Published in final edited form as:

Genesis. 2009 February ; 47(2): 74–84. doi:10.1002/dvg.20460.

Characterization of a *Dazl*-GFP Germ Cell-Specific Reporter

Cory R. Nicholas^{1,2,*}, Eugene Y. Xu³, Salman F. Banani², Robert E. Hammer⁴, F. Kent Hamra⁵, and Renee A. Reijo Pera^{1,2}

¹Department of Obstetrics and Gynecology, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Palo Alto, CA

²Department of Obstetrics and Gynecology, Center for Reproductive Sciences, University of California at San Francisco, San Francisco, California

³Department of Obstetrics and Gynecology, Northwestern University, Chicago, Illinois

⁴Department of Biochemistry and Cecil H. & Ida Green Center for Reproductive Biological Sciences, University of Texas Southwestern, Dallas, Texas

⁵Department of Pharmacology and Cecil H. & Ida Green Center for Reproductive Biological Sciences, University of Texas Southwestern, Dallas, Texas

Summary

In this study, we characterized the promoter activity of a 1.7 kb sequence in the 5' flanking region of the mouse *Deleted in Azoospermia-Like (Dazl)* gene. We found the 1.7 kb sequence sufficient to drive robust germ cell-specific expression of green fluorescent protein (GFP) in adult mouse testis and lower levels of expression in adult ovary and in fetal and newborn gonads of both sexes. This expression pattern was confirmed in two independently-derived transgenic mouse lines. In adult testis, *Dazl*-GFP exhibited a developmentally-regulated, stage-specific expression pattern during spermatogenesis. GFP was highly expressed in spermatocyte stages, with strongest expression in pachytene spermatocytes. Weaker expression was observed in round and elongating spermatids, as well as spermatogonial cells. In the fetal gonad, GFP transcript was detected by e12.5 in both sexes; however, GFP fluorescence was only detected during later embryonic stages. In addition, we produced mouse embryonic stem cell (ESC) lines harboring the *Dazl*-GFP reporter and used this reporter to isolate putative germ cell populations derived from mouse ESCs following embryoid body differentiation and fluorescence activated cell sorting.

Keywords

Dazl; GFP; germ cell; reporter; ES; embryonic stem; transgenic; testis; ovary; primordial germ

INTRODUCTION

The development of an *in vitro* system to robustly isolate germ cells that are differentiated from embryonic stem cell (ESC) lines would greatly facilitate the study and understanding of germ cell development. Such a system would be especially useful for the study of human germ cell development, where germ cell specification and commitment occur during early

fetal stages that are inaccessible (Moore, 2003). Because ESCs can spontaneously differentiate to form a heterogeneous mixture of various tissues, and because specific germ cell surface antigens to allow isolation are lacking, validated germ cell-specific reporters represent a promising strategy for identification and isolation of ESC-derived germ cells. In fact, recent studies have documented the isolation of germ cells from mouse ESCs (Geijsen *et al.*, 2004; Hubner *et al.*, 2003; Kerkis *et al.*, 2007; Nayernia *et al.*, 2006; Novak *et al.*, 2006; Qing *et al.*, 2007; Toyooka *et al.*, 2003), and two of these studies utilized germ cell-specific reporters to identify and isolate sperm (Nayernia *et al.*, 2006; Toyooka *et al.*, 2003). However, functional oocytes have yet to be isolated following mouse ESC differentiation, and the isolation of human germ cells following ESC differentiation has not yet been reported, thus necessitating the development of additional differentiation and identification strategies.

Here, we sought to characterize the germ cell specificity of a mouse *Dazl* promoter sequence driving the expression of green fluorescent protein (GFP), and to then use this reporter to isolate germ cells following ESC differentiation. The *DAZ* (*Deleted in AZoospermia*) gene family of *BOULE*, *DAZL*, and *DAZ* encodes RNA binding proteins that are exclusively expressed in germ cells and are required for germ cell differentiation across nonmammalian and mammalian species (Dorfman *et al.*, 1999; Haag, 2001; Reijo *et al.*, 1995; Xu *et al.*, 2001; Yen, 2004). In particular, the autosomal *DAZL* gene is expressed throughout germ cell development from embryonic stage primordial germ cells (PGC) to mature gametes in adults (Cooke *et al.*, 1996; Dorfman *et al.*, 1999; Reijo *et al.*, 1996, 2000; Seligman and Page, 1998). Mouse *Dazl* null mutants are sterile in both sexes, exhibiting a near complete lack of mature germ cells by birth and reduced germ cell numbers in mouse embryos (Lin and Page, 2005; Ruggiu *et al.*, 1997). Consequently, defining regulatory elements of the mouse *Dazl* gene represents an opportunity to design a pan-germ cell-specific reporter that would be expressed during essentially all stages of postspecification germ cell development in both sexes. The evolutionary conservation of the *DAZ* family may also allow the extension of the mouse *DAZL* reporter to a human system and similarly facilitate the isolation of germ cells following their differentiation from cultures of human ESCs (Clark *et al.*, 2004).

RESULTS

Production of *Dazl*-GFP Transgenic Mice

A *Dazl*-enhanced green fluorescent protein (eGFP) reporter was constructed using a 5' 1.7 kb sequence located upstream of the mouse *Dazl* translational start site that also included the 5' UTR. The 1.7 kb sequence was chosen based on limited flanking sequence available at the time and based on the size of several promoter constructs required for germ cell-specific expression. The elements required for such expression in spermatocytes or spermatids were usually present within ~ 2 kb or less from the coding region (Nayernia *et al.*, 1994; Reddi *et al.*, 1999; Robinson *et al.*, 1989; Sage *et al.*, 1999; Zambrowicz *et al.*, 1993). The putative promoter fragment was cloned upstream of the coding region for eGFP, which was followed by the SV40 polyadenylation sequence. To produce transgenic mice, the reporter vector was linearized, purified, and then microinjected into the maternal pronucleus of fertilized mouse FVB/N oocytes. The microinjected oocytes were transferred into the oviducts of pseudopregnant female recipients, which then gave birth to 35 pups; 12 of these 35 pups were identified as founder transgenic mice based on genomic PCR analysis for the *Dazl*-eGFP transgene. Nine of these founders were subsequently mated with wild-type FVB/N mice, and six of nine (66%) founders transmitted the transgene to offspring to generate the *Dazl*-eGFP lines. For comparison, the 1.7 kb *Dazl* sequence was also linked to a coding sequence for humanized renilla hrGFP followed by the SV40 polyadenylation sequence, and this construct was also used to generate lines of transgenic mice, termed *Dazl*-hrGFP mice.

Transgene Expression

Testes from mice bearing the *Dazl*-eGFP transgene were analyzed for eGFP expression by fluorescence microscopy at 2–3 months of age. Green fluorescence above wild-type was detected in three of six (50%) lines; these lines were designated *Dazl*-eGFP transgenic lines 5, 10, and 26 (Fig. 1a; line 5 and 26 data not shown). The *Dazl*-eGFP line (or line eGFP) in this report refers to line 10 which contained robust testicular transgene expression. Line 26 originally displayed high levels of transgene expression as well but was later observed to undergo transgene silencing upon colony expansion. Nevertheless, line 26 was characterized in parallel with line 10 and found to have an identical transgene expression pattern both before and after silencing, despite lower levels of expression after silencing. Line 5 exhibited very weak transgene expression and was not characterized further. *Dazl*-hrGFP transgenic mouse line 7-2 also showed high levels of transgene expression in the testis and was designated transgenic line *Dazl*-hrGFP (or line hrGFP) (Fig. 1a). Transgene expression could not be detected above background in intact ovarian tissues (data not shown). However, weak GFP transgene expression was detected above background in individual oocytes from disrupted adult ovarian tissues (Fig. 1b). Adult gonadal transgene expression was also examined by flow cytometry for GFP fluorescence and was detected above background in both adult testis and ovary. A total of 18.81% of cells exhibited eGFP fluorescence in transgenic line *Dazl*-eGFP adult testis, whereas only 0.34% of cells were fluorescent in transgenic adult ovary (Fig. 1c). Similarly, 18.18% of cells were hrGFP positive in transgenic line *Dazl*-hrGFP testis compared with 0.78% positive in line hrGFP ovary (data not shown).

To confirm these results, RT-PCR was performed to assay GFP transcript levels in the adult gonads of each mouse line. GFP expression was detected in adult testis and ovary from line *Dazl*-eGFP and -hrGFP (Fig. 2a; line eGFP data shown). Expression of GFP transcript was also detected in newborn and fetal (e12.5, e14.5, and e16.5 dpc) gonads from both sexes, resembling the expression pattern of endogenous *Dazl* transcript which has been detected as early as e11.5 in the fetal gonad and may be expressed at even earlier stages of embryonic development (Fig. 2b) (Lin and Page, 2005; Pan *et al.*, 2007). A similar expression profile for hrGFP was obtained when the gonads from the *Dazl*-hrGFP transgenic mouse line were analyzed by RT-PCR (data not shown).

Surprisingly, eGFP and hrGFP fluorescence from fetal and newborn transgenic gonads was not readily detectable above background by fluorescence microscopy, despite the presence of GFP transcript during these stages. Faint GFP fluorescence was identified above background in testes from e14.5 and e16.5 dpc; however, GFP fluorescence could not be detected in e12.5 testes or in ovary from any stage (data not shown). Furthermore, fetal gonads from e12.5 and e17.5 were analyzed by flow cytometry, and eGFP fluorescence was only observed above background in later stage testes (0.76%) and ovaries (0.03%) from e17.5 (see Fig. 3). Likewise, low levels of hrGFP fluorescence were only identified above background in e17.5 stage fetal testes (0.11%) and ovaries (0.02%) from *Dazl*-hrGFP transgenic mice (data not shown). Therefore, *Dazl*-GFP reporter transcriptional activity was initiated at the PGC stage in the gonads as early as e12.5, but translational activity was not detected until after germ cells committed to a sex-specific program as pro-spermatogonia/oocytes. However, we can not rule out alternative or additional mechanisms responsible for the absence of GFP protein in gonocytes such as transcript instability and targeted degradation.

Gonad-Specific Expression

Next, a panel of adult tissues from the transgenic lines was examined in comparison with wild-type mice to determine whether transgene expression was confined to the gonad. By

fluorescence microscopy, GFP fluorescence was only identified above background in the testis of transgenic mice (Fig. 4a; line hrGFP data shown). Similar expression patterns were observed in line eGFP (data not shown). Transgene expression was next assayed by fluorometry in a panel of tissues isolated from the *Dazl*-hrGFP mice. Again, GFP expression could only be detected in the testis of transgenic mice when compared with tissues from wild-type mice (Fig. 4b). Additionally, RT-PCR analysis confirmed the expression of GFP transcript in the testes and ovaries of line hrGFP transgenic mice (Fig. 4c; line eGFP data not shown). GFP transcript was also present in kidney tissue; however, GFP protein was not detected above background in the kidney by fluorometry.

Germ Cell-Specific and Stage-Specific Expression

To determine whether transgene expression from the *Dazl* promoter sequence was restricted to germ cells, adult testes from the transgenic lines were enzymatically disrupted into single cell suspensions and fluorescence activated cell sorted (FACS) into GFP positive and negative populations. Total RNA was isolated from each population and then analyzed by quantitative RT-PCR for the relative expression of germ and somatic cell marker transcripts. As illustrated in Figure 1c, approximately 18% of testicular cells were GFP positive in transgenic line eGFP when compared with testicular cells from age matched wild-type mice. The germ cell transcripts, *Dazl*, *Vasa*, and *Scp-3*, were expressed at similar levels in both the GFP positive and negative testis cell populations isolated from mouse line eGFP (Fig. 5a). The detection of germ cell-specific transcripts in the GFP negative population suggested that the *Dazl*-GFP transgene is not expressed in some germ cells of the testis and was indicative of stage-specific GFP expression in germ cells during spermatogenesis. In contrast, *Sox-9* and *Lhr*, markers for Sertoli and Leydig somatic cells respectively, were greatly enriched in the GFP negative population illustrating that testicular somatic cells lack robust GFP transgene expression (Fig. 5b). Thus, transgene expression from the *Dazl* promoter sequence was restricted to the germline with possible germ cell- and stage-specific regulation in the testis.

Transgene expression also appeared to be germ cell-specific in adult ovary. Faint GFP fluorescence above background was only detected in oocytes following the disruption of ovarian tissue (Fig. 1b). In addition, transgenic adult ovaries and e17.5 embryonic testes that were analyzed by flow cytometry in Figures 1C and 3 were also FACS sorted into GFP positive and negative populations and examined by RT-PCR for endogenous *Dazl* transcript expression. Notably, *Dazl* transcript was only detected in the GFP positive population and was not detected in the GFP negative population suggesting that GFP positive cells from adult ovary and embryonic testis are germ cells and that *Dazl* reporter activity is restricted to germ cells (Fig. 5C).

Subsequently, testis sections from transgenic lines eGFP and hrGFP were immunostained for GFP to confirm the results obtained by RT-PCR. Indeed, GFP expression was restricted to germ cells within the seminiferous tubules of transgenic mice (Fig. 6a, line eGFP data shown). However, germ cell staining was not uniform, as some tubules did not stain positive for GFP, and others exhibited partial staining. Consistent with RT-PCR analysis (Fig. 5a), these immunostaining results showed the *Dazl*-GFP reporter to be highly regulated during specific epithelial cycle stages of sperm development. Similar results were obtained with transgenic line hrGFP (data not shown). GFP expression could not be detected above background in transgenic adult ovary sections by immunostaining (data not shown).

Overall, *Dazl*-GFP expression in transgenic adult testis was primarily detected from three main stages—pachytene spermatocytes, round spermatids, and elongating spermatids, with strongest expression in mid-pachytene spermatocytes (Fig. 6d,e). Initial expression was found in early pachytene spermatocytes of epithelial cycle stage II, followed by strong

expression in mid-pachytene spermatocytes and spermatids of stage VI–VII. Expression decreased in late pachytene spermatocytes and elongating spermatids of stage VIII to IX, and completely disappeared by stage XII. Expression was not detected in leptotene or zygotene spermatocytes. Weak staining was observed in some spermatogonial cells, but further analysis is needed to determine the specific subtypes that express the *Dazl*-GFP transgene, potentially including spermatogonial stem cells.

Sertoli cells lining the basement membrane of tubules did not appear to be stained (Fig. 6e). Additionally, although interstitial Leydig cells appeared to be positive, negative control slides also illustrated Leydig cell staining (Fig. 6b,c). This result, combined with the above RT-PCR analysis showing a 20-fold increase in LHR transcript level in GFP negative testicular cells, demonstrated that the interstitial cell staining was nonspecific. Hence, GFP transgene expression, driven by the 1.7 kb mouse *Dazl* promoter, was germ cell-specific in adult testis. However, we cannot eliminate the possibility of low basal transgene expression in a subset of testicular somatic cells or other nontesticular somatic tissues that were not analyzed above.

Germ Cell Differentiation from ESCs

Following the validation of germ cell specificity in transgenic mouse gonad, mouse ESC lines were genetically modified to contain the *Dazl*-eGFP reporter transgene by electroporation and antibiotic selection. Three independent *Dazl*-eGFP clonal ESC lines were generated, which contained low (line 2), medium (line 17), and high (line 8) levels of transgene expression in undifferentiated ESC cultures (Fig. 7a). *Dazl* reporter expression in undifferentiated ESC cultures was anticipated because *Dazl* transcripts have previously been detected at high levels in ESCs (Geijsen *et al.*, 2004), and *Dazl* protein was also found in preimplantation embryos (Pan *et al.*, 2007). We reasoned, as confirmed by results below (see Fig. 7), that *Dazl* reporter expression should disappear in differentiating somatic cells and only persist in the germ cell lineage following ESC differentiation, if expression recapitulates that observed *in vivo*.

Dazl-eGFP ESC lines were differentiated in embryoid body suspension cultures for 1, 4, 9, or 15 days (Fig. 7b). Then, GFP positive and negative populations were isolated by FACS and analyzed by quantitative RT-PCR to determine germ cell or somatic cell identities (Fig. 7c). As expected, the percentage of eGFP positive cells in embryoid bodies declined dramatically in all three transgenic reporter lines during a 15 day time-course of differentiation (Table 1).

Analysis of somatic cell markers *Ncam*, *Afp*, and *Kdr* (markers of ectoderm, endoderm, and mesoderm, respectively) revealed a general decrease of somatic cell marker transcript levels in the eGFP positive population compared with the negative population over the time-course (Fig. 7c). In contrast, germ cell marker analysis of *Dazl*, *Vasa*, and *Scp-3* demonstrated a gradual increase in the fold change of all germ cell marker transcript levels in the eGFP positive population over the 15 day time-course of differentiation (Fig. 7c). Thus, *Dazl*-eGFP reporter expression becomes restricted to the germline during mouse ESC differentiation and can be used to identify and isolate the putative ESC-derived GFP positive germ cell populations.

DISCUSSION

We report the characterization of two independently-derived transgenic mouse lines containing an eGFP or hrGFP reporter downstream of a 5' sequence flanking the mouse *Dazl* gene. The 1.7 kb 5' sequence is sufficient to direct germ cell-specific expression of the GFP transgene in transgenic mice and following ESC differentiation. High levels of

transgene expression are detectable in germ cells from adult testis, and low levels of transgene are expressed in oocytes from adult ovary and in fetal and newborn gonads of both sexes. Specifically, the *Dazl* reporter is active in adult testis from pachytene spermatocyte to elongating spermatid stages, with strongest reporter expression observed in mid-pachytene spermatocytes, and in some spermatogonial cells. Although the expression of GFP transcript can be detected in PGC stage e12.5 gonads, the expression of GFP protein (via fluorescence) could only be found at low levels in fetal gonad from e14.5 to e17.5. The low levels of fluorescence observed at these later embryonic pro-spermatogonia/oocyte stages mirror the low levels of GFP protein and fluorescence expressed in adult spermatogonia and oocytes, respectively. This is in stark contrast to the robust transgene expression of GFP protein in pachytene spermatocytes from adult testis and is surprising given that e17.5 oocytes are also predominantly in a pachytene stage of meiotic prophase but do not robustly express GFP protein. As a result, our study highlights the regulated expression of *Dazl* during meiosis in the testis; other studies have suggested that this expression may be required for progression through meiotic prophase (Saunders *et al.*, 2003; Schrans-Stassen *et al.*, 2001).

However, *Dazl* is clearly required for premeiotic embryonic germ cell development in both males and females (Lin and Page, 2005; Ruggiu *et al.*, 1997). Notably, previous reports have found strong endogenous premeiotic *Dazl* expression (Brekman *et al.*, 2000; Niederberger *et al.*, 1997; Reijo *et al.*, 1996, 2000; Seligman and Page, 1998). Thus, the *Dazl*-GFP transgene protein expression observed here does not parallel endogenous protein expression patterns as previously reported and demonstrated by mouse gene disruption.

Robust spermatogonia, oocyte, and PGC protein expression may require additional regulatory sequence from the mouse *Dazl* locus. A sex-specific expression pattern was also observed in *Scp-1* reporter transgenic mice, where 1.8 kb of 5' flanking sequence was sufficient to drive transgene expression in testis but not in ovary (Sage *et al.*, 1999). Additionally, sex-specific transcriptional regulatory elements were identified in *Gdf-9* reporter transgenic mice including a 5' flanking E-box sequence required for ovary expression and a 3' flanking sequence that can repress testis expression (Yan *et al.*, 2006). Translational regulatory elements may also be required for endogenous expression patterning. The 3' UTR is essential for post-transcriptional regulation of many transcripts and developmental processes in mammalian and nonmammalian species (Kuersten and Goodwin, 2003; Leatherman and Jongens, 2003). In mice, *Dazl* protein can bind to sequences in the 3' UTR and can activate the translation of *Vasa* and *Scp-3* transcript in premeiotic germ cells (Reynolds *et al.*, 2007, 2005). *Dazl* transcript also contains an identical sequence in its 3' UTR and may undergo self-regulation to activate premeiotic translation. Similarly in mice, the *Nanos-2* 3' UTR functions as a translational repressor element in ovary but is an enhancer element in testis (Tsuda *et al.*, 2006). Likewise in *Drosophila*, *C. elegans*, and zebrafish, *Nanos* and *Dazl* translation is regulated by the 3' UTR (Dahanukar and Wharton, 1996; Jadhav *et al.*, 2008; Kosaka *et al.*, 2007). Therefore, the accurate recapitulation of endogenous expression patterns of the *Dazl* reporter transgene, and other germ cell-specific reporters, may require regulatory elements from the 3' UTR and from additional 5' and/or 3' flanking sequence.

Following the validation of germ cell specificity in transgenic mice, we used the *Dazl*-GFP reporter to identify and isolate putative germ cells that were differentiated from mouse ESCs. Recent studies have characterized germ cell reporter transgenic mice containing *Oct-4* (Yeom *et al.*, 1996; Yoshimizu *et al.*, 1999), *Blimp-1* (Ohinata *et al.*, 2005), *Fragilis* (Tanaka *et al.*, 2004), *Stella* (Payer *et al.*, 2006), *Tnap* (MacGregor *et al.*, 1995), *Vasa* (Gallardo *et al.*, 2007), *Stra-8* (Nayernia *et al.*, 2004), *Scp-1* (Vidal *et al.*, 1998), *Alf* (Han *et al.*, 2004), *Gdf-9*, *Zp-3*, *Msx-2* (Lan *et al.*, 2004), *Acrosin* (Nayernia *et al.*, 1992), and *Protamine* (Zambrowicz *et al.*, 1993) reporters, and mouse ESC-derived germ cell

populations utilizing *Oct-4* (Hubner *et al.*, 2003), *Stella* (Payer *et al.*, 2006), *Vasa* (Toyooka *et al.*, 2003), *Stra-8* and *Protamine-1* (Nayernia *et al.*, 2006) reporters. However, these reporters have limitations in that they may be expressed in somatic and germ cells, may be sex-specific, and/or may be limited in expression to brief stages of germ cell development. Although we sought to use the *Dazl*-GFP transgene to report various stages of ESC-derived male and, especially, female germ cell development, GFP fluorescence was difficult to detect in oocytes and absent or below detection in PGCs. Thus, the optimal use of the *Dazl*-GFP reporter presented here may be in the study of male germ cell development *in vitro* or *in vivo* in mice, and possibly in human stem cell systems. Additionally, the transgene may be useful for identifying and characterizing cis- and trans-elements that confer sex-specific and germ cell stage-specific regulation on the *Dazl* gene.

METHODS

Construction of *pDazl*-GFP

A 1.707 kb genomic fragment located at positions –15 to –1722 relative to the start codon for the *Dazl* open reading frame, and encompassing 176 bp of 5' UTR in the first exon, was amplified from C57BL/6 mouse genomic DNA by PCR (pfu-turbo, Stratagene, La Jolla, CA). The PCR primers used to amplify the genomic fragment (5'-ttcgggtgtaaaacctcg-3' and 5'-tcttccttcgagaattccag-3') were based on sequence information originally obtained from the Celera mouse genome data base. Limited to 4 kb of available 5' sequence flanking *Dazl*, multiple primer pairs were originally designed to amplify 1.5–3 kb of the promoter region. The 1.7 kb PCR product was the only amplicon successfully amplified and was purified from a 1% agarose gel and then ligated into pCR Blunt (Invitrogen, Carlsbad, CA). The 1.7 kb fragment was excised from pCR blunt using Nsi I and Not I, and then ligated into the Nsi I and Not I sites of pHRGFP (Stratagene) to generate *pDazl*-hrGFP. Then, the 1.7 kb fragment was excised from *pDazl*-hrGFP using Nsi I and Pst I, and ligated into the Pst I site of pEGFP-1 (Clontech, Mountain View, CA) to generate *pDazl*-eGFP.

Production of *Dazl*-GFP Transgenic Mice

The plasmid containing the *Dazl*-hrGFP transgene was digested with Nsi I and Mlu I to yield a 2.82 kb fragment free of most vector sequences, and which contained the *Dazl* 5'-flanking DNA driving the hrGFP expression cassette. The gel-purified transgene fragment was injected into the pronuclei of fertilized one-cell B6SJL F2/J mouse eggs, which after culturing to the two-cell stage, were transferred to the oviduct of day 1 pseudopregnant B6CBA F1/J recipients. Transgenic mice were identified by dot blot analysis of DNA isolated from a tail biopsy using a 32-P-labeled hrGFP probe. Among the 47 animals born from injected eggs, 14 were identified as transgenic. Transgenic lines were analyzed for the expression of hrGFP in testicular germ cells by viewing squash-preps of seminiferous tubules under a fluorescent microscope. Expression of hrGFP above wild-type levels was detected in ~57% of the lines analyzed. Transgenic line 7-2 expressed the highest relative levels of hrGFP in testicular germ cells, and therefore, was further maintained as a homozygous line that produced normal numbers of offspring/litter (Mean litter size = 6.5, SD $\pm$ 1.7, n = 15 litters). *Dazl*-eGFP transgenic mice were produced by similar methods (see Results section). Transgenic *Dazl*-eGFP founders and offspring were identified by PCR on tail-tip genomic DNA using Platinum Taq DNA Polymerase (Invitrogen) with primers: (F—GCCTATTGGCTGTAGCACGTCACG, R—CTTCAGCTCGATGCGGTTACCAG).

RT-PCR

Gonads and somatic tissues were dissected and digested using Collagenase IV/Dispase (1 mg/mL, Invitrogen) and DNaseI (0.1%, Roche, Basel, Switzerland) for 30 min at 37°C for adult gonads; or using 0.25% Trypsin (Invitrogen) and DNaseI (0.1%, Roche) for 10–15 min

at 37°C for newborn/fetal gonads and embryoid bodies. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA prepared with the SuperScript III Kit (Invitrogen). RT-PCR and quantitative RT-PCR were performed with iQ SYBR Green Supermix (BioRad, Hercules, CA) on a MyiQ system (BioRad) or with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7300 Real Time PCR System (Applied Biosystems). For relative expression analysis, sample Ct values were normalized to *UbiquitinB* by subtracting the *UbiquitinB* Ct and then by calculating two raised to the negative power of the difference in Ct values. For fold change analysis, *UbiquitinB* Ct values were again subtracted from sample Ct values, and the difference in the GFP[−] samples was then subtracted from the difference in the GFP⁺ samples. The fold change of GFP⁺ over GFP[−] was calculated as two raised to the negative power of this difference.

Primers: F/R: *eGFP*, AGCTGACCCTGAAGTTCATCTG/
TATAGACGTTGTGGCTGTTGTAGTT; *UbiquitinB*, GCGGTTTGTGCTTTCATCAC/
GGCAAAGATCAGCCTCT GCT; *Dazl*, ATGGCTCAGTAAAAGAAGTGAAGATAA/
AGTACATAAAATTTGTTTCCTGATTGC; *Vasa*,
CTAGGAAGACCAAATAGTGAATCTGAC/TCCAGAACCTGTTACTACTTCTTCATT;
Scp3, AGAAATGTATACCAAAGCTTCTTTCAA/
TTAGATAGTTTTCTCCTTGTTCTCTCA; *Sox9*, CACGGAACAGACTCACATCTCT/
CCTCTCGCTTCAGATCAACTTT; *Lhr*, TTTCCAAACAATGTGAAAGCAC/
CAACACCCTAAGGAAGGCATAG; *Ncam*, GAGGTGACCCCAGATTCAGA/
TCTGGCTCATCAAACTGCAC; *Afp*, GAAGCAAGCCCTGTGAACTC/
AGCTTGGCACAGATCCTTGT; *Kdr*, GGCGGTG GTGACAGTATCTT/
GTCAGTACAGAGGCGATGA.

Flow Cytometry and FACS Sorting

Mouse tissue and embryoid bodies were digested to single cell suspensions as above. Cells were washed and re-suspended in PBS (Invitrogen) with 1% BSA (Sigma-Aldrich, St. Louis, MO) or in differentiation media, filtered through 40 µm nylon cell strainer (BD Biosciences, San Jose, CA), and analyzed/sorted for GFP on MoFlo (DakoCytomation, Glostrup, Denmark) or Aria (BD Biosciences) machines. For embryonic gonad analysis, embryonic heads were first genotyped to identify transgenic embryos using the ZR Genomic DNA II kit (Zymo Research, Orange, CA) prior to processing and analysis. Embryonic sex was determined by gonad morphologies.

Fluorometric Analysis

Relative levels of fluorescence were measured in lysates prepared from various mouse tissues using a FL600 fluorescence microtiter plate reader (BioTek, Winooski, VT) equipped with filter wheel sets for maximal excitation at 485 nm and maximal emission at 516 nm, as previously described (Hamra *et al.*, 2004).

Immunohistochemistry

Adult testes were fixed in 4% para-formaldehyde (Sigma-Aldrich) overnight at 4°C, paraffin embedded, and sectioned (5 µm). Briefly, slides were deparaffinized and rehydrated, blocked in 10% hydrogen peroxide (Sigma-Aldrich) for 20 min, permeabilized with 0.1% Triton-X100 (Sigma-Aldrich) for 5 min, blocked in 4% normal horse serum (Sigma-Aldrich), incubated overnight at 4°C with 1:200 anti-GFP JL-8 antibody (Clontech), incubated for 30 min at room temperature with 1:200 Biotinylated horse anti-mouse IgG secondary antibody (Vector Labs, Burlingame, CA), 30 min at room temperature with VECTASTAIN ABC standard (Vector Labs), 3 min DAB (Vector Labs), counterstained with Mayer's hematoxylin (Sigma-Aldrich) for 30 s, and coverslips mounted with Prolong Gold (Invitrogen).

Mouse ESCs

Mouse ESC lines were maintained in Knockout DMEM (Invitrogen) ES media with 10% FBS (Hyclone, Logan, UT), 1% NEAA and 1% L-glutamine (both Invitrogen), 0.001% BME (Sigma), 1000 U/mL LIF (Chemicon—Millipore, Billerica, MA), and passaged with 0.05% Trypsin (Invitrogen) on MEFs or 0.1% Gelatin (Sigma-Aldrich). To generate transgenic ESC lines, *Dazl*-GFP reporter plasmids were linearized and purified by alcohol precipitation. Thirty microgram of DNA were electroporated into 20 million ESCs (F1-2.1.10B mouse ESC line from 129XCastaneus—gift from Barbara Panning) at 500 μ F, 250 V, 500°C. Transfected cells were replated onto gelatin coated plates and selected with 250 μ g/mL Geneticin (Invitrogen) for 1 week following transfection for 48 h. After selection, colonies were picked and expanded separately on MEFs. For embryoid body differentiation, mouse ESCs were passaged into differentiation media (same as knockout ES media but without LIF) and transferred to Costar ultra low attachment plates (Corning, Corning, NY) for suspension culture.

Acknowledgments

We thank Dr. Joanna Gonsalves for eGFP cloning assistance, and Dr. Fredrick Moore, Dr. Nigel Killeen, and Jason Dietrich from the UCSF Transgenic/Targeted Mutagenesis Core for generating the *Dazl*-eGFP transgenic mice. Additionally, we thank Margaret Mayes and Amarjeet Grewal for histology services, Shuwei Jiang and Patty Lovelace for FACS services, and members of the Reijo Pera laboratory for helpful conversations during this study.

Contract grant sponsor: National Institutes of Health; Contract grant numbers: RO1 HD047721, U54HD055764; Contract grant sponsor: California TRDRP; Contract grant numbers: 14RT-0159, 15DT-0187

LITERATURE CITED

- Brekthman V, Itskovitz-Eldor J, Yodko E, Deutsch M, Seligman J. The DAZL1 gene is expressed in human male and female embryonic gonads before meiosis. *Mol Hum Reprod* 2000;6:465–468. [PubMed: 10775651]
- Clark AT, Bodnar MS, Fox M, Rodriguez RT, Abeyta MJ, Firpo MT, Pera RA. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet* 2004;13:727–739. [PubMed: 14962983]
- Cooke HJ, Lee M, Kerr S, Ruggiu M. A murine homologue of the human DAZ gene is autosomal and expressed only in male and female gonads. *Hum Mol Genet* 1996;5:513–516. [PubMed: 8845845]
- Dahanukar A, Wharton RP. The Nanos gradient in *Drosophila* embryos is generated by translational regulation. *Genes Dev* 1996;10:2610–2620. [PubMed: 8895662]
- Dorfman DM, Genest DR, Reijo Pera RA. Human DAZL1 encodes a candidate fertility factor in women that localizes to the prenatal and postnatal germ cells. *Hum Reprod* 1999;14:2531–2536. [PubMed: 10527983]
- Gallardo T, Shirley L, John GB, Castrillon DH. Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre. *Genesis* 2007;45:413–417. [PubMed: 17551945]
- Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 2004;427:148–154. [PubMed: 14668819]
- Haag ES. Rolling back to BOULE. *Proc Natl Acad Sci USA* 2001;98:6983–6985. [PubMed: 11416173]
- Hamra FK, Schultz N, Chapman KM, Grellhesl DM, Cronkhite JT, Hammer RE, Garbers DL. Defining the spermatogonial stem cell. *Dev Biol* 2004;269:393–410. [PubMed: 15110708]
- Han S, Xie W, Kim SH, Yue L, DeJong J. A short core promoter drives expression of the ALF transcription factor in reproductive tissues of male and female mice. *Biol Reprod* 2004;71:933–941. [PubMed: 15151936]
- Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF III, Boiani M, Scholer HR. Derivation of oocytes from mouse embryonic stem cells. *Science* 2003;300:1251–1256. [PubMed: 12730498]

- Jadhav S, Rana M, Subramaniam K. Multiple maternal proteins coordinate to restrict the translation of *C. elegans* nanos-2 to primordial germ cells. *Development* 2008;135:1803–1812. [PubMed: 18417623]
- Kerkis A, Fonseca SA, Serafim RC, Lavagnoli TM, Abdelmassih S, Abdelmassih R, Kerkis I. In vitro differentiation of male mouse embryonic stem cells into both presumptive sperm cells and oocytes. *Cloning Stem Cells* 2007;9:535–548. [PubMed: 18154514]
- Kosaka K, Kawakami K, Sakamoto H, Inoue K. Spatiotemporal localization of germ plasm RNAs during zebrafish oogenesis. *Mech Dev* 2007;124:279–289. [PubMed: 17293094]
- Kuersten S, Goodwin EB. The power of the 3' Translational control and development. *Nat Rev Genet* 2003;4:626–637. [PubMed: 12897774]
- Lan ZJ, Xu X, Cooney AJ. Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. *Biol Reprod* 2004;71:1469–1474. [PubMed: 15215191]
- Leatherman JL, Jongens TA. Transcriptional silencing and translational control: Key features of early germline development. *Bioessays* 2003;25:326–335. [PubMed: 12655640]
- Lin Y, Page DC. Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. *Dev Biol* 2005;288:309–316. [PubMed: 16310179]
- MacGregor GR, Zambrowicz BP, Soriano P. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development* 1995;121:1487–1496. [PubMed: 7789278]
- Moore, KL.; Persaud, TVN. Before we were born: Essentials of embryology and birth defects. Philadelphia: W.B. Saunders Company; 2003. p. 530
- Nayernia K, Burkhardt E, Beimesche S, Keime S, Engel W. Germ cell-specific expression of a proacrosin-CAT fusion gene in transgenic mouse testis. *Mol Reprod Dev* 1992;31:241–248. [PubMed: 1571157]
- Nayernia K, Li M, Jaroszynski L, Khusainov R, Wulf G, Schwandt I, Korabiowska M, Michelmann HW, Meinhardt A, Engel W. Stem cell based therapeutical approach of male infertility by teratocarcinoma derived germ cells. *Hum Mol Genet* 2004;13:1451–1460. [PubMed: 15163638]
- Nayernia K, Nieter S, Kremling H, Oberwinkler H, Engel W. Functional and molecular characterization of the transcriptional regulatory region of the proacrosin gene. *J Biol Chem* 1994;269:32181–32186. [PubMed: 7798216]
- Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, Dev A, Wulf G, Ehrmann IE, Elliott DJ, Okpanyi V, Zechner U, Haaf T, Meinhardt A, Engel W. In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. *Dev Cell* 2006;11:125–132. [PubMed: 16824959]
- Niederberger C, Agulnik AI, Cho Y, Lamb D, Bishop CE. In situ hybridization shows that Dazl expression in mouse testis is restricted to premeiotic stages IV–VI of spermatogenesis. *Mamm Genome* 1997;8:277–278. [PubMed: 9096110]
- Novak I, Lightfoot DA, Wang H, Eriksson A, Mahdy E, Hoog C. Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis. *Stem Cells* 2006;24:1931–1936. [PubMed: 16644921]
- Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A, Saitou M, Surani MA. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 2005;436:207–213. [PubMed: 15937476]
- Pan HA, Liao RW, Chung CL, Teng YN, Lin YM, Kuo PL. DAZL protein expression in mouse preimplantation embryo. *Fertil Steril* 2008;89(5 Suppl):1324–1327. [PubMed: 17761180]
- Payer B, Chuva de Sousa Lopes SM, Barton SC, Lee C, Saitou M, Surani MA. Generation of stella-GFP transgenic mice: A novel tool to study germ cell development. *Genesis* 2006;44:75–83. [PubMed: 16437550]
- Qing T, Shi Y, Qin H, Ye X, Wei W, Liu H, Ding M, Deng H. Induction of oocyte-like cells from mouse embryonic stem cells by coculture with ovarian granulosa cells. *Differentiation* 2007;75:902–911. [PubMed: 17490416]

- Reddi PP, Flickinger CJ, Herr JC. Round spermatid-specific transcription of the mouse SP-10 gene is mediated by a 294-base pair proximal promoter. *Biol Reprod* 1999;61:1256–1266. [PubMed: 10529272]
- Reijo RA, Dorfman DM, Sleen R, Renshaw AA, Loughlin KR, Cooke H, Page DC. DAZ family proteins exist throughout male germ cell development and transit from nucleus to cytoplasm at meiosis in humans and mice. *Biol Reprod* 2000;63:1490–1496. [PubMed: 11058556]
- Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, Chapelle A, Silber S, Page DC. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet* 1995;10:383–393. [PubMed: 7670487]
- Reijo R, Seligman J, Dinulos MB, Jaffe T, Brown LG, Disteché CM, Page DC. Mouse autosomal homolog of DAZ, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. *Genomics* 1996;35:346–352. [PubMed: 8661148]
- Reynolds N, Collier B, Bingham V, Gray NK, Cooke HJ. Translation of the synaptonemal complex component Sycp3 is enhanced in vivo by the germ cell specific regulator Dazl. *RNA* 2007;13:974–981. [PubMed: 17526644]
- Reynolds N, Collier B, Maratou K, Bingham V, Speed RM, Taggart M, Semple CA, Gray NK, Cooke HJ. Dazl binds in vivo to specific transcripts and can regulate the pre-meiotic translation of Mvh in germ cells. *Hum Mol Genet* 2005;14:3899–3909. [PubMed: 16278232]
- Robinson MO, McCarrey JR, Simon MI. Transcriptional regulatory regions of testis-specific PGK2 defined in transgenic mice. *Proc Natl Acad Sci USA* 1989;86:8437–8441. [PubMed: 2813402]
- Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* 1997;389:73–77. [PubMed: 9288969]
- Sage J, Martin L, Meuwissen R, Heyting C, Cuzin F, Rassoulzadegan M. Temporal and spatial control of the Sycp1 gene transcription in the mouse meiosis: Regulatory elements active in the male are not sufficient for expression in the female gonad. *Mech Dev* 1999;80:29–39. [PubMed: 10096061]
- Saunders PT, Turner JM, Ruggiu M, Taggart M, Burgoyne PS, Elliott D, Cooke HJ. Absence of mDazl produces a final block on germ cell development at meiosis. *Reproduction* 2003;126:589–597. [PubMed: 14611631]
- Schrans-Stassen BH, Saunders PT, Cooke HJ, de Rooij DG. Nature of the spermatogenic arrest in Dazl $-/-$ mice. *Biol Reprod* 2001;65:771–776. [PubMed: 11514340]
- Seligman J, Page DC. The Dazh gene is expressed in male and female embryonic gonads before germ cell sex differentiation. *Biochem Biophys Res Commun* 1998;245:878–882. [PubMed: 9588208]
- Tanaka SS, Nagamatsu G, Tokitake Y, Kasa M, Tam PP, Matsui Y. Regulation of expression of mouse interferon-induced transmembrane protein like gene-3, Ifitm3 (mil-1, fragilis), in germ cells. *Dev Dyn* 2004;230:651–659. [PubMed: 15254899]
- Toyooka Y, Tsunekawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci USA* 2003;100:11457–11462. [PubMed: 14504407]
- Tsuda M, Kiso M, Saga Y. Implication of nanos2–3'UTR in the expression and function of nanos 2. *Mech Dev* 2006;123:440–449. [PubMed: 16806845]
- Vidal F, Sage J, Cuzin F, Rassoulzadegan M. Cre expression in primary spermatocytes: A tool for genetic engineering of the germ line. *Mol Reprod Dev* 1998;51:274–280. [PubMed: 9771647]
- Xu EY, Moore FL, Pera RA. A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans. *Proc Natl Acad Sci USA* 2001;98:7414–7419. [PubMed: 11390979]
- Yan C, Elvin JA, Lin YN, Hadsell LA, Wang J, DeMayo FJ, Matzuk MM. Regulation of growth differentiation factor 9 expression in oocytes in vivo: A key role of the E-box. *Biol Reprod* 2006;74:999–1006. [PubMed: 16495478]
- Yen PH. Putative biological functions of the DAZ family. *Int J Androl* 2004;27:125–129. [PubMed: 15139965]
- Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hubner K, Scholer HR. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* 1996;122:881–894. [PubMed: 8631266]

- Yoshimizu T, Sugiyama N, De Felice M, Yeom YI, Ohbo K, Masuko K, Obinata M, Abe K, Scholer HR, Matsui Y. Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. *Dev Growth Differ* 1999;41:675–684. [PubMed: 10646797]
- Zambrowicz BP, Harendza CJ, Zimmermann JW, Brinster RL, Palmiter RD. Analysis of the mouse protamine 1 promoter in transgenic mice. *Proc Natl Acad Sci USA* 1993;90:5071–5075. [PubMed: 8389466]

FIG. 1.

Dazl-GFP transgene expression in adult testis and ovary. **(a)** Brightfield and fluorescent microscopy pictures of adult testes from wild-type (WT), *Dazl*-eGFP (eGFP), and *Dazl*-hrGFP (hrGFP) transgenic mouse lines ($\times 100$). **(b)** Brightfield and fluorescent microscopy pictures of oocytes from wild-type (WT) and transgenic (line eGFP and hrGFP) adult mouse ovary ($\times 200$). **(c)** Flow cytometry analysis of adult gonads from wild-type (WT) and transgenic (line eGFP) mice showing the percentage of GFP positive cells (x -axis: GFP, y -axis: side scatter).

FIG. 2. GFP transcript expression in adult, newborn, and fetal gonad. **(a)** RT-PCR for GFP and *UbB* control transcripts in wild-type (WT) and transgenic line eGFP testis and ovary from adult mice. **(b)** RT-PCR for GFP and *UbB* control transcripts in wild-type (WT) and transgenic line eGFP testis (M) and ovary (F) from adult (Ad), newborn (Nb), and e12.5, e14.5, or e16.5 embryonic stage mice. Reverse transcriptase (RT) negative controls and *Dazl* positive controls are included.

FIG. 3. GFP fluorescence in fetal gonad. Flow cytometry analysis of e12.5 and e17.5 testes (M) and ovaries (F) from wild-type (WT) and transgenic (line eGFP) embryos (*x*-axis: GFP, *y*-axis: side scatter).

FIG. 4.

Gonad-specific expression. **(a)** Brightfield and fluorescent microscopy pictures of adult testis, kidney, liver, and spleen from wild-type and transgenic line hrGFP mice ($\times 100$). **(b)** Fluorometric absorbance analysis for hrGFP in a panel of adult tissues from wild-type (striped bars) and transgenic mice (solid bars) illustrating testis-specific transgene expression ($n = 3$). **(c)** RT-PCR analysis for hrGFP and *Ubb* control transcripts in adult tissues from wild-type (WT) and line hrGFP mice with reverse transcriptase (RT) negative controls. Lv, liver; He, heart; Lu, lung; Si, small intestines; Li, large intestines; Pa, pancreas; Sp, spleen; Ki, kidney; Te, testis; Ov, ovary.

FIG. 5.

Germ cell-specific expression. **(a)** Adult testes were analyzed for *Dazl*, *Vasa*, and *Scp-3* germ cell transcripts by quantitative RT-PCR analysis of FACS isolated GFP negative (light grey bars) and GFP positive (dark grey bars) populations from transgenic line eGFP, normalized to *Ubb* ($n = 4$). **(b)** FACS isolated GFP negative and positive populations from transgenic testes were also analyzed for *Sox-9* and *Lhr* somatic cell transcripts by quantitative RT-PCR analysis, normalized to *Ubb* ($n = 4$). **(c)** FACS sorted populations from transgenic line eGFP adult ovary and e17.5 testis analyzed by RT-PCR for *Dazl* and *Ubb* transcripts.

FIG. 6.

Germ cell-specific and meiotic stage-specific expression. **(a)** Immunohistochemistry (IHC) staining of an adult testis section from transgenic line eGFP with GFP antibody ($\times 100$). **(b)** Secondary antibody only control staining of a testis section from line eGFP ($\times 100$). **(c)** An adult testis section from wild-type mice stained with GFP antibody ($\times 200$). **(d)** An overview of *Dazl*-eGFP expression in line eGFP testis by IHC illustrating tubules in different stages of the epithelial cycle ($\times 200$). **(e)** Strongest GFP expression was observed in mid-pachytene spermatocytes, and was also detected at lower levels in round and elongating spermatids, but was not detectable in Sertoli cells and spermatocyte stages prior to pachytene ($\times 630$).

FIG. 7.

Dazl-eGFP mouse embryonic stem cell differentiation. (a) Brightfield and fluorescent microscopy pictures of transgenic clonal ESC lines 2, 8, and 17 ($\times 100$). (b) *Dazl*-eGFP ESCs were differentiated for up to 15 days as embryoid bodies and subsequently FACS sorted to isolate eGFP positive and negative populations ($\times 100$). (c) Germ cell (*Dazl*, *Vasa*, *Scp-3*) and somatic cell (*Ncam*, *Afp*, *Kdr*) marker transcript levels in the eGFP positive population from transgenic ESC line 8 embryoid bodies were analyzed by quantitative RT-PCR and compared with transcript levels in the eGFP negative population (x-axis: length of differentiation in days, y-axis: fold change of GFP+/GFP- normalized to *Ubb*, $n = 2$).

Table 1Percentage *Dazl*-eGFP Expression by FACS Following Differentiation

	2^a	8^a	17^a
d1 ^b (%)	29	49	31
d4 ^b (%)	18	30	17
d9 ^b (%)	2.8	11	2.5
d15 ^b (%)	0.5	2.8	0.2

^a *Dazl*-eGFP mouse embryonic stem cell clones.^b Days of embryoid body differentiation.