Murine inner cell mass-derived lineages depend on Sall4 function

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Sall4 is a mammalian Spalt transcription factor expressed by cells of the early embryo and germ cells, an expression pattern similar to that of both Oct4 and Sox2, which play essential roles during early murine development. We show that the activity of Sall4 is cell-autonomously required for the development of the epiblast and primitive endoderm from the inner cell mass. Furthermore, no embryonic or extraembryonic endoderm stem cell lines could be established from Sall4-deficient blastocysts. In contrast, neither the development of the trophoblast lineage nor the ability to generate trophoblast cell lines from murine blastocysts was impaired in the absence of Sall4. These data establish Sall4 as an essential transcription factor required for the early development of inner cell mass-derived cell lineages.

Mammalian development starts with the formation of the blastocyst, which subsequently implants to give rise to a newborn embryo and all its specialized organs (1, 2). The blastocyst already harbors three distinct tissue lineages; the epiblast, the extraembryonic endoderm, and the trophectoderm (3, 4). ES cells and extraembryonic endoderm stem cells have been established from the inner cell mass (ICM), and trophoblast stem (TS) cells from the trophectoderm (5, 6). Recently, major progress has been made in understanding the transcriptional regulatory circuitry that governs these early lineage decisions in the early mouse embryo and ES cells (7, 8). Genetic studies in mice demonstrated that the transcription factors Oct4/Pou5F1, Nanog, and Sox2 are crucial regulators of epiblast and ES cell identity (9–12). In contrast, Gata6 is a transcription factor required within the primitive endoderm at the epiblast stage (13), whereas, in the absence of Cdx2, trophectoderm fate is not maintained (14). Recently, a reciprocal interaction between Oct4 and Cdx2 has been shown to be essential for the establishment of the trophectoderm lineage (15). The Spalt/Sall transcription factor family was initially discovered in Drosophila and later found to be conserved in Caenorhabditis elegans, Xenopus, fish, mouse, and humans (16). In Drosophila, Spalt proteins possess homeotic function during development and are required in photoreceptor differentiation and planar cell polarity (17). Genetic studies in C. elegans demonstrated that the spalt-like gene sem-4 represses Hox and LIM domain containing transcription factors (18). In humans, mutations at the SALL4 locus result in a range of clinically overlapping phenotypes, including Okihiro syndrome, Holt–Oram syndrome, acrorenal-ocular syndrome, and thalidomide embryopathy (19). In this study we demonstrate that murine Sall4, like Oct4, Sox2, and Nanog, is important for ICM-derived cell lineages, the epiblast, and primitive endoderm, adding to our understanding of the transcriptional components underlying early mammalian development.

Results and Discussion

Sall4 protein is already seen at the two-cell stage because of maternal contribution and then appears again in some cells of the 8- to 16-cell-stage embryo after zygotic transcription has initiated. In late blastocysts, the Sall4 RNA and Sall4 protein become enriched in the ICM. Within the epiblast expression is maintained uniformly until the mid–late streak stages and is also evident in the derivatives of the primitive endoderm, including Reichert’s membrane. (Fig. 1 A–C and Fig. 6 A–C, which is published as supporting information on the PNAS web site). By 10.5 days postcoitum (dpc), Sall4 RNA is seen within progenitor populations of the brain, neural tube, pituitary gland, heart, liver, somites, limbs, and also in the female germ line (Fig. 6 D–F).

To define the roles of Sall4 in early mammalian development we generated two different Sall4 mutant alleles via homologous recombination in ES cells. In the first mutant allele, hereafter called Sall4floxed, exon 2, which encodes 80% of the Sall4 protein, was replaced by an internal ribosomal entry site (IRES) and a Histone2BEGFP coding sequence. In the second allele, Sall4homozygous we have flanked exon 2 of Sall4 with loxP sites which results in the allele, Sall4Δexon2 upon Cre expression, which has lost most of the Sall4 coding sequence including the first six zinc fingers of the Sall4 protein (Fig. 7, which is published as supporting information on the PNAS web site). Live-born homozygous Sall4 mutant pups from heterozygous intercross matings of our two mutant alleles were never observed. Even among prestreak embryos genotyped by PCR, none of the Sall4homozygous/Sall4homozygous or Sall4homozygous/Sall4Δexon2 genetic combinations were detected. However, empty implantation sites were observed regularly. Thus, Sall4 deficiency results in perimplantation lethality before egg cylinder formation.

Blastocysts from Sall4floxed/homozygous intercrosses displayed in roughly 1:2:1 ratio strong : intermediate : no GFP fluorescence. Embryos sorted by fluorescence and analyzed by RT-PCR, confirmed that embryos with bright fluorescence were Sall4Δexon2 homozygous embryos and lacked any Sall4 immunoreactivity (Fig. 1 D–H). Thus, introduction of the H2bEFP coding sequence into the Sall4 locus allows genotyping of 3.5-dpc Sall4 mutant blastocysts by fluorescence intensity without killing them. All future experiments were done with the Sall4Δexon2 allele. Immunofluorescence staining for Oct4, Nanog, Sox2, Cdx2, and Tra2a as well as RT-PCR analysis for Fgf4, Gat6, and PEM did not reveal significant differences between WT and homozygous Sall4Δexon2 mutant embryos (Fig. 1 I–O). Thus, development of the presumptive trophectoderm and ICM compartments, as well as proliferation and cell survival apparently proceeds normal in the absence of Sall4 protein until the blastocyst stage.

3.5-dpc embryos from Sall4Δexon2 heterozygous intercrosses were sorted according to their fluorescence intensity and reimplanted separately in pseudopregnant females. In all cases indistinguishable decidual swellings at 5.5 dpc were observed. Whereas implantation sites resulting from control blastocysts contained embryos of the expected age, no ICM-derived em-
counterstaining is blue.

Cdx2 (trophectoderm marker) (trophectoderm cell types in the postimplantation embryo in a reciprocal any obvious differences (marker analysis on WT and Sall4 immunostaining (express GFP fluorescence intensity and can be sorted into strong (Fig. 2 completely absent from mutant epiblast of sections from control implantation sites but was Sall4 RNA as shown by RT-PCR using actin as a control and do not show Sall4 immunostaining (D and E). Indirect immunofluorescence and RT-PCR marker analysis on WT and Sall4 mutant expanded blastocysts does not reveal any obvious differences (I–O). Nanog (epiblast marker) (U and M), Troma1 (trophectoderm/epithelial marker) (U and M), Oct4 (ICM marker) (X and N), Cdx2 (trophectoderm marker) (L and O), and Sox2 (L and O) are shown. DAPI counterstaining is blue.

bryonic structures were recognizable in Sall4-null blastocyst implantations (Fig. 2 A and B). This indicates that the empty implantation sites observed in heterozygous Sall4H2bEGFP intercrosses likely result from Sall4H2bEGFP homzygous blastocysts. RNA in situ hybridization was used to further characterize the Sall4 mutant phenotype. Oct4 showed a clear signal in the epiblast of sections from control implantation sites but was completely absent from mutant Sall4H2bEGFP implantation sites (Fig. 2 C and G). In contrast, H19, which stains all extraembryonic cell types in the postimplantation embryo in a reciprocal pattern to Oct4, was expressed within the area corresponding to Sall4H2bEGFP mutant implantation site, showing that extraembryonic cell types were present. Many positive cells invaded the uterine stroma, typical of trophoblast giant cells (Fig. 2 D and H). Although few Cdx2 and Gata6-positive cells at 5.5 dpc were present in mutant implantation sites (Fig. 2 I and J), they did not give rise to recognizable structures at later embryonic stages (Fig. 8, which is published as supporting information on the PNAS web site). These results further demonstrate that no ICM-derived structures can be detected after implantation of Sall4 mutant embryos.

The data presented so far suggested a specific defect in the developmental potential or viability of the ICM in Sall4H2bEGFP mutant embryos. Therefore, in vitro culture experiments were performed to assess the developmental potential of Sall4H2bEGFP mutant blastocysts. Blastocysts from Sall4H2bEGFP intercrosses were sorted by fluorescence intensity and placed in ES cell medium. All embryos readily outgrew a layer of trophoblast giant cells independent of their genotype (Fig. 3 A–D). In WT and heterozygous embryos distinctive ICM-derived cell masses appeared and continuously grew during the culture period (Fig. 3 A–C). In contrast, the ICM of homozygous mutant Sall4H2bEGFP embryos did not expand significantly (Fig. 3 D–F). Consistent with this observation, few mitotic cells were detected

Fig. 2. Sall4H2bEGFP mutant embryos lack ICM derivatives. Shown are paraffin sections of PAS-stained, presorted, and reimplanted control (A) and mutant (B) embryos at 5.5 dpc. Marker RNA in situ analysis was performed on reimplanted, pregenotyped embryos at 5.5 dpc (WT, C–F; mutant, G–J). Sall4 mutant embryos do not form epiblast or primitive endoderm derivatives. Shown are Oct4 (epiblast maker) (C and G) and Gata6 (primitive endoderm marker) (F and J). Disorganized extraembryonic tissue is present in Sall4 mutant implantation sites as shown by positive staining for Cdx2 (E and I). Giant cells are clearly visible in H19 staining (D and H).
in mutant explants by phospho-HistoneH3 staining after 5 days of culture, suggesting that proliferation of the ICM-derived cell masses had ceased (Fig. 3 G and M). Importantly, mutant cells did not show an increased rate of apoptosis, as determined by TUNEL staining (data not shown). Surprisingly, RNA in situ hybridization performed on the ICM-derived cell mass of Sall4 homozygous mutant blastocyst outgrowths showed a strong positive signal for H19, an extra embryonic cell marker, after 5 days of culture (Fig. 3 H and N). Likewise, a significant proportion of cells were positive for Cdx2 which was not seen in WT outgrowths (Fig. 3 L and R). In contrast, RNA in situ analysis for Gata6 and Hnf4 detected few primitive endoderm cells (Fig. 3 I, J, O, and P). Rarely, Oct4-positive cells were found after 5 days of culturing (Fig. 3 K and Q). In contrast, trophoblast cell cultures were readily obtained from WT and mutant blastocyst outgrowths cultured in medium supplemented with FGF4 and heparin (6), which differentiated into trophoblast giant cells after FGF4 withdrawal (Fig. 9, which is published as supporting information on the PNAS web site). These results demonstrate that Sall4 deficient blastocyst cells do not in general have a proliferation block.

To further characterize the homozygous mutant Sall4 blastocysts we used immunosurgery to remove the trophectoderm layer and to isolate the ICM from 3.75-dpc blastocysts (20). When ICMs of Sall4-null blastocysts were cultured in ES cell medium, trophoblast giant cells could be identified by their morphology whereas under the same conditions no trophoblast giant cells were seen in heterozygous Sall4 ICMS. Surprisingly, withdrawal of FGF4 from Sall4-null ICM-derived cultures yielded trophoblast giant cells (Fig. 4D). RT-PCR expression analysis further confirmed the observed morpholog-
ical differences. Primitive endoderm markers were absent in homozygous mutant Sall4\textsuperscript{-}\textsuperscript{floxed} cells (Fig. 4E). This experiment demonstrates that there is a cell-autonomous requirement for Sall4 function to establish extra embryonic endoderm cell lines from primitive endoderm. Although we cannot rule out that some trophectoderm cells survived the immunosurgery, we favor the interpretation that the observed trophectoderm cell lines originate from homozygous mutant Sall4 ICM cells (21). These results underscore the cell lineage-specific defect of Sall4-null blastocysts and suggest a possible switch in the developmental potential of the ICM.

To determine whether Sall4 is required cell-autonomously in the epiblast, we performed morula aggregation experiments (22). Sall4 mutant cells contributed to the ICM in chimeric blastocysts (Fig. 5A and Fig. 10A, which is published as supporting information on the PNAS web site). Chimeric blastocysts of a Sall4\textsuperscript{floxed}/heterozygous/Rosa26 or Sall4\textsuperscript{floxed}/null/Rosa26 genotype mix were implanted independently into pseudopregnant females. Embryos recovered at 9.5 dpc were stained for β-galactosidase activity. Sall4\textsuperscript{null} heterozygous/Rosa26 blastocysts resulted in embryos with various degree of chimerism (Figs. 5B and 10B). In contrast, all embryos recovered from Sall4\textsuperscript{null}/Rosa26 blastocysts were derived from Rosa26 cells only. (Figs. 5C and Fig. 10C) demonstrating that Sall4 is required cell-autonomously within the epiblast. In addition, epiblast-specific deletion of Sall4 using the well-established Sox2-Cre line (23) leads to a developmental arrest at the late primitive streak stage but clearly before somite formation (Fig. 5D and E) despite that all four murine Sall family members are expressed at this stage (Fig. 6G). This result further demonstrates that Sall4 is essential for epiblast development in vivo.

ES cells most closely resemble the properties of the epiblast in vitro. To assess whether Sall4 is continuously required for self-renewal of ES cells we established several independent ES cell lines from blastocyst outgrowths from Sall4\textsuperscript{floxed} heterozygous intercrosses. These ES cell lines are feeder-dependent and genotypically either heterozygous or homozygous for a Sall4\textsuperscript{null} allele. They express high levels of Sall4 which is down-regulated upon differentiation (Fig. 11, which is published as supporting information on the PNAS web site). Using either a pCAG-CreIRESNeo or a pCAG-CreIRESPuro vector we were unable to obtain any homozygous Sall4-null ES cell lines. In contrast deletion of one Sall4 allele or both Sall4 alleles in case a pCAG-HASall4IRESPuro rescue vector was present was successful (Fig. 11). Thus, we conclude that one functional Sall4 allele is indispensable for maintenance of the self-renewing ES cell phenotype.

Next we asked whether Sall4 is cell-autonomously required in the primitive endoderm. We therefore produced chimeras in which WT ES cells were introduced into mutant Sall4 embryos (Fig. 5F). In this situation ES cells do not normally contribute to the primitive endoderm or trophectoderm (22, 24). Thus defects within the primitive endoderm caused by defects within the epiblast will be rescued in this constellation. Sall4 heterozygous and homozygous mutant blastocysts were injected with Rosa26 ES cells or R1 CAG-LacZ ES cells (Table 1, which is published as supporting information on the PNAS web site). All decidua at 6.5 dpc from control and mutant Sall4\textsuperscript{floxed} genotypes were
stained for β-galactosidase activity followed by paraffin sectioning. Heterozygous Sall4 blastocyst implantation sites harbored embryos of the expected developmental stage with various amount of chimerism in the epiblast. As expected no LacZ+ blue cells contributed to extraembryonic ectoderm and primitive endoderm derivatives (Fig. 6G). In contrast, only embryos arrested in development at an earlier stage were found in decidual swellings resulting from injected Sall4 mutant blastocysts (Fig. 5H). In these chimeric embryos all cell types appeared to be present. Interestingly, the putative epiblast consisted of only blue WT cells, suggesting that the initially present Oct4/Nanog-positive, Sall4-negative cells may have differentiated into trophectoderm or primitive endoderm derivatives. The developmental arrest of the WT epiblast therefore strongly suggests a cell-autonomous defect within the Sall4 mutant primitive endoderm derivatives consistent with our observation that no extra embryonic endoderm cell lines could be established from Sall4 mutant outgrowths.

Sall4 expression parallels expression of Oct4 and Sox2 during early mouse development but precedes the expression of Nanog and FoxD3, two other transcription factors that have been shown to govern the undifferentiated state of ES cells (11, 25). Despite the parallel expression of Oct4, Sox2, and Sall4, there are notable differences in their loss-of-function phenotypes. Although homozygous mutant Oct4, Sox2 and Sall4 deciduals lack an epiblast, with only a few trophoblast cells present, blastocyst outgrowths of the three mutant genotypes show different behavior in vitro (refs. 9 and 12 and this work).

In contrast to Oct4 mutant blastocyst outgrowths which only yield trophoblast giant cells, Sall4 mutant blastocyst outgrowths contain some Oct4-positive cells and cells expressing endoderm-specific markers. However, the Sall4 mutant outgrowths do not show any significant expansion or apoptosis under ES cell culture conditions. In this respect the observed phenotype of Sall4 homozygous mutant blastocyst outgrowths more resembles the reported Sox2 and FoxD3 mutant phenotypes (9, 25). When one compares ICM outgrowths of the three mutant phenotypes the situation is reversed. In the Oct4 mutant ICM outgrowths giant cells appear immediately, whereas prolonged cultivation of the ICMs of Sall4 as well as Sox2-null mutants is required until giant cells can be observed. In contrast, when ICMs are cultivated in medium containing FGF4, Sall4 mutant ICMs give rise to TS cell lines that readily differentiated into giant cells after FGF withdrawal. This phenotype is similar to that observed in the Oct4 mutant situation but opposite to the Sox2 situation where no TS cell lines could be established even from blastocyst outgrowths (9, 12). Cdx2 has been postulated to respond in an autoregulatory manner to a yet unidentified apical signal (15). Sall4 may be required in this context to support Oct4 repression of Cdx2 expression, consistent with the much lower expression of Sall4 in the trophectoderm compared with the ICM. Thus, Sall4 loss may be permissive in this situation and an additional signaling event may be required to allow the observed cell fate change. How Sall4 modulates the reciprocal Oct4/Cdx2 interaction remains to be explored. Consistent with this idea, we also see up-regulation of H19 in Sall4-null blastocyst outgrowths pointing to another gene where Sall4 may function in a repressive mode. In addition our experiments have demonstrated that Sall4 is cell-autonomously required in the primitive endoderm. In contrast to Oct4 and Nanog, Sall4, like Sox2, is expressed later during development in various organ systems. At these later time points Sall4 expression is always correlated with uncommitted dividing stem and progenitor populations in various organ systems. Thus, the human Okihiro syndrome may result from a premature depletion of different progenitor cell pools depending on the genetic background.

In conclusion, the data presented here add Sall4 to the list of transcription factors including Oct4, Sox2, Nanog and Cdx2 that are required for early mammalian development. Further work should provide additional insights into the transcriptional cross-talk underlying murine ICM pluripotency and ES cell self-renewal.
Experimental Procedures

**Generation of Sall4\textsuperscript{flxed} and Sall4\textsuperscript{loxP/loxP} Mice.** Targeting strategy is described in Fig. 7. R1 ES cells were electroporated with the linearized construct. Positive clones were confirmed by Southern blot using 5\textsuperscript{th} and 3\textsuperscript{rd} outside probes. Sall4 heterozygous mice were maintained on a mixed 129/SvBgl6/Cd1 background. Mice were housed in specific pathogen-free and light-controlled, temperature-controlled (21°C), and humidity-controlled (50–60% relative humidity) conditions. Food and water were available ad libitum. The procedures for performing animal experiments were performed in accordance with the principles and guidelines of Laboratory Animal Resources/European Molecular Biology Laboratory.

**Immunohistochemistry and in Situ Hybridization.** For immunohistochemistry, blastocysts were fixed in 2% PFA for 5 min, washed and blocked for 30 min in PBS containing 0.2% Triton X-100, 1% glycine, 3% BSA, and 5% donkey serum, followed by primary antibody overnight at 4°C. Free-floating blastocysts were embedded in PBS by using spacers (Secure Seal; Molecular Products) and analyzed immediately by confocal microscopy (TCS SP2; Leica, Wetzlar, Germany).

Sall4 peptide antibody [produced against N-terminal 16 aa of Sall4 (KQAKPHINWEEGQGE) coupled to KLH and injected into rabbits] was used at a dilution of 1:500 after affinity purification. Troma1 antiserum (kindly provided by R. Kemler, Max Planck Institute, Freiburg, Germany) at 1:20, Oct4 antiserum (monoclonal mouse anti-Oct4, C10, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50, Nanog antiserum (CosmoBio, Tokyo, Japan) at 1:150, antiserum against Sox2 (kindly provided by R. Lovell-Badge, MRC National Institute for Medical Research, London, U.K.) at 1:250, monoclonal anti-Cdx2 (CDX2-88; BioGenex, San Ramon, CA) at 1:100, phospho-Histone H3 antiserum (rabbit; Upstate Biotechnology, Lake Placid, NY) at 1:300, anti-Cdx3 (56C1; NeoMarkers, Fremont, CA) at 1:30. Hybridization with 35S-labeled and nonradioactive antisense RNA probes was done as previously described (26).

**ES Cell, TS Cell, Immunosurgery, and Outgrowth Culture Experiments.** Culture and derivation of ES cell lines and of trophoblast cell lines from blastocysts was performed as described (27, 28). Immunosurgical isolation of ICMs was carried out on freshly flushed 3.75-dpc blastocysts or upon cultivation of such blastocysts in M16 medium over night (20). Unabsorbed rabbit anti-mouse T cell (Thy1) antiserum (Cedarlane, Hornby, ON, Canada) was used at 25% (vol/vol) in DMEM ( Gibco) to label cells with surface exposure. Embryos were incubated for 15 min at 37°C, washed through several drops of DMEM, and incubated for 15 min at 37°C in 25% (vol/vol) Standard Guinea Pig Complement (Cedarlane) to kill all cells decorated with antibody. Efficient killing was observed microscopically by swelling of trophectodermal cells. For ES cell or TS cell outgrowth formation, blastocysts or ICMs after immunosurgery were transferred to ES cell medium or TS cell medium and documented photographically (D1; Nikon, Munich, Germany) under phase contrast optics.

**Embryo Culture and Chimeric Aggregations.** Two cell stage to morula stage embryos were collected by flushing oviducts with M2 medium. Blastocysts were collected, by flushing the uterine horns with M2. Collected embryos were expanded in M16 at 37°C and 5% CO\textsubscript{2} for 24 h (27). ROSA26 morulae were aggregated with Sall4\textsuperscript{flxed} morulae as described (27). After transfer into pseudopregnant female mice, embryos were harvested at embryonic day 9.5. To generate ES cell–embryo chimera, ROSA26 and CAG-LacZ ES cells were injected into blastocysts obtained from Sall4\textsuperscript{flxed} heterozygous intercrosses. After transfer, embryos were harvested at embryonic day 6.5.

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