Sp1/Sp3 Compound Heterozygous Mice Are Not Viable: Impaired Erythropoiesis and Severe Placental Defects

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The ubiquitously expressed zinc finger transcription factors Sp1 and Sp3 play critical roles in embryonic development. *Sp1* knockout mice die around embryonic day 10.5. Mice lacking Sp3 are postnatal lethal. Mice heterozygous for either *Sp1* or *Sp3* are apparently normal, although slightly smaller. Here, we show that compound heterozygosity of *Sp1* and *Sp3* results in embryonic lethality accompanied by a spectrum of developmental abnormalities, including growth retardation, morphological alterations of the lung, impaired ossification, anemia, and placental defects. Anemia in *Sp1/Sp3* compound heterozygous mutant embryos is associated with impaired maturation of erythrocytes. Analyses of the placenta revealed a markedly reduced spongiotrophoblast layer and a severe disorganization of the labyrinth layer in *Sp1/Sp3* compound heterozygous as well as in Sp3-deficient mutant embryos. Our findings demonstrate that a threshold of Sp1 and Sp3 activity is required for normal embryonic development, suggesting that Sp1 and Sp3 act cooperatively to regulate downstream targets. *Developmental Dynamics 236:2235–2244, 2007.*

Key words: transcription factor; Sp1; Sp3; compound heterozygous mice; erythropoiesis; placenta

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INTRODUCTION

Sp transcription factors control the expression of a variety of different genes, including house keeping, tissue-specific, development-specific, and cell cycle-regulated genes (reviewed in Philipsen and Suske, 1999; Suske, 1999; Bouwman and Philipsen, 2002; Suske et al., 2005). Nine different *Sp* genes designated *Sp1* to *Sp9* have been identified in mammals (Suske et al., 2005). All Sp proteins share a highly conserved zinc finger DNA-binding domain at the C-terminus, the adjacent Buttonhead-box (btd-box), and the N-terminal Sp-box, a stretch of conserved amino acids of unknown function. Sp1 to Sp4 are further characterized by two glutamine-rich activation domains, whereas Sp5 to Sp9 contain proline-, alanine-, or serine/threonine-rich domains (Bouwman and Philipsen, 2002; Suske et al., 2005).

The ubiquitously expressed transcription factors Sp1 and Sp3 are structurally and evolutionary most closely related to each other, and they interact with GC- and GT-boxes with similar specificity and affinity (Hagen et al., 1992). The overall structural similarity between Sp1 and Sp3 and their ubiquitous expression pattern suggest that these two proteins are functionally equivalent. However, detailed biochemical and molecular studies have revealed significant differences. For instance, Sp1 but not Sp3 is a highly O-linked glycosylated

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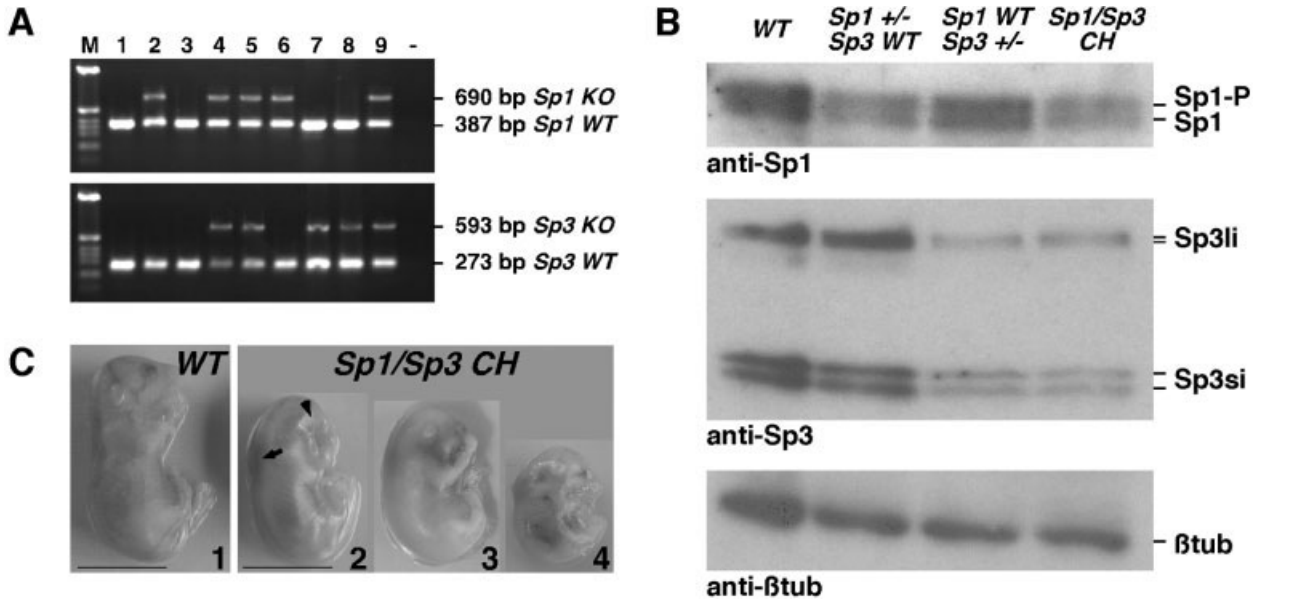

Fig. 1. Compound heterozygous (CH) *Sp1/Sp3* mutant embryos. **A:** Polymerase chain reaction analysis of littermates. DNA fragments amplified from wild-type (WT; *Sp1 WT* and *Sp3 WT*) and mutant alleles (*Sp1 KO* and *Sp3 KO*) are indicated. M, size marker. **B:** Immunoblot analysis of extracts (30 μg of protein) from embryonic day (E) 16.5 *WT*, $Sp1^{+/-}$, $Sp3^{+/-}$, and *Sp1/Sp3* compound heterozygous embryos. Signals referring to nonphosphorylated and phosphorylated Sp1 (Sp1 and Sp1-P) and long and short isoforms of Sp3 (Sp3li and Sp3si) are indicated. **C:** *Sp1/Sp3* compound heterozygous embryos at E18.5. Embryos 2, 3, and 4 represent typical $Sp1^{+/-}Sp3^{+/-}$ phenotypic variants. Viable embryos (embryo 2; 33.3%) are smaller and have characteristic blood-filled distensions on the back (arrow) and absent eyes (arrowhead). Dead embryos display a swollen body shape and a diffuse pink color (embryo 3; 41.7%), or different stages of resorption (embryos 4; 25.0%). Scale bar = 1 cm.

protein (Jackson and Tjian, 1988; Sapetschnig et al., 2004), and four expressed isoforms of Sp3 (Sapetschnig et al., 2004) are targets for SUMO modification mediating transcriptional repression (Ross et al., 2002; Sapetschnig et al., 2002, 2004).

Gene targeting experiments revealed that Sp1 and Sp3 also differ in their biological function, as Sp1- and Sp3-deficient mice exhibit distinct phenotypes. $Sp1^{-/-}$ embryos die around day 10.5 of embryonic development (E10.5) (Marin et al., 1997). Sp1-deficiency causes a cell-autonomous defect, because $Sp1^{-/-}$ ES cells injected in blastocysts contribute efficiently to early chimeric embryos, but after E11, these cells decline rapidly with no detectable contribution to any tissue of newborn mice (Marin et al., 1997).

$Sp3^{-/-}$ mice develop until the end of pregnancy but die immediately after birth due to respiratory failure (Bouwman et al., 2000). In addition, skeletal ossification and late tooth formation is impaired in Sp3-deficient E18.5 embryos (Bouwman et al., 2000; Göllner et al., 2001). Finally, the absence of Sp3 results in cell-autonomous differentiation defects of the erythroid and myeloid cell lineages (Van Loo et al., 2003), indicating an important function of Sp3 in hematopoiesis.

Disruption of the *Sp* genes revealed distinct functions of Sp1 and Sp3 in vivo. Sp1 and Sp3 may also have overlapping and cooperative functions at least at early developmental stages. Redundancy would explain why many GC/GT-box containing genes that were described previously as Sp1 and/or Sp3 target genes are not affected in individual *Sp*-targeted mice (Marin et al., 1997).

To elucidate the biological relationships and cooperative functions between Sp1 and Sp3, we investigated the effect of combined loss-of-function mutations in these *Sp* genes by intercrossing heterozygous *Sp1* and *Sp3* mutant mice to compound heterozygosity. $Sp1^{+/-}Sp3^{+/-}$ mice are not viable and the embryos display a variety of developmental abnormalities with variable penetrance. Most significantly, embryos suffer from anemia and display severe placental defects. Our results demonstrate that compound haploinsufficiency for Sp1 and Sp3 activity impedes normal embryonic development suggesting that Sp1 and Sp3 act cooperatively to regulate downstream targets in the developing placenta and other tissues.

RESULTS

Compound Heterozygous *Sp1/Sp3* Mice Are Not Viable

To investigate the functional cooperation and synergy between the transcription factors Sp1 and Sp3 in vivo, we generated compound *Sp1/Sp3* mutant mice by interbreeding $Sp1^{+/-}$ and $Sp3^{+/-}$ mice (Fig. 1A). Immunoblots with extracts from $Sp1^{+/-}Sp3^{+/-}$ embryonic tissues demonstrated that both proteins were reduced to the same extent as in the corresponding single heterozygous extracts (Fig. 1B).

Compound heterozygosity of Sp1 and Sp3 is lethal because no $Sp1^{+/-}Sp3^{+/-}$ mice were obtained at weaning. At E18.5, 20% of the pups were compound heterozygous, but only one-third was alive (Table 1). The appearance of the viable E18.5 embryos (Fig. 1C, embryo 2) resembled *Sp3 null* embryos. Consequently, we analyzed the lung and skeleton, both tissues affected in $Sp3^{-/-}$ embryos (Bouwman et al., 2000; Göllner et al., 2001). In

TABLE 1. Genotype Distribution of $Sp1^{+/-}$ x $Sp3^{+/-}$ Intercrossings[a]

	$Sp1^{+/-} \times Sp3^{+/-}$	$Sp1^{+/+}\ Sp3^{+/+}$	$Sp1^{+/-}\ Sp3^{+/+}$	$Sp1^{+/+}\ Sp3^{+/-}$	$Sp1^{+/-}\ Sp3^{+/-}$	
					alive	*dead*
P10	n = 62 (100%)	22 (35.5%)	18 (29.0%)	22 (35.5%)	0 (0.0%)	0 (0.0%)
E18.5	n = 60 (100%)	20 (33.3%)	19 (31.7%)	9 (15.0%)	4 (6.7%)	8 (13.3%)
E16.5	n = 157 (100%)	41 (26.1%)	47 (29.9%)	39 (24.9%)	30 (19.1%)	0 (0.0%)
E14.5	n = 183 (100%)	50 (27.3%)	48 (26.2%)	40 (21.9%)	45 (24.6%)	0 (0.0%)

[a]P, postnatal day; E, day of embryonic development; n, number of mice/embryos.

brief, the overall tissue structure of $Sp1^{+/-}Sp3^{+/-}$ E18.5 lungs is more compact compared with wild-type lung tissue (Supplementary Figure S1, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat). Ossification in the main skull bones as well as the ossification centers of the paws were reduced (Supplementary Figure S2). Both findings recapitulate the description of $Sp3^{-/-}$ embryos at the same stage of development (Bouwman et al., 2000; Göllner et al., 2001). Several viable E18.5 embryos were missing one or both eyes (Fig. 1C, embryo 2). Missing eyes were sometimes also observed in newborn $Sp1^{+/-}$ mice (unpublished observations).

Dead $Sp1^{+/-}Sp3^{+/-}$ embryos at E18.5 exhibited either a swollen body shape (five of eight pups) or were in different stages of resorption (Fig. 1C, embryos 3 and 4, respectively). At E14.5 almost 25% and at E16.5 20% of the embryos were *Sp1/Sp3* compound heterozygous (Table 1). The majority of the embryos died between E16.5 and E18.5 as revealed by a significant ($P < 0.05$) reduction in the number of living embryos at E18.5.

$Sp1^{+/-}Sp3^{+/-}$ Embryos Suffer From Anemia

To investigate the cause of lethality, we analyzed E16.5 and E14.5 *Sp1/Sp3* compound heterozygous embryos. These embryos displayed a pale body color (Fig. 2A,B), and the fetal livers were barely visible (E16.5) or smaller (E14.5) than the wild-type (Fig. 2A,B). In addition, approximately half of the $Sp1^{+/-}Sp3^{+/-}$ embryos developed edemas along the back. The body weight at E14.5 was only slightly reduced, and at E16.5 it was markedly reduced (67% of wild-type weight) (Fig. 2C). The relative weight of the fetal livers and placentas, but not of other organs (data not shown), was significantly reduced (Figs. 2D, 3A). Blood volume at E16.5 was also found to be markedly decreased in $Sp1^{+/-}$ $Sp3^{+/-}$ embryos (data not shown).

Histological sections of fetal livers from E16.5 revealed that $Sp1^{+/-}Sp3^{+/-}$ liver sinusoids were almost empty and erythrocytes were only present in the blood vessels (Fig. 2E). In wild-type and single heterozygous $Sp1^{+/-}$ or $Sp3^{+/-}$ liver sections the sinusoids were filled with erythrocytes (Fig. 2E, and data not shown). Analysis of E16.5 peripheral blood showed a major proportion of nucleated primitive, yolk sac-derived erythrocytes (34%, Fig. 2F) and a fourfold reduction of enucleated red blood cells (Fig. 2G), indicating an anemia in *Sp1/Sp3* compound heterozygous embryos at E16.5. A reduction of enucleated red blood cells or an increased number of nucleated primitive erythrocytes was observed neither in $Sp1^{+/-}$ nor in $Sp3^{+/-}$ single heterozygous embryos (Fig. 2G, and data not shown). The defect in erythroid development is already present at earlier stages. Flow cytometry analyses of $Sp1^{+/-}Sp3^{+/-}$ E14.5 fetal liver cells revealed altered cellular profiles relative to the wild-type and single heterozygous controls (Fig. 2H, and data not shown). The $CD71^{med\text{-}low}$ $Ter119^{high}$ population, which represents late stages of erythroid development (orthochromatic erythroblasts and reticulocytes; Zhang et al., 2003), is strongly reduced in the *Sp1/Sp3* compound heterozygous livers (5.4% of total fetal liver cells compared with 22.5% in wild-type). The portion of early progenitors is increased (78.7%) compared with wild-type fetal livers (60.3%), which may reflect a delay in the differentiation of erythroid progenitors.

Reduced Number of Erythroid Progenitors in E14.5 *Sp1/Sp3* Compound Heterozygous Fetal Livers

Colony-forming assays were performed to score erythroid progenitor cells in E14.5 fetal livers. A constant number of cells (5×10^5 cells per fetal liver) was seeded, thus compensating for differences in cellularity of the fetal livers. The number of erythroid burst-forming units (BFU-Es) was markedly reduced in $Sp1^{+/-}Sp3^{+/-}$ fetal liver cultures. Less BFU-Es were also found in $Sp1^{+/-}$ but not in $Sp3^{+/-}$ single heterozygous cultures (Fig. 2I). Erythroid colony-forming units (CFU-Es) were reduced in the $Sp1^{+/-}Sp3^{+/-}$ cultures but not in $Sp1^{+/-}$ and in $Sp3^{+/-}$ single heterozygous cultures (Fig. 2J). We also performed colony-forming assays for megakaryocyte progenitors (CFU-MKs), because E14.5 fetal liver sections indicated a reduced number of megakaryocytes in *Sp1/Sp3* compound heterozygous embryos (data not shown). We found that the number of CFU-MKs is twofold reduced in $Sp1^{+/-}Sp3^{+/-}$ and in $Sp1^{+/-}$ single heterozygous cultures as compared with wild-type cultures. A slight reduction of CFU-MKs was also found in $Sp3^{+/-}$ cultures (Fig. 2K). In conclusion, the frequency of erythroid BFU-E and CFU-E precursors as well as megakaryocyte CFU-MK precursors was markedly reduced in *Sp1/Sp3* compound heterozygous fetal livers. The reduced number of BFU-Es and CFU-Es is in accordance with the reduced number of erythrocytes in the circulation.

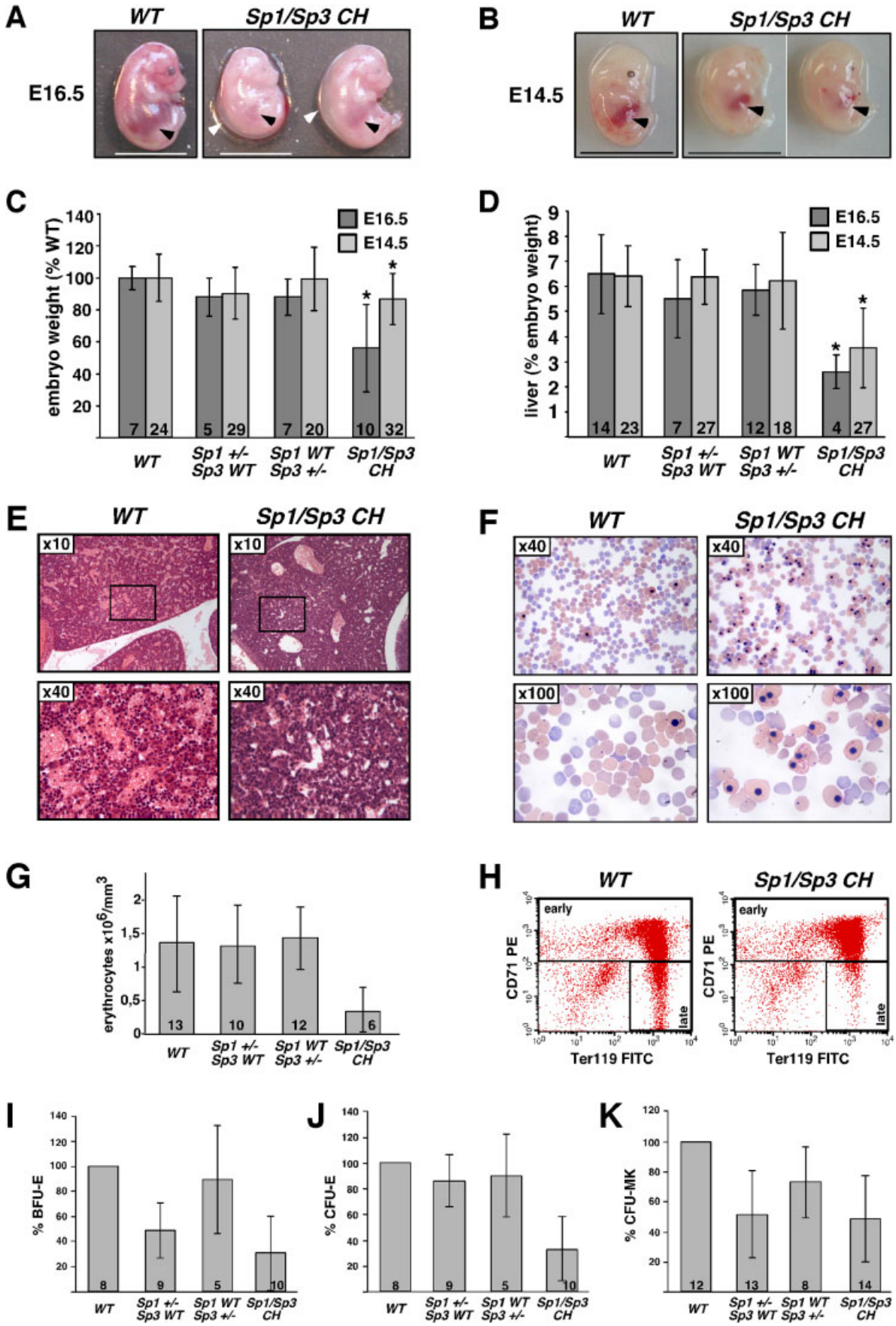
A
WT
Sp1/Sp3 CH
E16.5
B
WT
Sp1/Sp3 CH
E14.5
C
embryo weight (% WT)
E16.5
E14.5
7 24
5 29
7 20
10 32
WT
Sp1 +/- Sp3 WT
Sp1 WT Sp3 +/-
Sp1/Sp3 CH
D
liver (% embryo weight)
E16.5
E14.5
14 23
7 27
12 18
4 27
WT
Sp1 +/- Sp3 WT
Sp1 WT Sp3 +/-
Sp1/Sp3 CH
E
WT
Sp1/Sp3 CH
x10
x40
F
WT
Sp1/Sp3 CH
x40
x100
G
erythrocytes x10^6/mm^3
13
10
12
6
WT
Sp1 +/- Sp3 WT
Sp1 WT Sp3 +/-
Sp1/Sp3 CH
H
WT
Sp1/Sp3 CH
early
late
CD71 PE
Ter119 FITC
I
% BFU-E
8
9
5
10
J
% CFU-E
8
9
5
10
K
% CFU-MK
12
13
8
14

Fig. 2.

Highly Disorganized Placentae in *Sp1/Sp3* Compound Heterozygous and *Sp3 null* Mice

Because the relative placental weight in E14.5 $Sp1^{+/-}Sp3^{+/-}$ embryos was approximately 60% that of wild-type placentae (Fig. 3A) the placenta was analyzed in more detail. Several prominent layers are morphologically visible in murine placenta sections (Fig. 3B): the embryonic chorion plate; the labyrinth layer, where fetal and maternal blood meet for exchange of gas and nutrients; the spongiotrophoblast layer, which consists of spongiotrophoblast and glycogen-storing cells; and a layer of trophoblast giant cells separating the placenta from the maternal decidua (Fig. 3B). In E14.5 and E16.5 $Sp1^{+/-}Sp3^{+/-}$ placentae, all layers were present but the size of the spongiotrophoblast layer was visibly reduced (Fig. 3B,C). In 75% of the cases, the shape of the $Sp1^{+/-}Sp3^{+/-}$ placentae displayed a striking reversal of the curvature (Fig. 3C). In addition, large portions of the labyrinth layer appeared disorganized. Because these morphological abnormalities were more pronounced at E16.5, detailed analyses were performed at this stage. *Sp3 null* placentae were also included, as they have not been previously investigated.

Several trophoblast subtype-specific markers (Simmons and Cross, 2005) were monitored by in situ hybridization. *Trophoblast-specific-protein alpha* (*Tpbpa*) (Lescisin et al., 1988) is expressed in spongiotrophoblast cells and in trophoblast glycogen cells. *Tpbpa* staining confirmed that the spongiotrophoblast layer was thinner and of an irregular shape in all *Sp1/Sp3* compound heterozygous placentae (Fig. 3C, *Tpbpa*). A reduced spongiotrophoblast layer was also observed in *Sp3 null* placentae, although at lower penetrance (50%). No alterations were found in *Sp1* or *Sp3* single heterozygous placentae (data not shown).

We performed in situ hybridization with a probe for *prolactin-like protein C3* (*Prlpc3*), specifically expressed in spongiotrophoblast cells (D. Simmons, unpublished observations), and *protocadherin 12* (*Pcdh12*), specifically expressed in glycogen cells (Bouillot et al., 2006) of the spongiotrophoblast cell layer and the decidua. *Prlpc3* staining was significantly reduced, and only very few *Pcdh12*-positive glycogen cells were detected in *Sp1/Sp3* compound heterozygous and $Sp3^{-/-}$ placentae (Supplementary Figure S3), indicating a reduction in both spongiotrophoblast and trophoblast glycogen cells in *Sp1/Sp3* compound heterozygous and *Sp3 null* placentae. *Proliferin* (*Plf*) expression in trophoblast giant cells at the boundary of the maternal decidua and the spongiotrophoblast layer (Rossant and Cross, 2001) was also investigated. *Plf*-expressing trophoblast giant cells were present in *Sp1/Sp3* compound heterozygous as well as in $Sp3^{-/-}$ mutant placentae (Fig. 3C, *Plf*).

Obvious alterations were also visible in the labyrinth layer. The wild-type labyrinth is composed of highly branched maternal blood sinuses and fetal blood vessels in close proximity and has a regular porous appearance. The maternal blood spaces are separated from the fetal blood spaces by four layers of cells: a mononuclear trophoblast layer of sinusoidal trophoblast giants cells lining the maternal sinuses, two layers of syncytiotrophoblast cells, and a layer of fetal endothelial cells lining the fetal blood vessels (Simmons and Cross, 2005). In $Sp1^{+/-}Sp3^{+/-}$ mutants, the regular architecture of the labyrinth was highly disorganized in three of four cases (Fig. 3D). Clustered streaks with small nucleated cells were visible. Similar clusters of cells were also found in $Sp3^{-/-}$ labyrinth layers, although to a lesser extent (Fig 3D, HE). To investigate the organization of different cell types in the labyrinth, we performed in situ hybridization and immunohistochemical analyses with probes and antibodies specific for trophoblast and fetal cell types. In situ hybridization for *cathepsin q* (*Ctsq*) (Ishida et al., 2004), a gene specific for mononuclear trophoblasts (also called sinusoidal trophoblast giant cells) (Simmons et al., 2007) showed that the maternal blood spaces are enlarged and disorganized in *Sp1/Sp3* compound heterozygous and in *Sp3 null* placentae (Fig. 3D, *Ctsq*). *Glial cell missing 1* (*Gcm1*), expressed in some syncytiotrophoblast cells at E16.5 (Basyuk et al., 1999), was almost absent in E16.5 compound heterozygous *Sp1/Sp3* placentae and strongly reduced in *Sp3*-deficient placentae (Supplementary Figure S3). However, *Krt18* expression, a marker for all trophoblast cells (Hashido et al., 1991), indicated the presence of trophoblast cells other than $Ctsq^{+}$ si-

Fig. 2. Impaired erythropoiesis in *Sp1/Sp3* compound heterozygous (CH) mutant embryos (*Sp1/Sp3* CH) at embryonic day (E) 16.5 and E14.5. **A:** E16.5 wild-type (WT, left) and *Sp1/Sp3* compound heterozygous (right) littermates. $Sp1^{+/-}Sp3^{+/-}$ embryos are pale and developed edemas (white arrowheads) along the back. Fetal livers (black arrowheads) are only faintly visible. **B:** E14.5 wild-type (left) and $Sp1^{+/-}Sp3^{+/-}$ (right) littermates. Fetal livers (black arrowheads) are small. The indicated scales correspond to 1 cm. **C:** Relative embryo weights at E16.5 and E14.5, wild-type embryos were set to 100%. The number of mice weighed is indicated at the bottom of the bars. **D:** Relative liver weight per embryo showing that the liver weight of $Sp1^{+/-}Sp3^{+/-}$ embryos is markedly reduced. Error bars indicate the standard deviation. *$P < 0.05$ WT vs. CH. **E:** Hematoxylin and eosin–stained liver sections at ×10 and ×40 magnification. The framed areas correspond to those shown at higher magnification. Fetal liver sinusoids of wild-type embryos are filled with blood. In $Sp1^{+/-}Sp3^{+/-}$ fetal livers, the sinusoids are virtually empty. **F:** Blood smears of E16.5 peripheral blood at ×40 and ×100 magnification, stained with May-Grünwald/Giemsa solutions. In blood from $Sp1^{+/-}Sp3^{+/-}$ embryos, up to 34% of the erythrocytes were nucleated compared with approximately 1% in wild-type blood. **G:** Number of enucleated erythrocytes per mm^3 of peripheral blood. The number of blood samples counted from different mice is indicated at the bottom of the bars. **H:** Flow cytometry of E14.5 wild-type (left) and *Sp1/Sp3* compound heterozygous (right) fetal liver cells from representative littermates. Freshly isolated fetal liver cells were double-labeled with phycoerythrin (PE)-conjugated anti-CD71 and fluorescein isothiocyanate (FITC) -conjugated anti-Ter119 mouse antibodies. Dead 7-aminoactinomycin-D (7-AAD)-positive cells were excluded from the analysis. Axes indicate relative logarithmic fluorescence units for PE (*y*-axis) and FITC (*x*-axis). Early stages of erythrocyte maturation are highly CD71 positive, during maturation Ter119 expression increases and CD71 intensity declines. **I–K:** Colony-forming assays with E14.5 fetal liver cells from embryos with the indicated genotype. The number of erythroid burst-forming units (BFU-Es; I), erythroid colony-forming units (CFU-Es; J), and colony-forming assays for megakaryocyte progenitors (CFU-MKs; K) was determined with 5×10^5 liver cells per culture. Within each litter, the wild-type value was set to 100%. Error bars indicate the standard deviation.

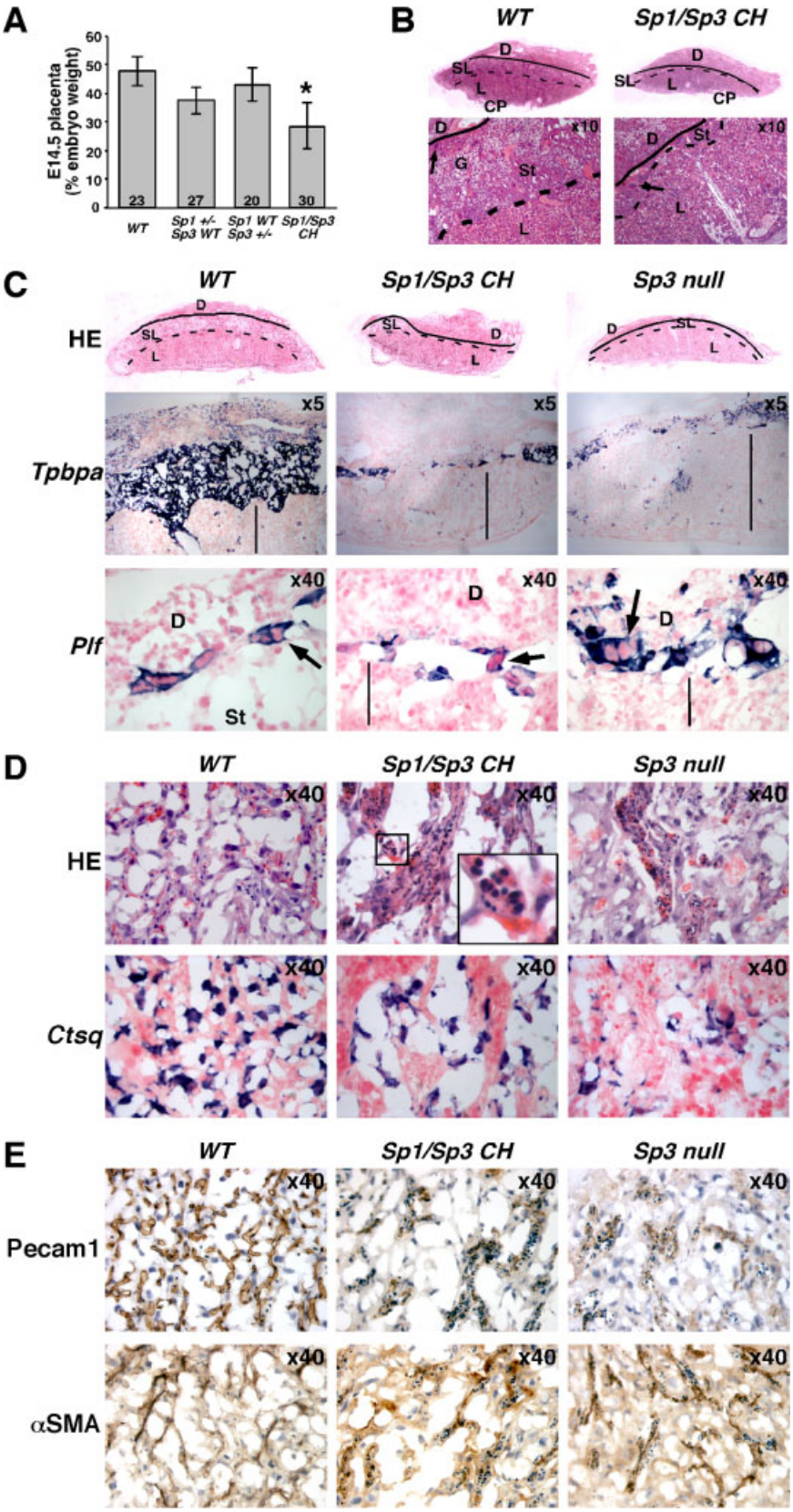

Fig. 3. Analyses of the placental spongiotrophoblast and labyrinth layer. **A:** Relative placenta weights at embryonic day (E) 14.5. The number of placentae weighed is indicated at the bottom of the bars. Error bars indicate the standard deviation. *$P < 0.05$ wild-type (WT) vs. compound heterozygous (CH). **B:** Hematoxylin and eosin (H&E) staining of E14.5 placentae from wild-type (left) and $Sp1^{+/-}Sp3^{+/-}$ (right) mice. D, decidua; SL, spongiotrophoblast layer; St, spongiotrophoblast cells; G, glycogen-storing cells; L, labyrinth layer; CP, chorion plate. The arrows point to trophoblast giant cells. Continuous lines depict the demarcations between the spongiotrophoblast layer and the decidua, and the dashed lines between the labyrinth layer and the spongiotrophoblast layer. **C:** Trophoblast cell type marker analyses of the spongiotrophoblast layer (SL) at E16.5 in wild-type, *Sp1/Sp3* compound heterozygous (*Sp1/Sp3 CH*), and *Sp3 null* mice by in situ hybridization (blue staining). Original magnifications are indicated. Abbreviations are as in B. The vertical lines indicate the dimension of the labyrinth. *Tpbpa*, *Trophoblast-specific protein alpha*, marker for spongiotrophoblast cells and trophoblast glycogen cells in the periphery; *Plf*, *proliferin* expressed in trophoblast giant cells at the boundary of the maternal decidua and the spongiotrophoblast layer, and in a subpopulation of spongiotrophoblast cells. The arrows point to trophoblast giant cells. **D:** Labyrinth layer of E16.5 placentae. The H&E-stained sections at ×5 and ×40 magnification indicate that the regular architecture of the labyrinth layer is disorganized in *Sp1/Sp3* compound heterozygous and $Sp3^{-/-}$ placentae. In situ hybridization for *Ctsq* (*cathepsin q*) is specific for mononuclear trophoblasts lining maternal sinusoids (sinusoidal giant cells, blue staining). **E:** Analyses of fetal-derived cells by immunohistochemistry (brown staining). Pecam1, platelet/endothelial cell adhesion molecule 1 is expressed in endothelial cells; αSMA, alpha smooth muscle actin in pericytes.

nusoidal giant cells within the labyrinth layer (Supplementary Figure S3). The Krt18 signals on *Sp1/Sp3* compound heterozygous and *Sp3 null* placenta sections were much stronger as compared with wild-type placentae, indicating that Sp3 might negatively regulate Krt18 expression as previously proposed (Apt et al., 1996).

TABLE 2. Phenotypes of Different *Sp1* and *Sp3* Mutants[a]

	$Sp1^{+/-}$	$Sp3^{+/-}$	$Sp1^{-/-}$	$Sp3^{-/-}$	$Sp1^{+/-}$ $Sp3^{+/-}$
Viability	Viable	Viable	E10.5 lethal	Postnatal lethal	E16.5-E18.5 lethal
Affected organs/ defects	Slightly growth retarded	Slightly growth retarded	Multiple abnormalities	E18.5: ossification, lung, tooth	E18.5: ossification, lung
				>E14.5: hematopoiesis	>E14.5: erythropoiesis
				>E14.5: placenta	>E14.5: placenta

[a]P, postnatal day; E, day of embryonic development.

The clustered streaks with small nucleated cells visible on $Sp1^{+/-}Sp3^{+/-}$ and $Sp3^{-/-}$ placenta sections were negative for trophoblast-specific marker genes. Immunohistological staining for Pecam1, a marker for endothelial cells (Ilan and Madri, 2003), and alpha smooth muscle actin (αSMA), a marker for smooth muscle cells/pericytes (McHugh, 1995), demonstrated that these structures contained fetal vessels (Fig. 3E). In conclusion, the morphological alterations within the labyrinth layer most likely result in an impaired exchange of gas, nutrients, and waste between embryo and mother.

DISCUSSION

Many genes described in the literature as Sp1 and/or Sp3 target genes are not affected in individual Sp-targeted mice, suggesting redundancy in vivo. To address this issue, we sought originally to generate embryos deficient in both proteins. However, we found that already compound heterozygosity is lethal at late embryonic stages. *Sp1/Sp3* compound heterozygous mice display some striking similarities to Sp3-deficient mice (Table 2). The aberrant morphology of the lungs and impaired bone ossification was observed in both mutant mice. However, compound haploinsufficient *Sp1/Sp3* mice did not result in a delay in the formation of the ameloblast layer of the developing teeth (data not shown), a hallmark of $Sp3^{-/-}$ E18.5 embryos (Bouwman et al., 2000). In both, *Sp3 null* (Van Loo et al., 2003) and *Sp1/Sp3* compound heterozygous mutants, markedly more nucleated erythrocytes were observed in blood smears. Such a high portion of nucleated erythrocytes is typical for anemia when the number of mature, enucleated definitive erythrocytes is not sufficient. The reduced number of enucleated mature erythrocytes could originate from an intrinsic defect in the developmental profession of definitive erythrocytes. In *Sp3 null* livers the delay in the formation of definitive erythrocytes is very likely due to an intrinsic developmental defect of erythroid cells because the BFU-Es and the CFU-Es were comparable to wild-type livers (Van Loo et al., 2003). This finding appears to be different in *Sp1/Sp3* compound heterozygous mice. Here, the amount of BFU-Es and CFU-Es were markedly reduced. Because also fewer CFU-MKs were present in *Sp1/Sp3* compound heterozygous fetal livers, this finding suggests that the formation of hematopoietic progenitor cells (erythroid/megakaryocyte progenitors, EMP) is impaired.

The placenta is crucial for mammalian development as it functions as the fetal–maternal interface for nutrient, gas, and waste exchange, and in addition produces several hormones, which direct maternal responses to pregnancy. Some of the defects observed in *Sp1/Sp3* compound heterozygous may thus be secondary to the placental defects, as nutrient and oxygen deprivation as well as altered placental hormone secretion may contribute to a delay of various differentiation processes. Placental abnormalities as the primary cause for other developmental abnormalities have been shown for Rb-deficient mice (Wu et al., 2003). The most pronounced phenotype of *Sp1/Sp3* compound heterozygous placentae is a markedly reduced spongiotrophoblast layer and a disorganized labyrinth layer. Within the spongiotrophoblast layer both spongiotrophoblast cells and trophoblast glycogen cells are reduced. This finding suggests that the dose of Sp1 and Sp3 is not critical for the formation of spongiotrophoblast or trophoblast glycogen cells per se but rather for the proliferation or maintenance of a common precursor.

A dramatically reduced spongiotrophoblast layer has also been observed in several other lethal knockout mice, including *Bruce* (Lotz et al., 2004), *Hif1* (Xiao et al., 1999), and *Rap250* (Antonson et al., 2003). Whether expression of any of these genes is reduced in *Sp1/Sp3* compound heterozygous embryos and might thus contribute to the observed phenotype remains to be established. For the *Hif1* gene, it was shown that *Hif1A* gene transcription is dependent on *Sp* binding sites (Minet et al., 1999).

Haploinsufficiency of both Sp1 and Sp3 also leads to a severe disruption of the normal labyrinth layer architecture. The normal wild-type labyrinth has a sponge-like porous appearance consisting predominantly of syncytiotrophoblast and sinusoidal giant cells surrounding maternal blood sinuses and fetal vessels. Syncytiotrophoblast cells form the major nutrient transport surfaces within the labyrinth layer. In *Sp1/Sp3* compound heterozygous mice, the normally well-organized structure of the labyrinth is severely disrupted. Although disorganized, fetal endothelial cells, pericytes, and sinusoidal trophoblast giant cells are all present. Interestingly, *Gcm1*, normally expressed in at least some syncytiotrophoblast cells until E17.7 (Basyuk et al., 1999) was clearly detectable in E16.5 wild-type placentae but not in *Sp1/Sp3* compound heterozygous mice. It is unclear whether syncytiotrophoblast cells in *Sp1/Sp3* compound heterozygous placentae are reduced in number, as measured by *Gcm1* expression, or whether they have an altered gene ex-

pression profile, because *Krt18*$^+$ trophoblast cells, other than *ctsq*$^+$ sinusoidal trophoblast giant cells, are clearly present. More accurate markers of the syncytiotrophoblast layers, particularly at later gestational ages, are needed to further clarify this point.

Our results demonstrate that a threshold of Sp1 and Sp3 activity is required for normal embryonic development. Two scenarios might explain this observation. One possibility would be redundancy. In this scenario, Sp1 and Sp3 would activate or repress the same set of target genes by binding to the same promoter elements. Because numerous promoters contain Sp1/Sp3 binding sites, a twofold decrease in Sp1 and Sp3 levels could alter the expression levels of many different genes leading to pronounced physiological defects in vivo. The alternative scenario would be that Sp1 and Sp3 regulate different sets of genes. A slight deregulation of each set of genes would not cause severe developmental defects because single heterozygous *Sp1* and *Sp3* mice are viable and fertile. However, deregulation of both sets of genes would have synergistic effects leading to the severe abnormalities observed in *Sp1/Sp3* compound heterozygous mice. The two scenarios are not mutually exclusive, and at present we cannot distinguish between these two possibilities. Very likely, both redundancy and synergy, account for the observed defects in *Sp1/Sp3* compound heterozygous mice as a great many of genes contain functionally important Sp-binding sites in their promoters and other regulatory elements. Redundancy and synergy could explain why the Sp factors regulating specific genes in vivo are still largely unknown.

There are numerous studies demonstrating that Sp1 and Sp3 differ in their ability to activate GC-box containing promoters in reporter assays. In addition, molecular and biochemical investigations have shown marked differences between Sp1 and Sp3 at the molecular and biochemical level (Suske, 1999; Bouwman and Philipsen, 2002). How these biochemical properties contribute to the different phenotypes of targeted *Sp1* and *Sp3* mice, as well as to the defects in *Sp1/Sp3* compound heterozygous mice described in this study remains to be clarified.

EXPERIMENTAL PROCEDURES

Mouse Handling and Breedings

Sp1 and *Sp3* single heterozygous mice (Marin et al., 1997; Bouwman et al., 2000) back-crossed to the C57BL/6 background were intercrossed to obtain compound heterozygous progeny. For timed pregnancies, animals were mated overnight and females examined for a vaginal plug in the morning. Noon of the day of vaginal plug appearance was considered day 0.5 postcoitum (E0.5). Pregnant mice were killed by cervical dislocation, and embryos were dissected from uteri at different time points of pregnancy.

Genotyping of Mice by Polymerase Chain Reaction

DNA was prepared from tail snips with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and analyzed in two independent polymerase chain reaction (PCR) reactions for the presence of the *Sp1* and *Sp3* wild-type and targeted alleles. PCR conditions were 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C for 31 cycles. For genotyping of each *Sp* wild-type and targeted allele, three primers were used: a common primer specific for the *neomycin resistance* gene (5′-CATCGCCTTCTATCGCCTTCTTGA-3′), and two *Sp* gene-specific primers. *Sp1* sense primer, 5′-AATTTGCCTGCCCTGAGTGC-′3; *Sp1* antisense primer, 5′-TTGGACCCATGCTACCTTGC-3′. *Sp3* sense primer, 5′-TGTTCAGACACTCACGCTTGGTCA-3′; *Sp3* antisense primer, 5′-GGACGATTCTATGATGCCTCCTAC-3′. The sizes of the amplified fragments are as follows: *Sp1WT*, 387 bp; *Sp1KO*, 690 bp; *Sp3WT*, 273 bp; and *Sp3KO*, 593bp.

Immunoblot Analysis

The E16.5 tissue was frozen in liquid nitrogen, pulverized with a frozen mortar and pestle, and homogenized in sodium dodecyl sulfate (SDS) lysis buffer (Sapetschnig et al., 2002). Lysates were boiled and then centrifuged for 10 min, and supernatants were stored at −80°C. A total of 30 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and probed with the appropriate antibodies. The following antibodies were used: rabbit anti-Sp1 polyclonal IgG (Hagen et al., 1994), 1:3,000; rabbit anti-Sp3 polyclonal IgG (Santa Cruz Biotechnology), 1:3,000; rabbit anti–β-tubulin polyclonal IgG (Santa Cruz Biotechnology), 1:15,000; and horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham Life Science), 1:30,000. The peroxidase reaction was visualized by chemiluminescent substrate (Immobilon Western, Millipore, Billerica, MA).

Histological Analysis

Embryos and placentae were dissected at different days of embryonic development (E14.5, E16.5, E18.5), cut in half longitudinally (E18.5, placentae) or directly fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) at 4°C overnight, and subsequently embedded in paraffin according to standard procedures. Sections were stained with hematoxylin and eosin (H&E-staining) according to standard procedures.

Colony Forming Assays

Single-cell suspensions of E14.5 fetal livers were prepared using a 20-gauche needle and syringe and washed in Iscove's modified Dulbecco's medium containing 2% fetal bovine serum (StemCell Technologies, Vancouver, Canada). Cultures for BFU-E and CFU-E were performed using methylcellulose medium Methocult M3334 containing recombinant human erythropoietin (3 U/ml; StemCell Technologies, Vancouver, Canada). CFU-MK colonies were cultured in methylcellulose medium Methocult M3231 supplemented with recombinant murine thrombopoietin (5 ng/μl; StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions. A total of 5×10^5 cells per liver were plated on 35-mm tissue culture dishes in dupli-

cate and cultured at 37°C, 5% CO_2. Colonies were scored by morphology after 3 (CFU-E) and 8 (BFU-E, CFU-MK) days in culture.

Flow Cytometry Analysis

Single-cell suspensions of E14.5 fetal livers were incubated with phycoerythrin conjugated rat anti-mouse CD71 antibody and fluorescein isothiocyanate–conjugated rat anti-mouse Ter119 antibody (BD Biosciences Pharmingen). The 7-aminoactinomycin-D (7-AAD; BD Biosciences Pharmingen) staining was used to exclude dead cells from the analysis. Flow cytometry was carried out on a FACSCalibur (BD Biosciences, Heidelberg, Germany) with 5×10^4 events counted per sample.

Bone and Cartilage Staining

The E18.5 embryos were skinned and eviscerated, and the carcasses were stained with Alcian Blue (cartilage) and Alizarin Red (bone) using standard procedures (Hogan et al., 1994).

Blood Smears and Blood Cell Counting

Blood samples at E16.5 were collected by bleeding dissected embryos. Coagulation was prevented by adding one-fifth volume of 0.05 M ethylenediaminetetraacetic acid (EDTA). Blood smears were prepared on slides with 5 to 20 μl of blood. Smears were dried at room temperature for 2 hr, stained with May-Grünwald solution for 5 min, washed in water, stained in 4% Giemsa solution for 20 min and finally washed in water. Slides were dried overnight at room temperature. Per blood smear, a minimum of 4,700 cells were counted. Fifteen microliters of EDTA–blood were used for blood cell counting by Coulter Counter (Scil Vet abc, Scil animal care company GmbH, Viernheim, Germany).

In Situ Hybridization of Placental Slides

In situ hybridizations are described in detail in Simmons et al. (2007).

Statistics

The statistical significance of weight differences (Figs. 2C,D, 3A) was determined using an unpaired Student's *t*-test. Data are presented as mean ± standard deviation of the mean. The Fisher's exact test was used to calculate the significance of the reduction of living $Sp1^{+/-}Sp3^{+/-}$ embryos between E16.5 and E18.5 (Table 1).

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