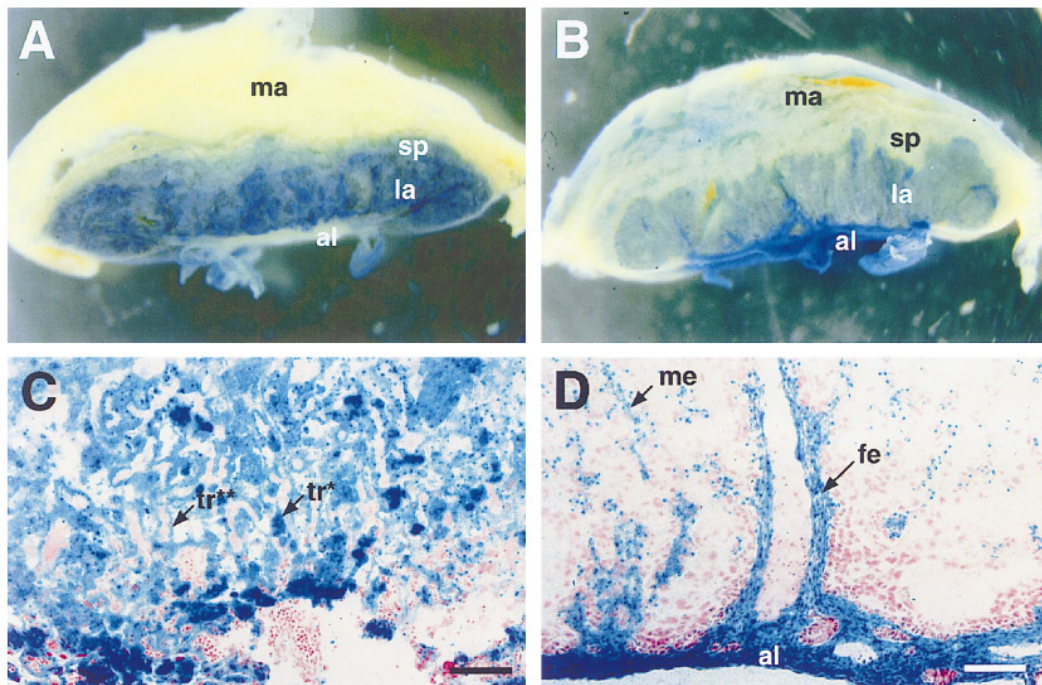We observed two morphologically distinct populations of

FIG. 2. Analysis of trophoblast and nontrophoblast cell contribution in ES cell ↔ tetraploid embryo chimeric placentae. An R1 ES cell ↔ ROSA26/+ tetraploid embryo chimeric placenta at 12.5 d.p.c. (A) Shows lacZ staining in trophoblast cell lineages (i.e., the spongiotrophoblast (sp) and the labyrinthine trophoblast (la) layers), but not in nontrophoblast cell lineages (i.e., allantois (al), maternal decidua (ma)). A section of the same sample (C) shows spatial distribution of trophoblast cells in the labyrinthine layer. There are two morphologically distinct populations of labyrinthine trophoblast observed (tr*, tr**). In contrast, a ROSA26/+ ES cell ↔ CD-1 tetraploid embryo chimeric placenta at 12.5 d.p.c. (B) shows a complementary pattern to (A), with lacZ staining specifically in fetally derived nontrophoblast cells. Sections of these placentae (D) show spatial distribution of nontrophoblast cells (fetal blood vessels (fe), mesenchyme cells (me)) in the labyrinthine layer (la). Scale bar, 200 μm.

trophoblast cells in the labyrinthine layer of the R1 ES cell ↔ ROSA26/+ tetraploid embryo chimeric placenta (Fig. 2C). One is a population of clumps of cuboidal mononuclear cells associated with dark lacZ staining (Fig. 2C, tr*), and the other was a population with elongated morphology (Fig. 2C, tr**), surrounding the maternal blood sinuses. With lacZ staining, we found that it was relatively easy to identify trophoblast cells in the labyrinthine layer, especially the clumps of trophoblast cells because of their dark staining.

Mash2 Mutant Cells Are Excluded from the Spongiotrophoblast Layer of Chimeric Placentae at 12.5 d.p.c.

To assess the specificity of Mash2 function in different trophoblast lineages, chimeras were generated between wild-type embryos and ROSA26-labeled mutant embryos (*Mash2*−/+) (Fig. 1), and contributions of mutant cells were analyzed in the trophoblast and nontrophoblast (mesoderm-derived) cells of the chimeric placentae (Table 1) as compared

to results derived from ES cell ↔ tetraploid embryo aggregations shown in Fig. 2. A range of mosaicism was observed in control chimeric placentae at 12.5 d.p.c., but lacZ-positive cells were observed in all three layers of the placenta in 15 of 16 samples (Table 1, Figs. 3C and 3D), where contributions from the labeled cells were observed in the trophoblast. We found one placenta with relatively low contribution of lacZ-positive cells in the labyrinthine trophoblast layer which showed no blue cell contribution in either the spongiotrophoblast or giant cell layer. There was some evidence for segregation of labeled and unlabeled cells into broad sectors within the placenta, suggestive of some degree of coherent growth of clonal descendants (James *et al.,* 1993; West *et al.,* 1995). In mutant (*Mash2*−/+) ↔ wild-type (*Mash2*+/+) chimeras, the distribution of labeled and unlabeled cells was significantly different. Independent of the extent of overall contribution of mutant cells to the trophoblast, mutant cells appeared to be excluded from the spongiotrophoblast layer, which was mostly unlabeled (Figs. 3E–3H). This observation was the case in all of 11 samples
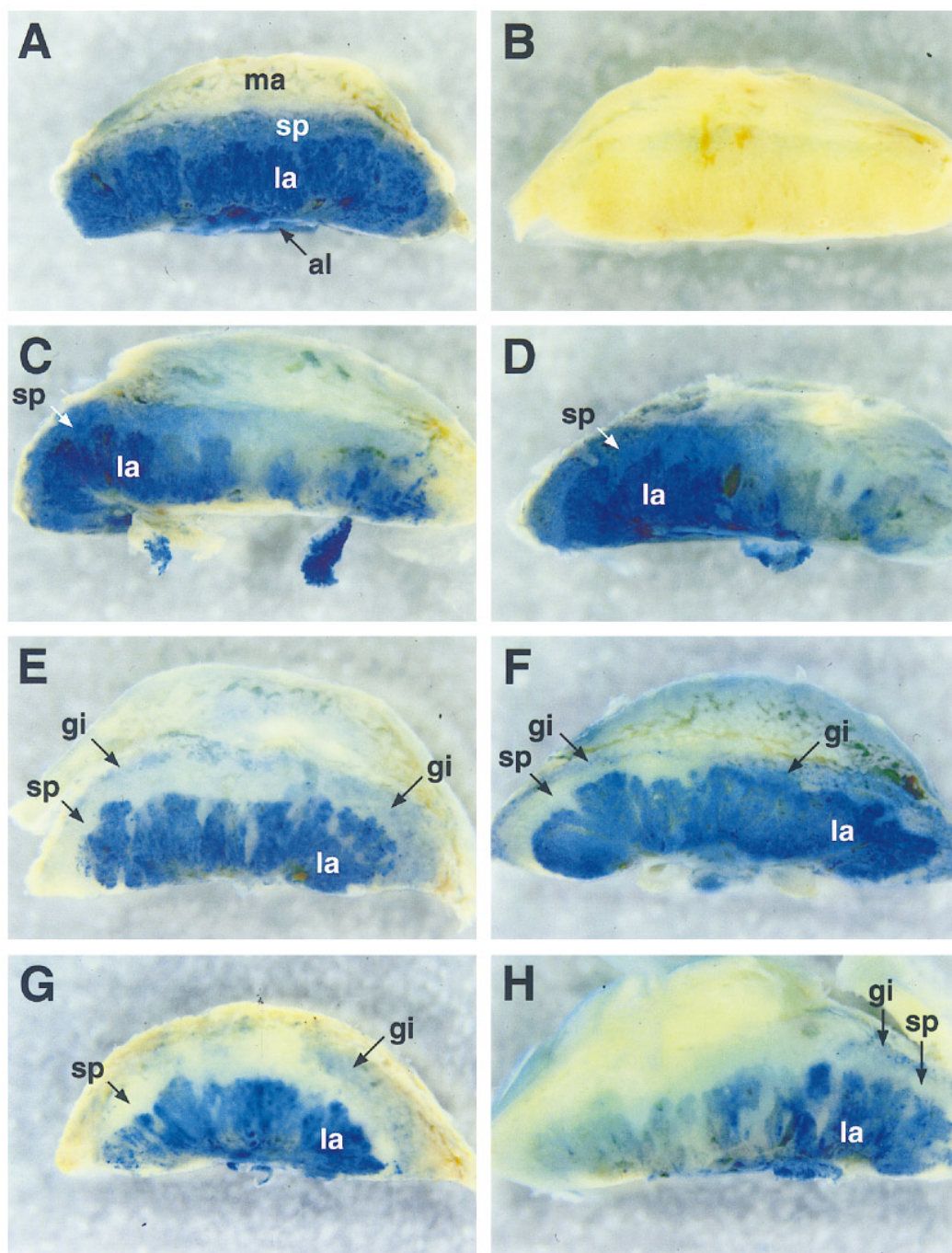
FIG. 3. Analysis of *Mash2* mutant cell contribution in chimeric placentae at 12.5 d.p.c. A ROSA26/+ placenta (A) shows positive control staining in all fetal components including the labyrinthine (la) and spongiotrophoblast (sp) layers but not in maternal decidua (ma), while negative control CD-1 placenta (B) shows minimal background staining. *Mash2*+/+; ROSA26/+ ↔ wild-type CD-1 chimeric placentae (C,D) show contribution of blue cells to both the labyrinthine (la) and spongiotrophoblast (sp) layers. In contrast, *Mash2*−/+; ROSA26/+ ↔ wild-type CD-1 chimeric placentae (E–H) show significantly low contribution of blue cells (*Mash2* mutant) to spongiotrophoblast layer (sp), whereas high mutant cell contribution is observed in the labyrinthine trophoblast (la) and giant cell layer (gi). Some blue cells seen in the vicinity of the spongiotrophoblast layer (E,F) turned out to be giant cells by analysis of sections (Figs. 4D and 4E). al, allantois.
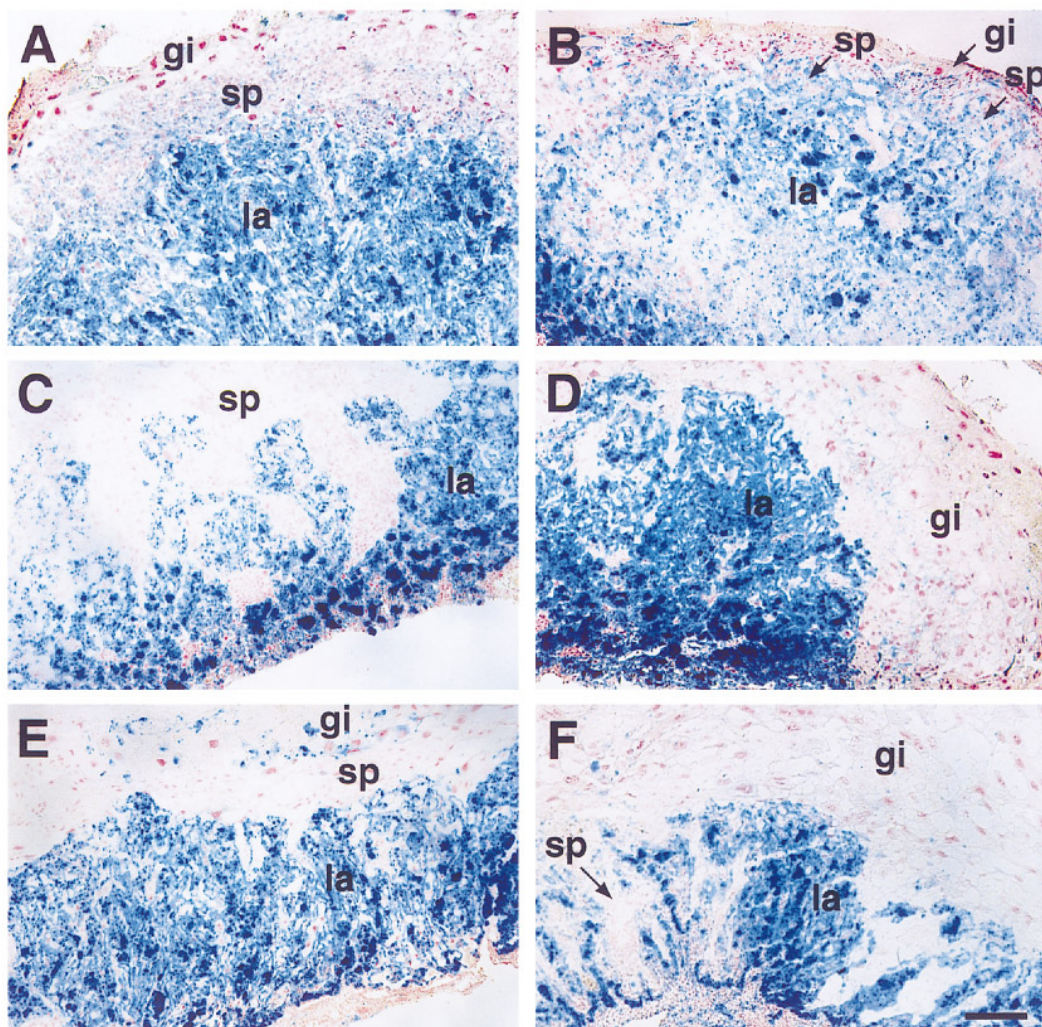
FIG. 4. Histological analysis of chimeric placentae at 12.5 d.p.c. Sections of the placentae shown in Fig. 3 (A,C,E–G). (A) A section of the ROSA26/+ placenta (Fig. 3A) showing lacZ staining in all trophoblast layers (i.e., the labyrinthine trophoblast (la), spongiotrophoblast (sp), and giant cell (gi) layers). (B) A section of the *Mash2*+/+; ROSA26/+ ↔ wild-type CD-1 chimeric placenta (Fig. 3C) showing lacZ-positive cell contribution in all trophoblast layers. (C–F) Sections of the *Mash2*–/+; ROSA26/+ ↔ wild-type CD-1 chimeric placentae corresponding to those shown in Figs. 3E–3G (4C and 4D to 3E, 4E to 3F, 4F to 3G). No blue cell (*Mash2* mutant) contribution to the spongiotrophoblast (sp) is observed, whereas mutant cell contribution is high in the labyrinthine trophoblast layer (la). A thickened giant cell layer is observed in D and F. Scale bar, 200 μm.

which showed mutant cell contribution to the other trophoblast layers (Table 1).

Cryosections of those placentae revealed that mutant cells were totally absent from the spongiotrophoblast layer (Figs. 4C–4F), whereas wild-type blue cells were capable of contributing to the spongiotrophoblast in control placentae (Figs. 4A and 4B). Mutant blue cells observed in the vicinity of the spongiotrophoblast layer (Figs. 3E–3G) turned out to be giant cells (Figs. 4D–4F). There appeared to be no restriction on the ability of *Mash2* mutant cells to contrib-

ute to the giant cell layer, even when the mutant cell contribution was high (Figs. 3E–3G and 4D–4F).

Blue mutant cells were also observed in the labyrinthine layer of experimental chimeras (Figs. 4C–4F). By comparison with the sections of control placentae derived from ES cell ↔ tetraploid embryo aggregations in which only the primitive ectoderm-derived components or trophoblast components were labeled (Figs. 2C and 2D), it was clear that mutant cells contributed to both trophoblast and nontrophoblast cells in the labyrinthine layer. This is most

TABLE 1

Number of Conceptuses in the Chimeric Analysis

	12.5 d.p.c.		10.5 d.p.c.	
	Mash2−/+ ROSA26/+ ↔ *Mash2*+/+	*Mash2*+/+ ROSA26/+ ↔ *Mash2*+/+	*Mash2*−/+ ROSA26/+ ↔ *Mash2*+/+	*Mash2*+/+ ROSA26/+ ↔ *Mash2*+/+
Chimeric trophoblast + chimeric embryo proper	11	16	10	11
Chimeric trophoblast + non-chimeric embryo proper	0	0	0	0
Nonchimeric blue trophoblast + chimeric embryo proper	0	2	0	2
Nonchimeric white trophoblast + chimeric embryo proper	15	16	12	8
Nonchimeric blue conceptuses	0	1	4	16[a]
Nonchimeric white conceptuses	3[b]		16[a,b]	
Number of conceptuses analysed	26	35	26	37

Note. Embryo proper includes the yolk sac and fetally derived nontrophoblast components of the placenta.

[a] Resulted from unsuccessful aggregation in one experiment.

[b] Not included in the total number.

apparent in the chimeric placentae with the highest mutant cell contribution, where nearly the entire labyrinth can be derived from mutant cells, with the spongiotrophoblast still remaining wild-type in origin (Figs. 3E–3G and 4C–4F). Sections of these placentae (Figs. 4C–4F) revealed mutant cell contribution to both of the two types of labyrinthine trophoblast shown in Fig. 2C, with no evidence of any qualitative differences in contribution to either cell type, as well as mutant cell contributions to endothelial and mesenchyme cell types (Fig. 2D).

It thus appears that there is a cell-autonomous requirement for Mash2 in the spongiotrophoblast but that Mash2 is not required for giant cell and labyrinthine trophoblast development.

Exclusion of Mash2 Mutant Cells from the Spongiotrophoblast Layer of Chimeric Placentae Occurs before 10.5 d.p.c.

To examine the onset of exclusion of mutant cells from the spongiotrophoblast layer, chimeras were also examined at 10.5 d.p.c., when *Mash2*−/− and *Mash2*−/+ embryos die (Guillemot *et al.,* 1995, 1994). Positive control ROSA26/+ placentae showed lacZ staining in all fetal components (Figs. 5A and 6A) and negative control CD-1 placentae showed minimal background (Fig. 5B). In chimeric placentae, we observed that labeled mutant cells were excluded from the spongiotrophoblast layer but were present in the giant cell and labyrinthine layer (Figs. 5E, 5F, 6C, and 6D) in all of 10 placentae which had lacZ-positive cell contribution to the trophoblast (Table 1). There were mutant cells contributing to both trophoblast and nontrophoblast cells in the labyrinthine layer, based on the comparison with

a ROSA26/+ ES cell ↔ CD-1 tetraploid embryo chimeric placenta at 10.5 d.p.c. (data not shown). Labeled wild-type cells were capable of contributing highly to the spongiotrophoblast in all of 11 control chimeric placentae which had blue cell contribution to the trophoblast (Figs. 5A, 5C, 5D, 6A, and 6B, Table 1), suggesting that the exclusion of *Mash2* mutant cells from the spongiotrophoblast layer occurs before 10.5 d.p.c.

DISCUSSION

Mash2 is a lineage-specific bHLH transcription factor that is strongly expressed in the diploid trophoblast cell lineage and plays an essential role in trophoblast cell development (Guillemot *et al.,* 1994). *Mash2* null mutant mice generated by gene targeting die around 10.5 d.p.c. due to a placental defect which is characterized by complete absence of the spongiotrophoblast. The labyrinthine trophoblast layer is reduced but not absent (Guillemot *et al.,* 1994). Based on the expression pattern of *Mash2* transcripts, Mash2 could be required for development of both spongiotrophoblast and labyrinthine trophoblast, since transcripts are detected in precursors of both these cell lineages and persist through their early development (Guillemot *et al.,* 1994). However, it is also possible that the defect in the labyrinthine layer in *Mash2* mutants is secondary to the primary loss of spongiotrophoblast or, alternatively, the defect in the labyrinthine layer could be the primary cause of the absence of the spongiotrophoblast.

To elucidate the cell autonomous requirement of Mash2 in development of these trophoblast cell lineages, we have performed a chimeric analysis of *Mash2* mutant embryos
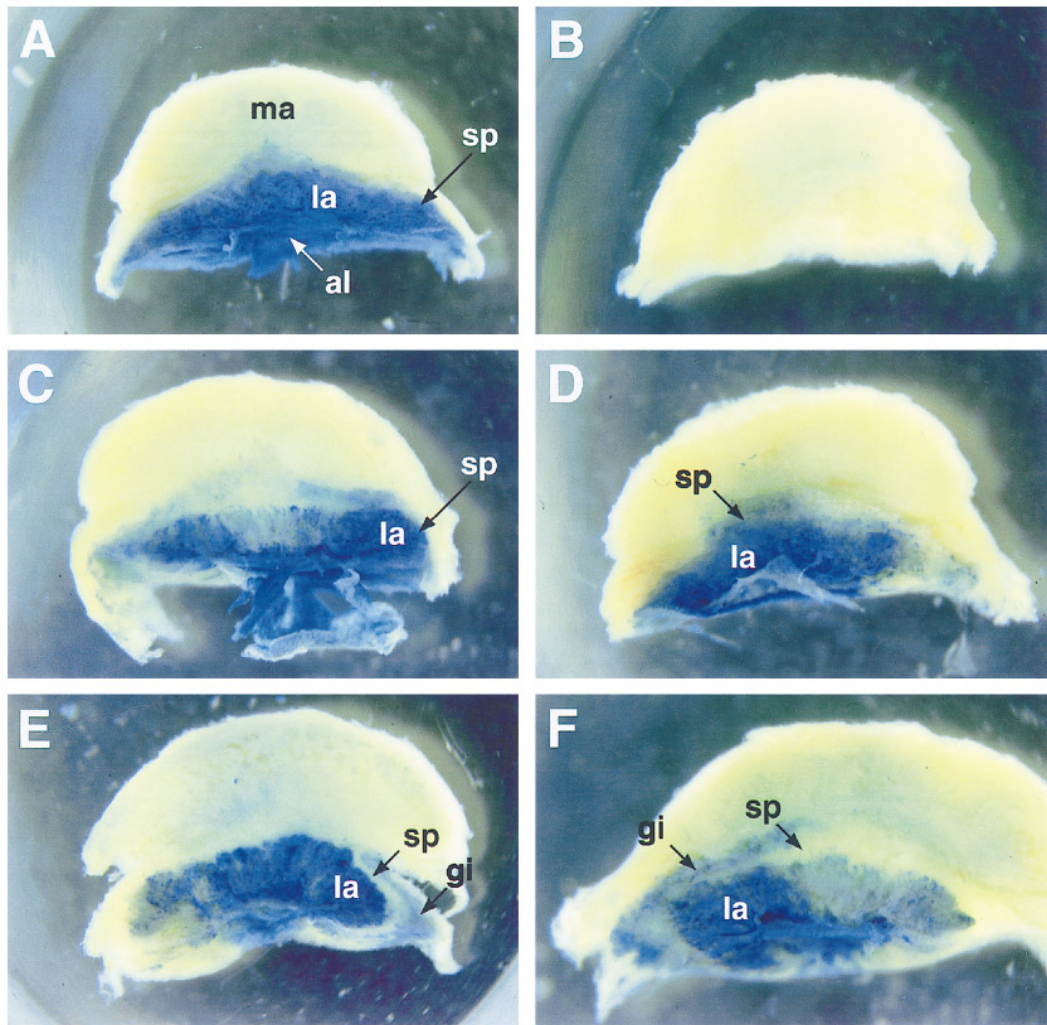
FIG. 5.   Analysis of *Mash2* mutant cell contribution in chimeric placentae at 10.5 d.p.c. A ROSA26/+ placenta (A) shows positive control staining in all fetal components including the labyrinthine (la) and spongiotrophoblast (sp) layers but not in maternal decidua (ma), whereas a negative control CD-1 placenta (B) shows minimal background staining. *Mash2*+/+; ROSA26/+ ↔ wild-type CD-1 chimeric placentae (C,D) show blue cells contributing to both the labyrinthine (la) and the spongiotrophoblast (sp) layers. In contrast, *Mash2*−/+; ROSA26/ + ↔ wild-type CD-1 chimeric placentae (E,F) show low contribution of blue cells in the spongiotrophoblast layer (sp), whereas high contribution of blue cells is observed in the labyrinthine (la) and giant cell (gi) layers. Histological analysis (Figs. 6C and 6D) revealed that all blue cells outside of the labyrinthine layer were giant cells. al, allantois.

by taking advantage of the fact that the *Mash2* gene is imprinted (Guillemot *et al.,* 1995). *Mash2* has been genetically and physically mapped to the distal region of mouse chromosome 7 (Guillemot *et al.,* 1995), which is syntenic to human chromosome 11p15, within a cluster of imprinted genes (Bartolomei *et al.,* 1991; De Chiara *et al.,* 1991; Giddings *et al.,* 1994; Hatada and Mukai, 1995; Hatada *et al.,* 1996; Hoovers *et al.,* 1995; Jones *et al.,* 1992; Lee *et al.,* 1997; Mannens and Wilde, 1997; Matsuoka *et al.,* 1996; Zemel *et al.,* 1992). Genomic imprinting of *Mash2* has been reported to result in a repression of transcription from the

paternally inherited allele, which seems to be the direct cause of the lethality of embryos with paternal duplication of mouse distal chromosome 7 (McLaughlin *et al.,* 1996). As a consequence of genomic imprinting, embryos which carry a maternally inherited null allele and a paternally inherited wild-type allele have an identical phenotype to null mutant embryos based on histology (Guillemot *et al.,* 1995). Thus we considered these embryos as functionally equivalent to null mutant embryos and analyzed the behavior of such cells in chimeric placentae produced by aggregation with wild-type embryos.
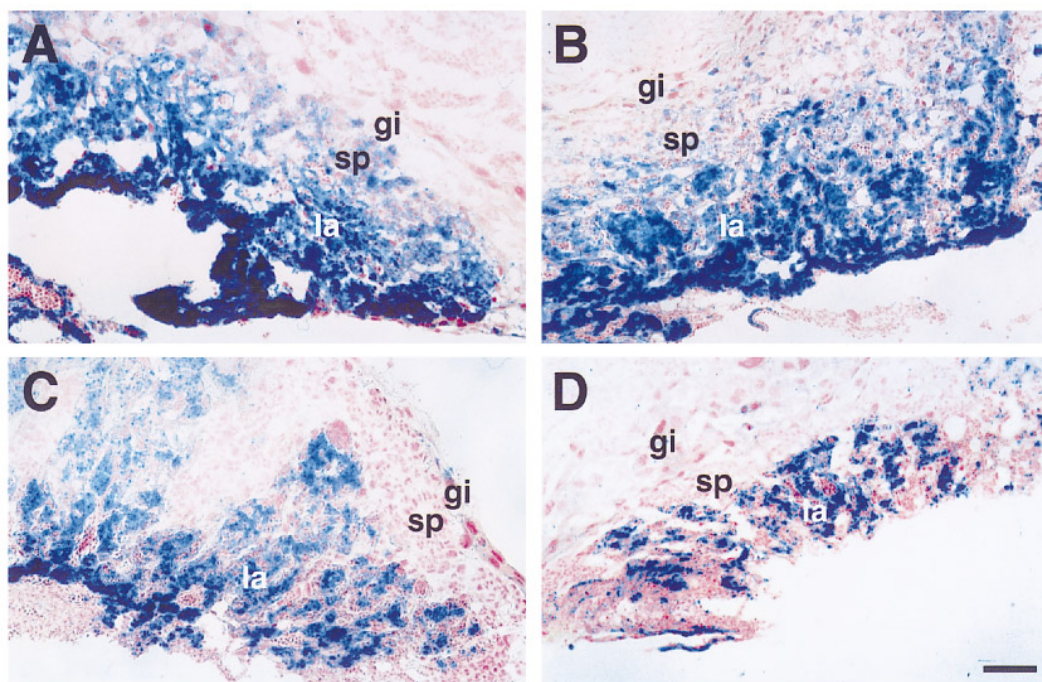
FIG. 6. Histological analysis of chimeric placentae at 10.5 d.p.c. Sections of the placentae shown in Figs. 5A and 5D–5F. (A) A section of the ROSA26/+ placenta (Fig. 5A) shows lacZ staining in all trophoblast layers (i.e., the labyrinthine trophoblast (la), spongiotrophoblast (sp), and giant cell (gi) layers). (B) A section of the *Mash2*+/+; ROSA26/+ ↔ wild-type CD-1 chimeric placenta (Fig. 5D) showing blue cell contribution to all trophoblast layers. (C,D) Sections of the *Mash2*−/+; ROSA26/+ ↔ wild-type CD-1 chimeric placentae corresponding to those shown in Figs. 5E and 5F. No mutant cell contribution to the spongiotrophoblast (sp) is observed, whereas mutant cell contribution is observed in both the labyrinthine trophoblast (la) and giant cell (gi) layers. Scale bar, 200 μm.

Our chimeric analysis showed no contribution of mutant cells to the spongiotrophoblast layer at either 12.5 or 10.5 d.p.c., suggesting that Mash2 is required in a cell autonomous manner for the proper development of spongiotrophoblast. Since *Mash2* transcripts are abundant in EPC at 8.5 d.p.c., Mash2 function seems to be required in diploid trophoblast cells of the EPC to allow development of the spongiotrophoblast layer by 10.5 d.p.c. Interestingly, in both *Mash2* null mutants and chimeric placentae, giant cells of mutant cell origin are observed (Guillemot *et al.,* 1994). Since secondary giant cells arise from the diploid trophoblast cells in EPC (Ilgren, 1981), these results suggest that *Mash2* mutant cells may differentiate into giant cells preferentially, instead of contributing to spongiotrophoblast cells (Fig. 7). In normal placentae, Mash2 would then play a cell autonomous role in maintaining the diploid trophoblast population of the spongiotrophoblast layer by either supporting cell proliferation or inhibiting differentiation of these cells into giant cells (Fig. 7), as suggested also by *in vitro* experiments with Rcho-1 cells (Cross *et al.,* 1995).

We observed a high contribution of mutant cells to the labyrinthine trophoblast layer in chimeric placentae, despite the morphological defects in this layer in mutant pla-

centae (Guillemot *et al.,* 1995, 1994). Two morphologically distinct populations of labyrinthine trophoblast cells could be recognized in both control ES cell ↔ tetraploid aggregates and experimental chimeric placentae. One of these was a population of cuboidal mononuclear cells, which were intensively lacZ positive, and the other was a set of cells with elongated morphology surrounding the maternal blood sinuses. It may be possible that the former population of the trophoblast in the labyrinth is the stem cell for the latter, although cell lineage analysis is needed to confirm this hypothesis. Recently, biochemical evidence for cell fusion has been reported in the labyrinth, very likely in the latter population (West *et al.,* 1995). Thus we cannot exclude the possibility that some *Mash2* mutant cells were rescued by cell fusion with wild-type cells in a subset of the labyrinthine trophoblast. It seems unlikely that cell fusion can explain the persistence of all mutant labyrinthine trophoblast cells, since some experimental chimeric placentae had a labyrinthine trophoblast layer which was almost entirely mutant derived. Observation of the mutant cell contribution to the mononuclear labyrinthine trophoblast in experimental chimeric placentae strongly suggests that Mash2 function is not essential for development of these

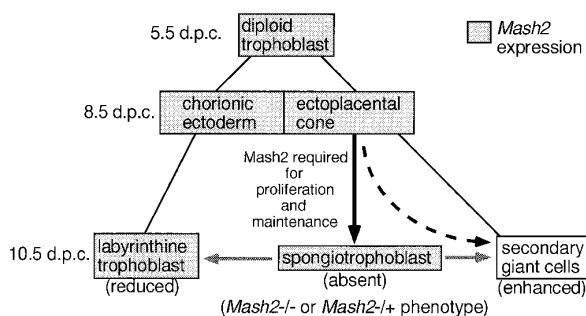**FIG. 7.** A model of Mash2 function in development of the trophoblast cell lineage. See details in text.

cells despite the homogenous expression of the *Mash2* transcripts in their precursors (Guillemot *et al.,* 1995, 1994).

Taken together, our results suggest that the absence of spongiotrophoblast observed in *Mash2* null mutant placentae is a primary cell autonomous defect and that the reduced labyrinthine layer, which is considered to be the cause of lethality, is a secondary and non-cell autonomous defect (Fig. 7). This implies that an intact spongiotrophoblast layer is required for normal development of the labyrinthine trophoblast layer. This is unlikely to result from a direct lineal contribution of cells of the spongiotrophoblast layer to the labyrinthine trophoblast layer, since chimeras can be obtained in which essentially all of the labyrinth is *Mash2* mutant derived, while all of the spongiotrophoblast is wild-type. More likely, the spongiotrophoblast could be the source of specific signals which promote the formation of the labyrinthine layer or it could provide structural support for the labyrinth to develop.

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