Maspin plays an essential role in early embryonic development

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Summary

Maspin (Mp) is a member of the serpin family with inhibitory functions against cell migration, metastasis and angiogenesis. To identify its role in embryonic development in vivo, we generated maspin knockout mice by gene targeting. In this study, we showed that homozygous loss of maspin expression was lethal at the peri-implantation stage. Maspin was specifically expressed in the visceral endoderm after implantation; deletion of maspin interfered with the formation of the endodermal cell layer, thereby disrupting the morphogenesis of the epiblast. In vitro, the ICM of the $Mp^{-/-}$ blastocysts failed to grow out appropriately. Data from embryoid body formation studies indicated that the $Mp^{-/-}$ EBs had a disorganized, endodermal cell mass and lacked a basement membrane layer. We showed that the embryonic ectoderm lineage was lost in the $Mp^{-/-}$ EBs, compared with that of the $Mp^{+/+}$ EBs. Re-expression of maspin partially rescued the defects observed in the $Mp^{-/-}$ EBs, as evidenced by the appearance of ectoderm cells and a layer of endoderm cells surrounding the ectoderm. In addition, a maspin antibody specifically blocked normal EB formation, indicating that maspin controls the process through a cell surface event. Furthermore, we showed that maspin directly increased endodermal cell adhesion to laminin matrix but not to fibronectin. $Mp^{+/-}$ endodermal cells grew significantly slower than $Mp^{+/+}$ endodermal cells on laminin substrate. We conclude that deletion of maspin affects VE function by reducing cell proliferation and adhesion, thereby controlling early embryonic development.

Supplemental data available online

Key words: Maspin, Mouse, Endoderm, Homozygous lethality, Embryonic development

Introduction

Morphogenesis of the peri-implantation mouse embryo involves cell differentiation, migration and extensive cell-cell and cell-matrix interaction. Shortly after implantation to the uterine tissues, the embryos develop into three cell lineages: the extra-embryonic ectoderm, the primitive endoderm and the epiblast that forms the embryo proper (Tam and Beddington, 1992). The extra-embryonic ectoderm contributes to the formation of the placenta and the ectodermal component of the chorion. After implantation, the primitive endoderm appears as a layer of cells on the blastocoelic surface of the inner cell mass; later on, these cells contribute to the endoderm of the extra-embryonic tissues of the conceptus. The primitive endoderm rapidly gives rise to two cell lineages: the visceral endoderm and the parietal endoderm. The presence of the extra-embryonic visceral and parietal endoderms support the development of the embryo, but does not contribute to its physical structure (Gardner, 1982; Gardner, 1983). Extra-embryonic endoderm cells are believed to facilitate filtration and nutrient exchange (Ang et al., 1993; Dufort et al., 1998). Recently, the extra-embryonic visceral endoderm (VE) has been shown to play roles in other embryonic processes such as ectoderm cavitation (Beddington and Robertson, 1998; Bielinska et al., 1999; Coucouvanis and Martin, 1999; Lu et al., 2001). Several genes have been implicated in the VE differentiation, including the GATA transcription factor family of proteins, hepatic nuclear factor transcription factors and BMP proteins (Barbacci et al., 1999; Coucouvanis and Martin, 1999; Kuo et al., 1997; Morrisey et al., 1998).

Maspin (Serpinb5 – Mouse Genome Informatics) is a member of the serine protease inhibitor family with tumor suppressing activity (Zou et al., 1994). Initially identified from normal mammary epithelial cells, the maspin gene is neither mutated nor deleted, but it is transcriptionally downregulated or silenced by epigenetic changes in breast cancer (Futscher et al., 2002; Zhang et al., 1997a). Maspin protein, made in either *E. coli*, yeast, or insect cells, inhibits breast tumor cell migration and invasion (Sheng et al., 1996; Zhang et al., 1997b) in vitro. It also inhibits mammary tumor progression and metastasis in maspin transgenic mice (Zhang et al., 2000a) and in a syngeneic mammary tumor model (Shi et al., 2001). Maspin has also been shown to inhibit angiogenesis in the rat cornea and xenograft models (Zhang et al., 2000b). Like many other serpins, maspin exerts its inhibitory role against cell migration and invasion through a functional domain named the reactive site loop (RSL). However, as a member of the non-inhibitory serpin family, maspin acts in the absence of a protease inhibitor activity (Bass et al., 2002). Evidence from recent studies have indicated a role for maspin in cell adhesion (Abraham et al., 2003; Ngamkitidechakul et al., 2003; Seftor et al., 1998).

The processes of tumor cell invasion and metastasis shares many features with early embryonic development. To understand the role of maspin in normal embryonic development, we disrupted the maspin gene by a gene targeting strategy. Homozygosity for the maspin mutant resulted in an embryonic lethality in the mouse that occurred at the peri-implantation stage. The presence of empty decidua indicated that the $Mp^{-/-}$ embryos were implanted into the uterine wall, but failed to develop into gastrulated embryos thereafter, resulting in embryonic death at the peri-implantation stage. Further experiments proved that the organization of the endodermal layer and specification of ectoderm cells were affected in $Mp^{-/-}$ embryoid bodies. In vitro embryo outgrowth studies showed that the inner cell mass from the $Mp^{-/-}$ embryos failed to grow appropriately. Endoderm development requires the attachment of endodermal cells to the extracellular matrix. This interaction was compromised in the absence of maspin. These results indicate that maspin plays an essential role in early embryonic development and it does so by controlling the function of the extra-embryonic endoderm, thereby affecting epiblast morphogenesis.

Materials and methods

Targeted disruption of the maspin gene

We isolated and characterized an 18 kb lambda genomic clone from a 129/sv mouse genomic library (Strategene) using the murine maspin cDNA (Zhang et al., 1997b). This clone contains nearly all of the exons and introns of the maspin gene except the non-coding exon 1 and part of intron 1. To generate the targeting vector, we deleted a 2 kb region of the maspin gene that contains exon 7 (encodes the reactive site loop), part of intron 6 and the entire 3′-UTR region, and replaced it with a neomycin gene cassette. The construct was electroporated into ES cells and neomycin-resistant clones were selected in G418-containing medium. To confirm recombination, Southern blot analysis was carried out using the *Sac*I-*Sac*I (3.6 kb) fragment as a probe. Since the construction of the vector changed the *Sac*I site to *Bam*HI, digestion of recombinant +/– ES cell clone DNAs generated both the 3.6 kb and 12 kb fragments. Two clones were injected into C57BL/6 blastocysts to obtain chimeric mice for germ line transmission.

To select for $Mp^{-/-}$ ES cells, $Mp^{+/-}$ ES cells were cultured in ES medium with a high concentration of G418 (3 μg/μl). ES cell clones were isolated and transferred to 24-well plates without a feeder cell layer for genotyping purposes. Two $Mp^{+/+}$ and two $Mp^{-/-}$ ES clones were used for EB formation. The genotype analysis of the animals, embryos and embryo outgrowths were carried out by PCR using the following primers: wild-type sense 5′-gatggtggtgagtccatc-3′ and antisense 5′-tgacaaatgaagagcac-3′; knockout sense 5′-gccttctt-gacgagttct-3′ and antisense 5′-tgacaaatgaagagcac-3′. RT-PCR was performed using the sense primer 5′-gcttttgctgttgacttgttc-3′ (exon 2) and the antisense primer 5′-ttggtgtcttctgtcttgctgatt-3′ (exon 5).

Embryo recovery and outgrowth

Superovulated females were caged overnight with males and plugs were checked the following morning. Fertilization was assumed to occur at midnight and embryos were staged accordingly (the time of noon on day 1 is termed E0.5). Embryos at the two-cell stage were flushed from the oviducts of the superovulated females at E1.5 and blastocysts were flushed from uteri at E3.5. The embryos were cultured in a few microdrops of M2 medium (Sigma) and covered with mineral oil (Fisher Scientific). For the outgrowths, the flushed blastocysts were cultured on 60 mm dishes. After a few days in culture, the hatched embryos attached to the dishes and the ICM continued to proliferate and form large cell masses.

Isolation of endoderm cells from blastocysts

The blastocysts from the heterozygous intercrosses were collected at E3.5 and cultured in ES medium with a feeder cell layer for 7-8 days. The cell masses were picked, trypsinized with 0.25% trypsin, and transferred individually to 96-well plates with feeder cell layers. These cells were cultured for 3-4 days before being trypsinized and transferred to a new plate. After several passages on a feeder cell layer, these blastocyst cells frequently developed into endoderm cells rather than ES cells. These small, round endodermal cells grew quickly and spread throughout the plates. At this point, some cells were harvested for an endoderm adhesion assay. Aliquots of the cells were passed to dishes without feeder cell layers for three times and DNAs were made from the $Mp^{+/+}$ and $Mp^{+/-}$ genotypes. RNAs were also isolated and analyzed by RT-PCR for the expression of VE and PE markers, HNF4, and follistatin.

ES cell in vitro differentiation

ES cells were cultured on a layer of feeder cells for 3-4 days before they were trypsinized and transferred to petri dishes for EB formation. Adenovirus-maspin or control adenovirus was added to the ES cells before they were transferred to the petri dishes for EB formation. The adenovirus was diluted to 5 MOI (multiplicities of infection) with serum-free DMEM medium and incubated with the ES cells for 1 hour at 37°C. The cells were fed with new ES medium after the adenovirus infection. For the maspin antibody blocking experiment, anti-maspin serum (1:100 dilution) was added to the medium either at the beginning or during the process of EB formation. After 8-12 days in culture, the embryoid bodies were fixed in 4% PBS (pH 7.4) buffered formaldehyde for 1-2 hours, embedded with 2% agarose and sectioned.

Histology: in situ hybridization and immunohistochemistry

Implanted uteri at 4.5-7.5 days of gestation (E4.5-E7.5) were fixed in 4% PBS (pH 7.4) buffered formaldehyde for 20-24 hours and embedded in paraffin wax. The embryoid bodies were fixed in 4% PBS (pH 7.4) buffered polyformaldehyde for 1-2 hours and embedded in paraffin wax. Blocks were serially sectioned at 5 μm and mounted on poly-l-lysine-coated slides. The sections were blocked with 5% normal goat serum before staining with antibody. Primary antibodies to maspin (1:400), GATA4 (Santa Cruz Biotechnology, CA, 1:100), laminin 1 (NeoMarkers, CA, 1:2000), fibronectin (Novus Biologicals, CA, 1:200), and Oct-4 (Santa Cruz Biotechnology, CA, 1:1000) were incubated for 1 hour, followed by three washes with PBS. Samples were incubated with a 1:400 dilution of biotin conjugated secondary antibody (Vector Laboratories) for 45 minutes, washed in PBS, and incubated with the avidin and biotin solution for 45 minutes. The color was developed with a DAB Kit (Vector Laboratories) and images were captured with a Leica microscope equipped with a Spot digital camera. For in situ hybridization, the HNF4 probe (from Dr Fred Pereira, Baylor) was labeled by digoxigenin according to the manufacturer's instructions (Roche, Germany). Hybridization was carried out according to the conditions described by Duncan et al. (Duncan et al., 1997). The mitotic index for proliferation was analyzed using samples from embryo outgrowths and EBs. The embryo outgrowths were fixed with 4% polyformaldehyde for 1-2 hours at 4°C and then incubated with 0.1% Triton X-100 for 10 minutes. Both the outgrowth slides and EB sections were blocked with 5% normal serum before being incubated with the anti-phosphorylated histone 3 antibody (Upstate Biotech, NY, 1:200) for 1 hour. The images were captured with a Leica microscope equipped with a Spot digital camera.

TUNEL assay

The TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was performed according to the manufacturer's specifications (Roche). Briefly, slides were incubated

with 50 μl of the TUNEL reaction mixture for 30 minutes at 37°C. The slides were then rinsed three times with PBS and counterstained with DAPI. The mounted slides were analyzed using fluorescence microscopy. Apoptosis was quantified by counting the number of apoptotic positive cells in four slides with a 20×objective.

Endoderm cell adhesion and growth on ECM

Ninety-six-well plates were coated with 5 μg/cm^2 of fibronectin or 25 μg/cm^2 of laminin for 1 hour at room temperature as described by others (Streuli and Gilmore, 1999). After washing with PBS, the coated wells were treated with 10 mg/ml BSA for 1 hour. Equal numbers of $Mp^{+/+}$ or $Mp^{+/-}$ endoderm cells were seeded in each well. For the antibody blocking experiments, anti-maspin or preimmune antiserum was added to the wells at a 1:100 dilution. After 1 hour incubation, the plates were washed with PBS, trypsinized and counted with a hemacytometer. For the endoderm cell growth assay, the 96-well plates were coated with 25 μg/cm^2 of laminin in PBS overnight at 4°C. A total number of 10,000 cells ($Mp^{+/+}$ or $Mp^{+/-}$) were seeded in each well. The cells were cultured for 5 days and counted daily.

Results

Targeted disruption of the maspin gene in the mouse

We isolated a maspin genomic clone from the mouse 129/sv genomic library. This clone contained exon 2 and exon 7 as well as the 3′-flanking region (Fig. 1). The reactive site loop, a crucial functional domain of maspin is located in exon 6. To generate a mutant lacking maspin function, murine 129 embryonic stem (ES) cells were transfected with a targeting vector in which the exon encoding the reactive center loop and the entire 3′-UTR region were deleted (Fig. 1A). Of about 200 G418-resistent ES clones, 15 displayed maspin gene replacement. Positive clones were confirmed by Southern blot analysis using a genomic DNA fragment as the probe (Fig. 1B) and by polymerase chain reaction (PCR) analysis using primers to detect the junction between the genomic DNA and the Neo cassette (data not shown). To determine whether this design generated a non-functional mutant or a loss of maspin expression (due to the deletion of the 3′-UTR of the maspin gene which renders the truncated RNA unstable), ES cells homozygous for maspin gene replacement were isolated and used in embryoid body (EB) formation assays. Using a pair of oligos positioned between exon 2 and exon 5, we detected maspin mRNA in the wild-type EBs by RT-PCR. However, maspin mRNA was hardly detectable in the homozygous EBs (Fig. 1C). Further analysis by immunostaining confirmed that no maspin protein was made in the $Mp^{-/-}$ EBs (Fig. 4B). Differentiated cells from the maspin heterozygous background had reduced expression of maspin compared with that from the wild-type background (data not shown).

Maspin deficiency causes embryonic lethality shortly after embryo implantation

The heterozygous $Mp^{+/-}$ mice appeared to be normal morphologically after birth. However, when they were crossed, no homozygous maspin deletion progeny were obtained at birth. Among the 571 live-born offspring, 343 were heterozygotes and 228 were wild-type mice (see Table S1 at http://dev.biologists.org/supplemental/). This indicates that the homozygous maspin null mice are lethal during embryonic development. Interestingly, some of the heterozygous mice die

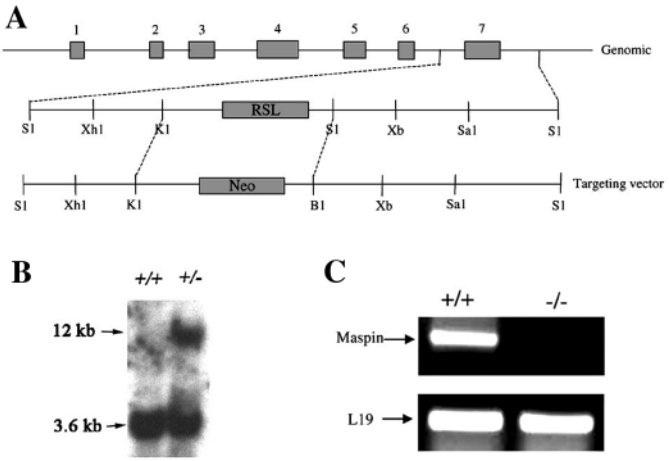

Fig. 1. Targeted disruption of maspin. (A) Targeting strategy. Homologous recombination replaces the seventh exon with the neomycin resistance (neo) gene. Note the change of *Sac*I (S1) into *Bam*HI in the construct. Restriction site are (S1) *Sac*I; (Xh1) *Xho*I; (K1) *Kpn*I; (B1) *Bam*HI; (Xb) *Xba*I; (Sa1) *Sal*I. (B) Southern blot analysis of $Mp^{+/+}$ and $Mp^{+/-}$ ES cells. The genomic DNAs were digested with a *Sac*I enzyme and the blot was probed with ^{32}P-labeled *Sac*I-*Sac*I fragment (3.6 kb). The 3.6 kb band was from the wild-type allele. A novel 12 kb band indicated the presence of the targeted allele. (C) RT-PCR analysis of maspin mRNA expression in $Mp^{+/+}$ and $Mp^{-/-}$ embryoid bodies.

at particular stage(s) of embryonic development as well. To determine whether the $Mp^{-/-}$ embryos die before implantation, $Mp^{+/-}$ mice were intercrossed; embryos at different developmental stages were cultured in vitro, examined for developmental defects and genotyped by PCR. The percentage of embryos undergoing normal development at the 2-cell, 4-cell and blastocyst stages were compared (see Table S2 at http://dev.biologists.org/supplemental/). Both $Mp^{-/-}$ and $Mp^{+/+}$ embryos were observed by the late blastocyst stage (data not shown). In addition, the $Mp^{-/-}$ blastocysts hatched without any noticeable defects (see Table S2 at http://dev.biologists.org/supplemental/). These results indicate that the $Mp^{-/-}$ embryos do not die before implantation. To further determine the time of embryonic death, in utero embryos ranging from 4.5 dpc to late gestation were microdissected and genotyped by either PCR using the yolk sac (8.5-12 dpc) or by immunostaining (4.5-7.5 dpc). All yolk sac samples were found to be derived from either $Mp^{+/+}$ or $Mp^{+/-}$ embryos. To identify the genotypes of the embryos at the peri-implantation stage, uterine decidua from 4.5 to 6.5 dpc were serially sectioned and stained with the maspin antibody for immunohistochemistry. As shown in Fig. 2A,B, $Mp^{+/+}$ embryos developed into egg cylinders of normal size with a layer of visceral endoderm (Fig. 2A, part a, Fig. 2B, parts a-h). The trophectoderm cells migrated along the endometrial layer and both the extra-embryonic ectoderm cells and the embryonic ectoderm cells were in close contact with the VE. In addition, a proamniotic cavity was present in the embryonic region and both the ectoplacental cone and the parietal endoderm were properly developed in the maspin wild-type embryos at 5.5 dpc. By contrast, no $Mp^{-/-}$ embryos were observed after 5.5 dpc (Fig. 2A). Among the 36 embryos examined, only two presumed $Mp^{-/-}$ embryos at 5.5 dpc were

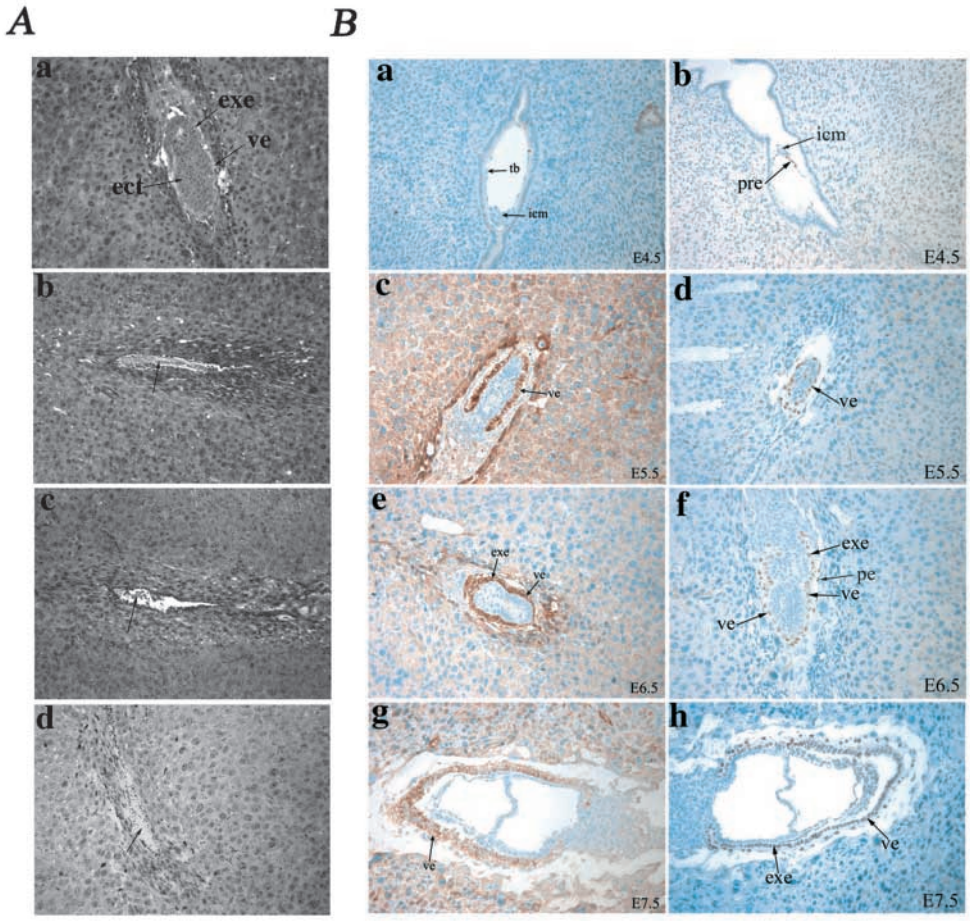

Fig. 2. Histological analysis and localization of maspin in early embryonic development.
(A) Histological analysis of $Mp^{+/+}$ and $Mp^{-/-}$ embryos at 5.5 dpc. (a) $Mp^{+/+}$ embryo. (b,c) Abnormal $Mp^{-/-}$ embryos undergoing resorption. (d) Empty decidua from the implantation of presumed $Mp^{-/-}$ embryos. Arrows indicate the debris of the embryos. (B) Localization of maspin (a,c,e,g) and GATA4 (b,d,f,h) in early embryonic development from E4.5 to E7.5. Note that maspin is specifically expressed in the visceral endoderm from E5.5 to E7.5. GATA4 is present in the ve, pre and pe. exe, extra-embryonic endoderm; ve, visceral endoderm; ect, ectoderm; Tb, trophoblast cell; icm, inner cell mess; pe, paretial endoderm; pre, primitive endoderm.

identified; both of these embryos failed to positively stain for maspin and were undergoing the process of resorption (Fig. 2A, parts b and c). One of the two presumed $Mp^{-/-}$ embryos had induced a uterine reaction and had a completely deformed embryo without a visible VE layer (Fig. 2A, part b). Extensive resorption was observed in the other embryo, which had no visible embryo structures except for the cell mass (Fig. 2A, part c). These types of resorbed $Mp^{-/-}$ embryos were not observed in other serially sectioned empty decidua (as shown in Fig. 2A, part d), indicating that most of the resorption happened at a stage prior to 5.5 dpc. Approximately 25% of the decidua were found to be empty (Fig. 2A, part d, Table 1), consistent with the ratio of null embryos (Table S1 at http://dev.biologists.org/supplemental). The presence of decidualization indicates that the maspin null embryos can implant into the uterus but die shortly after implantation.

Table 1. Result of the decidua sections

Age	Number of decidua	Empty	Normal			Ratio
			+/+	+/–	–/–	
E4.5	15	1		14		93.3%
E5.5	33	9	24		0	72.7%
E6.5	46	13	33		0	71.7%

The genotype was examined by immunostaining. E4.5 embryos do not express maspin protein and the genotype at this stage is unknown. All embryos of E5.5 and E6.5 express maspin in the endoderm, except the empty decidua, suggesting that they are either $Mp^{+/+}$ or $Mp^{+/-}$ embryos at these stages.

As $Mp^{-/-}$ mice die between 4.5 and 5.5 dpc, it is of interest to determine the temporal and spatial patterns of maspin expression during the peri-implantation stage. At 4.5 dpc, maspin was not expressed in the inner cell mass, the primitive endoderm, or in the uterine cells surrounding the embryo (Fig. 2B, part a). However, decidualization induced maspin expression in the inner zone of the decidua reaction of the uterus by 5.5 dpc. Maspin protein appeared in the visceral endoderm (VE) at 5.5 dpc (Fig. 2B, part c). This expression was restricted to both the embryonic and the extra-embryonic visceral endoderm at 6.5 dpc and 7.5 dpc (Fig. 2B, parts e,g). Maspin expression was not observed in the parietal endoderm, the ectoplacental cone, the chorion and the amnion. GATA4 is a transcription factor with a known role in endoderm differentiation in the mouse embryo (Kuo et al., 1997). Immunohistochemistry of embryo sections with the GATA4 antibody showed a pattern of staining in the visceral endoderm similar to that of the maspin antibody (Fig. 2B, parts b,d,f,h). However, GATA4 was expressed earlier than maspin, at 4.5 dpc in the primitive endoderm (Fig. 2B, part b), suggesting that GATA4 might be required for an early event of endoderm differentiation as an upstream transcription factor. Interestingly, GATA4 was expressed in the parietal endoderm whereas maspin was not expressed in this cell layer (Fig. 2B, part h). In addition, GATA4 was not induced in the uterus during the decidua reaction. As an early event of embryonic development, the generation of the visceral endoderm provides the embryo with nutritional and hematopoietic functions (Ang et al., 1993; Dufort et al., 1998). As maspin is specifically expressed in the visceral endoderm cells after implantation, its deletion seems to be destructive to the formation of the endodermal cell layer and for the morphogenesis of the epiblast, thus preventing further embryo development.

Mp$^{-/-}$ blastocysts display an inner cell mass failure during outgrowth and differentiation

The *Mp*$^{-/-}$ embryos may die of a generalized growth failure due to the lack of an organized layer of VE cells. To determine the extent to which mutant preimplantation embryos can proliferate, E3.5 blastocysts derived from *Mp*$^{+/-}$ matings were cultured individually in vitro. During the outgrowth process, blastocysts undergo both cell growth and differentiation. Trophoblasts from *Mp*$^{-/-}$ embryos differentiated and expanded without any noticeable defects. Among the 30 embryos plated, all of the *Mp*$^{-/-}$ embryos developed a trophoblast region equal to the size of the *Mp*$^{+/+}$ embryos, suggesting that the initial trophoblast differentiation, as well as the morphogenesis, was normal in the *Mp*$^{-/-}$ embryos. In contrast to the trophoblast, the morphogenesis and survival of the ICM in blastocyst outgrowths of *Mp*$^{-/-}$ and *Mp*$^{+/+}$ embryos showed a significant difference after 3 days of cell culture. Although the ICM regions of the *Mp*$^{-/-}$ and *Mp*$^{+/+}$ embryos were indistinguishable up to 48 hours of culturing (data not shown), a clear difference was observed in the growth rate of the ICM regions between these two embryo types by 96 hours (Fig. 3). This difference became more dramatic between the fourth and eighth day in culture as the ICM cells from the *Mp*$^{-/-}$ embryos failed to proliferate while the wild-type ICM cells continued to expand. The inner cell mass outgrowth failure occurred in 100% of the embryos identified by PCR as being *Mp*$^{-/-}$. Outgrowth penetration did not occur in the *Mp*$^{-/-}$ blastocysts; thus supporting the observed lethality of the *Mp*$^{-/-}$ homozygous embryos in vivo. Morphologically, most of the *Mp*$^{+/+}$ outgrowths were spherical while the *Mp*$^{-/-}$ outgrowths were irregular in shape (Fig. 3). Immunostaining with the GATA4 antibody confirmed that the *Mp*$^{+/+}$ embryos had more GATA4-positive endoderm cells than the *Mp*$^{-/-}$ embryos had at day 4 of culture (data not shown). Under the microscope, the *Mp*$^{+/+}$ inner cell mass showed a continuous layer of small and round endodermal cells during days 4-8 of culture. However, this monolayer of endodermal cells was not obvious in any of the *Mp*$^{-/-}$ ICM regions (Fig. 3). To examine whether the inner cell mass failure resulted from a defect in proliferation, embryo outgrowths from days 6 and 8 of culture were harvested and stained with the mitotic marker, phosphorylated histone 3. The cells from the *Mp*$^{-/-}$ blastocysts had a dramatic reduction in their rate of proliferation compared to that of the wild type blastocysts (data not shown).

Mp$^{-/-}$ embryoid bodies exhibit a disorganized layer of visceral endoderm and defective cavitation

Embryonic stem cells can be induced to aggregate and differentiate into embryoid bodies that are covered by a layer of visceral endoderm, a process which mimics embryonic development in the uterus (Coucouvanis and Martin, 1995; Tremblay et al., 2000). To further explore the role of maspin in early embryonic differentiation, *Mp*$^{-/-}$ and *Mp*$^{+/+}$ ES cells were induced to form embryoid bodies. Most of the *Mp*$^{+/+}$ ES cells differentiated into embryoid bodies with a large lumen in the center after 8 days in culture (Fig. 4A). The formation of lumen, also termed EB cavitation, is a process involving the organized apoptosis of ectodermal cells in the center of the EB. In addition to the presence of the lumen, the *Mp*$^{+/+}$ EBs showed a well-organized layer of visceral endoderm (Fig. 4B,C). By contrast, the *Mp*$^{-/-}$ ES cells formed EBs of a smaller size consisting of disorganized cell masses (Fig. 4A). As in the embryo, maspin is expressed in the layer of the visceral endoderm (Fig. 4B). The endoderm lineage cells in *Mp*$^{+/+}$ EB were confirmed by immunostaining with GATA4 antibody (Fig. 4C). However, the GATA4-positive cells in the *Mp*$^{-/-}$ embryoid bodies were scattered throughout. Interestingly, most cells in the *Mp*$^{-/-}$ embryoid bodies were GATA4 positive, including the superficial cells. As GATA4-positive cells can originate from the primitive endoderm, we used a specific VE marker, HNF4, to determine the cell lineage in both *Mp*$^{+/+}$ and *Mp*$^{-/-}$ EBs. The expression pattern of HNF4 by in situ hybridization was very similar to that of GATA-4 by immunostaining studies (Fig. 4D). We further stained the EBs with an antibody against Troma1, a marker for the PrE lineage (Duprey et al., 1985; Verheijen et al., 1999). Not a single cell in either the *Mp*$^{+/+}$ or the *Mp*$^{-/-}$ EBs stained positively for Troma1 (data not shown). These data suggested that early differentiation from primitive endoderm to visceral endoderm was not blocked in the *Mp*$^{-/-}$ EBs. In *Mp*$^{+/+}$ EBs, the embryonic ectoderm cells were adjacent to the basement membrane and surrounded by the VE layer. These embryonic ectoderm cells

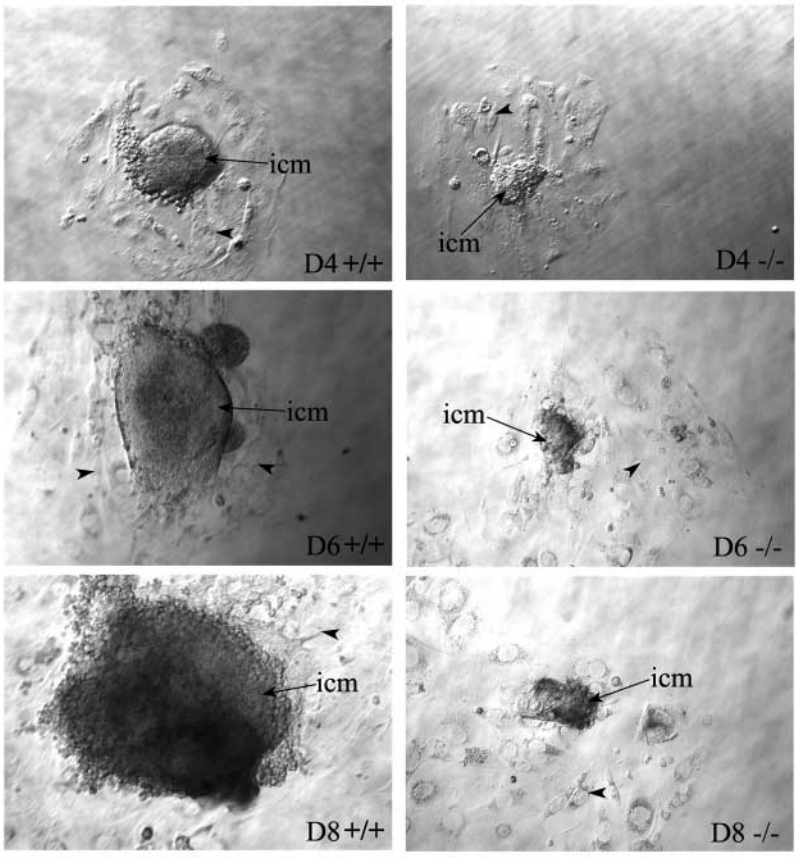

Fig. 3. Phase-contrast micrographs of *Mp*$^{+/+}$ (left) and *Mp*$^{-/-}$ (right) embryonic outgrowths at different stages. Arrows indicate the inner cell mass and arrowheads indicate the trophoblast giant cell. icm, inner cell mass.

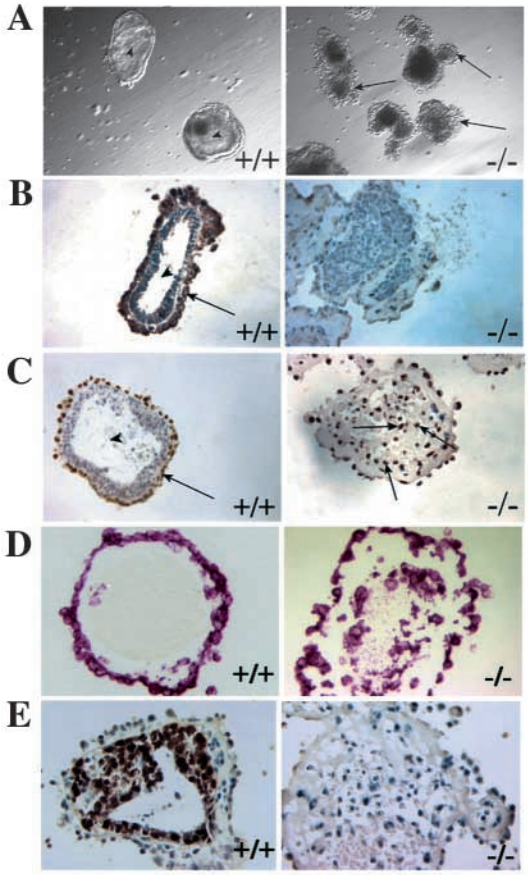

Fig. 4. Defective embryoid body formation in the $Mp^{-/-}$ ES cells. (A-D) Phase contrast micrographs of $Mp^{+/+}$ and $Mp^{-/-}$ embryoid bodies. Arrowheads show the lumen formed in the $Mp^{+/+}$ embryoid body and arrows show the outer layer of cells detaching from the $Mp^{-/-}$ embryoid bodies. (B) Maspin expression in $Mp^{+/+}$ and $Mp^{-/-}$ embryoid bodies analyzed by immunostaining. Arrowhead shows the lumen in the $Mp^{+/+}$ EB; arrow shows the endodermal cells which express maspin. Note no maspin protein is detected in $Mp^{-/-}$ EB. (C) GATA4 immunostaining of $Mp^{+/+}$ and $Mp^{-/-}$ EBs. Arrows indicate the endodermal cells and arrowhead indicates the lumen of the $Mp^{+/+}$ EBs. (D) HNF4 expression (fast-red labeled cells) of $Mp^{+/+}$ and $Mp^{-/-}$ EBs analyzed by in situ hybridization. (E) Oct4 immunostaining of $Mp^{+/+}$ and $Mp^{-/-}$ EBs.

were positive for the Oct4 marker (Fig. 4E). However, almost no cells in the $Mp^{-/-}$ EBs were positive for Oct4 (Fig. 4E). This result indicates that the development of the embryonic ectoderm or epiblast is defective in the absence of maspin. This ectoderm differentiation defect may result from the lack of a continuous layer of VE cells lining the outside of the ectoderm.

Defective apoptosis pattern and reduced rate of proliferation in $Mp^{-/-}$ EBs

To further characterize the defect in VE development in $Mp^{-/-}$ EBs, the TUNEL (TdT-mediated dUTP-biotin nick end labeling) assay was carried out using both the $Mp^{+/+}$ and $Mp^{-/-}$ embryoid bodies. As shown in Fig. 5A, apoptotic cells were observed in the center of the $Mp^{+/+}$ embryoid bodies where the presumed lumens would be formed. By contrast, the TUNEL-positive cells were scattered throughout the $Mp^{-/-}$ embryoid bodies. In fact, the lumen frequently did not form in the $Mp^{-/-}$ EBs. However, statistical analysis did not show a significant difference in the rate of apoptosis between the $Mp^{+/+}$ and $Mp^{-/-}$ EBs (Fig. 5A).

We also examined the rate of cell proliferation in the $Mp^{+/+}$ and $Mp^{-/-}$ EBs (Fig. 5B). EB sections were stained with an antibody to the mitotic marker, phosphorylated histone 3. The immunostaining confirmed that the rate of cell proliferation in the $Mp^{-/-}$ EBs was significantly reduced than that of the $Mp^{+/+}$ EBs (Fig. 5B). This is consistent with the prior observation of an inner cell mass failure in the $Mp^{-/-}$ blastocyst outgrowths.

Gain and loss of maspin function in the embryoid body

To prove that the EB formation defects in the $Mp^{-/-}$ ES cells are maspin specific, the maspin gene was introduced into $Mp^{-/-}$ ES cells by adenovirus infection. After adenovirus-maspin infection, ES cells were induced to form embryoid bodies. As shown in Fig. 6A, embryoid bodies with small lumens in the center were formed. This is in contrast to the $Mp^{-/-}$ EBs, which were never found to contain a lumen structure (Fig. 4, Fig. 6A). Additionally, some of the GATA4-positive endoderm cells were re-organized into a layer outside of the embryoid body (Fig. 6B). Concurrently, Oct4 positive ectoderm cells were able to align directly under the VE layer, confirming that the lack of maspin resulted in a defect in ectoderm development (Fig. 4). This partial rescue of the defect in the $Mp^{-/-}$ EBs by adenovirus-maspin infection indicates that maspin is directly involved in the organization of the endoderm layer as well as the development of the embryonic ectoderm.

It has been shown that during early cell fate specification, extensive signaling crosstalk exists between the ectoderm and the visceral endoderm. For example, in the pre-cavitated embryo or EB, the BMP4 signal produced in the ectoderm acts on the primitive endoderm to promote visceral endoderm differentiation, while BMP2 produced in the endoderm acts on ectoderm to promote apoptosis (Coucouvanis and Martin, 1999). The disorganization of the $Mp^{-/-}$ embryoid bodies might be due to a defect in either the cell-cell or the cell-matrix interactions, which are intact in the $Mp^{+/+}$ EBs through the action of cell surface-bound maspin. To test whether the VE produced maspin acts through either an intracellular or an extracellular event, ES cells were treated with a maspin anti-serum, which recognizes the reactive site loop of maspin, either during or after the formation of the $Mp^{+/+}$ embryoid bodies. When the maspin anti-serum was added after EB formation, the treated cells flattened at the periphery as they gave rise to coherent outgrowths (Fig. 6C). When the anti-serum was added during EB formation, EBs with normal lumen were unable to form. The morphology of these anti-serum treated EBs was similar to that of the EBs from the $Mp^{-/-}$ ES cells. Pre-immune serum had no effect on the formation and morphology of the $Mp^{+/+}$ embryoid bodies (Fig. 6C). This finding suggests that maspin controls the process of EB formation through an extracellular event. It may either act on the endoderm cell surface or be secreted to act on the adjacent ectoderm cells. This action is essential for the organization of the endodermal cell layer and subsequent embryonic development.

Maspin-expressing endoderm cells had increased binding to laminin 1 and a higher cell growth rate

As maspin acts in an extracellular manner, it may regulate cell attachment to the extracellular matrix. To test whether maspin

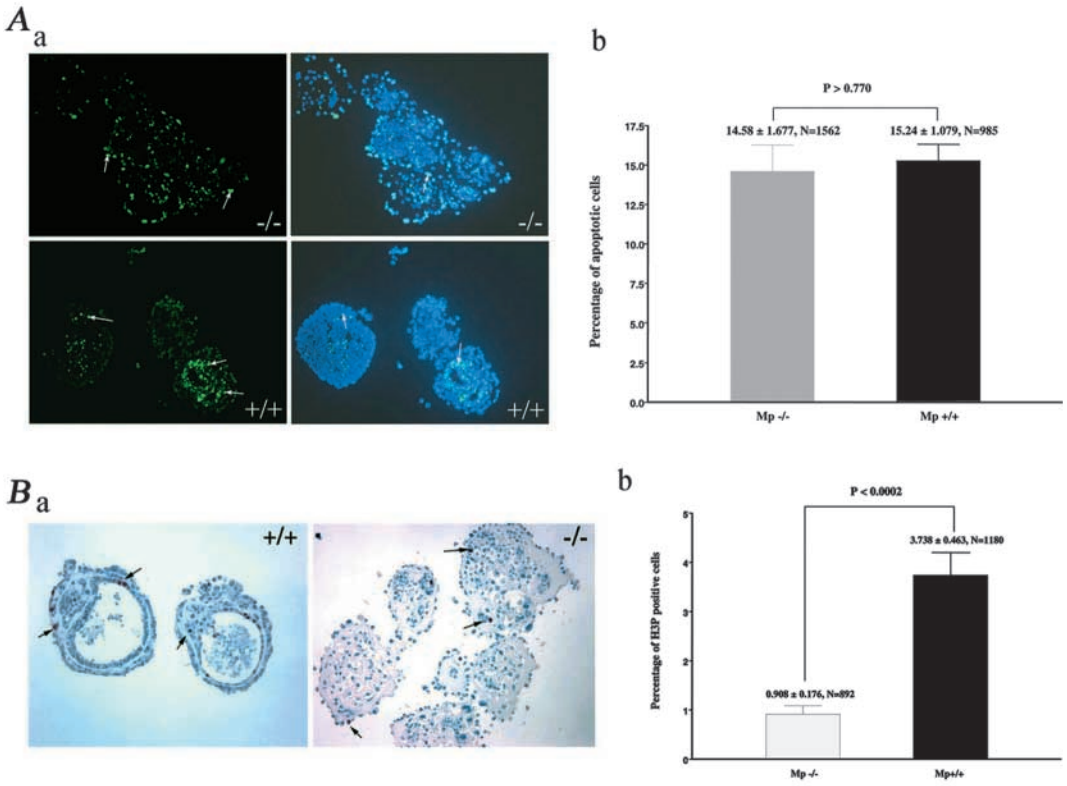

Fig. 5. Apoptosis and proliferation analysis of $Mp^{+/+}$ and $Mp^{-/-}$ EBs. (A) TUNEL analysis of the $Mp^{+/+}$ and $Mp^{-/-}$ EBs. (a) TUNEL staining. Fluorescent green cells are TUNEL-positive apoptotic cells. Left panels are TUNEL staining; right panels are the overlapped images of DAPI and TUNEL staining. Note the pattern of apoptosis in $Mp^{+/+}$ (below) and $Mp^{-/-}$ (above) EBs. Arrows indicate the apoptotic cells. (b) Analysis of TUNEL-positive apoptotic cells in $Mp^{+/+}$ and $Mp^{-/-}$ EBs. N, the number of cells counted. No significant difference is observed between the two classes of EBs. (B) $Mp^{-/-}$ EBs have a reduced rate of cell proliferation. (a) The mitotic index of EBs was determined by immunostaining with anti-phosphorylated histone 3 (H3P). Arrows indicate the proliferating cells. (b) Statistical analysis of the percentage of H3P-positive cells in $Mp^{+/+}$ and $Mp^{-/-}$ EBs. N, the number of cells counted. Note that there is a significant difference between the two classes of EBs.

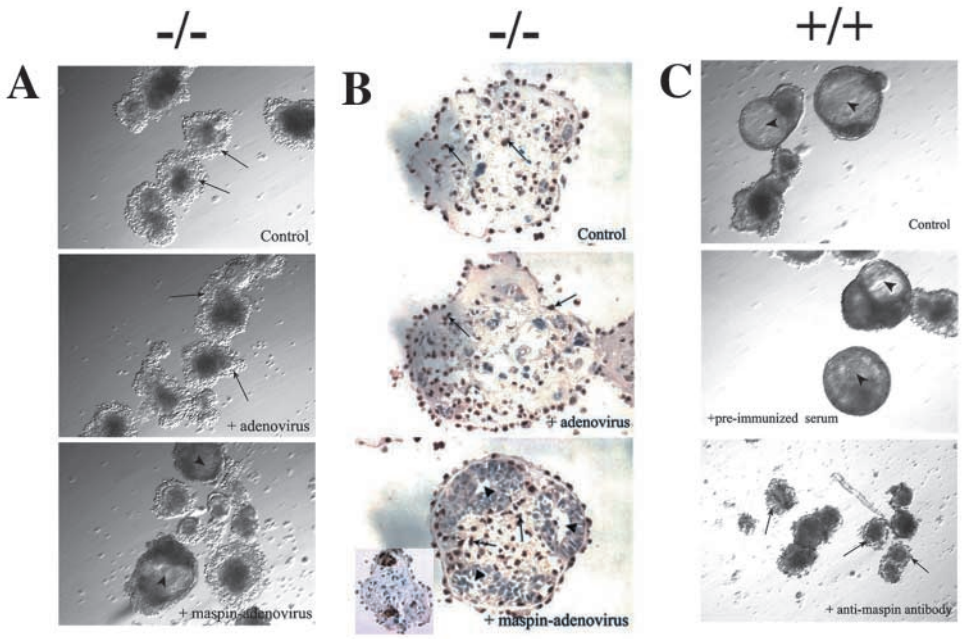

Fig. 6. Gain and loss of maspin function in embryoid body formation. (A-C) Rescue of defective EBs from $Mp^{-/-}$ ES cells using adenovirus-maspin. Note that the adenovirus control has no effect on lumen formation (arrows). (b) GATA4 staining of the adenovirus-maspin treated EBs. Arrows indicate GATA4-positive endodermal cells and arrowheads indicate the small lumen formed after adenovirus-maspin infection. Inset is Oct4 immunostaining of the adenovirus-maspin treated $Mp^{-/-}$ EBs. (C) Blocking of EB formation using a maspin antibody. Note that the pre-immune serum has no effect on $Mp^{+/+}$ EBs. Arrows indicate the layer of cells that are detaching.

regulates endodermal cell adhesion, the components of the extracellular matrix that surround the VE cells in embryos and EBs were examined (Fig. 7A, parts a-f). As shown in Fig. 7A, both laminin 1 and fibronectin were present in the basement membrane adjacent to the VE layer (Fig. 7A, parts a-f). The VE cells made and secreted these components to deposit them on the basement membrane to set the boundary between the VE and the ectoderm cells. Although a large amount of fibronectin and laminin 1 were present in the $Mp^{-/-}$ EBs, a normal, continuous layer of basement membrane was not formed, but rather the basement membrane layer was spread throughout the scattered endodermal cells (Fig. 7A, parts g,h).

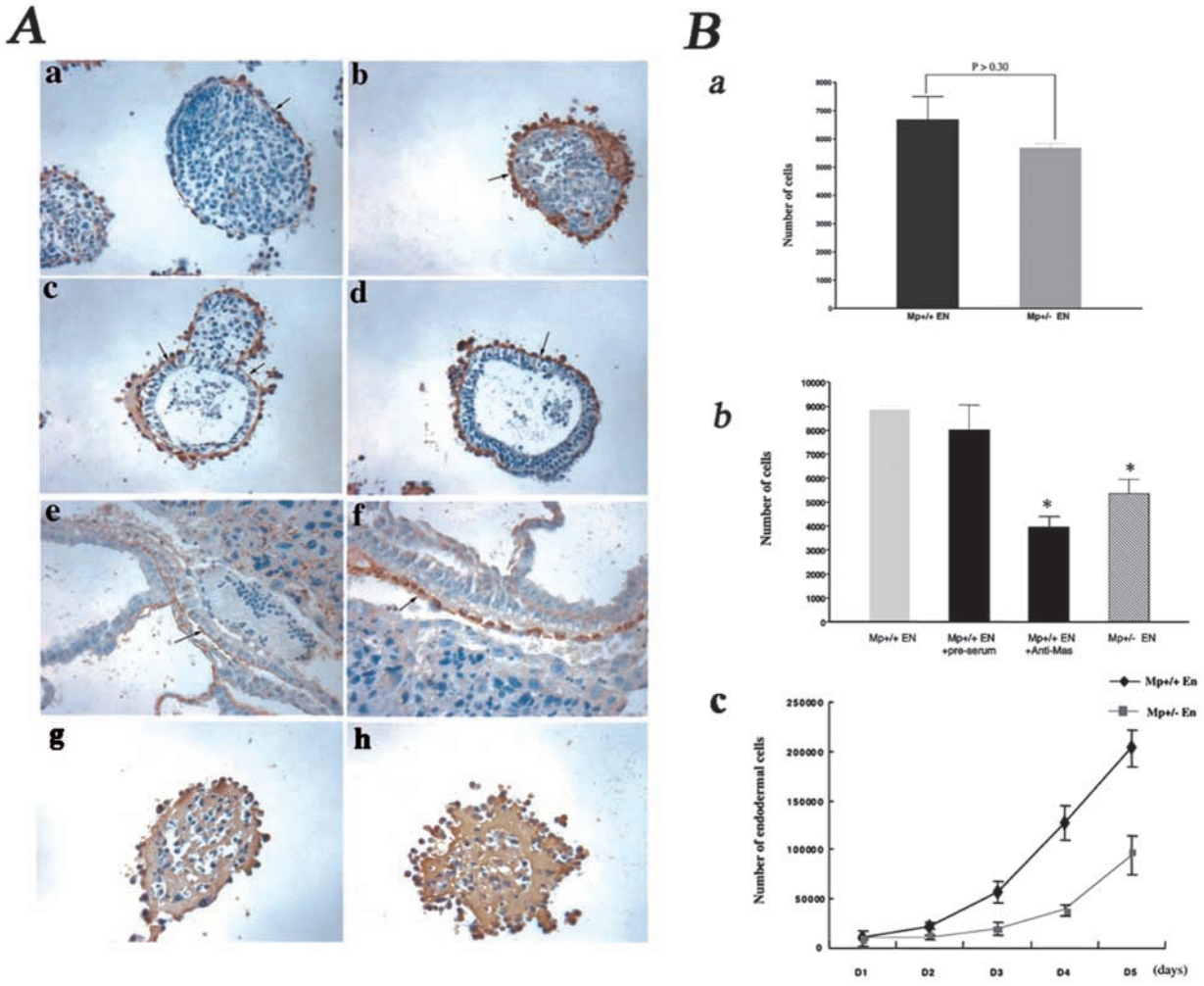

Fig. 7. (A) Expression of fibronectin (a,c,e,g) and laminin (b,d,f,h) in $Mp^{+/+}$ EBs, $Mp^{+/+}$ E7.5 embryos, and $Mp^{-/-}$ EBs. (a,b) $Mp^{+/+}$ EB sections after 3 days of cell culture. (c,d) $Mp^{+/+}$ EBs after 8 days of cell culture. (e,f) $Mp^{+/+}$ embryos at E7.5; (g,h) $Mp^{-/-}$ EBs after 8 days of cell culture. (B) Maspin affects endodermal cell adhesion. (a) Endodermal cell adhesion to fibronectin. (b) Endodermal cell adhesion to laminin matrix. $Mp^{+/+}$ endodermal cells were treated with either preimmune serum or anti-maspin antiserum. Significant differences in cell adhesion were observed when the $Mp^{+/+}$EN, +anti-Mas and $Mp^{+/-}$ EN cells to $Mp^{+/+}$ EN cells (indicated by an asterisk) were compared. (c) Decreased rate of cell proliferation in the $Mp^{+/-}$ EN cells. Note the difference in the cell doubling times for the $Mp^{+/+}$ and $Mp^{+/-}$ cells.

We then tested the ability of the endoderm cells to adhere to the matrix of either laminin 1 or fibronectin. Because it was impossible to isolate $Mp^{-/-}$ endoderm cells from blastocyst outgrowths, owing to the inner cell mass failure, we isolated $Mp^{+/+}$ and $Mp^{+/-}$ endoderm cells for the cell adhesion analysis. In the presence of fibronectin, both $Mp^{+/+}$ and $Mp^{+/-}$ endoderm cells displayed a similar ability to attach to the matrix (Fig. 7B, part a). However, significant differences were observed when the cells were plated on the laminin 1 matrix (Fig. 7B, part b). $Mp^{+/+}$ endoderm cells attached to laminin better than $Mp^{+/-}$ cells, indicating that maspin selectively increases VE attachment to laminin. To prove that this increased adhesion to laminin is maspin specific, we treated $Mp^{+/+}$ endoderm cells with an anti-maspin antibody. This treatment significantly decreased cell adhesion to laminin ($P<0.01$). Treatment with preimmune serum had no adverse effect on cell adhesion ($P \gg 0.05$). To test whether this increased attachment resulted in an increase in cell survival and growth, endoderm cells were plated on laminin to observe the growth rate. As shown in Fig. 7B (part c), $Mp^{+/+}$ endoderm cells had a significant growth advantage over the $Mp^{+/-}$ cells.

Discussion

Early embryonic development is characterized by a series of cell-fate decisions that restrict developmental potentials in an asymmetric fashion. At the peri-implantation stage, the trophectoderm lineage, which is located on the outside of the morula, starts to migrate along the endometrial layer. In blastocyst culture, this lineage of cells spread quickly in the culture dishes. We showed that trophectoderm cells were able to function normally independent of maspin expression (Fig. 3). $Mp^{-/-}$ embryos developed a trophoblast region equal to the size of the $Mp^{+/+}$ embryos (Fig. 3), suggesting that the initial trophoblast differentiation, as well as the morphogenesis, was normal in the $Mp^{-/-}$ embryos. Shortly after implantation, the inner cell mass is specified into two lineages, the embryonic ectoderm (epiblast), which gives rise to all cell types of the

embryo, and the extra-embryonic (primitive) endoderm, which further differentiates into the visceral and parietal endoderms and contributes to the yolk sac. The most important finding from this study is that homozygous deletion of the maspin gene causes the embryo to die shortly after implantation. As the lethality occurred at such an early stage, we chose to characterize the phenotype by in vitro methods. Both blastocyst outgrowths and embryoid body formation assays were used to assess the role of maspin during the in vitro equivalent of peri-implantation development. $Mp^{-/-}$ blastocysts display an inner cell mass failure during outgrowth and differentiation. The initial outgrowth of the $Mp^{-/-}$ blastocysts seemed to be normal, but a defect appeared around day 4 of cell culture. Although the wild type ICM cells continued to expand, the $Mp^{-/-}$ ICM cells failed to proliferate. Morphologically, the $Mp^{+/+}$ inner cell mass showed a continuous layer of small and round endoderm cells, confirmed by immunostaining with the GATA4 antibody, while the $Mp^{-/-}$ embryos had a few scattered cells positive for GATA4 (data not shown). After 4-8 days in culture, the epiblast (embryonic ectoderm) was gradually lost in the $Mp^{-/-}$ embryos. Likewise, the $Mp^{-/-}$ EBs appeared to be normal morphologically at the beginning of EB formation (data not shown). However, after 4 days in cell culture, the $Mp^{-/-}$ EBs began to display a different growth pattern than that of the $Mp^{+/+}$ EBs. $Mp^{-/-}$ ES cells formed EBs of a much smaller size. Histological analysis showed that the $Mp^{-/-}$ EBs consisted of disorganized cell masses (Fig. 4). Most importantly, cells of the embryonic ectoderm origin were not present in the $Mp^{-/-}$ EBs. Instead, the $Mp^{-/-}$ EBs retained cells of the endoderm lineage. These data indicate that the embryonic ectoderm is initially normal in the maspin mutants, but it is subsequently lost, presumably at a time when dependence on the VE is first manifested. To determine whether these cells belong to the lineages of either the primitive endoderm, the VE or the PE, we examined the expression of HNF4 and Troma1 in the $Mp^{-/-}$ EBs. Troma1 (Duprey et al., 1985; Verheijen et al., 1999), a marker for PrE, did not stain any of the $Mp^{-/-}$ EB cells. However, HNF4 was present in most of the $Mp^{-/-}$ EB cells. We therefore assume that most of the cells in the $Mp^{-/-}$ EBs might be visceral endoderm (Fig. 4). We also carried out RT-PCR analysis of RNA isolated from the $Mp^{+/+}$ and $Mp^{-/-}$ EBs using primers for follistatin, a PE marker gene (Veltmaat et al., 2000). Both the $Mp^{+/+}$ and $Mp^{-/-}$ EBs expressed the follistatin gene (data not shown), suggesting that these EBs do contain PE cells. During mouse development, the parietal endoderm (PE) forms from both the primitive endoderm (PrE) and the visceral endoderm (VE) (Veltmaat et al., 2000). These data suggest that early endoderm differentiation (from primitive endoderm to VE or to PE) is not blocked in the absence of maspin, but rather the loss of the embryonic ectoderm cells account for the defect in the $Mp^{-/-}$ embryos. Furthermore, treatment with adenovirus-maspin partially rescued the defect in the $Mp^{-/-}$ EBs. Most notably, the embryonic ectoderm appeared complete with a layer of VE cells in the $Mp^{-/-}$ EBs (Fig. 6B). These data, along with the inner cell mass failure and the embryonic lethality before E5.5 collectively demonstrate that maspin plays a key role in the development of the early embryonic ectoderm.

How does maspin regulate the development of the embryonic ectoderm? Apparently, maspin expression is restricted to the visceral endoderm in the wild-type EB but not in the E5.5 epiblast and the PE cells (Figs 2, 4). Our data indicate that maspin exerts its effect through the VE cells. Numerous reports demonstrate that the three early embryonic lineages depend on each other for survival, patterning and differentiation. BMP molecules have been shown to mediate the signaling cascade between the endoderm and ectoderm cells (Coucouvanis and Martin, 1999). Loss of several these BMP transcription factors expressed in the VE result in an altered epiblast morphology (Ang et al., 1996; Dufort et al., 1998; Tremblay et al., 2001). The defect in the $Mp^{-/-}$ embryos is more severe in comparison to the transcription factor mutants. The development of VE cells was clearly affected by the lack of maspin in the $Mp^{-/-}$ embryos. The VE cells in both the $Mp^{-/-}$ EBs and blastocyst outgrowths had decreased proliferation (Fig. 5). The isolated $Mp^{+/-}$ endoderm cells also proliferated slower than the $Mp^{+/+}$ cells (Fig. 7B, part c). In addition, unlike the $Mp^{+/+}$ EBs, the $Mp^{+/-}$ EBs did not form an organized VE cell layer. Rather, the cells were scattered and surrounded by ECM molecules (Fig. 7A, parts g,h). The disordered formation of the VE layer upon the basement membrane is indicative of incomplete differentiation of the VE at a late stage of embryogenesis (Duncan et al., 1997). For example, the early specification for VE is normal in the $Hnf4^{-/-}$ EBs, but further differentiation of the VE is defective (Duncan et al., 1997). The VE from the $Mp^{-/-}$ EBs might not be able to undergo further differentiation. The gradual disappearance of the ectoderm cells could contribute to the defect for further VE development because of the lack of cell signaling between the VE and the ectoderm. Conversely, defective VE cells could then send destructive signals to the remaining ectoderm cells, eventually resulting in the elimination of the ectoderm cells. In this regard, although maspin is not essential for early endoderm specification, the possibility for its involvement in late VE differentiation cannot be excluded.

As the formation of wild-type EBs depends on the VE cells lying on an intact layer of basement membrane and the maspin antibody treatment was able to block normal EB formation, we suspect that maspin exerts its function at least partially through a cell-ECM interaction. We showed that the VE cells from the $Mp^{+/-}$ embryos had a significant reduction in their adhesion and growth rate in comparison to the $Mp^{+/+}$ VE cells when plated on laminin. This reduction caused by the loss of one copy of maspin seems to affect embryonic development in vivo, as we observed that some of the heterozygous mice die at certain stage(s) of embryonic development (see Table S1 at http://dev.biologists.org/supplemental). Therefore, defective VE development could result from reduced cell adhesion to matrix and reduced proliferation. The effect on adhesion and proliferation may not be mutually exclusive as many cells require appropriate cell adhesion to unique ECM components for survival. ECM proteins are generally assembled in basement membranes and recent data indicates that the regulation of this process is crucial for embryo development (Colognato and Yurchenco, 2000; Li et al., 2002). The basement membranes formed between the visceral endoderm, the developing epiblast and the parietal endoderm (Reichert's membrane), which extends over the trophectoderm, are the first membranes formed during embryogenesis (Leivo et al., 1980). Although differentiation of the primitive endoderm cells precedes basement membrane assembly, the processes of epiblast differentiation and proamniotic cavitation require the

completion of basement membrane assembly to be entirely functional (Murray and Edgar, 2000; Murray and Edgar, 2001). Recent experiments have demonstrated that the laminin 1-null and β1-integrin-null cells are unable to form basement membranes or undergo epiblast differentiation and cavitation (Li et al., 2003; Li et al., 2002). Not surprisingly, in the $Mp^{+/+}$ EBs and embryos that stained positive for laminin and fibronectin expression, the basement membrane layers were intact (Fig. 7A). However, this distinctive basement membrane layer as well as the visceral endoderm layer was diminished in the $Mp^{-/-}$ EBs (Figs 4, 6). Therefore, maspin produced in the VE may be involved in the ECM assembly, which controls further embryonic development.

The importance of cell-ECM interactions during early embryonic development is highlighted by several mouse mutants that lack integrin molecules, the cell-surface adhesion receptors for ECM. One of these integrin molecules, αV integrin, is present in blastocysts and associates with the β3 subunit. Through its interactions with the other ECM components, the αVβ3 integrin mediates the initial blastocyst-uterine interaction during implantation (Cross et al., 1994). In addition, deletion of either the α5 or the α4 integrin subunits results in an embryonic lethality because of a defect in mesoderm development (Yang et al., 1993; Yang et al., 1995). The defects observed in the integrin β1 mutants during embryonic development because of the similarity to the maspin mutant. In the β1-null embryos, endoderm morphogenesis was defective and the embryo died at E5.5 (Fassler and Meyer, 1995; Stephens et al., 1995). Blastocyst outgrowths for the β1-null embryos were blocked because of an inner cell mass failure. However, the trophoblast function in the β1-null embryos was largely normal; both the decidual reaction was induced and outgrowths on fibronectin coated substrates were observed. These features were faithfully duplicated in the maspin null embryos. Current investigations are focusing on whether the functional similarities of these two deletion mutants are the result of an interaction between these two molecules during embryonic development.

As maspin was discovered as a putative tumor suppressor gene, we have carried out a series of animal experiments demonstrating that maspin is capable of inhibiting mammary tumor growth and metastasis. The observed suppression of primary tumor growth is probably due to the inhibition of angiogenesis and an increase in apoptosis. However, the mechanism responsible for the inhibition of metastasis is not fully understood. Initially, it was thought that maspin might inhibit tumor metastasis by inhibiting certain proteases. However, recent evidence has suggested that maspin functions independent of protease inhibition (Bass et al., 2002; Zhang et al., 1999). The study of maspin in early embryonic development provides a definitive answer to one of these mechanisms of maspin action. Tumor metastasis requires the detachment of tumor cells from the extracellular matrix as well as extensive invasion through the basement membrane and stroma (Liotta et al., 1991; Stetler-Stevenson, 1993). The increased cell adhesion caused by maspin could hinder such a process and thereby prevent tumor metastasis. Further experiments on the role of maspin in endoderm differentiation and cell-cell interactions during embryonic development will probably shed more light on our understanding of its role in tumor invasion and metastasis.

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