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GASZ promotes germ cell derivation from embryonic stem cells

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Abstract Primordial germ cells (PGCs) are the first germ-line population that forms from the proximal epiblast of the developing embryo. Despite their biological importance, the regulatory networks whereby PGCs arise, migrate, and differentiate into gametes during embryonic development remains elusive, largely due to the limited number of germ cells in the early embryo. To elucidate the molecular mechanisms that govern early germ cell development, we utilized an in vitro differentiation model of embryonic stem cells (ESCs) and screened a series of candidate genes with specific expression in the adult reproductive organs. We discovered that gain of function of *Gasz*, a gene previously reported to participate in meiosis of postnatal spermatocytes, led to the most robust upregulation of PGC formation from both human and murine ESCs. In contrast, *Gasz* deficiency resulted in pronounced reduction of germ cells during ESC differentiation and decreased expression of MVH and DAZL in genital ridges during early embryonic development. Further analyses demonstrated that GASZ interacted with DAZL, a key germ cell regulator, to synergistically promote germ cell derivation from ESCs. Thus, our data reveal a potential role of GASZ during embryonic germ cell development and provide a powerful in vitro system for dissecting the molecular pathways in early germ cell formation during embryogenesis.
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Introduction

Maintenance of the germ cell lineage ensures the passage of genetic information through generations. The initial germ cell population, known as PGCs, arises from proximal epiblast at 6.5 days postcoitum (dpc) in mice (Ginsburg et al., 1990). These cells subsequently migrate to the genital ridge, and later differentiate into oocytes or spermatozoa (Ewen and Koopman, 2010; Hayashi et al., 2007; Ohinata et al., 2009; Saga, 2008; Saitou, 2009). Although the eventual process of

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gamete production has been intensively studied over the last century, the regulatory networks during the early stages of germ cell development, including the emergence, fate specification, migration, and differentiation of PGCs into sex-dependent gametes, remain elusive, largely due to the technical difficulties to access early embryos and the restricted number of germ cells during embryogenesis.

Although several new PGC-specific genes were recently identified by gene expression analyses (Mise et al., 2008; Wang et al., 2001; Yabuta et al., 2006), to date only a few of them have been confirmed to participate in early germ cell development by gene targeting experiments, including PR-domain containing transcriptional regulators, PRDM1/BLIMP1 and PRDM14, and the DAZ family of RNA binding proteins (Ohinata et al., 2005; Ruggiu et al., 1997; Saitou, 2009; Yamaji et al., 2008). The *DAZ* gene family consists of *BOULE*, *DAZ-like* (*DAZL*), and *DAZ*, according to their evolutionary hierarchy. Among them, *Dazl* starts to express at 11.5 dpc in mice, and its deficiency results in female and male infertility (Ruggiu et al., 1997). In addition, gene targeting analysis with *Dazl*−/− mice on a C57BL/6 inbred background revealed a pronounced reduction of germ cells by 14.5 dpc, thus suggesting that Dazl participated in early germ cell development (Lin and Page, 2005). Gill and colleagues recently showed that DAZL was required for transition of post-migrating PGCs to "gametogenesis competent cells", a process they called "licensing" (Gill et al., 2011). PGCs in the absence of DAZL could not develop into either male or female gametes (Gill et al., 2011).

A number of reports have demonstrated that pluripotent ESCs can form embryoid bodies (EBs) and spontaneously differentiate into PGCs and haploid spermatids in vitro (Geijsen et al., 2004; Hayashi et al., 2011; Kee et al., 2009; Toyooka et al., 2003), thereby providing an alternative model to investigate the signaling networks during early germ cell formation. Pluripotency related genes, *Oct4* and *Nanog*, which are expressed at high levels in ESCs, are also enriched in PGCs, and therefore can serve as markers with SSEA1 surface antigen for ESC-derived PGCs (Geijsen et al., 2004; West et al., 2009). In addition, loss of genomic imprinting occurs solely in germ cell lineages, and thus DNA methylation erasure of imprinted genes has been a unique in vitro surrogate to confirm the identity of PGCs (Geijsen et al., 2004; West et al., 2009). In two recent papers, DAZ family members were shown to promote PGC formation from both mouse and human ESCs, but also to function in the later stages of meiosis and gamete formation in vitro (Kee et al., 2009; Yu et al., 2009). LIN28, a RNA binding protein, enhances BLIMP1 expression by blocking the maturation of *let-7*, a miRNA regulator of BLIMP1, and thus promotes PGC derivation from mouse ESCs indirectly (West et al., 2009). Therefore, the ESC in vitro differentiation model provides a useful system to dissect early germ cell development during embryogenesis.

GASZ (also called ASZ1) is a recently identified *G*erm cell specific protein with four *A*nkyrin repeats, a *S*terile alpha motif, and a putative leucine *Z*ipper (Ma et al., 2009; Yan et al., 2004; Yan et al., 2002). In a previous report, we demonstrated that the loss of function of *Gasz* leads to male infertility due to defects in post-natal meiosis as well as abnormal piRNA biosynthesis and retrotransposon expression (Ma et al., 2009). However, the biological function of GASZ in early germ cell development has yet to be elucidated. In the present study, using in vitro differentiation models of human and mouse ESCs, we demonstrate that GASZ promotes PGC derivation and enhances the expression of genes involved in early germ cell development. In addition, we found that GASZ was highly enriched in post-migrating PGCs, and its deficiency led to defects in expression of early germ cell markers during embryogenesis in vivo. Further analyses reveal that GASZ interacts with DAZL to synergistically enhance PGC formation from ESCs. Therefore, our data demonstrate a novel role of GASZ in mammalian germ cell development both from ESCs and during early embryogenesis.

Materials and methods

Cell culture and ESC differentiation

Mouse ESCs were maintained and differentiated according to published protocols (Wang et al., 2008; Wang et al., 2005). Human ESCs, H1 or BG01 (WiCell Research Institute), were cultured in DMEM/F12, supplemented with 20% knockout serum replacement (KSR) and 4 ng/ml recombinant β-FGF (Invitrogen). To differentiate, human ESCs were dissociated into small clumps (<100 cells per clump) with collagenase IV (1 mg/ml, Sigma), and plated onto gelatin-coated plates in differentiation medium (DMEM with 15% fetal bovine serum, FBS, Invitrogen). Cells were treated with 20 ng/ml activin A (R&D) for 36 h, followed by 20 ng/ml human BMP4 (R&D) for four days, maintained in differentiation medium afterwards, and collected at various time points. Stable ESC lines were established either by sorting of GFP+ cells or by selection of hygromycine resistant ESCs after viral transduction. All other cell lines were cultured in high glucose DMEM with 10% FBS.

Flow cytometry analysis

Single cells dissociated from EBs or differentiated human ESCs were incubated with a monoclonal antibody against SSEA1 (Hybridoma bank) for an hour at 4 °C in DMEM with 10% FBS (D10). Cells were washed twice with ice-cold D10, and then reacted with rat anti-mouse APC-IgM (eBioscience) or rat anti-mouse PE-IgM (BD) for 30 min at 4 °C. Stained cells were analyzed with FACSCalibur (BD Biosciences) or sorted with FACSAria II (BD Biosciences).

Embryonic germ cell colony formation and alkaline phosphatase staining

1–5 × 10^4 whole EB cells or 5 × 10^3 SSEA1+ cells purified by flow cytometry-based sorting were plated onto mouse embryonic fibroblasts (MEFs) with mouse ESC culture media containing 15 ng/ml of β-FGF (Invitrogen) and 30 ng/ml of SCF (Peprotech), plus 2 μM trans-retinoic acid (RA, Sigma). Individual embryonic germ cell (EGC) colonies were picked and expanded in medium described above without RA for imprinting analysis. Alternatively, EGC colonies were harvested directly after 7 days post-plating for total RNA collection, or fixed in 4% paraformaldehyde, staining for tissue-nonspecific alkaline phosphatase activity.

Plasmid construction

Mouse *Dazl*, *Gpbox*, *Psx1*, *Pem*, *Rbmy*, and *Gasz* were amplified from a cDNA library of mouse testis and cloned into

pMSCV–IRES-GFP or pMSCV–IRES-hygromycine for retroviral infections of ESCs. Human *GASZ* was amplified from a human testis cDNA library purchased from Shanxi Chaoying Biotechnology Company (Shanxi, China). A PCR fragment containing human *DAZL* cDNA was amplified from pBub-IRES-GFP-DAZL plasmid (a kind gift from Dr. Lixin Feng, Shanghai Jiaotong University), and further cloned into a lentiviral vector (pLenti). For constructs used in co-immunoprecipitation, *Gasz* and its deletion mutants were cloned into pLenti and fused with either FLAG or GFP. Mouse *Dazl* was cloned into pSG5-HA2 (a kind gift from Dr. Xiaotao Li, East China Normal University), in fusion with HA tag. A lentiviral vector, Pll3.7, was used for cloning all shRNAs in gene knockdown assays. Stable ESC lines containing shRNA were established by drug selection of hygromycine. All primers used for gene cloning, PCR, RT-PCR, and sequences for shRNA were provided in the Supplementary material Table 1.

Genomic DNA preparation and southern blots

Cells were lysed in digestion buffer (100 mmol/l NaCl, 10 mmol/l Tris–HCl, pH 8.0,25 mmol/l EDTA, 1% SDS, 0.1 mg/ml proteinase K) for 6 h. Genomic DNA was purified with phenol/chloroform extraction, precipitated with ethanol, and dissolved in DNase-free water. DNA was digested with appropriate restriction enzymes (Fig. 2E) overnight, separated in 0.8% agarose gels, and blotted following the standard procedures. Probe labeling and further detection were performed with DIG High Prime DNA Labeling and Detection Kits (Roche).

Bisulphite sequencing

0.5 μg of genomic DNA was treated by adding 1 μl of 6N NaOH, and followed by incubation for 15 min at 37 °C. 120 μl conversion solution (107 μl of 4.04M $NaHSO_3$, 7 μl of 10mM hydroquinone, and 6 μl of 6N NaOH) was then added. Fifteen cycles of DNA denaturing for 30 s at 95 °C and bisulphite treatment for 15 min at 50 °C were performed. Nested PCR was then used to amplify the *Snrpn* DMR as previously described (Lucifero et al., 2002). Ten clones from each sample were sequenced. Sample treatment and processing were performed simultaneously for quality control.

Total RNA preparation, RT-PCR and quantitative real-time PCR

Total RNAs were extracted with TRIzol (Invitrogen) following the manufacturer's instructions. cDNAs were synthesized using a PrimeScript® RT reagent Kit (TaKaRa Biotechnology Co., Ltd.). cDNAs from human tissues were purchase from Shanxi Chaoying Biotechnology Co., Ltd (Shanxi, China). Real-time PCR was performed on Stratagene MX3000P instrument and analyzed as described previously (Wang et al., 2005). Primers are listed in Supplementary material Table 1.

Co-immunoprecipitation (Co-IP), Western Blot, and mass spectrometry analysis

Cells were harvested in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100, with protease inhibitor cocktail from Santa Cruz Biotechnology Inc.), incubated with primary antibodies for 6 h at 4 °C, and then precipitated with protein A-agarose beads (Santa Cruz Biotechnology Inc.). For Mass Spectrometry analysis, peptides were extracted from destained SDS-PAGE gel and analyzed on LTQ XL (Thermo Fisher Scientific) in Data Dependent Acquisition modes. The spectra were analyzed using SEQUEST against the Uniprot Swiss-Prot Database and the International Protein Index (IPI) Mouse Database (version 3.87). Antibodies used in Co-IP and Western Blot: HA (1:1000, Santa Cruz Biotechnology Inc.); FLAG (1:5000, Sigma); GFP (1:1000, Santa Cruz Biotechnology Inc.); Dazl (1:1000, Abcam); Antibody against GASZ was made from guinea pigs as previously reported (Ma et al., 2009).

Whole mount in situ hybridization (WISH), immunohistofluorescence (IHF), immunohistochemistry (IHC)

Embryos from C57BL/6 mice were used for ISH. *Gasz* knockout mice were reported previously (Ma et al., 2009). The age of embryo was defined as 0.5 dpc at noon on the day of vaginal plug observation. Gonads were fixed overnight at 4 °C in 4% paraformaldehyde. WISH, IHF, and IHC were performed according to standard protocols (Ma et al., 2009). Full length mouse *Gasz* or *Dazl* sense and anti-sense probes were labeled and detected according to instructions provided with DIG RNA Labeling Kit and DIG Nucleic Acid Detection Kit (Roche). For IHF and IHC, gonadal sections were blotted with guinea pig anti-GASZ (1:50), rabbit anti-MVH (1:500, Abcam), or rabbit anti-DAZL (1:1000, Abcam), and followed with FITC-conjugated anti-guinea pig (1:100), TRITC-conjugated anti-rabbit (1:500, all from Jackson ImmunoResearch) antibodies. All animal experimental procedures were conducted in accordance with the local Animal Welfare Act and Public Health Service Policy.

Teratoma formation and histological analysis

2×10^6 ESCs were injected into nude mice anesthetized with diethyl ether. Mice were sacrificed three weeks after injection. Surgically removed tumors were fixed in 4% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin following standard protocols.

Statistical analysis

All data were presented as group means ± one standard errors (s.e.m.). *p* values were calculated from Student's *t*-test for comparisons when indicated.

Results

GASZ is highly expressed in mouse PGCs

To identify novel genes that participate in early germ cell formation, we interrogated several published arrays to reveal genes with differential expression levels between mouse ESCs and PGCs (Mise et al., 2008; Wang et al., 2001; Yabuta et al., 2006). We next examined their expression profiles by RT-PCR

on various mouse tissues and on whole EB populations during mouse ESC differentiation. Among several genes that we assessed, *Gasz*, several *Rhox* family members including *Pem*, *Gpbox*, and *Psx1*, and an RNA binding protein, *Rbmy*, were specifically expressed in testis and/or ovary (Fig. 1A). In addition, expression of *Gpbox*, *Psx1*, and *Gasz* increased gradually during ESC differentiation (Fig. 1B), similar to known germ cell specific genes, *Mvh* and *Dazl*, indicating their possible roles during embryonic development.

Since SSEA1 was reported to mark ESC-derived PGCs in vitro (Geijsen et al., 2004; Hayashi et al., 2011; Vincent et al., 2011), we sorted SSEA1 + cells from day 9 mouse EBs by flow cytometry, and evaluated the expression of candidate genes and known germ cell markers by real-time RT-PCR. Pluripotency related genes (i.e., *Oct4* and *Nanog*) as well as key regulators of PGC specification (i.e., *Blimp1* and *Stella*), germ cell-specific genes in post-migrating PGCs (i.e., *Dazl* and *Mvh*) (Saitou, 2009), were all significantly upregulated in SSEA1 + cells compared to SSEA1-fraction, suggesting that SSEA1 + cells were indeed enriched for PGCs (Fig. 1C). More importantly, *Dazl*, *Mvh*, and *Gasz* were highly expressed in SSEA1 + population, compared to ESCs, whereas *Blimp1* and *Stella* demonstrated similar levels (Fig. 1C). These data indicate that GASZ can serve as a potential marker to distinguish PGCs from mouse ESCs, in addition to the known germ cell genes, DAZL and MVH.

We previously reported that GASZ was expressed in pachytene spermatocytes and oocytes in adult mice and other species (Ma et al., 2009; Yan et al., 2004; Yan et al., 2002). To investigate if GASZ was also expressed in early germ cells during embryonic development, we performed whole mount in situ hybridization (WISH) as well as immunohistofluorescence (IHF) on genital ridges from mouse embryos at 11.5–13.5 dpc. We confirmed that GASZ was first detectable in post-migrating PGCs at 12.5 dpc and largely co-localized with MVH and DAZL in both male and female genital ridges (Figs. 1D–E, Supplementary Fig. 1B). Taken together, these data reveal that GASZ is specifically expressed in PGCs from both ESC-derived populations and developing murine gonads, suggesting a potential role for GASZ in early germ cell formation.

GASZ stimulates PGC formation from mouse ESCs

PGCs arise from a rare SSEA1 + population of differentiated ESCs and can further develop into ESC-like colonies called embryonic germ cells (EGCs) when cultured on feeder cells in the presence of β-FGF, LIF, and retinoic acid (Geijsen et al., 2004). These EGCs express SSEA1 surface antigen and are positive for tissue-non-specific alkaline phosphatase (TNAP). We demonstrated above that *Gasz*, *Pem*, *Gpbox*, *Psx1*, and *Rbmy* were all specifically expressed in adult reproductive organs (Fig. 1A). To investigate if the candidate genes participate in PGC formation, we established stable mouse ESC lines engineered to ectopically express these genes (Supplementary Fig. 1C) and explored their PGC developmental potential. Among the five candidate genes, gain of function of *Gasz* led to the most robust upregulation of PGC derivation, as demonstrated by enhanced percentage of SSEA1 + cells from day 5 to day 9 EBs with flow cytometry analyses and increased number of EGC colonies with TNAP staining (Figs. 2A–B, Supplementary Fig. 1D–E). Similar results were observed in two female ESC lines with C57BL/6 background overexpressing GASZ (Figs. 2C–D, Supplementary Fig. 1F–G), supporting a common role for GASZ in germ cell development from both male and female ESCs.

As loss of imprinting occurs solely in germ cell lineages, we examined DNA methylation of imprinted genes with Southern Blot analyses on individually picked mouse EGC colonies (Fig. 2E, Supplementary Fig. 2I). Indeed, most of EGC colonies from day 9 EBs with either empty vector or with GASZ expression lost imprinting at the *Kvlqt1* and *Rasgrf1* loci (Fig. 2E). In addition, these ESC-derived EGCs showed extensive loss of methylation at *Snrpn* imprinted loci, as examined by bisulphite sequencing, compared to their parental ESCs (Fig. 2F). Furthermore, quantitative gene expression analyses by real-time RT-PCR also revealed that compared with SSEA1-fraction sorted from day 9 EBs, somatic markers such as *HoxA1* and *HoxB1* were significantly downregulated in SSEA1 + populations, either in control cells or in cells overexpressing GASZ (Fig. 2G). In this assay, we utilized a mouse ESC line stably expressing ectopic DAZL, a germ cell specific gene known to promote PGC formation from ESCs (Kee et al., 2009; Yu et al., 2009). We observed similar pattern in DAZL overexpressing cells, in which *Hox* transcript levels remained unaffected, compared to control SSEA1 + population. Therefore, the ESC-derived SSEA1 + populations were indeed PGCs, and their biological properties were maintained with ectopic GASZ or DAZL expression.

To further elucidate the role of GASZ in promoting PGC formation, we examined gene expression affected by GASZ with real-time RT-PCR analyses. Notably, *Oct4* and *Nanog*, as well as germ cell specific markers including *Stella* and *Mvh*, were all upregulated upon GASZ overexpression in day 9 whole EB populations, but the levels of genes representing three germ layers (*Sox17*, *AML1*, *Nkx2.5*, and *Pax6*) were not altered (Fig. 2H), consistent with a selectively increased germ cell population when ectopic GASZ was expressed. In addition, we confirmed that ESCs with ectopic GASZ or DAZL expression displayed normal differentiation potential into three germ layers by teratoma formation (Fig. 2I). More importantly, the expression levels of several regulators for germ cell formation, such as *Oct4*, *Dazl*, *Mvh*, *Miwi*, *Mili* and *Stra8*, were markedly upregulated upon GASZ overexpression in sorted SSEA1 + cells isolated from whole EBs, but not in the SSEA1- fraction (Fig. 2J), suggesting that GASZ may specifically regulate these genes in mouse germ cells.

GASZ deficiency leads to reduced germ cell derivation from mouse ESCs

To corroborate the physiological function of GASZ in early germ cell formation, endogenous *Gasz* was knocked down by delivering short hairpin RNAs (shRNA) via a lentiviral vector into ESCs (Fig. 3A), and the germ cell forming potential upon GASZ deficiency was explored. TNAP + colony formation analyses from differentiated ESCs revealed a pronounced reduction of EGC colonies upon *Gasz* knockdown (Fig. 3B). Similar phenotype was observed with two different shRNAs against *Gasz* (Supplementary Fig. 2C, D), indicating that the decreased germ cell formation was not due to off target effects of *Gasz* RNA interference.

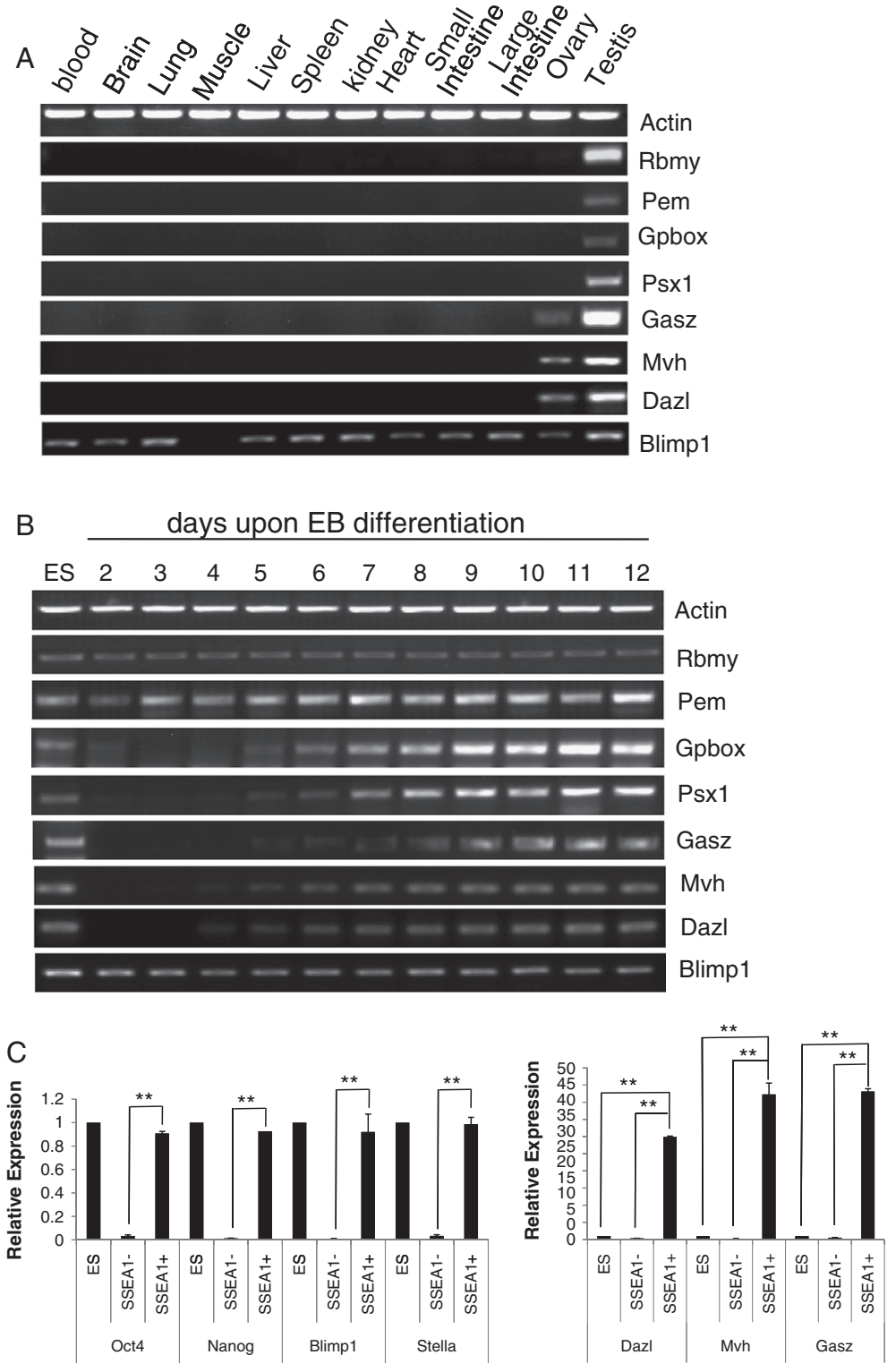


Figure 1 GASZ was enriched in mouse ESC-derived PGCs (A–B). Candidate gene expression analyzed with RT-PCR on various adult tissues (A) or whole EBs at different time points during ESC differentiation (B). (C) Real-time RT-PCR performed on ESC, SSEA1−, and SSEA1+ populations isolated from day 9 EBs. Data represents mean of fold changes (relative to ESCs) ± one s.e.m. from four independent SSEA1 sorting assays. **: $P < 0.01$. (D) Whole mount in situ hybridization with a *Gasz* anti-sense probe on genital ridges isolated from 11.5 to 13.5 dpc mouse embryos. (E) Immunohistofluorescence for GASZ, MVH, or DAZL expression on genital ridges isolated from mouse embryos at 13.5 dpc. Scale bar: 50 μM.

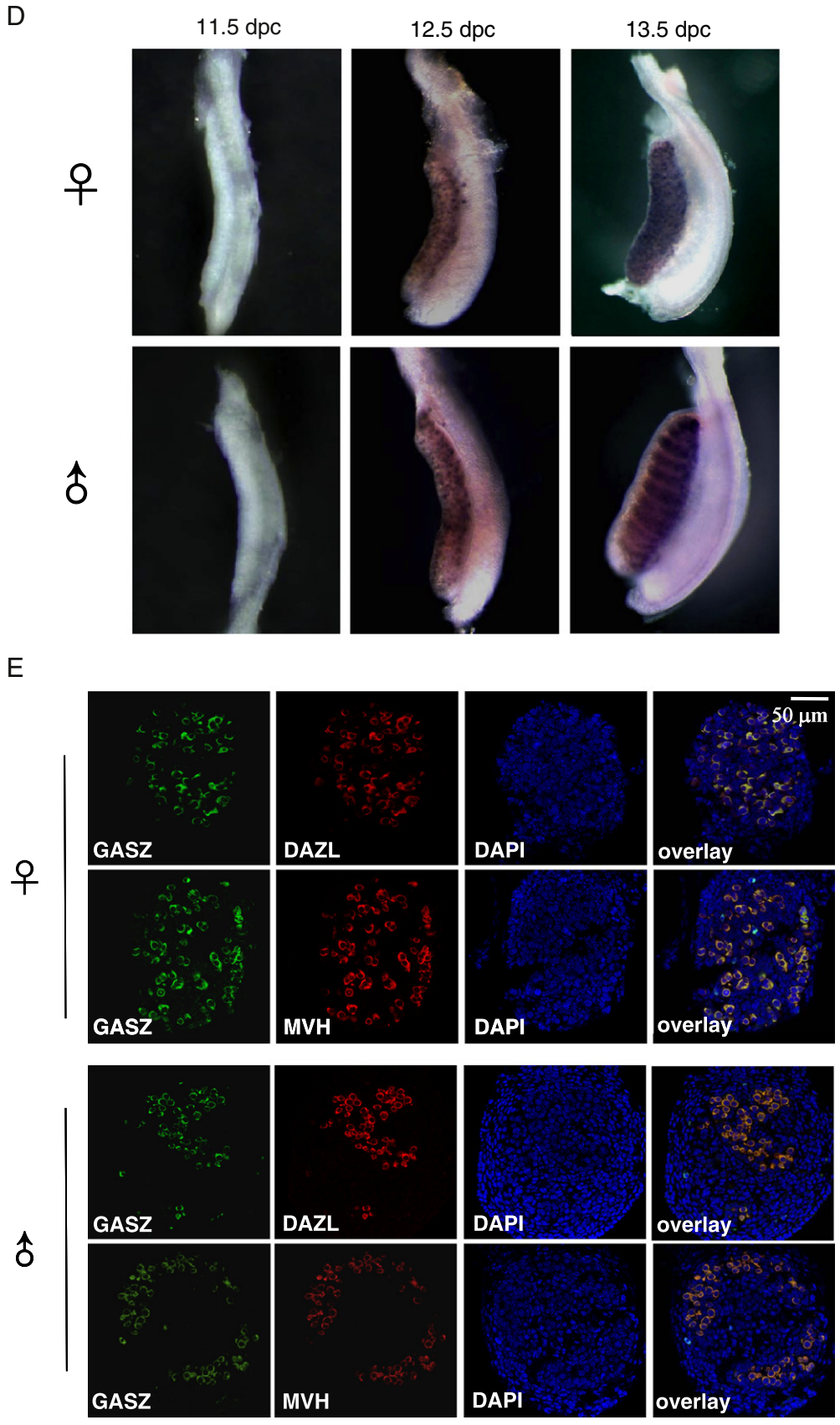


Figure 1 (*continued*).

We next investigated the effect of GASZ deficiency on the expression of genes involved in germ cell development. Similar to the results observed in gain of function of GASZ, expression levels of key post-migrating PGC markers, such as *Dazl*, *Mvh*, and *Mili*, and premeiotic marker *Stra8*, were dramatically decreased in SSEA1+ population but not in SSEA1- cells upon *Gasz* deficiency (Fig. 3C), while the genes for PGC specification (such as *Oct4*, *Nanog*, and *Blimp1*) remained largely unchanged (Fig. 3C, Supplementary Fig. 2G). We further examined the imprinting status of EGC clones by Southern Blot analyses. As shown in Fig. 3D, all individually picked EGC clones from day 9 control EBs or from EBs upon

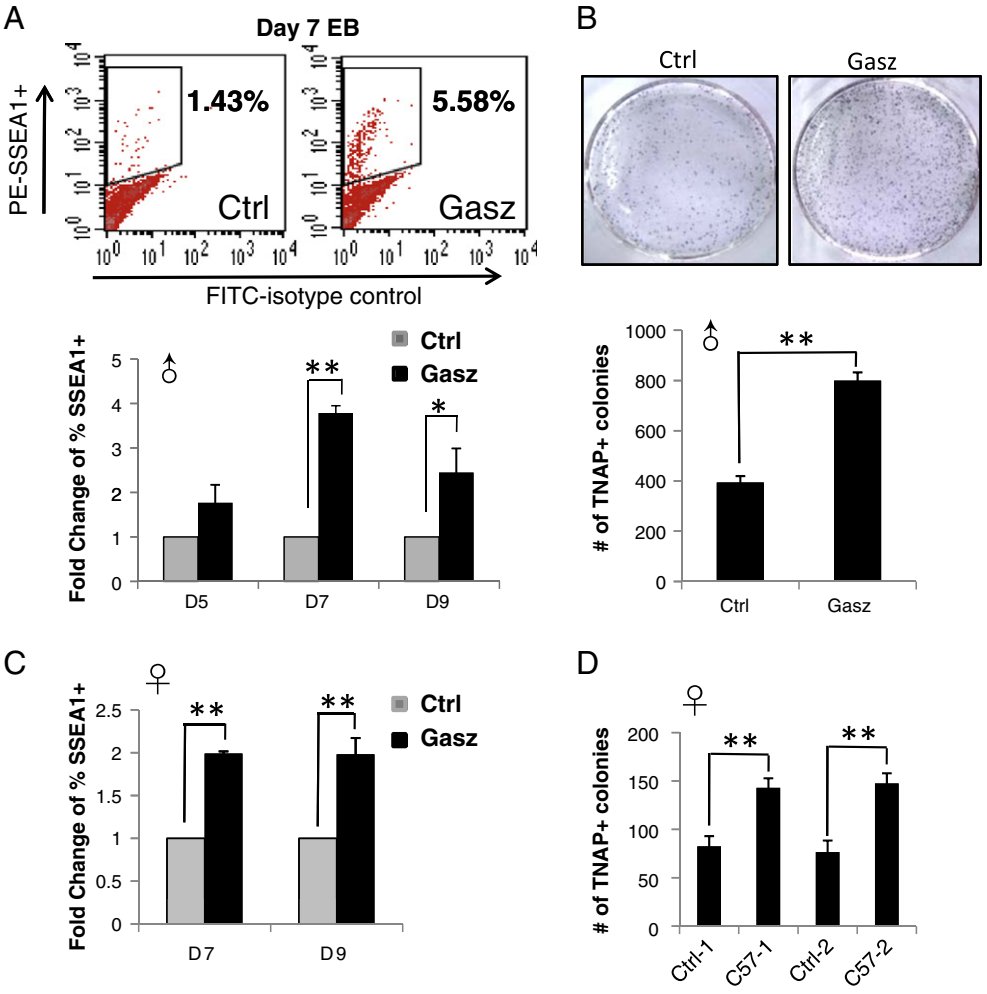


Figure 2 GASZ enhanced PGC derivation from mouse ESCs. (A) Flow cytometry analyses of SSEA1 staining of dissociated EB cells at various days along ESC differentiation. Typical SSEA1 staining of control and GASZ overexpressing EBs was shown above the panel. Fold changes on the percentage (%) of SSEA1+ cells in GASZ overexpressing EBs over control were averaged from three independent experiments. (B) TNAP + EGC forming assay on day 9 EBs differentiated from control or GASZ overexpressing ESCs. Number of TNAP + EGC colonies was calculated from duplicates of a representative experiment, and results were reproduced in three independent assays. (C) The percentage of SSEA1+ cells measured by flow cytometry during EB differentiation. Fold changes in GASZ overexpressing EBs over control were averaged from four assays on two female ESC lines, C57-1&2. (D) Number of TNAP + EGC colonies examined on day 9 EBs from C57-1&2. Results shown are mean ± one s.e.m for four replicates from two independent assays. (E) Imprinting status at *Kvlqt1* and *Rasgrf1* loci examined by Southern Blot on individual EGC colonies cultured from day 9 EB cells and their parental ESCs. A diagram for the restriction enzymes and probes used in Southern Blot was shown above the panel. Eag1 and Not1 are methylation sensitive enzymes, DMR: DNA methylated region. (F) Bisulphite sequencing on *Snrpn* imprinted locus. White circles, unmethylated CpG dinucleotides; black circles, methylated CpGs. Percentages of CpG dinucleotide methylation are given. (G) Quantification of *Hox* gene expression by real-time RT-PCR on SSEA1− (Ctrl-) and SSEA1+ fractions (+) sorted from day 9 EBs of control (Ctrl+), GASZ or DAZL overexpressing cells. (H) Real-time RT-PCR analyses for gene expression on whole EBs along differentiation of control (Ctrl) or GASZ overexpressing ESCs. (I) Histological studies of teratoma sections of control (left panel), DAZL (middle panel) or GASZ (right panel) overexpressing ESCs. (J) Real-time RT-PCR data on SSEA1− or SSEA1+ cells sorted from day 9 EBs of control (Ctrl), GASZ or DAZL overexpressing cells. (G–H, J): Relative expression levels over control cells were calculated from 2 to 4 independent RNA preparations. day: D (D5, D7, or D9). All data represent mean ± one s.e.m. *: $P < 0.05$; **: $P < 0.01$.

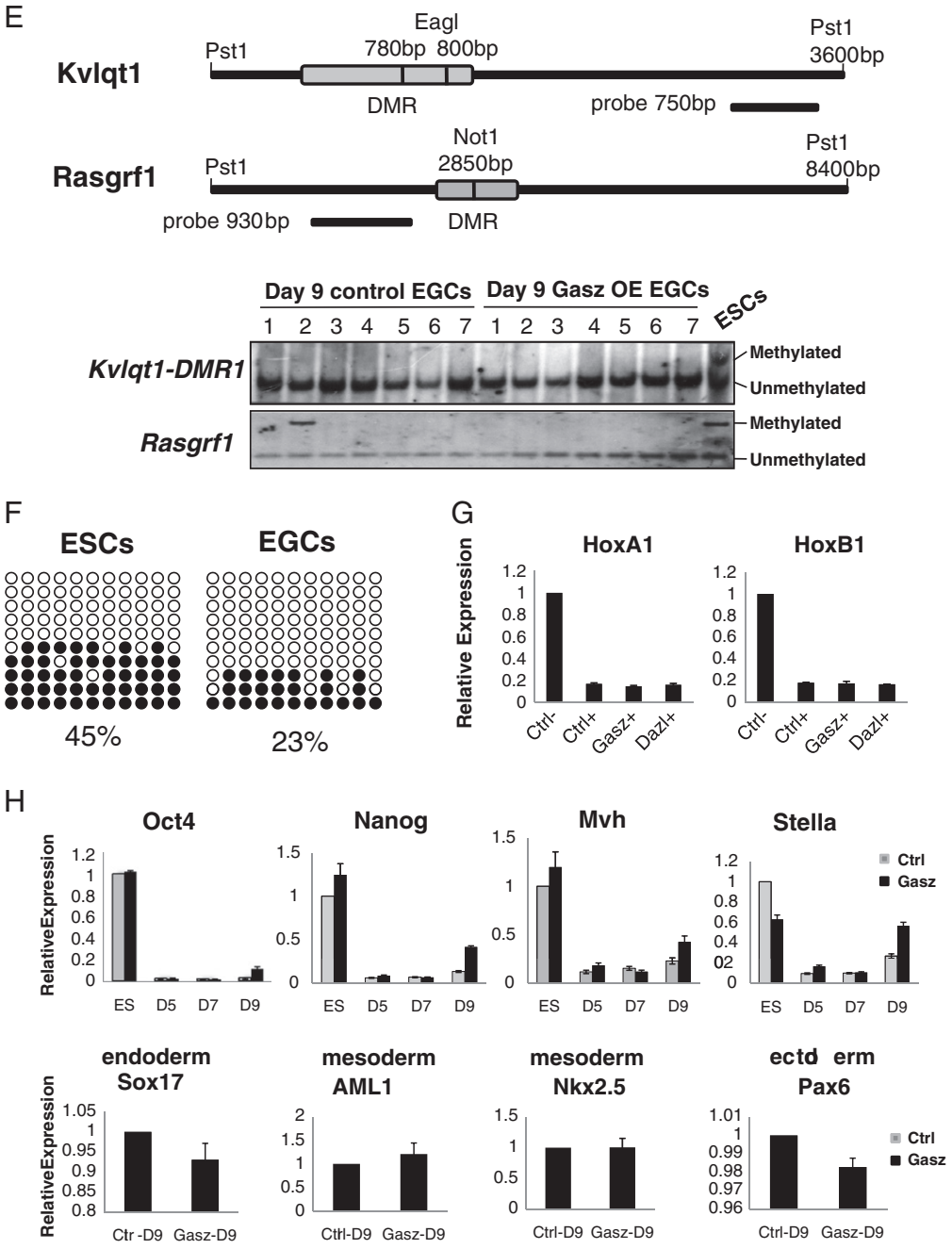


Figure 2 (*continued*).

Gasz knockdown demonstrated imprinting erasures at both *Kvlqt1* and *Rasgrf1* loci, suggesting that GASZ depletion did not affect PGC specification or epigenetic reprogramming.

To further dissect the role of GASZ in early germ cell development in vivo, we utilized a *Gasz* knockout mouse model we previously established (Ma et al., 2009), and performed real-time RT-PCR assays and immunohistochemistry (IHC) for MVH and DAZL expression on genital ridges from isolated embryos. At 14.5 dpc, we indeed observed decreased transcript levels and a marked reduction of staining in MVH and DAZL proteins in male *Gasz*−/− gonads (Fig. 3E, Supplementary Fig. 2H). Taken together, these data suggest a potential requirement for GASZ in male embryonic germ cell development under physiological conditions.

GASZ interacts with DAZL to promote mouse germ cell derivation

To further explore the molecular mechanisms by which GASZ promotes germ cell formation, we sought to identify interaction partners of GASZ in EGCs by co-immunoprecipitation (Co-IP) and further examined the proteins that were co-purified with GASZ by a mass spectrometry analysis. In this

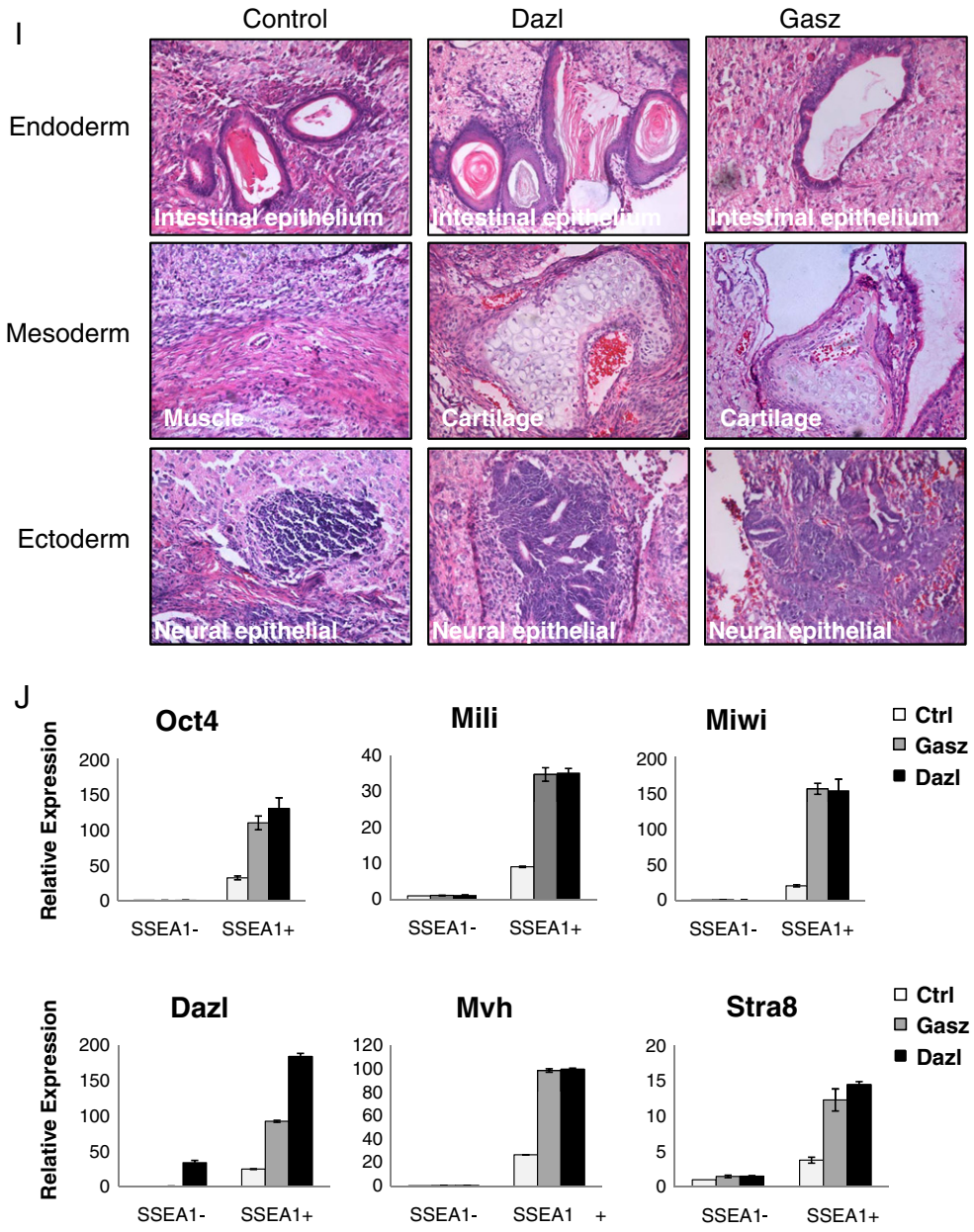


Figure 2 (*continued*).

assay, DAZL, a known protein in germ cell development, was identified (Fig. 4A), and was further verified to be associated with GASZ in both EGCs (Fig. 4B) and testis with Co-IP analyses (Fig. 4C).

Given the link of DAZL and germ cell formation, we evaluated if GASZ could synergistically work with DAZL to stimulate PGC formation from ESCs, and if the interaction of GASZ with DAZL was important for its function in germ cell development. We therefore performed the following experiments (Supplementary Fig. 4A): (1) ectopically expressed both GASZ and DAZL proteins, (2) knocked down *Dazl* in GASZ overexpressing ESCs, and assessed PGC forming efficiency from these ESC lines. Indeed, we observed an increased SSEA1+ population as well as more EGC colonies formed from differentiated ESCs when GASZ and DAZL were both ectopically expressed (Fig. 4D, Supplementary Fig. 4B). In addition, *Dazl* knockdown markedly compromised the ability of GASZ to enhance PGC development from ESCs, compared to GASZ overexpressing samples (Fig. 4D, Supplementary material Fig. 4B). Consistent with this result, gene expression analysis confirmed that GASZ and DAZL worked together to upregulate germ cell specific genes including *Oct4*, *Stella*, and *Mvh* from whole EBs at day 9 post differentiation (Fig. 4E). Taken together, these data suggest that GASZ synergistically works with DAZL to enhance PGC derivation from ESCs, and DAZL appears to be required for the function of GASZ in this process.

GASZ contains four ankyrin repeats (ANK), a sterile alpha motif (SAM), and a putative basic leucine zipper (b-Zip) (Ma et al., 2009; Yan et al., 2004; Yan et al., 2002). To examine

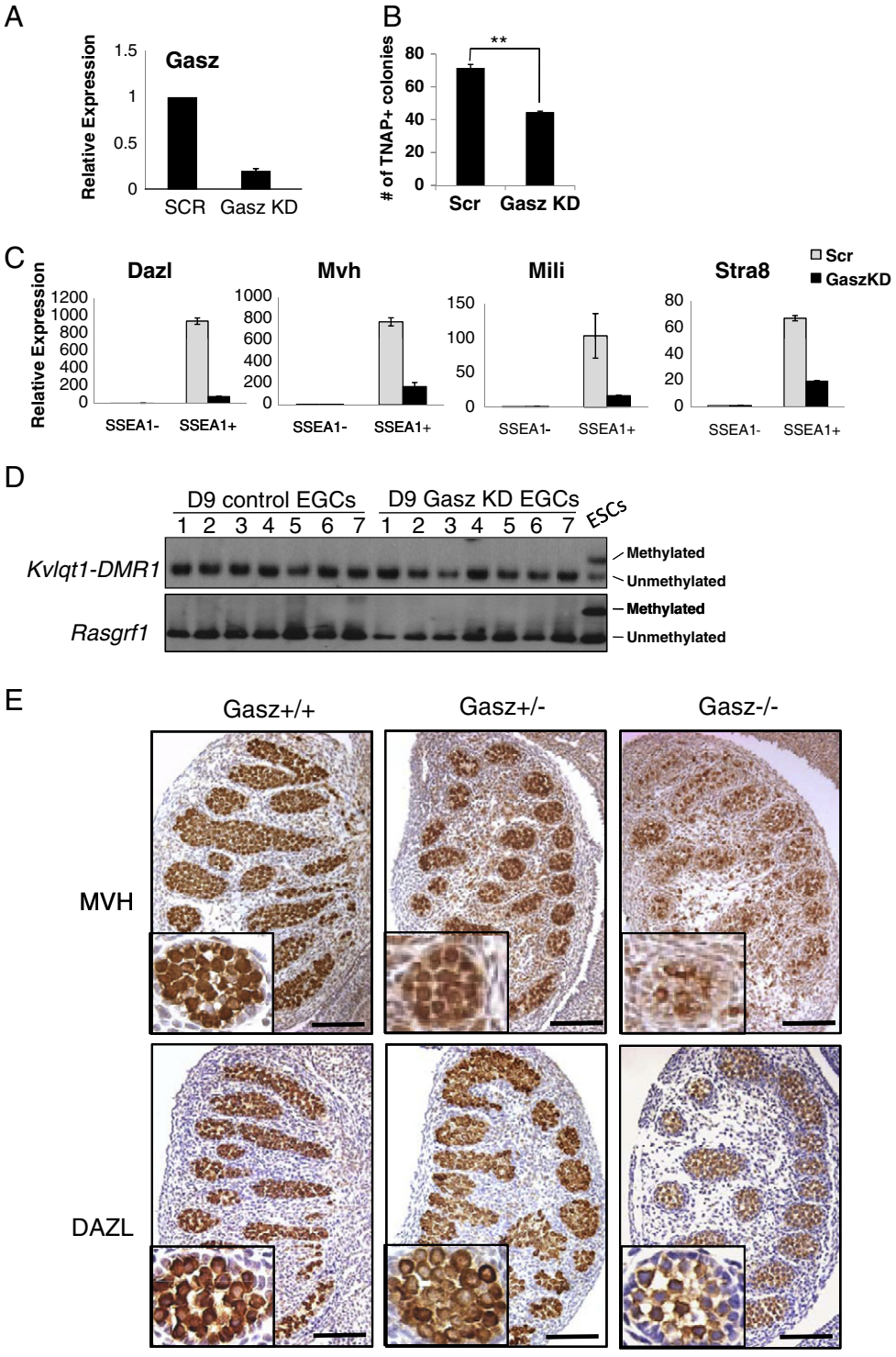


Figure 3 GASZ deficiency led to reduced germ cell formation from ESCs. (A) ESC lines with stable *Gasz* knockdown were confirmed by RT-PCR. (B) TNAP + EGC colony forming assay on day 7 EBs. Number of TNAP + EGC colonies was calculated from four replicates in two independent experiments. (C) Relative gene expression levels measured by real-time RT-PCR in *Gasz* knockdown (Gasz KD) samples compared to control (Scr: scrambled shRNA). RNA samples were collected from sorted SSEA1− and SSEA1+ populations from day 9 EBs. (B–C) All data represent mean ± one s.e.m. **:$P < 0.01$. (D) Imprinting status at *Kvlqt1* and *Rasgrf1* loci examined by Southern Blot on individual EGC colonies cultured from day 9 EB cells and their parental ESCs. (E) Immunohistochemistry with antibodies against MVH or DAZL on male genital ridges isolated from 14.5 dpc *Gasz*+/+ (wildtype), *Gasz*−/+ (heterozygote knockout) and *Gasz*−/− (homozygote knockout) embryos. Scale bars: 100 μM. Insets show images with higher magnification.

the functional domains of GASZ that are required for its interaction with DAZL, a series of deletion mutants of GASZ were transfected into 293T cells and Co-IP analyses were performed (Fig. 5A). We found that deletion of ANK or b-ZIP domain did not affect the association of GASZ with DAZL, whereas deletion of SAM or ANK and SAM domains completely abolished the interaction of GASZ with DAZL, suggesting that the SAM domain of GASZ was responsible for its association with DAZL (Fig. 5A). We then analyzed if deletion of SAM domain could affect the ability of GASZ to enhance germ cell derivation from ESCs. An ESC line overexpressing SAM deletion mutant was then established (Supplementary Fig. 4A). Consistent with previous findings, full-length GASZ increased the percentage of SSEA1+ cells, number of TNAP+ EGC colonies, and up-regulated expression of germ cell specific genes from whole EBs at day 9 post differentiation, but its ability was significantly compromised upon the deletion of SAM domain (Figs. 5B–D), suggesting that interaction with DAZL mediated by SAM domain of GASZ is important for its function in germ cell development from ESCs.

GASZ promotes germ cell formation from human ESCs

To investigate if GASZ also participates in human germ cell formation, we first analyzed its expression in different human adult tissues and various human cell lines, including HAF (fibroblasts), 293T (immortalized embryonic kidney cells), Hela (cervical cancer), H1299 (lung carcinoma), HCT116 (colorectal adenoma), HepG2 (heptocellular carcinoma), and MCF7 (breast carcinoma). Interestingly, we observed that *GASZ*, *DAZL*, and *MVH*, were all selectively expressed in human testis, but not in other somatic tissues or cell lines (Fig. 6A), implicating potential roles for these genes in human germ cell development.

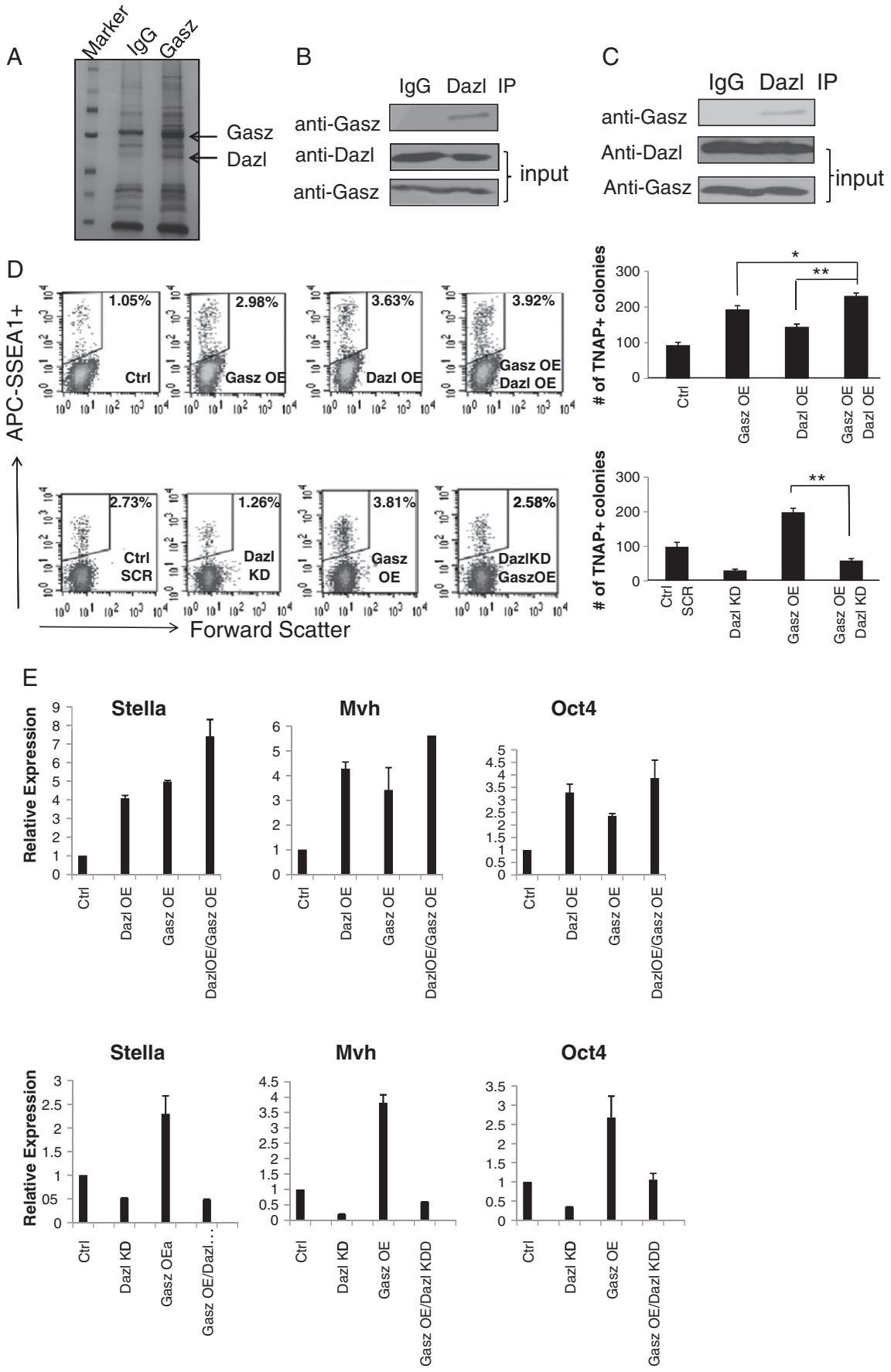
It was reported that DAZ family members and BMP4 stimulated germ cell development from human ESCs (Kee et al., 2009; Kee et al., 2006). In addition, SSEA1 is only expressed in differentiated human ESCs and has been reported as a surface marker for putative PGCs (Tilgner et al., 2008). We thus established stable human ESC lines with shRNAs against *DAZL*, and applied an induction system by treating human ESCs with activin A and sequentially with BMP4, following several modified protocols for germ cell derivation from mESCs and hESCs (Clark et al., 2004; Park et al., 2009; Tilgner et al., 2008) (Hayashi et al., 2011). Under this condition, we observed that DAZL deficiency led to significant reduction of *MVH* and *GASZ* expression, as well as decreased SSEA1+ putative PGC formation during ESC differentiation (Figs. 6B–C). These findings are consistent with published studies (Kee et al., 2009), and thus suggest that our in vitro differentiation system from human ESCs can be used to monitor the functions of candidate genes in early germ cell development.

Next we sought to evaluate the germ cell forming potential from DAZL or GASZ overexpressed human ESC lines (Supplementary Fig. 5A). We found that both DAZL and GASZ could dramatically promote SSEA1+ putative PGC formation from two different human ESC lines (Fig. 6D, left panels, Supplementary Fig. 5D). In addition, we also observed an up-regulation of *GASZ* by ectopic DAZL expression, and an increase of *DAZL* level upon GASZ overexpression (Fig. 6D, right panels), indicating a regulatory relationship between these two proteins in human germ cell development. Taken together, our results implicate a conserved role for GASZ to stimulate germ cell development from both mouse and human ESCs.

Discussion

The ESC differentiation system is a powerful, sensitive, and assessable in vitro model to explore the molecular pathways in lineage determination during early embryonic development. So far, several studies suggested that germ cells can be spontaneously developed from ESCs under appropriate conditions and can be further enriched by sorting them with SSEA1, CXCR4, c-kit or CD61 antigens (Bucay et al., 2009; Hayashi et al., 2011; Park et al., 2009; Tilgner et al., 2008) or by GFP under germ cell specific promoters (Hayashi et al., 2011; Kee et al., 2009). In this study, we utilized ESC differentiation systems to show that ectopic expression of *Gasz*, a gene known to be involved in post-natal spermatogenesis, could stimulate germ cell derivation from both human and mouse ESCs. Our study thus proposes a potential usage of ectopic gene manipulation in improving germ cell differentiation from ESCs. In addition, we revealed that *Gasz* deficiency impaired the germ cell derivation from ESCs and confirmed its requirement for expression of key PGC regulators, DAZL and MVH, with a *Gasz* knockout mouse model. We further demonstrated a functional interaction of GASZ and DAZL in promoting PGCs from ESCs. Therefore, our findings implicate a novel role of GASZ in early germ cell development in mammals, and also provide a useful system for investigating the molecular pathways in embryonic germ cell formation.

Due to the limited numbers of PGCs in mouse embryos, it is difficult to dissect the molecular mechanisms of early germ cell development. Recently, using ESC in vitro differentiation models, a number of studies demonstrated that *Dazl*, *Blimp1*, *Lin28*, and possibly *Rhox6/Psx1*, participated in PGC formation from ESCs (Kee et al., 2009; Liu et al., 2011; West et al., 2009; Yu et al., 2009). However, because of the pluripotency features shared by ESCs and PGCs, many germ cell specific genes, including SSEA1 surface antigen, *Oct4*, *Blimp1*, and *Stella*, are all highly expressed in ESCs. So far, there is a lack of definitive markers to distinguish PGCs and their derivative, EGCs, from ESCs except methylation erasure of imprinted loci. In our study, after day 5 of EB differentiation, EGC colonies were solely derived from SSEA1+ cells (data not shown), and our methylation analyses displayed imprinting erasure in EGC colonies (Figs. 2E, F), proving these SSEA1+ EGCs were indeed from germ cell lineage. Additionally, expression data demonstrated that SSEA1+ cells were enriched with pluripotency related genes (*Oct4* and *Nanog*), key regulators in PGC fate specification (*Blimp1* and *Prdm14*), and other PGC markers, (*Stella* and *Fragilis*), as well as germ cell genes that expressed in PGCs after migration around 10.5–11.5 dpc (*Mvh* and *Dazl*) (Fig. 1C) (Tanaka et al., 2000). Given that EGC culture can only be established from PGCs before sex determination in vivo (McLaren, 2003), SSEA1+ cells in our assays recapitulated the expression profiles and biological properties of post-migrating PGCs before sex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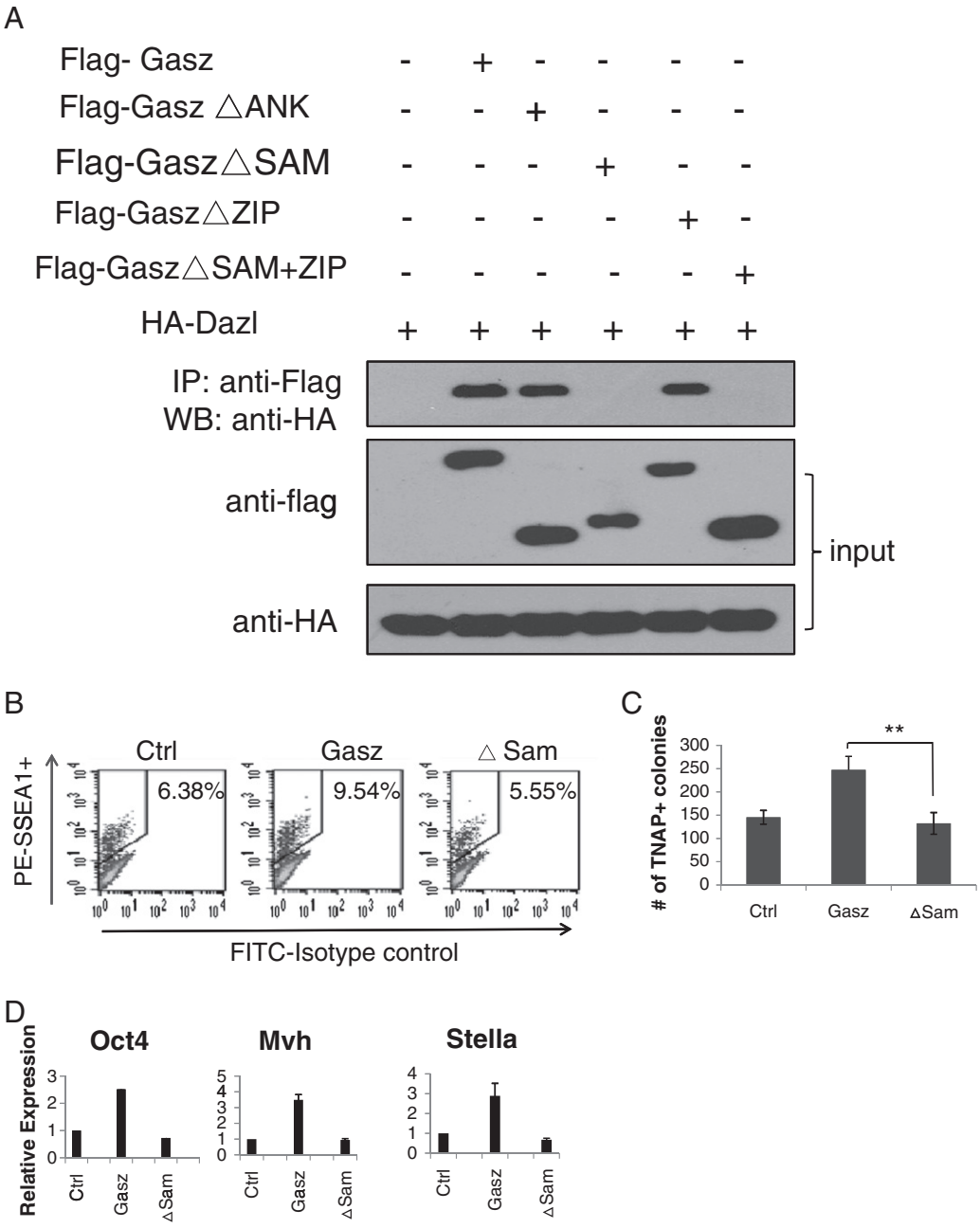
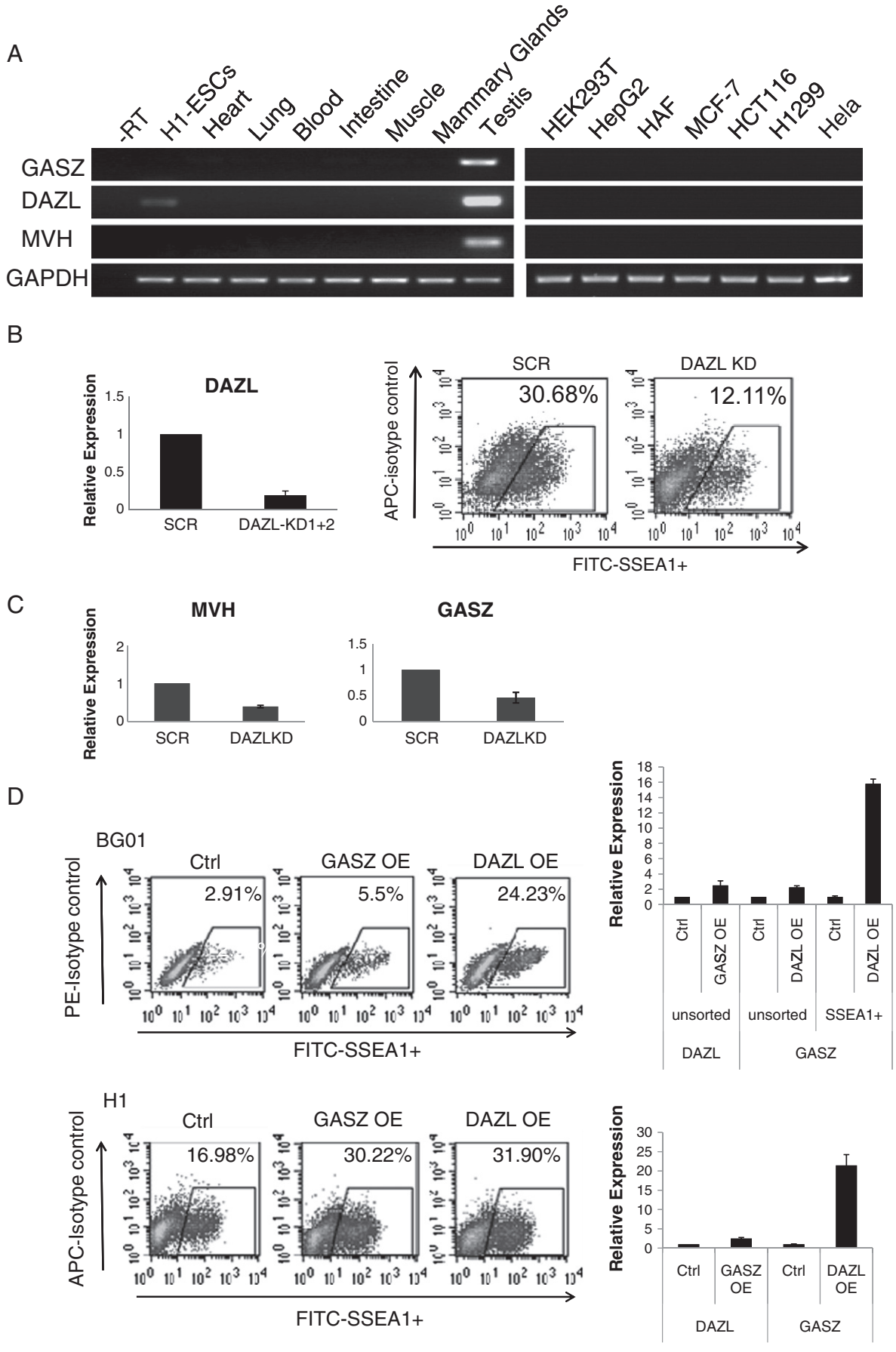


Figure 5 SAM domain of GASZ is required for its interaction with DAZL. (A) Co-IP of FLAG-tagged GASZ deletion mutants with HA-tagged DAZL in 293T cells. The antibodies for Western Blots (WB) or co-IP (IP) were indicated on the left. +: cDNA transfected; −: without the cDNA indicated. (B–D) Whole day 9 EBs differentiated from ESC lines with ectopic expression of full-length GASZ or Sam deletion mutant (Sam) examined with SSEA1 staining by flow cytometry (B), TNAP + colony formation assay (C), and quantitative expression analyses of germ cell specific genes by real-time RT-PCR (D). Data represent mean ± one s.e.m. calculated from four independent experiments (C) or duplicates in one representative assay (D).

Figure 4 GASZ interacted with DAZL to promote PGC derivation from mouse ESCs. (A) Co-IP with an antibody against GASZ. A silver staining image of GASZ pull-down sample was shown. (B–C) Co-IP with either control IgG or a DAZL antibody on cell lysates from GASZ overexpressing EGCs (B) or from testis (C). The antibodies for Western Blots were indicated on the left. (D) Flow cytometry analyses of SSEA1 staining (left panels) and TNAP + EGC colony formation assays (right panels) on day 9 EBs. Control: Ctrl; overexpression: OE; knockdown: KD; *: $p < 0.05$; **: $p < 0.01$. Data represent mean ± one s.e.m. calculated from four independent experiments. (E) Real-time RT-PCR analyses on day 9 EB samples from (D). Data were shown as mean ± one s.e.m. calculated from duplicates in one representative assay.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HCT116
H1299
Hela
GASZ
DAZL
MVH
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Relative Expression
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DAZL-KD1+2
SCR
DAZL KD
30.68%
12.11%
APC-isotype control
FITC-SSEA1+
C
MVH
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determination. *Gasz* was highly enriched in these SSEA1+ PGCs derived from differentiated ESCs, in contrast to its low expression level in ESCs (Fig. 1C). This result was consistent with its first appearance around 12.5 dpc in genital ridges (Figs. 1D–E). PGCs are segregated from somatic cells in mice at 6.5 dpc, and settle at the genital ridges before 11 dpc (Ginsburg et al., 1990; McLaren, 2003). Therefore, GASZ can complement DAZL and MVH as a unique and useful marker to distinguish post-migrating PGCs from ESCs.

We previously reported that a null mutation of *Gasz* on a C57BL6/J/129 hybrid background led to male infertility through increased expression of retrotransposons and block of meiosis in post-natal spermatocytes (Ma et al., 2009). Our data revealed that GASZ overexpression promotes SSEA1+ PGC derivation from ESCs, and its knockdown compromises EGC formation (Figs. 2A–D, 3B). Altered GASZ expression specifically affects transcript levels of genes in post-migrating PGCs, such as *Dazl*, *Mvh*, *Mili*, *Miwi*, and premeiotic marker *Stra8* in SSEA1+ cells. Results were reproduced in several independent experiments with different ESC lines from E14, C57/BL6, and CCE (Fig. 2, Supplementary Fig. 1H, 2B, 2D), arguing against an artificial phenomenon due to gene manipulation. Consistently with these in vitro findings, we observed that GASZ started to express in post-migrating PGCs from 12.5 dpc, and the expression levels of DAZL and MVH were decreased in GASZ$^{-/-}$ gonads at 14.5 dpc (Fig. 3E, Supplementary Fig. 2H), suggesting that GASZ is required for maintaining the expression of these key post-migrating PGC regulators during embryonic development. Therefore, our data proposed a previously under-appreciated role of GASZ during embryonic germ cell development. Many genes have been implicated during terminal differentiation and maturation of germ cells during adulthood, while fewer genes were found to participate in the maintenance of post-migrating PGCs before birth. It is possible that mild defects during embryonic germ cell formation may not be manifested due to current technical limitation. In addition, the degree of impaired fertility varies frequently among different genetic backgrounds in gene targeting analyses. Notably, gene knockout analyses of *Dazl* or *Pin1* yield no embryonic abnormality prior to backcrossing with C57/BL6 mice, where both XY and XX *Dazl* null or *Pin1* deficient embryos displayed defects in early germ cell development (Atchison et al., 2003; Lin and Page, 2005). Although no obvious gross abnormalities were detected in GASZ$^{-/-}$ gonads at 14.5 dpc, given the interaction and functional relationship between GASZ and DAZL, further studies in *Gasz* knockout mice with different genetic backgrounds may help us understand the physiological requirement of GASZ during embryonic germ cell development.

Modulation of GASZ by ectopic overexpression or by RNA interference in ESCs affected several key germ cell markers, such as *Oct4*, *Dazl*, *Mvh*, *Miwi*, and *Mili*, which may in turn contribute to the altered PGC or EGC derivation from differentiated EBs. Since GASZ is localized in the intermitochondria cement of spermatocytes, and our sequence analysis did not detect any nuclear localization/export signals either (data not shown), GASZ does not appear to be a transcription factor to directly regulate those germ cell specific genes. Instead, GASZ contains several protein interaction domains, such as ANK and SAM. Our data demonstrated that GASZ interacted with RNA-binding proteins including DAZL (Figs. 4–5) and MVH (Ma et al., 2009). Therefore, it is plausible that GASZ may modulate the stability and/or expression of these target genes indirectly through interaction with its partners. It will be of great interest to explore if GASZ actually possesses the ability for direct association with other mRNAs. Given the links between GASZ and RNA-binding proteins, findings from the current study will shed light on our understanding of how protein interactions and RNA modifications contribute to early germ cell development.

Conclusion

In this study, using an ESC in vitro differentiation model, we report that GASZ affects germ cell development with both gain of function and gene knockdown assays. In addition, we demonstrated that Gasz is expressed specifically in reproductive organs from both human and mouse, and is highly enriched in PGCs derived from mouse ESCs or PGCs from mouse genital ridges in vivo. Our study further documents that GASZ interacts with DAZL and synergistically stimulates germ cell derivation from mouse ESCs. Thus, our data provide new insights that GASZ functions with RNA-binding proteins in early germ cell development and support ESC in vitro differentiation system as an accessible model for investigating the molecular pathways during embryogenesis.

Acknowledgments

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Figure 6 GASZ was specifically expressed in human testis and stimulated germ cell formation from human ESCs. (A) Expression of *GASZ*, *DAZL* and *MVH* examined on cDNAs from various human tissues and cell lines with RT-PCR. (B) Human ESC lines with stable *DAZL* knockdown were confirmed with the expression of *DAZL* by real-time RT-PCR (left panel). SSEA1 staining by flow cytometry analyses (right panel) from differentiated human ESCs either with a scrambled shRNA (SCR), or with shRNAs against *DAZL*. (C) Relative gene expression of MVH and GASZ measured by real-time RT-PCR on differentiated cells from control or *DAZL* knockdown (KD) ESCs. (D) SSEA1 staining on differentiated cells from control (Ctrl), GASZ or DAZL overexpressing (OE) ESCs (left panel). Quantification of *GASZ* or *DAZL* transcript levels by real-time RT-PCR from either human ESCs 7 days post differentiation or sorted SSEA1+ population (right panel). All data represent mean ± one s.e.m. from 2 to 4 independent assays.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.05.012.

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