Temporal changes of *Sall4* lineage contribution in developing embryos and the contribution of *Sall4*-lineages to postnatal germ cells in mice

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Mutations in the *SALL4* gene cause human syndromes with defects in multiple organs. *Sall4* expression declines rapidly in post-gastrulation mouse embryos, and our understanding of the requirement of *Sall4* in animal development is still limited. To assess the contributions of *Sall4* expressing cells to developing mouse embryos, we monitored temporal changes of the contribution of *Sall4* lineages using a *Sall4 GFP-CreERT²* knock-in mouse line and recombination-dependent reporter lines. By administering tamoxifen at various time points we observed that the contributions of *Sall4* lineages to the axial level were rapidly restricted from the entire body to the posterior part of the body. The contribution to forelimbs, hindlimbs, craniofacial structures and external genitalia also declined after gastrulation with different temporal dynamics. We also detected *Sall4* lineage contributions to the extra-embryonic tissues, such as the yolk sac and umbilical cord, in a temporal manner. These *Sall4* lineage contributions provide insights into potential roles of *Sall4* during mammalian embryonic development. In postnatal males, long-term lineage tracing detected *Sall4* lineage contributions to the spermatogonial stem cell pool during spermatogenesis. The *Sall4 GFP-CreERT²* line can serve as a tool to monitor spatial-temporal contributions of *Sall4* lineages as well as to perform gene manipulations in *Sall4*-expressing lineages.

*Sall4* is one of four *Sall* genes that encode zinc finger transcription factors¹⁻². Heterozygous mutations in the human *SALL4* gene cause Duane-radial ray syndrome (also known as Okihiro syndrome), an autosomal dominant disorder³. *SALL4* mutations are also found in Acro-renal-ocular syndrome⁴. It is considered that these syndromes are caused by *SALL4* haploinsufficiency⁵. The patients exhibit upper limb deformities and aberrant ocular movements due to defects in specific nerves. Other symptoms include renal agenesis, unilateral deafness, choanal atresia, external ear malformations, and ventricular septal defect with varying degrees⁶⁻⁷. The radial ray malformations are observed in the anterior forelimbs and include hypoplasia or aplasia of the thumbs and/or the radius, triphalangeal thumbs, and preaxial polydactyly.

Heterozygous *Sall4* mutant mouse phenotypes partially recapitulate human patients' symptoms. For instance, *Sall4*²⁻/- mice exhibit anal stenosis, ventricular septum defects, exencephaly, hypoplastic kidney, anogenital tract abnormalities, conductive deafness, neural tube closure defects and kinked tails with different penetrance of phenotypes⁸⁻¹⁰. In contrast, limb defects were not observed in *Sall4*²⁻/- mice. The human patient symptoms and *Sall4*²⁻/- mouse phenotypes together indicate that *SALL4* plays important roles in the development and function of a variety of tissues and organs and that many of these functions are likely to be conserved among mammals. However, our understanding of *Sall4* functions during mammalian development remains incomplete, mainly due to the peri-implantation lethality of *Sall4* null mouse embryos, which has hampered analysis of *Sall4* post-implantation functions¹¹.

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The *Sall4* expression pattern during mouse embryonic development provides insights into likely *Sall4* functions during mammalian development. In pre-implantation stages, *SALL4* protein is detected in the two-cell stage embryos due to maternal contribution. After zygotic gene expression starts, *Sall4* transcripts are detected in early cleavage stages. In blastocysts, *Sall4* is expressed in the inner cell mass and trophoectoderm. During these stages, *Sall4* activity contributes to proliferation of cells in the inner cell mass of blastocysts. In addition, a recent report using single cell technology demonstrated that *Sall4* modulates gene regulatory networks to promote commitment of inner cell mass cells to pluripotent epiblast or primitive endoderm.

After implantation, *Sall4* is expressed uniformly in the epiblast until mid-streak stages (embryonic day (E) 6.5). *Sall4* null embryos arrest around the peri-implantation stage, indicating its critical role in the epiblast during this period. *Sall4* is widely expressed in E7.5 embryos, and strong expression is gradually confined to the head and primitive streak while weaker expression is maintained broadly in E8.5 embryos. After completion of gastrulation, *Sall4* is highly expressed in the posterior body, such as the tail bud and in the presomitic mesoderm, the un-segmented posterior paraxial mesoderm, at E9.5–E12.5. During these stages, *Sall4* expression in most other areas of embryos rapidly declines and expression becomes confined to small domains.

During limb development, *Sall4* expression is detected in the mesenchyme from the beginning of outgrowth in both forelimb and hindlimb buds. *Sall4* expression is confined to the distal mesenchyme by E10.5, becomes anteriorly biased at E11.5, and is confined to the narrow, distal-most region by E12.5.

Other than tail and limb buds, *Sall4* is expressed in craniofacial structures. *Sall4* is expressed in the frontonasal mesenchyme, the midbrain, the mandibular arch and maxillary arch at E9.5–E10.5. At E11.5–E12.5, *Sall4* expression rapidly declines but remains detectable in the midbrain. *Sall4* is also expressed in the developing genital tubercle at E11.5–E12.5.

In postnatal mice, *Sall4* is expressed in undifferentiated spermatogonia in the testis. Functional analysis showed that *Sall4* is essential for maintenance of undifferentiated spermatogonia. In female germ cells, *Sall4* is highly expressed in oocytes at different developmental stages. Deletion of *Sall4* in the primary follicle stage or primordial follicle stage oocytes causes oocyte immaturity and infertility, demonstrating the requirement for *Sall4* in female germ cells.

These expression patterns suggest that *Sall4* plays roles in various tissues and organs during embryonic development and in germ cells. As described above, however, the functions of *Sall4* in tissue/organ development remain largely unknown in post-implantation mouse embryos due to the early lethality of *Sall4* null mutants. Previous studies of *Sall4* expression patterns have provided some insights into probable *Sall4* functions during post-gastrulation mouse development. However, because *Sall4* expression declines rapidly in post-gastrulation stages in mouse embryos, it is likely that *Sall4*-expressing cells dynamically change their contributions to developing tissues and organs in mouse embryos. Therefore, we sought to gain insights into the roles of *Sall4* by determining the contributions of *Sall4*-expressing cells in early post-gastrulation stages through mid-gestation stage mouse embryos. For this purpose, we performed genetic tracing of *Sall4*-expressing cells using an inducible Cre cassette knocked into the *Sall4* 1st exon together with recombination-dependent reporter mouse lines that monitor *Sall4*-Cre activity.

**Results**

The GFP signals of *Sall4 GCE* knockin mice reflect high levels of *Sall4* mRNA expression. To detect contribution of *Sall4* expressing cells at a given time of development, we generated a novel allele, in which GFP-CreER<sup>26</sup> (GCE) is knocked into the 1<sup>st</sup> exon of the *Sall4* gene (Fig. 1a). Loss of *Sall4* leads to embryonic lethality and as expected *Sall4<sup>GCE/GCE</sup>* homozygous mutant pups were not recovered from crosses of heterozygotes (not shown). We first compared *Sall4* mRNA expression pattern and the GFP signals at E7.5–E12.5. At E7.5, both *Sall4* mRNA and the GFP signals were broadly detected (Fig. 1b,c). At E8.5, the GFP signals were readily detectable only in the anterior and posterior parts of the body (Fig. 1f,f'). At this stage, *Sall4* is broadly expressed, and high levels of *Sall4* mRNA expression are confined to the anterior and posterior parts of the body (Fig. 1d,d'). The GFP signals were not detected in tissues with weaker *Sall4* mRNA signals. At E9.5, strong GFP signals were detected in the posterior part of the body (Fig. 1i,i'), where strong *Sall4* mRNA signals were also detected (Fig. 1g,g'). Higher magnification images show that signals were in the posterior neural plate and paraxial and lateral plate mesoderm tissues (Fig. 1g'). At E10.5, the GFP signals were detected in the posterior tip of the tail bud, posterior neural tube, anterior presomitic mesoderm and somites (Fig. 1j,j'). *Sall4* mRNA is expressed at high levels (Fig. 1j,j'). The GFP signals and *Sall4* mRNA were also detected in the genital primordium (Fig. 1j,j'). Although *Sall4* mRNA is expressed in the distal portions of forelimb and hindlimb buds (Fig. 1f,f'), the GFP signals were undetectable in limb buds at E10.5 (Fig. 1i,i'). At E11.5, the GFP signals were detected in the tail bud, presomitic mesoderm and somites (Fig. 1o,o'), consistent with strong expression of *Sall4* mRNA in the tail bud and anterior presomitic mesoderm (Fig. 1m,m'). The GFP signal and *Sall4* mRNA were also detected in the genital primordium (Fig. 1m,m'). Similar to E10.5 embryos, the GFP signals were undetectable in limb buds, although *Sall4* mRNA is expressed (Fig. 1m,m'). At E12.5, *Sall4* mRNA is expressed in the anterior presomitic mesoderm, while the expression is significantly reduced in the tail bud tip and distal-most limb mesenchyme (Fig. 1p,p'). At this stage, weak GFP signals were detected in the posterior somites (Fig. 1r). Strong GFP signals were maintained in the genital primordium (Fig. 1r'), consistent with *Sall4* mRNA expression (Fig. 1p,p').

These signal comparisons indicate that the GFP signals in *Sall4 GCE* embryos are highly congruent with endogenous *Sall4* mRNA expression but detectable only in cells/tissues where high levels of *Sall4* mRNA are expressed. In particular, the GFP signals were consistently detectable in the posterior tissues (tail bud, neural tube, presomitic mesoderm) and the external genitalia. In contrast, the GFP signals became undetectable during the early outgrowth stages of the limb bud.
Figure 1. The GFP signals of Sall4 GCE embryos were detectable in cells/tissues with high levels of Sall4 mRNA expression. (a) Schematic of targeting strategy to knock-in the GCE cassette into the exon 1 of the Sall4 gene. (b,d,g,j–j",m–m",p–p") Sall4 mRNA expression pattern of indicated stages by whole mount in situ hybridization. Bright field images (e,h,k–k",n–n",q,q") and the GFP images (f,i,l–l",o–o",r,r") of Sall4 GCE embryos at indicated stages. At E8.5 (d–f") and E9.5 (g–i"), arrowheads and arrows point to the head and the posterior tip of the body. Panels in b,c,d,e,f,g,h and i show lateral views of the whole embryo. Panels in d',e',f',g',h' and i' show dorsal views of the posterior part of the body. At E10.5–E12.5, dashed black arrows point to the signal at the distal part of the limb buds in dorsal views with the anterior to the top (j,j",m,m",p,p"). Arrows point to the posterior tip of the tail (j,j",l,l",m,m",o,o"). Red arrowheads point to the anterior presomitic mesoderm in dorsal views (j",l",m",o",p"). Yellow arrowheads point to the external genital primordium (j""i",l",m",o",p""q",r). The genital primordia are shown in the ventral views (j",p") or lateral views (k",l",m",n",o",q",r"). Asterisks in j",l",m",o",p" and r indicate signals in the somites. Abbreviations. an: anterior, f: forelimb bud, g: external genital primordium, h: hindlimb bud, l: lateral plate mesoderm, n: neural tube, p: paraxial mesoderm, po: posterior, s: somites.
The \textit{Sall4} lineage contribution is rapidly restricted to the posterior of the body in post-gastrulation stages. To compare contributions of \textit{Sall4}-expressing cells at different stages, we crossed \textit{Sall4 GCE} males with \textit{R26-LacZ} females, and administered tamoxifen to the pregnant females at different time points. Embryos were then collected and stained for LacZ activities at E13.5 (Fig. 2a). Consistent with the broad expression of \textit{Sall4} mRNA and GFP from the \textit{GCE} allele at E7.5 (Fig. 1b,c), tamoxifen injection at E7.5 resulted in essentially ubiquitous LacZ staining (Fig. 2b). LacZ-labelled \textit{Sall4} lineage contribution decreased in the anterior of the body by E8.5, with staining detected mainly posterior to the middle of the trunk (Fig. 2c). The anterior border of the \textit{Sall4} lineage contribution was anterior to the hindlimb when tamoxifen injection was performed at E9.5 (Fig. 2d), while injection at E10.5 shifted the strong LacZ staining domain posterior to the hindlimb, with weaker LacZ staining detected in the flank immediately anterior to the hindlimb (Fig. 2e). The contribution became restricted to the tail tip by E11.5 (Fig. 2f) and was undetectable in the tail at E12.5 (Fig. 2g). These results show a rapid and spatially dynamic reduction of \textit{Sall4} lineage contribution to the posterior of the body during E8.5–E11.5. It has been reported that \textit{Sall4} \textit{−/−} embryos exhibit exencephaly at low penetrance\cite{8}. Because the exon 1 is replaced with the \textit{GCE} cassette in the \textit{Sall4 GCE} allele (Fig. 1a), we also observed low penetrance exencephaly in \textit{Sall4 GCE}; \textit{R26-LacZ} embryos, as expected if the \textit{GCE} allele is null or severely hypomorphic (Fig. 2e).

Reduction of \textit{Sall4} lineage contributions is different in forelimbs and hindlimbs. The limb develops from specific regions of lateral plate mesoderm, arising from the 7–12 somite levels for forelimbs and the 25–29 somite levels for hindlimbs. We found that \textit{Sall4} lineages at E7.5, before formation of the limb-forming regions, contribute to both forelimbs and hindlimbs (Fig. 2b’–g’). At E8.5 (8–12 somite stage), the forelimb-forming region is specified but the hindlimb-forming region has not been established. At this stage we found that the \textit{Sall4} lineage sparsely contributed to forelimbs with more contributions to the anterior portion (Fig. 2c’). In contrast precursors of hindlimbs are still in the posterior lateral plate mesoderm at this stage, and \textit{Sall4} mRNA is highly expressed in this region. Similarly, \textit{Sall4} lineages at E8.5 broadly contributed to the hindlimbs (Fig. 2c”). At E9.5 (20–24 somite stage), forelimb buds have been formed, but hindlimb buds are just initiating outgrowth in the posterior region of the elongating body. The contribution of \textit{Sall4} expressing cells to forelimbs at this stage was detected in a sparse and anteriorly biased manner, and the contribution was reduced relative to E8.5 (Fig. 2d’). The contribution to hindlimbs was still broadly detected, also with less LacZ signal intensity than at E8.5 (Fig. 2d”). At E10.5, both forelimb buds and hindlimb buds are developed, and \textit{Sall4} mRNA is expressed in both types of limb buds. \textit{Sall4} contribution from the \textit{GCE} allele was detected in a very sparse manner in the autopod of forelimbs (Fig. 2e’), but the contribution to hindlimbs was still broadly detected (Fig. 2e”). At E11.5 and E12.5, \textit{Sall4} lineage contributions to forelimbs were not detected (Fig. 2f’–g”), although \textit{Sall4} mRNA is expressed at these stages (Fig. 1m’–p’\cite{4,6}). \textit{Sall4} lineage contribution at E11.5 to hindlimbs was detected in the anterior zeugopod and the most anterior digit (Fig. 2f”). \textit{Sall4} contributions to both forelimbs and hindlimbs became undetectable at E12.5 (Fig. 2g’–g”).

In summary, in both forelimbs and hindlimbs, \textit{Sall4} lineages contributed broadly to the limb when tamoxifen was injected before specification of limb progenitors (Fig. 2b’–g’). When tamoxifen was injected around the time...
of specification of forelimb progenitors (~E8.5) and hindlimb progenitors (~E9.5), Sall4 lineage contribution to the limb declined. Even with such similarities, Sall4 lineage contribution to hindlimbs persisted longer than that to forelimbs.

**Sall4 lineage contribution to the craniofacial structures.** Sall4 is expressed in craniofacial structures. At E8.5, Sall4 mRNA is expressed in the forebrain (Fig. 3b,b'), and Sall4 lineage contribution at E8.5 was detected in the nasal structure and lower jaw (Fig. 3e,e'). At E9.5, Sall4 mRNA expression is evident in the midbrain, nasal process and the first branchial arch (Fig. 3c,c'). Sall4 lineage contribution at E9.5 was detected as slightly broader than that at E8.5, and the LacZ signal was more intense than at E8.5 (Fig. 3e,e',f,f'). At E10.5, Sall4 mRNA is expressed in the front nasal process (Fig. 3d,d'). Sall4 lineage contribution at this stage was detected in the distal tip of the nasal structure (Fig. 3g,g') and more weakly in the midbrain. After E11.5, Sall4 lineage contribution was not detected in the nasal structure (Fig. 2f,g).

These results show that Sall4 lineage contribution to the craniofacial structures becomes slightly broader from E8.5 to E9.5; however, between E9.5 and E10.5, the contribution declines rapidly, similar to the contribution to the axial level.
Sall4 lineage contribution to the external genitalia. While Sall4 mRNA expression rapidly declines in post-gastrulation stages, the external genital primordium remains a strong Sall4 expressing domain (Fig. 1j’,l’,m’,o’,p’). During E7.5–E9.5, when Sall4 lineages contribute to the posterior body (Fig. 2b,c,d), Sall4 lineages were detected in the entire external genitalia (Fig. 4b,b’,c,c’,d,d’). In contrast, Sall4 lineage contribution was restricted to the posterior half of the genital primordia at E10.5 and E11.5 (Fig. 4e,e’,f,f’). At E12.5 Sall4 lineage contribution was still detected in the posterior of external genitalia, but with reduced signal intensities (Fig. 4g,g’).

The Sall4 lineage contributes to the extra-embryonic tissues, such as yolk sac and umbilical cord. Sall4 expression in extra-embryonic tissues has not been reported. Unexpectedly, we found that Sall4 lineages contribute to extra-embryonic tissues such as the yolk sac in a very transient manner. Labeling Sall4 lineages at E7.5 resulted in patchy LacZ staining in the yolk sac (Fig. 5b). When tamoxifen injection was performed at E8.0, LacZ staining was broadly detected in the yolk sac (Fig. 5c). However, tamoxifen injection at E8.5, E9.0 and E9.5 resulted in little LacZ signal (Fig. 5d,e,f). This staining pattern demonstrates a very narrow time window during which Sall4 lineages contribute to the yolk sac.

To determine whether Sall4 lineages contribute to hematopoietic or endothelial cells in the yolk sac at E13.5, we performed immunofluorescence analysis. For this purpose, we used R26-tdTomato, instead of R26-LacZ, and co-detected tdTomato by mCherry antibodies together with anti-PECAM1 (endothelial marker, Fig. 5i) or anti-TER119 (blood cell marker, Fig. 5m). Fluorescent images showed that tdTomato-positive Sall4 lineages did not overlap with PECAM1 (Fig. 5g–j) and TER119 (Fig. 5k–n). These results indicate that Sall4 lineages contribute to the yolk sac stroma during a very narrow time window around E8.0.

To determine the origin of the contribution of Sall4 lineages to the extra-embryonic tissues, we re-examined Sall4 expression by in situ hybridization. Due to a lag from tamoxifen injection to CreER-dependent recombination21 (see Discussion), we used E8.5 embryos and detected Sall4 expression in the yolk sac and allantois (Fig. 5o,p). The expression pattern supports the idea that Sall4 expressing cells in the yolk sac contribute to the yolk sac stroma at later stages.

The Sall4 lineage contributes to the peri-vascular tissues in the umbilical cord. The allantois is the precursor tissue of the umbilical cord22. Expression of Sall4 in the allantois, found in this study (Fig. 5o,p) suggests that Sall4-expressing cells contribute to umbilical cord. Therefore, we examined the Sall4 lineage contribution to the umbilical cord by sectioning LacZ-stained E12.5 umbilical cord. We found that tamoxifen injection at E7.5 resulted in broad LacZ signals around the two vessels of the umbilical cord (Fig. 6b). When tamoxifen injection was done at E8.5, Sall4 lineages contributed less to the tissue around the vessels (Fig. 6c). No contribution to tissues around the umbilical vessels was detectable when tamoxifen was administered at E10.5 (Fig. 6d). Immunofluorescence of cross sections of the umbilical cord at E13.5, labelled at E8.0, showed that tdTomato-positive Sall4-lineage did not overlap with VEGFR2, a vascular endothelial cell marker (Fig. 6e–h). These results support the idea that the Sall4 lineage transiently contributes to the peri-vascular mesenchyme in the umbilical cord.

A previous lineage tracing experiment demonstrated that Tbx4-expressing cells also contribute to the peri-vascular mesenchyme in the umbilical cord23, suggesting that Sall4 and Tbx4 are co-expressed in the

Figure 4. Sall4 lineage contribution to the external genital primordium. Schematic of tamoxifen injections at different time points of embryonic development. (b–g’) Lateral views (b–g) and frontal views (b’–g’) of external genitalia of LacZ stained E13.5 embryos. Black arrowheads point to the broadly stained external genitalia, labelled at E7.5–E9.5. Arrows point to the LacZ-stained posterior of external genitalia, labeled at E10.5–12.5. Scale bar in panel b: 1 mm. Panels b to g’ are in the same scale.
Figure 5. Sall4 lineage contribution to the yolk sac stroma in a narrow time window. Schematic of tamoxifen injections at different time points of embryonic development. (b–f) LacZ-stained yolk sac. The staining was broadly detected when tamoxifen was injected at E8.0. Scale bar in panel b: 1 mm. Panels b to f are in the same scale. (g–n) Immunofluorescence images of DAPI (g,k), tdTomato (h,l), PECAM (i) and TER119 (m). (j) and (n) show merged images of (g–i) and (k–m), respectively. The tdTomato signals do not overlap with PECAM and TER119. Scale bar in panel g: 100 µm. Panels g to n are in the same scale. (o,p) Lateral views of E8.5 embryos hybridized with antisense (o) or sense (p) Sall4 probes. Sall4 is expressed in the yolk sac (ys) and the allantois (al), in addition to the embryo. Control sense probe generated no signals. Abbreviations. al: allantois, ys: yolk sac.
Figure 6. Sall4 lineage contribution to peri-vascular mesenchyme in the umbilical cord. Schematic of tamoxifen injections at different time points of embryonic development. (b–d) Cross section of LacZ-stained umbilical cord. Scale bar in panel b: 100 µm. Panels b to d are in the same scale. (e–h) Immunofluorescence images of DAPI (e), tdTomato (f), VEGFR2 (g) and merged image (h). The tdTomato signals do not overlap with VEGFR2. Scale bar in panel e: 100 µm. Panels e to h are in the same scale. (i–l) Whole mount in situ hybridization of Sall4 (i,k) and Tbx4 (j,l) of E8.5 embryos. Ventral views (i,j) and lateral views (k,l) of the allantois are shown. (m–q) Double detection of SALL4 immunoreactivities (magenta) and Tbx4 mRNA (green) on allantois sections. Panels n–q show closeup of the dotted square in (m). Panels n, o and p are shown in a black/white mode. Dotted lines in (o–q) indicate the border between the allantois and the embryo. Scale bar in m: 200 µm. Abbreviation. al: allantois, em: embryo, ne: neuroectoderm of the head region, ve: vessel.
precursor of umbilical cord. To address this possibility we compared expression patterns of \(Sall4\) and \(Tbx4\). \(Sall4\) is expressed in the proximal part of the allantois at E8.5, while \(Tbx4\) is more broadly expressed in the allantois (Fig. 6i–l). Therefore, the expression patterns of \(Sall4\) and \(Tbx4\) overlap at the proximal part of the allantois. To further clarify their co-expression, we performed fluorescent in situ hybridization of \(Tbx4\) in combination with \(SALL4\) immunofluorescence on sections of the E8.5 allantois. We detected nuclear \(SALL4\) signals associated with cytoplasmic \(Tbx4\) mRNA signals in the allantois (Fig. m–q). These results demonstrate that cells expressing \(Sall4\) and \(Tbx4\) in the proximal allantois contribute to peri-vascular mesenchyme tissue in the umbilical cord.

\(Sall4\) GCE labels spermatogonia and spermatogonial stem cells in the postnatal testis. In the testis \(SALL4\) is expressed mostly in undifferentiated spermatogonia, including spermatogonial stem cells (SSCs)\(^{17-19}\). To determine whether \(Sall4\) GCE is active in undifferentiated spermatogonia and SSCs, we injected neonatal mice with tamoxifen and monitored CreER activity using the \(R26-tdTomato\) transgene at 7 and 60 days post tamoxifen injection. The earlier time point allowed identification of the initial labeled germ cell population and the later time point allowed identification of SSCs, which persist long-term. We used anti-red fluorescent protein (RFP) antibody to visualize tdTomato-positive cells.

To identify undifferentiated spermatogonia, we performed immunofluorescence on whole-mount seminiferous tubules from control and experimental mice 7 days post tamoxifen injection using anti-\(SALL4\) and anti-PLZF antibodies. In controls lacking the GCE transgene, \(SALL4\)- and PLZF-positive cells were negative for tdTomato, and no \(Tbx4\) expression was detected in the entire gonad (Fig. 7a–a’). In experimental animals, all \(SALL4\)- and PLZF-positive cells were positive for tdTomato (Fig. 7b–b’). We also confirmed that the expression of \(Tbx4\) was limited to germ cells by co-staining tdTomato with the Sertoli cell markers SOX9 and GATA4 (Fig. 7c–c’). Together, our data indicate that \(Sall4\) GCE is active in undifferentiated spermatogonia, but not in Sertoli cells.

To assess the activity of \(Sall4\) GCE in SSCs, we examined testes 60-days post-tamoxifen injection. A cycle of spermatogenesis takes 35–40 days to complete and allows SSCs to produce differentiated progeny that give rise to all germ cell types\(^{24,25}\). If \(Sall4\) GCE is active in SSCs, most germ cell types will be positive for tdTomato 60-days post activation of CreER, importantly including undifferentiated spermatogonia. We co-stained the testis with anti-RFP along with anti-\(SALL4\) and anti-DMRT6 to label undifferentiated and differentiating spermatogonia, respectively. All \(SALL4\) and DMRT6-positive cells were positive for tdTomato (Fig. 8a–a’). We also used anti-small ubiquitin-related modifier-1 (SUMO1) and anti-linker histone H1T to label spermatocytes. These meiotic germ cells were positive for tdTomato, although these differentiated germ cell types have weaker tdTomato expression relative to spermatogonia (Fig. 8c–c’). Together, these data suggest that \(Sall4\) GCE is active in

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**Figure 7.** \(Sall4\) GCE is active in undifferentiated spermatogonia. Immunofluorescence of wholemount seminiferous tubules. (a–a’) \(Rosa26-tdTomato\) and (b–b’,c–c’) \(Sall4\) GCE; \(R26-tdTomato\) testis 7 days post-tamoxifen treatment stained for tdTomato (red), \(SALL4\) (green), SOX9 (green), PLZF (blue), GATA4 (blue). Scale bars in panels a,b,c: 100 \(\mu\)m. All panels are in the same scale.
Discussion
In this study, we sought to determine contributions of \textit{Sall4}-expressing cells during the period of post-gastrulation mouse development when \textit{Sall4} mRNA expression rapidly declines. LacZ staining at E13.5 showed drastic restrictions of LacZ-positive regions in embryos with tamoxifen injection at E8.5–E12.5. In particular, \textit{Sall4} lineage contributions to the axial level declined rapidly. Moreover, whole mount analysis showed reduction of \textit{Sall4} lineage contributions during these stages in other tissues, including the limb, craniofacial structures, and external genitalia in the embryo. Such genetic labeling to detect contributions of labelled cells is a well established approach, and is particularly useful in the case that gene expression patterns rapidly change/decline in developing embryos. However, an important caveat to note is that tamoxifen must be converted into an active
metabolite to induce activation of CreER protein. Consequently, there is 0.5–0.75 day lag from tamoxifen injection to CreER-dependent recombination. Given that Sall4 expression pattern changes in the stages we analyzed, the LacZ staining pattern includes a lag to the corresponding Sall4 mRNA expression pattern. In addition to the LacZ signals, we also have to note the characteristics of the GFP signals from the GCE cassette. The GFP signals are detectable only in the cells/tissues where Sall4 is highly expressed, compared to Sall4 mRNA expression in this study and previous reports, suggesting that the GCE cassette is a less sensitive indicator of expression than in situ analysis. This low intensity of the GFP signals may be derived from the engineered GCE cassette. Although the GFP reporter signal is low, its expression can still act as a useful tool to detect and/or isolate high levels of Sall4-expressing cells.

Around E8.0–8.5, the forelimb field is determined and Tbx5, a marker of forelimb progenitors, starts its expression in the lateral plate mesoderm. This timing of forelimb progenitor specification is correlated with the rapid decline of the contribution of Sall4 lineages to the forelimb between E7.5 and E8.5. In hindlimbs, we found that LacZ signal intensity declined when tamoxifen was injected around the stage of hindlimb progenitor specification (E9.5). The temporal difference of reduction of Sall4 lineage contribution to forelimbs and hindlimbs seems to involve differences in developmental timing between two types of limbs during body elongation: the broad contributions of Sall4 lineages to hindlimbs persisted until E10.5, while the LacZ signal intensity gradually declined from E7.5–E10.5. As a consequence, the Sall4 lineage contribution seems to be more significant in hindlimbs than forelimbs. Our previous study showed that conditional inactivation of Sall4 by TCre caused subtle defects in forelimbs, while hindlimbs exhibited severe skeletal defects. The rapid decline of Sall4 lineage contribution to the forelimb and continued contribution to hindlimbs during E8.5–E9.5 provides a likely reason for this phenotypic difference.

Another interesting aspect with respect to Sall4 conditional knockout phenotypes is that the Sall4 lineage at E11.5 contributes to the anterior zeugopod and most anterior digit in hindlimbs. These elements are defective in Sall4 conditional knockout phenotypes and exhibiting defects in the anterior limb elements. However, most of human patients exhibit limb defects primarily in forelimbs, with just a few cases in hindlimbs. Anterior skeletal defects and Sall4 contribution to the anterior part of the limb at E11.5 support the role of Sall4 in formation of the anterior limb skeletal elements. A major unanswered question is the difference in limb types affected in human patients with SALL4 heterozygous mutations versus mouse models of Sall4 conditional knockout. The duration of Sall4 lineage contribution to forelimbs and hindlimbs in this study correlates well with the severity of limb defects in Sall4 conditional knockout in our previous study. Therefore, one possibility is that SALL4-expressing cells might contribute longer to forelimb buds than to hindlimb buds in human embryos. Studies of post-implantation human developmental processes, which may be obtained by differentiation of pluripotent stem cells in vitro, could help clarify the difference of limb phenotypes between human patients and mouse mutants.

It has been reported that several patients with SALL4 mutations also exhibit distinctive facial appearances. For instance, hypertelorism (increased distance between the eyes) as well as epicantlic folds that could be caused by defects in the branchial arch. Our finding that Sall4 lineages contribute to the craniofacial region suggests that functional studies in mouse models, such as cell type-specific inducible knockout of Sall4, in the future would likely identify more detailed functions of Sall4 in the craniofacial development, and these might be relevant to defects in human patients with SALL4 mutations.

Neural tube closure defects, including exencephaly, are caused by complex genetic mechanisms. A previous study showed that mutations in Sall2 synergize with Sall2 mutations to cause exencephaly in mouse embryos. Interestingly, Sall4 lineage contribution to the midbrain persisted even when tamoxifen was given at E10.5, when endogenous Sall4 mRNA expression is low. This observation suggests that Sall4 expressing cells continue to proliferate in the midbrain. The Sall4 lineage contribution in the midbrain was observed as stronger LacZ signals with broader LacZ-positive domain when embryos exhibited an exencephaly phenotype. Such a LacZ staining pattern in exencephaly embryos suggests that over-proliferation of Sall4-expressing cells in the Sall4 heterozygous genotype may contribute to exencephaly, in addition to functional interaction with Sall2.

Sall4 lineage analysis in this study identified novel contribution of Sall4 lineages to the extra-embryonic tissues, such as the yolk sac and the peri-vascular tissue in the umbilical cord. This finding led to identification of previously unappreciated expression of Sall4 in the yolk sac and allantois. Interestingly, a previous genetic lineage tracing experiment led to the finding that Tbx4-expressing cells also contribute to the umbilical cord. Both Sall4 lineages and Tbx4 lineages contribute to the mesenchyme of the cord but not to the endothelium. Moreover, both Sall4 lineages and Tbx4 lineages contribute to the external genitalia. These similarities suggest that Sall4 lineages and Tbx4 lineages share a common origin. In another extra-embryonic tissue, yolk sac, Sall4 lineages also do not contribute to the endothelium. These observations highlight the distinct origin of the endothelium in the extraembryonic tissue.

Although Sall4 expression in the genital primordium in mouse embryos was reported previously, dynamic changes of Sall4 lineage contribution to the external genitalia are a novel finding of this study. Restriction of Sall4 lineage contribution to the posterior of the genital primordium during E10.5 and E12.5 suggests that roles of Sall4 in the external genital primordium change during these stages. Sex determination in mice occurs around E10.5 when the Sry gene on the Y chromosome is briefly expressed. However, the morphological differences of the external genitalia between males and females do not become evident until E16.5. Our analysis of Sall4 lineage contribution was at E13.5, before these differences appear. Studying Sall4 lineage contributions to later development would likely identify more detailed functions of Sall4 in the craniofacial development, and these might be relevant to defects in human patients with SALL4 mutations.

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stages could provide further insights into the roles of Sall4 in genital organ development, including those specific to the male or female.

Sall4 is expressed in spermatogonia and plays a role in SSC maintenance and spermatogonial differentiation\(^{17,20}\). By short (7 days) and long (60 days) lineage tracing in the testis, we found that Sall4-expressing cells contributed to not only undifferentiated spermatogonia but also differentiating spermatogonia and spermatocytes, confirming that Sall4 expression marks spermatogonial stem cells. The lineage tracing also revealed that the contribution of Sall4-expressing cells is specific to germ cells. Sall4 is expressed in all stages of undifferentiated spermatogonia, including A-single, A-paired and A-aligned spermatogonia\(^{18}\). Therefore, Sall4 GCE could be a tool to induce recombination in all these stages of spermatogonia.

In this study, we investigated Sall4 lineage contribution to mid-gestation mouse embryos mostly by whole-mount LacZ staining. In addition to the tissues and organs characterized in this study, human patients with SALL4 mutations exhibit symptoms in the heart, kidney and inner ear\(^{3-7}\). Detailed histological examinations of Sall4 lineage in sectioned samples could help provide insights into roles of Sall4 in these tissues and organs in the future. Moreover, as shown in the germ cells in the testis, the Sall4 GCE mouse line serves as an efficient lineage tracing tool in the postnatal mice. Therefore, Sall4 GCE could be used to determine contributions of Sall4 lineages and elucidate roles of Sall4 in the postnatal mice, providing detailed histological insights into the Sall4 lineage contributions in the adult human body.

**Methods**

**Generation of Sall4 GCE line.** Knocking in the GFP-CreERT\(^{22}\) (GCE)-Neo cassette to replace the 1\(^{st}\) exon of the Sall4 in C57BL/6 mouse embryonic stem cells and chimera production by blastocyst-injection were done at the University of Rochester Medical Center. The chimeric mice were bred with B6 albino, and germline transmission was confirmed by genomic PCR for Cre. The Sall4 GCE-Neo mice were bred with the PGK-FLp mouse line (B6.Cg-Tg(Pgk1-flp)10Sykr/j)\(^{39}\) to eliminate the Neo cassette. The Sall4 GCE mouse line is maintained on the C57BL/6 background.

**Breeding with reporter lines and tamoxifen injection.** The Sall4 GCE mice were bred with R26-LacZ (Gt(ROSA)26Sor\(^{11,38}\))\(^{38}\) or R26-tdTomato (Gt(ROSA)26Sortm14(CAG-tdTomato)Hze\(^{39}\))\(^{39}\) reporter mouse lines. Noon of the day that vaginal plug is found is referred as embryonic day (E) 0.5. Tamoxifen (10 mg/ml, 100 µl) was administered to pregnant mice by intraperitoneal injection\(^{40}\). To neonatal pups, tamoxifen (10 mg/ml, 100 µl) was injected under the back skin on the day of birth.

**Whole-mount in situ hybridization and LacZ staining.** Whole-mount in situ hybridization and whole mount LacZ staining on embryos were performed according to published procedures\(^{35,41}\). More than 10 embryos/stage were examined for in situ hybridization. For LacZ staining, 3–8 embryos were examined for each experimental setting of tamoxifen injection and LacZ staining.

**Immunofluorescence of yolk sac and umbilical cord.** For whole mount immunofluorescence analysis of yolk sac, the yolk sac was fixed in 4% paraformaldehyde overnight at 4 °C, washed with phosphate buffered saline (PBS), and dehydrated with methanol. After rehydration, the yolk sac was blocked with 5% donkey serum in PBS + 0.1% Triton X-100, incubated with primary antibodies, washed and incubated with secondary antibodies. After washing, yolk sac was mounted on glass slides with 4',6-diamidino-2-phenylindole (DAPI) fluoromount-G. For umbilical cord, the tissues were fixed, washed, dehydrated and rehydrated, and then cryo-sectioned at 14 µm. The slides were stained similar to the yolk sac samples. Fluorescent images were acquired with Zeiss LSM710 confocal microscopy. Primary antibodies used are shown in Supplementary Table 1. Alexa fluorophore-conjugated secondary antibodies (Invitrogen, 1:500) were used as secondary antibodies. Two samples were examined for both yolk sac and umbilical cord.

**Double detection of Tbx4 mRNA and SALL4 immunoreactivities in the allantois.** SALL4 immunofluorescence in combination with Tbx4 mRNA fluorescent in situ hybridization was performed on cryo-sections of E8.5 allantois with modifications to our previously reported method\(^{42}\). Blocking of endogenous peroxidase (POD) was done with 3% hydrogen peroxide in PBS at room temperature for 30 minutes. Section in situ hybridization was performed using digoxigenin (DIG)-labelled Tbx4 probe. After blocking with 10% heat-inactivated sheep serum, the slides were treated with anti-DIG-POD (Roche, Cat# 1120773910, 1:1000 dilution), and the signals were developed by the Alexa 488 Tyramid reagent (Invitrogen, Cat# B40953) by incubating the slides for 1 hour at 4 °C followed by 2 hours at room temperature. Then, the slides were incubated with anti-SALL4 antibody at 4 °C overnight, and the SALL4 signals were detected by Alexa594 goat anti-mouse IgG (Invitrogen, A-11005, 1:500). Sections from two embryos were examined.

**Immunofluorescence of testis sections.** The testis was fixed in 4% paraformaldehyde overnight at 4 °C. After washing with PBS, the testes were processed to prepare 5 µm paraffin sections. Slides with paraffin sections were rehydrated and boiled with 10 mM of citric acid (pH 6.0). Slides were blocked with 10% serum (goat or donkey depending on the secondary antibody used) in PBS + 0.1% Triton X-100 at room temperature for 1 h and incubated with primary antibody (Supplementary Table 1) overnight at room temperature. The slides were washed, followed by 2 h incubation with secondary antibody. After washing, nuclei were stained with DAPI, and fluorescent images were captured with a Zeiss Imager Z1 microscope using a Zeiss MRm camera. Two testes were examined at P60.
Immunofluorescence of whole mount seminiferous tubules. Whole mount immunofluorescence of seminiferous tubules was performed as previously described. All images were captured with a Zeiss Imager Z1 microscope using a Zeiss MRm camera. Two testes at P7 and two testes at P60 were examined for this study.

Experimental methods guideline statement. Animal experiments were performed according to the approval by the Institutional Animal Care and Use Committee of the University of Minnesota. Methods were carried out in accordance with relevant guidelines and regulations.

Data Availability Statement

Data generated or analyzed during this study are included in this published article. Additional datasets generated and analyzed during the study are available from the corresponding author on reasonable request.

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