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Generation of functional multipotent adult stem cells from GPR125+ germline progenitors

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Abstract

Adult mammalian testis is a source of pluripotent stem cells[1]. However, the lack of specific surface markers has hampered identification and tracking of the unrecognized subset of germ cells that gives rise to multipotent cells[2]. Although embryonic-like cells can be derived from adult testis cultures after only several weeks *in vitro*[1], it is not known whether adult self-renewing spermatogonia in long-term culture can generate such stem cells as well. Here, we show that highly proliferative adult spermatogonial progenitor cells (SPCs) can be efficiently obtained by cultivation on mitotically inactivated testicular feeders containing $CD34^+$ stromal cells. SPCs exhibit testicular repopulating activity *in vivo* and maintain the ability in long-term culture to give rise to multi-potent adult spermatogonial-derived stem cells (MASCs). Furthermore, both SPCs and MASCs express GPR125, an orphan adhesion-type G-protein-coupled receptor. In knock-in mice bearing a GPR125–β-galactosidase (LacZ) fusion protein under control of the native *Gpr125* promoter (GPR125–LacZ), expression in the testis was detected exclusively in spermatogonia and not in differentiated germ cells. Primary GPR125–LacZ SPC lines retained GPR125 expression, underwent clonal expansion, maintained the phenotype of germline stem cells, and reconstituted spermatogenesis in busulphan-treated mice. Long-term cultures of $GPR125^+$ SPCs (GSPCs) also converted into $GPR125^+$ MASC colonies. $GPR125^+$MASCs generated derivatives of the three germ layers and contributed to chimaeric embryos, with concomitant downregulation of GPR125 during differentiation into $GPR125^-$ cells. MASCs also differentiated into contractile cardiac tissue *in vitro* and formed functional blood vessels *in vivo*. Molecular book marking by GPR125 in the adult mouse and, ultimately, in the human testis could enrich for a population of SPCs for derivation of $GPR125^+$

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MASCs, which may be employed for genetic manipulation, tissue regeneration and revascularization of ischaemic organs.

The genetic and phenotypic profile of the specific subset of spermatogonial cells that converts into multipotent adult cells is poorly defined. We discovered a potential stem and progenitor cell surface marker (GPR125) expressed on the adult testis, while evaluating a large series of mouse knockouts[3]. The endogenous *Gpr125* locus was altered by joining the amino-terminal putative extracellular and first transmembrane domains to β-galactosidase (Supplementary Fig. 2). Homozygous mice were grossly normal and fertile. Histochemical examination of the post-natal testis with the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) revealed that GPR125 expression was restricted to the seminiferous tubules and was confined within the first layer of cells adjacent to the basement membrane of the peritubular cells (Fig. 1a–c). Immunohistochemistry revealed GPR125 expression only in spermatogonia (Fig. 1e).

As spermatogenesis proceeds along the length of the seminiferous tubule, characteristic sets of differentiating cell types are seen together in a given cross-section, allowing such cross-sections to be categorized into twelve stages[4]. Expression of GPR125 was greatest at later stages (that is, VII–VIII) with a nadir in earlier stages (that is, IV–V), as analysed either by promoter activity (X-gal) or by immunostaining (in wild-type mice; Fig. 1c–e). To quantify expression of GPR125–LacZ in the $Gpr125^{lacZ/lacZ}$ spermatogonia, staining was performed with fluorescein di-D-galactopyranoside (FDG), followed by flow cytometry. Freshly dissociated adult $Gpr125^{lacZ/lacZ}$ seminiferous tubules yielded ~35% $LacZ^+$ cells (Fig. 1f). The high yield of $LacZ^+GPR125^+$ cells may be a result of our preparation of testicular tissue—in which contaminating interstitial somatic cells and spermatids are lost during washing steps—combined with the high sensitivity of the FDG assay[5].

To determine whether $LacZ^+GPR125^+$ cells represent self-renewing spermatogonial cells with the capacity to generate MASCs, we sought to recapitulate *in vitro* the native niche that supports efficient self-renewal of these cells. We discovered that the $LacZ^+GPR125^+$ cells reside in close proximity to the $CD34^+$ peritubular cells[6], indicating that interaction of these two cell types may be essential for expansion of the $GPR125^+$ SPCs (Fig. 1g and Supplementary Fig. 3a, b). To culture $GPR125^+$ cells, we established primary mitotically inactivated adult mouse testicular stromal cells containing $CD34^+$ putative peritubular cells ($CD34^+$MTS), because our initial attempts using mouse embryo fibroblasts (MEFs) were unsuccessful. Amongst the $CD34^+$ stromal cells were also α-smooth-muscle-actin-positive ($ACTA2^+$) and vimentin-positive (VIM^+) cells that together supported derivation and long-term proliferation of adult SPCs from mouse testes of various ages (up to 1 yr) and genetic backgrounds in >90% of attempts (Fig. 1g, inset and Supplementary Fig. 3c, d). The adult spermatogonial cultures displayed heterogeneous colony size, with frequent formation of massive proliferating colonies, exponential overall growth, and ~30% of cells in S phase (Fig. 1h, j, and Supplementary Fig. 4a–c). Adult SPC lines were also derived from mice displaying green fluorescence in all tissues (see ref. [7]) and were serially passaged six times in typical fashion on $CD34^+$MTS. This revealed expansion of SPCs and near total (>99%) depletion of any somatic green-fluorescent-protein-positive (GFP^+) cells outside of the characteristic spermatogonial stem cell-like colonies, suggesting loss of the non-germline contaminants (Supplementary Fig. 4c). The SPC lines expressed typical mouse germ-lineage markers, including germ-cell nuclear antigen (GCNA1), DAZL and MVH (also known as DDX4) (Supplementary Fig. 4d–f)[8–10]. Notably, the colonies expressed the well-characterized marker PLZF (also known as ZBTB16), which identifies undifferentiated spermatogonia (Fig. 1i)[11, 12]. Evidence of bona fide stem cell activity within the SPC pool (cultured for more than one

year) was revealed by the ability of cells to participate in reconstitution of spermatogenesis in busulphan-treated host mice (see Fig. 2)[13].

Prior studies have found that embryonic stem cell (ESC)-like cells arose in culture either from neonatal testicular cells by spontaneous conversion in the presence of glial-cell-line-derived neurotrophic factor (GDNF) and leukaemia inhibitory factor (LIF) on MEFs[14], or in adult spermatogonial stem cells (SSCs) maintained in the absence of GDNF within four weeks after the initiation of spermatogonial colonies[1]. We found that long-term culture of adult SPCs generated distinct colonies of MASCs from cells that were originally cultured on the $CD34^+$MTS feeder layers for more than three months (Fig. 1k, l). The emergence of MASC colonies was heralded by a distinct morphologic change in a subset of SPC colonies (Supplementary Fig. 4g). Putative MASC colonies, resembling ESCs, were mechanically transferred from $CD34^+$MTS to MEFs for MASC expansion in the undifferentiated state (Fig. 1k, and Supplementary Fig. 1)[15]. Whereas the pluripotency marker OCT4 (also known as POU5F1) protein was undetectable in SPCs (data not shown), we found unequivocal OCT4 expression in the nuclei of MASCs that were expanded (15 passages before cryopreservation) on MEFs (Fig. 1l) and that were capable of differentiation into multiple lineages *in vitro*, including rhythmically contractile cardiogenic tissue (Supplementary Fig. 5a–d and Supplementary Movie 1). MASCs gave rise to teratomas (in 9/9 attempts) when injected subcutaneously in NOD-SCID mice (Supplementary Fig. 5e–h). The expression of LacZ in both *Gt(ROSA26)Sor-lacZ*[16] MASCs, and the resultant teratomas, excluded the possibility of a multipotent mesenchymal cell originating from the wild-type, mitomycin-C-inactivated feeders. Furthermore, MASCs cloned from single cells were similarly competent to form tri-lineage teratomas and to contribute to chimaeric embryos on blastocyst injection (see Fig. 3).

To determine whether GPR125 is expressed on SPCs, testes from $Gpr125^{+/lacZ}$ and $Gpr125^{lacZ/lacZ}$ mice were used to derive SPC lines that were propagated on $CD34^+$MTS. Refractile, cobblestone colonies reminiscent of SSCs appeared within one week, and large proliferative colonies were seen within 3–4 weeks that exhibited exponential clonal growth, and culture wells could be de-populated with complete re-growth of colonies (Fig. 2a, b). Maintenance of the germ-cell phenotype was confirmed by immunohistochemistry for GCNA and DAZL (Fig. 2c)[17], but c-KIT was absent by flow cytometry (Supplementary Fig. 6a). Strikingly, GPR125–LacZ SPCs maintained GPR125 expression after multiple passages *in vitro* (Fig. 2a, inset) and are hereafter referred to as $GPR125^+$ SPCs (GSPCs). To determine the frequency of repopulating cells, limiting dilution analysis was performed using GFP-labelled GPR125–LacZ GSPCs (from $Gpr125^{lacZ/lacZ}$ mice) on cells that were cultured beyond 9 months, revealing 0.23 (95% confidence interval, 0.19–0.27) colony forming units (c.f.u.) per cell or 1 c.f.u. for every 4–5 GSPCs (Fig. 2d). All emerging colonies derived from the testes of $Gpr125^{lacZ/lacZ}$ mice expressed *lacZ*, suggesting that the GSPCs are clonagenic (Fig. 2d).

The molecular identity of $Gpr125^{lacZ/lacZ}$ GSPCs in long-term culture was confirmed by quantitative PCR (Fig. 2e, and Supplementary Fig. 7). Among the transcripts expressed in $Gpr125^{lacZ/lacZ}$ GSPC cultures were germ-cell-specific genes, including *Dazl* and *Mvh*[10,17]. To rule out spontaneous spermatogenic differentiation of the cultured GSPCs[18], we surveyed transcripts characteristic of differentiated germ cells and noted diminished or absent levels for transcripts such as *Sox17*, *Tnp1*, *Adam2*, *Prm1* and *Pgk2* (ref. [19]). These data suggested that repopulating GSPCs were of germ-cell origin but remained undifferentiated. Even after *in vitro* propagation for over one year, GSPCs revealed a transcriptional profile highly reminiscent of spermatogonial stem cells (Fig. 2e, and Supplementary Fig. 7). Various cell surface markers used for isolation of SSCs were increased at the mRNA level in GSPCs: *Itga6* (~18-fold), *Tacstd1* (~5-fold), *Cd9* (~15-fold) and *Gfra1* (~128-fold)[20–22]. Similarly, genes with preferential promoter activity in undifferentiated cells, including *Stra8* and *Oct4*,

were detectable albeit at lower levels in the GPR125^{+} cells. Therefore, our culture technique yields undifferentiated spermatogonia, which like spermatogonia *in vivo*, express GPR125.

To interrogate the repopulating potential of GSPCs *in vivo*, we then evaluated the capacity of GFP-labelled *Gpr125*$^{lacZ/lacZ}$ GSPCs to restore spermatogenesis within busulphan-treated C57Bl6 host mouse testes[13]. Within 2–3 months after transplantation, robust GFP^{+} *Gpr125*$^{lacZ/lacZ}$ germ-cell colonies were detectable within the host seminiferous tubules (Fig. 2f, and Supplementary Fig. 8a). These colonies contained populations of GFP^{+} cells along the basement membrane, exhibiting typical spermatogonial morphology, and smaller round GFP^{+} cells located more centrally to tubular lumen (Fig. 2f, and Supplementary Fig. 8b–g). X-gal staining confirmed co-expression of GPR125 (LacZ^{+}) in a small subset of the GFP-labelled, transplanted cells along the basement membrane (Fig. 2g, and Supplementary Fig. 9a–e), recapitulating the spatial expression pattern in the GPR125–LacZ testes (see Fig. 1). Importantly, GFP^{+} spermatids were seen in donor-colonized tubules but not in adjacent tubules containing residual, host-derived spermatogenesis, confirming the presence of true stem cell activity within the long-term *Gpr125*$^{lacZ/lacZ}$ GSPC cultures (Fig. 2h, and Supplementary Fig. 8h). PCR for GFP detected donor-derived sperm in the epididymis draining the transplanted testis but not in negative controls (data not shown).

The origin of multipotent stem cells in the adult testis is not clear[23]. Therefore, we sought to formally prove that GSPCs could indeed generate multipotent cells, even after long-term expansion *in vitro*. The spontaneous emergence of MASCs was observed in the *Gpr125*$^{lacZ/lacZ}$ cultures that were initially propagated for more than 3 months. These *Gpr125*$^{lacZ/lacZ}$ MASCs had a high nuclear-to-cytoplasmic ratio, formed refractile colonies, and could be split in a ratio of ~1:8 in mouse embryonic stem cell medium every 2–3 days (Fig. 2i; passaged >30 times before cryopreservation). The majority of cells had a normal karyotype, and no evidence of clonal cytogenetic abnormalities was found for either *Gpr125*$^{lacZ/lacZ}$ MASCs or *Gt(Rosa26)Sor-lacZ* MASCs (data not shown). Notably, the majority of cells within the colonies were highly positive for GPR125 expression and also uniformly immuno-positive for OCT4 within the nucleus (Fig. 2j). FDG-labelling revealed more than 99% of both *Gpr125*$^{lacZ/lacZ}$ GSPCs and MASCs to be GPR125^{+}by β-galactosidase activity (Fig. 2k), suggesting that GPR125 is associated more universally with the stem- and progenitor-cell phenotype.

The multipotency of these *Gpr125*$^{lacZ/lacZ}$ MASCs was first assessed by formation and differentiation of embryoid bodies *in vitro*[24]. Within seven days after re-plating, embryoid bodies exhibited a distinct pattern of GPR125 expression, with distinct borders between GPR125^{+} and GPR125^{-} areas (Supplementary Fig. 10a, b). The resultant colonies contained HNF3β^{+} (FOXA2^{+}) cells derived from endoderm or ectoderm, cytokeratin-positive (KRT^{+}) or GFAP^{+} cells derived from ectoderm, and brachyury-positive or skeletal-muscle-myosin-positive (MYH2^{+}) cells derived from mesoderm (Fig. 3a, b).

When GPR125$^{lacZ/lacZ}$ MASCs were implanted subcutaneously in NOD-SCID mice, the resultant teratomas (14/14 attempts) similarly exhibited GPR125 expression in a lineage-specific manner, implying loss of GPR125 in certain differentiated cell types (Fig. 3c and Supplementary Fig. 11). In fact, these teratomas were reminiscent of GPR125–LacZ embryos, in which GPR125 expression is present in most but not all tissues and subsequently lost over time (see Fig. 3h, Supplementary Fig. 12). Lineage analysis of MASC teratomas demonstrated morphologic and immunologic evidence for tissue derivatives of all three germ layers, including mucin-positive (Muc5ac^{+}) endoderm, GFAP^{+} neuroectoderm, and mesodermal chondrocytic, myoid, and vascular cells (Fig. 3d–f).

The ability to form chimaeric animals has been used to demonstrate multipotency of germ-cell derivatives[2]. We therefore performed blastocyst injections with cloned *Gpr125*$^{lacZ/lacZ}$ MASCs and found 8 chimaeric embryos out of 37 evaluated (22%). Importantly, the expression pattern of GPR125 in the C57Bl6 (host)/*Gpr125*$^{lacZ/lacZ}$ (donor) chimaeric embryos partially recapitulated what was seen in heterozygous knock-in *Gpr125*$^{+/lacZ}$ embryos, with prominent signal in developing ossification centres (Fig. 3g, h, and Supplementary Fig. 12e, f). In addition, LacZ^{+} cells were also detected in the chimaeric gut and other tissues that are known to harbour GPR125^{+} cells in non-chimaeric embryos (Supplementary Table 2 and Supplementary Fig. 12). These data indicate that generation of GPR125^{+} MASCs from GSPCs results in the maintenance of the expected global expression pattern of the *Gpr125* gene. As such, lineage-specific derivatives of MASCs may have the essential set of genetic and epigenetic instructions that are critical for autologous organ regeneration.

To this end, we examined the ability of MASCs to differentiate into endothelial cells. An extensive network of vessel-like, lumen-containing VE-cadherin^{+} (CDH5^{+}) structures were formed *in vitro* from MASC embryoid bodies after 22 days of differentiation (Fig. 3i, and data not shown). To determine whether GPR125^{+}MASCs could differentiate into functional vessels *in vivo*, GPR125^{+} MASCs were transduced with a lentiviral vector expressing GFP under control of the promoter for the endothelial-specific marker VE-cadherin (ref. [25]). Teratomas formed in NOD-SCID mice from such transduced MASCs contained donor-derived GFP^{+} blood vessels, continuous with the host circulation, as shown by perfusion-based staining and the presence of red blood cells within the vessels (Fig. 3j–l).

We asked next whether MASCs use the same molecular machinery for multipotency as ESCs. Expression analysis of *Gpr125*$^{lacZ/lacZ}$ MASCs compared with mouse ESCs, GSPCs or MEFs revealed high levels of *Oct4*, *Nanog* and *Sox2* in both MASCs and ESCs (Fig. 4a). Minimal expression of typical SSC markers, including *Plzf*, *Ret* and *Stra8*, was seen in MASCs, which, as expected, were high in *Gpr125*$^{lacZ/lacZ}$ GSPCs. Unexpectedly, certain key germ-lineage transcripts (for example, *Dazl*) were nearly absent in MASCs, as were some canonical mouse ESC transcripts (for example, *Gdf3*, *Esg1* (also known as *Dppa5*) and *Rex1*; Fig. 4b). The differences in expression of these genes and others (for example, *Nog* and *brachyury*) suggest that MASCs constitute a distinct stem cell type from that reported in ref. [1].

Here, we have identified GPR125 as a surface marker for self-renewing, clonagenic, cKIT^{-}PLZF^{+} spermatogonial progenitor cells (GSPCs), with the capacity for both repopulating the testis and generating GPR125^{+} MASCs. Recent evidence indicates that spermatogonial progenitor cells can manifest stem cell activity[26]. This indicates that GPR125^{+}cKIT^{-}PLZF^{+}DAZL^{+} GSPCs may not only be endowed with spermatogonial stem cell activity but may also perform as undifferentiated spermatogonial cells that can convert into GPR125^{+}cKIT^{+}PLZF^{-}DAZL^{-}OCT4^{+}MASCs. These data pinpoint GPR125^{+} spermatogonial cells as the cellular ancestors of MASCs (see Supplementary Fig. 1). Differentiation of GPR125^{+} MASCs into GPR125^{-} tissues qualifies GPR125 expression as a useful marker for tracking differentiation and lineage-specification of stem and progenitor cells.

The precise molecular and cellular pathways governing the emergence of MASC colonies remain unclear. Although MASCs and ESCs have identical morphological characteristics and are both multi-potent, capable of giving rise to teratomas and chimaeric animals, there are major differences at the transcriptional level that distinguish these two cell types (Fig. 4c). Notably, unlike the ESC-like cells derived from STRA8^{+} SSCs[1], GPR125^{+} MASCs lack the molecular signature of ESCs but mimic other multipotent adult stem cells, such as multipotent adult progenitor cells (MAPCs)[27]. Our data, therefore, imply that multipotency may be driven by multiple unique sets of signals, even in the absence of gene products typically associated

with stem cells (for example, *Gdf3*, *Esg1* and *Rex1*). Also, in contrast to a prior report[1], the maintenance of long-term cultures of GSPCs was dependent on GDNF and was therefore necessary for the subsequent emergence of MASCs. Therefore, culture conditions may influence the ultimate multipotent phenotype.

GPR125 expression in undifferentiated cells and early progenitors and its subsequent downregulation on terminal differentiation raises the intriguing possibility of exploiting surface expression of GPR125 to isolate human SSCs and GSPCs. Recent data demonstrated the *in vitro* differentiation of endothelium from multipotent cells derived from the neonatal testis[28]. We extend these observations by showing that GPR125^{+} MASCs can generate functional vasculature *in vivo*. Taken together, these data indicate that GPR125^{+} MASCs could be used therapeutically for the generation of functional autologous vessels for revascularization. However, as germline stem and progenitor cells may have alterations in genomic imprinting in certain genes compared with adult somatic cells[14], the use of these cells for therapeutic purposes should proceed with caution and extensive pre-clinical experimentation.

METHODS SUMMARY

SSC, MASC and feeder-cell culture

C57Bl6 mice aged 4–12 wks served as donors for mixed primary testicular feeder cells, which were expanded following enzymatic digestion of the seminiferous tubules. Feeder cells were treated with mitomycin-C before use for stem cell culture. Mouse GSPCs were obtained from enzymatically dissociated seminiferous tubules from mice aged 3 weeks to 8 months and were plated in StemPro-34 (Invitrogen) with the modifications of ref. [29]. GSPCs were serially passaged onto fresh mitomycin-C-treated feeders every 2–8 wks. Morphologically atypical transitional colonies of GSPCs were mechanically removed from the plate after >2 wks in culture and re-plated in the same medium or embryonic stem cell (ESC) medium on mitomycin-C-inactivated MEF to obtain MASC lines.

GPR125–LacZ mice

VelociGene technology was employed for production of *Gpr125*$^{lacZ/lacZ}$ mice as previously described[3]. Briefly, targeting vectors were generated using a bacterial artificial chromosome (BAC) and contained *Gpr125* in which exons 16–19 were deleted and replaced in-frame with *lacZ*, as a reporter gene, and neomycin, as a selectable marker. Targeting vectors were electroporated into ESCs. Clones that were properly targeted were confirmed by real-time PCR-based loss-of-native-allele assay[3], using primers listed in Supplementary Information. Chimaeric mice were generated by blastocyst injection of ESCs and backcrossed to C57Bl6/J to produce heterozygote breeding pairs.

Additional methods

Additional methods, including histochemistry, immunostaining, flow cytometry following FDG-labelling, quantitative (q)PCR, lentivirus preparation and spermatogonial transplantation are presented in the Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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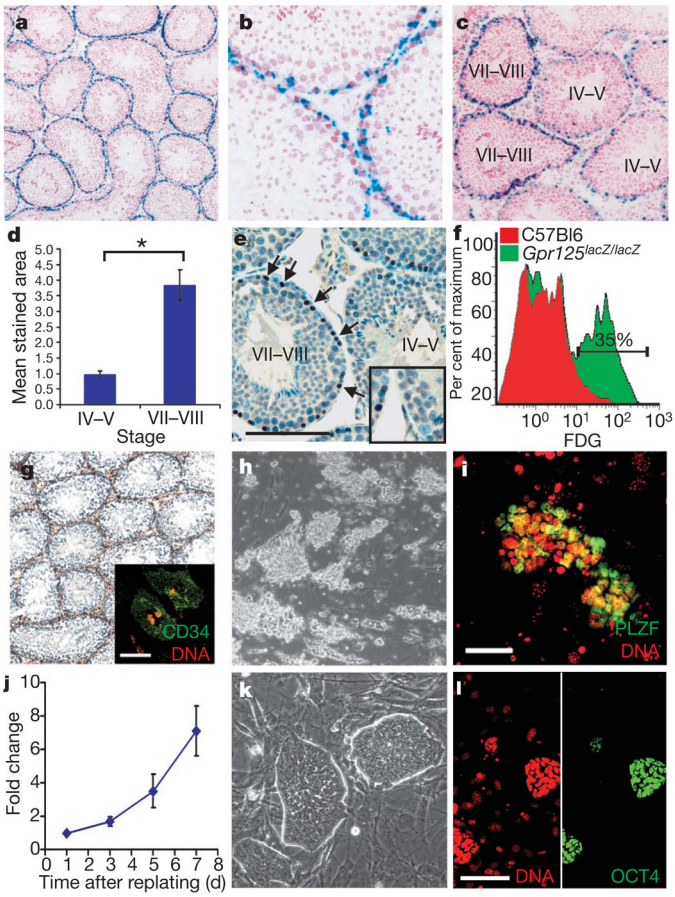

Figure 1. Restricted GPR125 expression in adult mouse testis and derivation of multipotent cells from spermatogonial progenitor cells (SPCs)

a–c, X-gal staining of adult GPR125–LacZ mouse testis. **d**, Quantification of X-gal staining (arbitrary units) in tubules grouped as stages IV–V (0.98 ± 0.11 (mean ± s.e.m.); $n = 30$ tubules) versus stages VII–VIII (3.84 ± 0.49; $n = 28$; *P <0.001 by Wilcoxon test). **e**, Anti-GPR125 staining (brown, arrows) of adult mouse testis. **f**, Flow cytometry on freshly dissociated adult *Gpr125*$^{lacZ/lacZ}$ testis. Roman numerals in **c**–**e** denote approximate stages of the seminiferous tubules[4]. **g**, Anti-CD34 staining (brown) of peritubular/interstitial mouse cells, which remain CD34^{+} (inset, green staining) following *in vitro* expansion. **h**, **i**, Highly proliferative SPC colonies (**h**) that express PLZF (**i**, green staining) after expansion on inactivated CD34^{+}MTS. **j**, The number (mean ± s.d.) of SPCs doubled every ~2 days. **k**, **l**, The appearance of MASCs derived from *Gt(Rosa26)Sor-lacZ* SPCs following transfer to MEF for expansion and antibody staining, revealing OCT4 expression in the nucleus (green, right panel in **l**). Scale bars, 50 μm. Nuclei are shown in red (**a**–**c**, **i**, **l**) or blue (**e**, **g**).

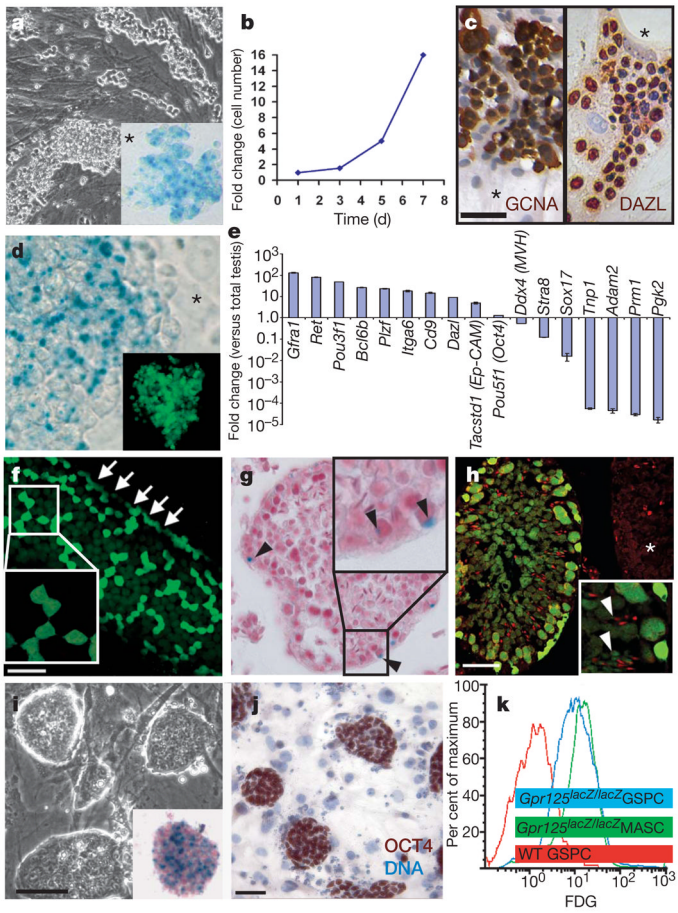

Figure 2. Characterization and multipotent derivatives of *Gpr125*$^{lacZ/lacZ}$ SPC lines

a, Morphology of SPC colonies and expression of GPR125 by X-gal staining (blue, inset). **b**, Proliferation of GPR125^{+} SPCs (GSPCs) in culture. **c**, Immunolabelling by germ-cell markers GCNA (brown, left panel), and anti-DAZL (brown, right panel). Absent staining in feeders is denoted by asterisks. **d**, Expression of GPR125–LacZ in cloned GSPCs (tracked by GFP (green, inset), using lentivirus). **e**, Quantitative PCR of *Gpr125*$^{lacZ/lacZ}$ GSPCs compared to *Gpr125*$^{lacZ/lacZ}$ total testis. Bars depict fold change (± scaled s.d.) of gene expression (compared with total testis) of genes associated with GSPCs or differentiating spermatogenic cells. **f**–**h**, Engraftment of *Gpr125*$^{lacZ/lacZ}$ GSPCs microinjected into busulphan-treated testes. **f**, Confocal slices (~1 μm, inset) distinguishing areas with GFP^{bright} spermatogonia along the basement membrane (arrows) from centrally located areas containing smaller, round GFP^{dim} differentiating cells, in the projection of 32 slices. **g**, GPR125 expression by X-gal staining (blue, arrowheads) present in engrafted cells along the basement membrane. **h**, Differentiation of donor-derived GFP^{+} cells and GFP^{-} non-engrafted tubules (arrowheads, GFP^{+} spermatids; asterisk, non-engrafted tubule). **i**, Derivation of GPR125^{+}MASCs (blue staining in inset, X-gal) from GSPCs. **j**, Nuclear labelling by anti-OCT4 (brown). **k**, Flow cytometry for GPR125 expression in *Gpr125*$^{lacZ/lacZ}$ MASCs or GSPCs by FDG staining (mean fluorescence intensity: 22.1 or 18.2, respectively, versus 2.2 in wild-type GSPC control). Scale bars, 50 μm.

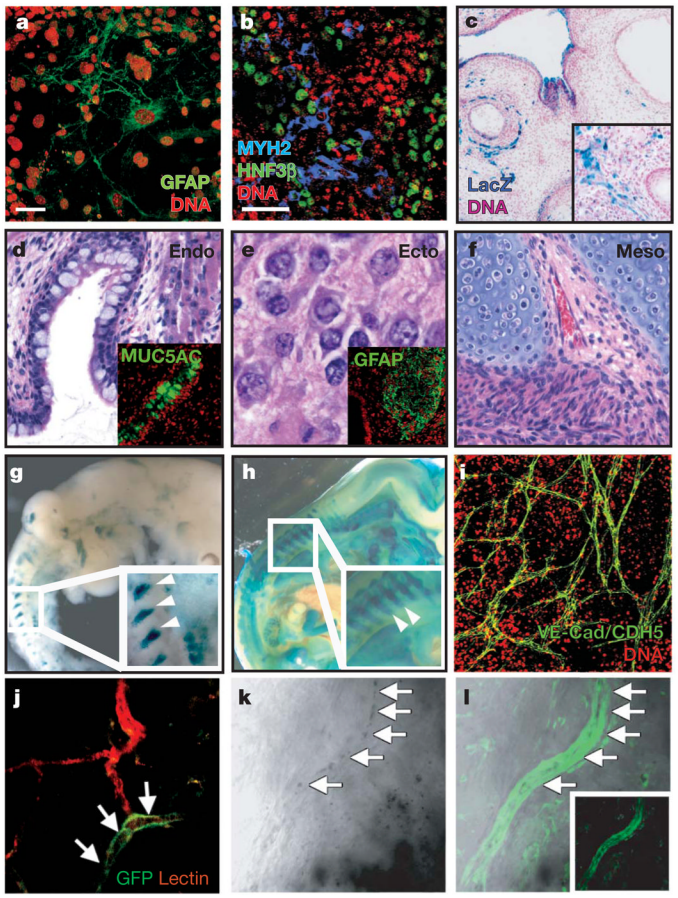

Figure 3. GPR125–LacZ MASCs exhibit multipotency and can form functional vessels

a, b, Embryoid bodies differentiated *in vitro* and immunolabelled for neuroectoderm (anti-GFAP, green; **a**); mesoderm (anti-myosin heavy chain, myosin HC, blue; **b**); and endoderm or ectoderm (using anti-HNF3β, green; **b**). **c**, X-gal-stained GPR125–LacZ MASC teratoma formed in NOD-SCID mice. **d**–**f**, Teratoma histology showing endodermal (**d**), ectodermal (**e**), and mesodermal (**f**) elements. Immunofluorescence (green) in insets: **d**, anti-Muc5ac; **e**, anti-GFAP. **g**, **h**, Whole-mount embryo X-gal staining. **g**, E13.5 GPR125–LacZ MASC chimaera formed by blastocyst injection. **h**, E14.5 full heterozygous *Gpr125*$^{+/lacZ}$ embryo. **g**, **h**, Arrowheads denote putative ossification centres. **i**, GPR125–LacZ MASCs differentiated *in vitro* (22 days) and stained with anti-VE-cadherin/CDH5 (green). **j**–**l**, Cloned MASCs that were previously transduced *in vitro* with a VE-cadherin (*Cdh5*) promoter fragment driving *GFP* (green) in a lentiviral vector, form functional teratoma vessels *in vivo*, demonstrated by perfusion with mouse endothelial specific lectin (red in **j**) or by the presence of blood in GFP^{+} vessels (black in **k**, **l**; inset in **l** shows GFP alone). Arrows denote donor-derived vessels. Nucleic acid counter stain in **a**–**c**, **d** (inset), **e** (inset), and **i** is red. Scale bars, 50 μm.

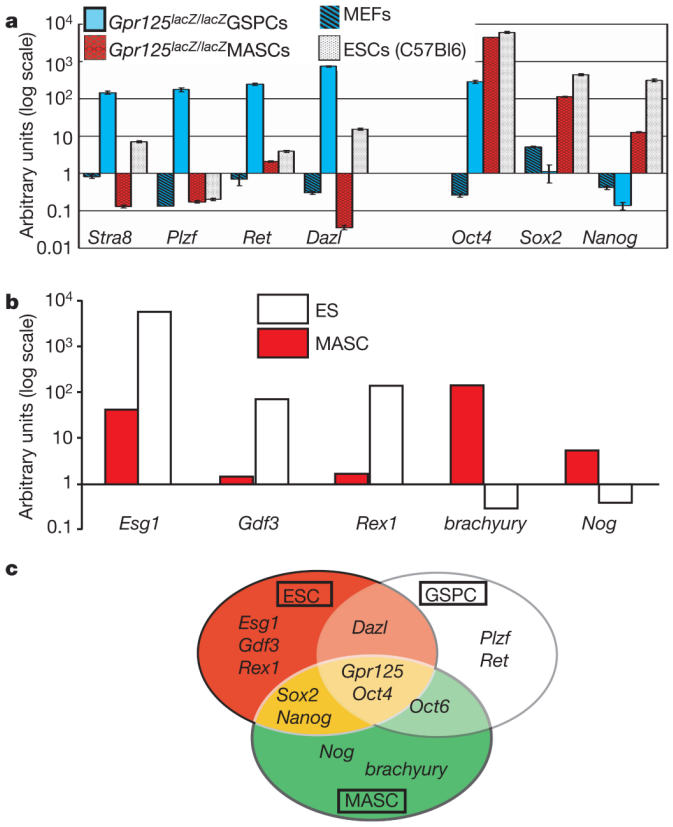

Figure 4. *Gpr125*$^{lacZ/lacZ}$ MASCs have an expression profile different from mouse embryonic stem cells

a, b, Quantitative PCR comparing expression of relevant genes *in vitro* in *Gpr125*$^{lacZ/lacZ}$ MASCs versus wild-type ESCs, *Gpr125*$^{lacZ/lacZ}$ GSPCs, and MEFs. **c**, Venn diagram illustrating transcripts unique or common to GSPCs, MASCs and ESCs.