Mammalian germ cells are determined after PGC colonization of the nascent gonad

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Mammalian primordial germ cells (PGCs) are induced in the embryonic epiblast, before migrating to the nascent gonads. In fish, frogs, and birds, the germ line segregates even earlier, through the action of maternally inherited germ plasm. Across vertebrates, migrating PGCs retain a broad developmental potential, regardless of whether they were induced or maternally segregated. In mammals, this potential is indicated by expression of pluripotency factors and the ability to generate teratomas and pluripotent cell lines. How the germ line loses this developmental potential remains unknown. Our genome-wide analyses of embryonic human and mouse germ lines reveal a conserved transcriptional program, initiated in PGCs after gonadal colonization, that differentiates germ cells from their germline precursors and from somatic lineages. Through genetic studies in mice and pigs, we demonstrate that one such gonad-induced factor, the RNA-binding protein DAZL, is necessary in vivo to restrict the developmental potential of the germ line: DAZL's absence prolongs expression of a Nanog pluripotency reporter, facilitates derivation of pluripotent cell lines, and causes spontaneous gonadal teratomas. Based on these observations in humans, mice, and pigs, we propose that germ cells are committed after gonadal colonization in mammals. We suggest that germ cell determination was induced late in embryogenesis—after organogenesis has begun—in the common ancestor of all vertebrates, as in modern mammals, where this transition is induced by somatic cells of the gonad. We suggest that failure of this process of germ cell determination likely accounts for the origin of human testis cancer.

germ cell | commitment | teratoma | pluripotency | DAZL

During embryogenesis, cells segregate into germ line and somatic lineages. In mammals, this split is first evident around the time of gastrulation, when intercellular signaling induces the formation of primordial germ cells (PGCs) (1, 2). Comparative studies reveal that an inductive method of germ line segregation likely existed in the common ancestor of all vertebrates (3). However, some vertebrates, such as fish, frogs, and birds, have acquired a different approach to germ line segregation. It occurs much earlier in these species—during the first cell divisions of the zygote—through the action of maternally supplied RNAs known as germ plasm (4).

Despite these different strategies for PGC formation, emerging evidence suggests that migratory PGCs of nonmammalian vertebrates remain developmentally uncommitted to gametogenesis, retaining the capacity for somatic differentiation. In frogs, PGCs arise via germ plasm readily differentiate into somatic cells when transplanted into host embryos (5). Similarly, in fish, migrarnated PGCs readily adopt somatic fates if depleted of Dnmt1 (6). In salamanders, where PGCs arise through inductive processes, irreversible commitment of the germ line occurs late in development, long after gastrulation is complete and somatic lineages are established (7). In mammals, migratory PGCs can form teratomas if transplanted to ectopic sites (8) and give rise to pluripotent cell lines in culture (9–11). It has also been suggested that presumptive PGCs (labeled genetically by Prdm1-Cre) in the posterior region of the embryo during allantoic elongation may contribute to nongametogenic lineages (12, 13). Taken together, these observations suggest that migratory PGCs of vertebrates retain a broad developmental potential, regardless of their mode of segregation. That is, migratory PGCs, while clearly cells of the germ line (the entire lineage from zygote to gamete), may not yet be germ cells, which, by definition, are committed to producing gametes and no other cell types (14).

To better understand germ line commitment in mammals, we examine the transition that occurs as PGCs invade the nascent gonads. We find a transcriptional program, initiated in human and mouse PGCs after colonization of the gonad, that distinguishes germ cells from their migratory germ line precursors, and from soma. Through genetic studies, we demonstrate that this program is necessary for germ cell commitment in mammals. In embryonic mice deficient in one factor induced at PGC colonization—the


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RNA-binding protein Dazl—the germline remains developmentally uncommitted, retaining expression of a network of pluripotency factors, the capacity for pluripotent cell line derivation, and the potential to form gonadal teratomas in mice and pigs.

Results

Germ Cell Determinants of Nonmammalian Vertebrates Are Expressed in Mice and Humans upon PGC Colonization of the Nascent Gonads. We searched for changes in gene expression that accompany PGC colonization of gonads in mammals. To this end, we reanalyzed published transcriptomes of migratory and gonadal germline cells from mouse (15) and human (16) embryos. Our analyses in mice identified 74 genes whose expression increased robustly after PGCs colonized the gonads (fold change of >4 [presented as log2 transformed] from E9.5 to E11.5, false discovery rate (FDR) < 0.05; Fig. 1A and Datasets S1 and S2). To determine whether this program of gene expression is similarly induced in human embryos, we reanalyzed the transcriptomes of single cells within a comparable developmental window (XY: weeks 4 to 9; XX: weeks 5 to 7 and 8; Dataset S3 and SI Appendix, Fig. S1). Of the 74 genes induced in mice, 44 have one-to-one orthologs in humans. As a set, these 44 genes are up-regulated in both XY and XX human germline cells after gonadal colonization, indicating that the program induced in the mouse germline at colonization is conserved in humans (Fig. 1B). Of particular importance, 13 genes up-regulated in mice were also significantly up-regulated in both the XY and XX human germlines (Fig. 1C).

Might this conserved program of gene expression, initiated after PGCs colonize the nascent gonads, serve to distinguish germ cells from other cell types in vivo? To address this, we examined, across diverse tetrapods, the expression breadth of these 13 genes. By reanalyzing RNA sequencing (RNA-seq) datasets of nine tissues from five mammals, as well as chicken and frog, we found that 10 of the 13 genes were predominantly or exclusively expressed in the adult testis, regardless of whether the germline is segregated by induction (as occurs in the mammalian epiblast) or via germ plasm (as occurs in the frog and chicken; Fig. 1D). In both mouse and human, each of these 10 gonad-specific genes is expressed predominantly in germ cells of embryonic gonads (Fig. 1E and F). Notably, several factors activated on PGC colonization are components of germ plasm in nonmammalian animals, including DAZL, DDX4 (the mammalian ortholog of Vasa), MAEL, and TDRD12.

By comparison, the set of genes expressed in migratory PGCs of mouse and human embryos, immediately prior to gonadal colonization, does not display such gonad-specific expression in tetrapods; instead, these genes are expressed across adult tissues (SI Appendix, Fig. S2A–D). The same is true for a curated set of PGC-defining factors gleaned from the literature, and also for a set of genes activated on PGC-like cell derivation (SI Appendix, Fig. S2A–D). Further, analysis of 500,000 random-sampled gene sets found none that displayed gonad specificity comparable to that of the 13 genes up-regulated as PGCs colonize the gonads (SI Appendix, Fig. S2E).

In sum, a set of genes whose expression defines germ cells of vertebrates is first activated in the mouse and human germlines after embryonic PGCs colonize the gonads. These genes include orthologs of germ plasm components critical to germline commitment in diverse nonmammalian metazoas (Dataset S2), raising the possibility that one or more of these genes direct germl cell commitment in mammals, and that this occurs following gonadal colonization.

If this is true, then the transcriptional profiles of the germline should provide evidence of its uncommitted nature prior to gonadal entry. Indeed, we find that, in both humans and mice, migratory and newly colonized germline cells express naïve and general pluripotency factors, which identify developmentally uncommitted cells in vivo (in the inner cell mass) and in vitro (in embryonic stem [ES] cells) (17–19) (SI Appendix, Fig. S3). [Pluripotency factors are similarly expressed in migratory PGCs of fish and birds (20, 21).] Further, in humans and mice of both sexes, these pluripotency factors are markedly down-regulated after PGC colonization and induction of the germ cell-defining program of gene expression (SI Appendix, Fig. S3). Might activation of this program be necessary to down-regulate pluripotency factors and restrict the developmental potential of the germline?

Expression of Pluripotency Factors, and the Capacity for Deriving Pluripotent Cell Lines, Are Prolonged in Dazl-Deficient Mice. We considered whether the program of gene expression induced after PGCs colonize the gonads functions in germ cell commitment. Evidence from a range of vertebrates suggests that DAZL, encoding an RNA-binding protein, might contribute to this function. For example, DAZL orthologs function in the germ plasm of fish (22, 23), frogs (24, 25), and birds (26). In C57BL/6 mice (B6), Dazl is necessary for licensing—the acquisition of meiotic and gametogenic competence—after PGCs colonize the gonads (27), and studies of mouse and human ES cells have suggested that DAZL limits the expression of pluripotency factors in vitro (28, 29). An opposing view—that Dazl serves to maintain germline pluripotency—emerges from reports that pluripotent embryonic germ (EG) cell lines could not be derived from Dazl-deficient embryos (30), and that the Dazl-deficient germline is unable to form gonadal teratomas (28), which arise when pluripotent cells differentiate to generate tissues of all three germ layers.

To reexamine the relationship between DAZL expression and germline commitment, we generated a reporter allele of DAZL expression (where both Dazl and tdTomato are translated from a single nascent RNA, referred to as Dazl-tdTomato; SI Appendix, Fig. S4A), and intercrossed this with a second fluorescent reporter allele, Nanog:GFP (a reporter of uncommitted cells). Flow cytometry revealed an abundance of Nanog:GFP-positive cells in E11.0 embryos (~12 tail somites [ts]) carrying both reporters, with few if any of these cells also expressing the DAZL reporter (Fig. 2A and SI Appendix, Fig. S4B). By 15 ts, however, we began to detect DAZL reporter expression in a small population of Nanog:GFP-positive cells. With increasing embryonic age, we continued to find a small group of cells expressing both reporters, while an increasing proportion expressed the DAZL reporter alone (no longer positive for Nanog:GFP). By 27 ts (~E12.5), very few cells were Nanog:GFP-positive, irrespective of the chromosomal sex of the embryo. These findings demonstrate, at cellular resolution, that the onset of DAZL expression is tightly correlated with the subsequent restriction of Nanog:GFP expression, across the entire population, within 36 h.

Might Dazl be necessary for this restriction? To test this, we crossed the Nanog:GFP reporter to both B6 and 129S4/SvJae (129S4) backgrounds, and monitored expression in each strain (SI Appendix, SI Discussion and Fig. S5). At E15.5, germline expression of the Nanog:GFP reporter was absent from the gonads of control embryos, but maintained in Dazl-deficient gonads, regardless of the embryo’s genetic background or sex (Fig. 2B and SI Appendix, Fig. S4C). In support, reanalysis of published RNA-seq data from Dazl-deficient mouse ovaries (31) shows that a set of “general” and “naïve” pluripotency factors (19) remain expressed at E14.5 (SI Appendix, Fig. S4D). These observations indicate that Dazl is necessary, in vivo, to extinguish the expression of key markers of uncommitted cells.

To test whether Dazl is necessary to restrict the developmental potential of the germline, we attempted to derive pluripotent cell lines from control and Dazl-deficient B6 embryos. Germline cells isolated from control E10.5 embryos readily gave rise to EG cell colonies, with a mean derivation efficiency of 10.0 ± 3.4 colonies per 100 EGFP-positive cells plated (mean ± SD, n = 8 embryos; Fig. 2C and SI Appendix, Table S1). Colony formation declined precipitously with increasing embryonic age, irrespective of the
At E12.5, only 5 of 18 control embryos gave rise to any EG colonies (0.03 ± 0.05 colonies), and no EG colonies were derived from E13.5 onward, irrespective of sex (n = 43 embryos). These observations were as expected, given prior reports that EG colony formation declines sharply after PGCs arrive at the gonads in mice (32), and the similarly transient capacity to derive pluripotent-like cells from human gonads (33).
Our experimental observations with Dazl-deficient embryos differed strikingly from littermate controls. At E10.5 (before Dazl is expressed), Dazl-deficient embryos gave rise to EG colonies at a frequency comparable to controls (10.8 ± 1.5 colonies per 100 EGFP-positive cells, n = 3 embryos; Fig. 2C). However, all Dazl-deficient embryos retained the capacity to generate EG colonies beyond E12.5 (n = 10 embryos, combined from E13.5 to E15.5), regardless of the embryo’s sex. Importantly, we first observed a significant difference between control and Dazl-deficient embryos at E11.5 (5.0 ± 4.2 colonies per 100 EGFP-positive cells, n = 7 embryos), concomitant with initiation of Dazl expression in the germine (Fig. 2A). Likewise, Dazl-deficient embryos isolated from an F1 cross between 129S4 and B6 mice retained the capacity to give rise to EG cell colonies until at least E15.5; the pluripotency of these cell lines was confirmed by injection into recipient blastocysts and resultant chimerism (SI Appendix, Fig. S4 E–H).

Taken together, these data establish that the Dazl-deficient germine continues to express pluripotency factors, and retains a PGC-like capacity for the derivation of pluripotent cell lines, even several days after colonization of the genital ridges in mice. We conclude that Dazl is necessary to restrict the developmental potential of the mouse germine after colonization of the gonads, regardless of genetic background, and prior to sexual differentiation of the germine.

Are other germ cell-defining factors (Fig. 1D) also required for this restriction? To address this question, we collected germine cells immediately prior to and shortly after gonadal entry, from control and Dazl-deficient embryos (34). RNA-seq analysis of these cells revealed that, apart from Dazl, expression of each of the other germ cell-defining factors is initiated upon gonadal entry in both control and Dazl-deficient embryos, indicating that DAZL is not required for their expression (SI Appendix, Fig. S4I). Further, this demonstrates that their expression is not sufficient to restrict Nanog/GFP expression in the Dazl-deficient germine—nor does their expression preclude derivation of EG cell lines (Fig. 2B and C).

### Spontaneous Gonadal Teratomas in Dazl-Deficient Mice of both Sexes

We next considered the fate of the Dazl-deficient germine in mice. In wild-type 129 males (but not females), teratomas arise spontaneously, at low but significant frequency, from germine cells (35). We hypothesized that these tumors arise from PGCs that enter the gonads but nonetheless remain uncommitted, retaining their teratoma-forming potential.

We collected 129S4 Dazl-deficient males and examined the testes for spontaneous teratomas. In control 129S4 mice at 4 mo of age, we found two teratomas among 127 males (Fig. 3 A–C), consistent with the low incidence reported in this strain (35). By contrast, among Dazl-deficient males, 19 of 69 (28%) exhibited testicular teratomas by 4 mo of age. To determine whether teratomas were present even earlier in postnatal life, we collected 129S4 mice at 4 wk of age. In controls, we found one teratoma in 131 males, 20 of 65 (31%) displayed testicular teratomas. Clearly, teratomas form at a markedly elevated rate in 129S4 Dazl-deficient males early in postnatal life, if not before.

Our earlier studies of EG cell derivation and Nanog reporter expression indicated that germine cells become restricted in their developmental potential independent of and prior to their sexual differentiation. Accordingly, we asked whether the germine in 129S4 Dazl-deficient ovaries can give rise to spontaneous teratomas. At 2 mo of age, we found no ovarian teratomas in 131 control females (Fig. 3B). By contrast, among Dazl-deficient males, 20 of 65 (31%) displayed testicular teratomas. Clearly, teratomas form at a markedly elevated rate in 129S4 Dazl-deficient males early in postnatal life, if not before.

Do these tumors arise from pluripotent mitotic cells (such as PGCs), or from cells that have completed meiosis I and have undergone parthenogenetic activation of pluripotency? In humans, ovarian teratomas may arise from either mitotic or meiotic
Collectively, these studies demonstrate that Dazl is necessary for the commitment of germ cells, in the gonads, from their uncommitted precursors, independent of sex. Dazl-deficient gonadal teratomas do not reflect the parthenogenetic reactivation of pluripotency in mitotic cells, but instead arise from mitotic germline cells that have retained broad developmental potential.

Are other germ cell-defining factors also required for this commitment? Like Dazl, Ddx4 and Gcna are germ plasm constituents, first expressed after PGC colonization of the gonads in humans and mice (Fig. 1 and refs. 38 and 39). To determine whether Ddx4 or Gcna is similarly necessary for germ cell commitment, we crossed null alleles for Ddx4 [Ddx4-Cre (40)] and Gcna (38) to a 129S4 background and measured teratoma incidence. We found no gonadal teratomas in 129S4.Ddx4-deficient mice, and a single tumor in a 129S4.Gcna-deficient male (Ddx4: n = 25 males, 23 females; Gcna: n = 50 males; SI Appendix, Fig. S6 C and D), indicating that, while necessary for the completion of spermotogenesis (38, 41), both Ddx4 and Gcna are dispensable for germ cell commitment.

**Sex Reversal Shows That the Testis Is a Favorable Site for Teratoma Formation.** Despite the common developmental origin of teratomas in Dazl-deficient males and females, their incidence is higher in males (Fig. 3B). To test whether this male bias reflected an intrinsic difference in the developmental potency of XY germ cells, or the testicular environment to which XY PGCs migrate, we reversed the gonadal sex of both XX and XY animals. In mammals, gonadal sex is determined by expression of a Y-linked gene, Sry. An Sry transgene (TgSry) can induce male development in XX embryos (42), and disruption of Sry results in female development of XY embryos.

We generated sex-reversed Dazl-deficient mice and assessed teratoma incidence. Teratomas were observed much less frequently in Dazl-deficient 129S4 females (either XX or XY) than in Dazl-deficient XY males (Fig. 4A). Strikingly, we observed testicular teratomas in all XX male mice (XX TgSry, n = 34 animals, 33 with bilateral teratomas). Thus, the mouse testis is a more favorable environment than the ovary for teratomas to arise from either the XX or XY Dazl-deficient germline. We conclude that XX germline cells are as susceptible to teratoma formation as their XY counterparts, if not more so, given an equivalent gonadal environment. Our data, and the fact that in the absence of Dazl, both XX and XY germline cells can form spontaneous teratomas, overturning the view that a Y-linked gene is essential for teratoma formation in male mice (43).

**Ablating Bax-Mediated Cell Death Increases Teratoma Formation in Dazl-Deficient Male Mice.** Our findings led us to hypothesize that many (or most) gonadal germline cells either die or form spontaneous teratomas in the absence of Dazl function. Indeed, these two fates—cell death or teratoma—might represent alternative outcomes, in vivo, of germline cells whose developmental potential has not been restricted. If this were the case, then curtailing cell death pathways in Dazl-deficient mice should increase the incidence of spontaneous gonadal teratomas. To attenuate apoptotic cell death, we intercrossed mice carrying the Dazl null allele with mice carrying a Bax null allele (44, 45). Among Dazl-deficient:Bax-heterozygous males, we observed a dramatically increased incidence of teratomas (68%; including 23 with bilateral teratomas, from 50 mice; Fig. 4B and SI Appendix, Fig. S6E) compared with Dazl-deficient:Bax wild-type male littermates (42%; including one with bilateral teratomas, from 26 males). Strikingly, we observed bilateral teratomas in all 17 double-knockout males examined (compared with none in 51 Bax-deficient males, where at least one copy of Dazl remained intact). We conclude that, in the testes of 129S4.Dazl-deficient males, the failure to restrict the developmental potential of the germline usually leads to Bax-mediated cell death. By genetically

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 attenuating or eliminating Bax-mediated cell death, the Dazl-deficient germ line’s broad developmental potential—and, thereby, its capacity for teratoma formation—is revealed more fully in vivo. In females, however, we observed no significant effect of Bax deficiency on the incidence of ovarian teratomas in Dazl-deficient mice (Fig. 4B and SI Appendix, Fig. S6F). This finding parallels our observations in sex-reversed mice, demonstrating again that the sexual identity of the somatic gonad strongly influences the likelihood of teratoma formation (Fig. 4A).

**DAZL-Deficient Pigs Develop Spontaneous Teratomas.** Given that Dazl is necessary for germ cell commitment in mice, we assessed whether this holds true in other mammals. We tested this in pigs, an outgroup to primates and rodents (Fig. 5A). Using a transcription-activator-like effector nucleases (TALEN)-mediated gene editing strategy, we generated female pigs with targeted disruptions of DAZL (SI Appendix, Table S3). We examined the ovaries of 20 DAZL-deficient females that were at least 6 mo old: 13 had large ovarian tumors, and 3 of the 13 had bilateral tumors (Fig. 5B and C). Histological examination revealed that these tumors were teratomas, containing disorganized mixtures of tissues derived from all three germ layers (Fig. 5D), similar to our findings in Dazl-deficient mice. We did not observe evidence of ovarian teratomas in 151 female controls of similar age [nor did we observe testicular teratomas in any of three previously generated (46) DAZL-deficient male pigs analyzed at 11 wk or 9 mo of age].

To assay whether DAZL is necessary for survival of germ line cells in the testes of pigs, as it is in diverse strains of mice (SI Appendix, Fig. S5), we examined testes of DAZL-deficient pigs at 11 wk and at 9 mo of age. In all gonads analyzed, we found no evidence of germ cells in either ovaries or testes (Fig. 5D and E). Consistent with this, immunohistological analysis revealed that all cells within the seminiferous epithelium expressed the Sertoli cell factor SOX9 (Fig. 5F). These data indicate that DAZL is required for the survival of germ line cells in the swine testis.

Combined with our data in humans and mice, these observations in pigs demonstrate, across eutherian mammals, that DAZL is necessary to restrict the developmental potential of PGCs after their arrival at the gonads.

**Embryonic Dazl Expression Is Sufficient for Germ Line Survival and Oogenesis.** Finally, we assessed whether a brief period of Dazl expression is sufficient to initiate germ cell commitment, after which Dazl might be dispensable for gametogenesis in mice. We constructed a conditional Dazl allele on a B6 background (referred to as B6.Dazl-2L), and then generated a null allele (Dazl-1L) using a germ line-specific Cre recombinase (40) (Ddx4-Cre; SI Appendix, Fig. S7A and B). Next, we transiently ablated Dazl from E14.5 onward, using Ddx4-Cre (Fig. 6A). This ablation occurred after PGCs extinguished Nanog-GFP and their potential to give rise to EG cells (Fig. 2A and C), and after sexual differentiation had commenced in both sexes and meiosis had been initiated in females. In conditional knockout mice (B6.Dazl-1L/2L.Cre, referred to as Dazl cKO), we observed germ cells in ovaries and testes at all ages tested, through 8 mo of age (Fig 6B–D). In Dazl cKO ovaries, we confirmed DAZL’s absence in germ cells at birth (marked by the LSL-tdTomato reporter, recombined in germ cells by Ddx4-Cre; Fig. 6E). In Dazl cKO testes, we confirmed the loss of DAZL expression in spermatogonia (marked by the Oct4-EGFP transgene and the germ cell marker GCNA; some tubules retained DAZL expression and exhibited variable spermatogenesis). These data suggest that, once germ cell commitment has occurred, Dazl is no longer necessary for germ cell viability in mice on a B6 background (SI Appendix, SI Discussion).

To test whether Dazl is dispensable during the remainder of gametogenesis, we assessed the fertility of Dazl cKO mice. Dazl cKO females were fertile through at least 8 mo of age (n = 5 females, with the recombined allele transmitted to all progeny; Fig. 6F). In contrast, Dazl cKO males were sterile, with no spermatozoa observed in the epididymis of adults (n = 5 males; Fig. 6G). Thus, Dazl has additional functions in postnatal spermatogenesis, consistent with previous descriptions on a mixed genetic background (47–49).

We conclude that a brief period of Dazl expression, after PGC colonization of the gonads, is sufficient for germ line commitment and the initiation of gametogenesis in mice, as shown by the survival of germ cells in the gonads of both sexes of Dazl cKO mice, and by the completion of oogenesis and fertility in females.

**Discussion**

Our studies sought to answer a fundamental question: When, where, and how does the mammalian germine restrict its developmental potential and irreversibly commit to gametogenesis? One view has been that mammalian PGCs are unipotent germ cells—only capable of giving rise to gametes (50–54). This view is challenged by several lines of evidence that reveal the broad developmental potential of migratory PGCs, including the expression of a network of pluripotency factors, the ability to

**Fig. 4.** Teratoma formation in Dazl-deficient mice is affected by sex reversal, and by ablation of Bax-mediated cell death. (A) Incidence of gonadal teratomas in sex-reversed mice. Mutation of Sry (Sry<sup>mmi</sup>) causes XY embryos to develop as anatomic females. Expression of Sry transgene (TgSry) causes XX embryos to develop as anatomic males. (B) Incidence of testicular teratomas (left) and ovarian teratomas (right) in Dazl-deficient mice (+/−) who were either homozygous wild-type (+/+), heterozygous (+/−), or deficient (−/−) for Bax; n = number of animals examined. **P value < 0.01, ***<0.0001, ns = not significant using Fisher’s exact test.
produce pluripotent cell lines in culture, the occurrence of spontaneous gonadal teratomas, and, most recently, evidence that presumptive PGCs may contribute to the allantois (12, 13). Comparable observations in fish (6), frogs (5), and salamanders (7) corroborate the view that, among vertebrates, migratory-stage PGCs are not yet irreversibly committed, but instead retain the capacity for somatic differentiation, regardless of whether germline segregation occurs via induction, or by germl plasm.

Our present studies in mammals provide definitive genetic evidence that the germ line’s broad developmental potential is restricted after PGC colonization of the gonads, and that Dazl is necessary for this to occur. We find that, in mouse embryos lacking Dazl function, PGCs migrate to the gonads but maintain expression of a network of pluripotency factors, and retain the ability to give rise to pluripotent cell lines until at least E15.5 in both sexes (Fig. 2 and SI Appendix, Fig. S4). The incidence was even higher in certain circumstances. For example, 33 of 34 Dazl-deficient, sex-reversed (XX) males developed bilateral gonadal teratomas, as did 17 Dazl-deficient:Bac-deficient XY males (Fig. 4). Strikingly, we also discovered ovarian teratomas in 35 of 300 Dazl-deficient females, compared to none in 426 control mice (SI Appendix, Table S4). The incidence was even higher in certain circumstances. For example, 33 of 34 Dazl-deficient, sex-reversed (XX) males developed bilateral gonadal teratomas, as did 17 Dazl-deficient:Bac-deficient XY males (Fig. 4). Strikingly, we also discovered ovarian teratomas in 35 of 300 Dazl-deficient females, compared to none in 426 control mice, demonstrating that Dazl is necessary for germ cell commitment in both sexes.

Taken together with published reports, our findings suggest a sequence of commitment steps during mammalian germline development. In developmental biology, a cell is “specified” when it will develop autonomously after isolation from the embryo; specified cells are not yet irreversibly committed, and may adopt other fates if transplanted to a new position (55). For example, the fate of specified trophoderm or of the inner cell mass may be altered upon relocation within the preimplantation embryo (56). Similarly, mammalian PGCs appear to be specified shortly after their induction (1, 2), without being irreversibly committed to gametogenesis.

In contrast, a cell is “determined” (fully committed) when its fate cannot be reversed by grafting (55). By these definitions, cells are determined when their potential is restricted, regardless of environment. It is informative here to revisit the work of Leroy Stevens, who discovered that PGCs can give rise to teratomas in mice (35, 57). [Likewise, migratory-stage PGCs from amphibians can give rise to somatic cell lineages if transplanted (5) or in cell culture (7).] Stevens demonstrated, by grafting PGCs, that they lose the capacity to form teratomas after colonizing the gonads (8, 58). Combining Stevens’ observations with our own, we conclude that germ cells are determined in mice after PGCs colonize the gonads, and that the induction of Dazl is necessary for this commitment (Fig. 7). Utilizing a conditional allele, we show that a brief period of Dazl expression is sufficient for this commitment to gametogenesis.

This model is further corroborated by our findings in DAZL-deficient pigs, where the germline similarly retains the capacity for teratoma formation after gonadal colonization. These findings—in two species whose most recent common ancestor lived about 95 million years ago—strongly suggest that DAZL-dependent commitment, occurring after gonadal colonization, operated in the last common ancestor of all eutherian mammals. Moreover, in fish (22, 23), frogs (24, 25), and birds (26), orthologs of DAZL are essential constituents of germ plasm, which functions in segregating germline from soma. Thus, DAZL is a key factor in germline commitment, regardless of whether germline segregation occurs by induction or germl plasm.

**Distinguishing Gonadal Germ Cells from Their Migratory Germline Precursors.** If the germline’s broad developmental potential is extinguished only after PGC colonization of the gonads, then the roles of key regulators expressed in PGCs prior to gonadal entry must also be considered. Here we will incorporate findings from both mammals and nonmammalian vertebrates to suggest that these critical regulators of migratory PGCs repress the cells’ capacity for somatic differentiation, insulating them from inductive cues.

Consider Nanos and Dnd1, which are expressed in migratory PGCs of many vertebrates and encode repressors of cellular differentiation. When dnd1 is knocked down in fish, PGCs adopt development. In developmental biology, a cell is “specified” when it will develop autonomously after isolation from the embryo; specified cells are not yet irreversibly committed, and may adopt other fates if transplanted to a new position (55). For example, the fate of specified trophoderm or of the inner cell mass may be altered upon relocation within the preimplantation embryo (56). Similarly, mammalian PGCs appear to be specified shortly after their induction (1, 2), without being irreversibly committed to gametogenesis.

In contrast, a cell is “determined” (fully committed) when its fate cannot be reversed by grafting (55). By these definitions, cells are determined when their potential is restricted, regardless of environment. It is informative here to revisit the work of Leroy Stevens, who discovered that PGCs can give rise to teratomas in mice (35, 57). [Likewise, migratory-stage PGCs from amphibians can give rise to somatic cell lineages if transplanted (5) or in cell culture (7).] Stevens demonstrated, by grafting PGCs, that they lose the capacity to form teratomas after colonizing the gonads (8, 58). Combining Stevens’ observations with our own, we conclude that germ cells are determined in mice after PGCs colonize the gonads, and that the induction of Dazl is necessary for this commitment (Fig. 7). Utilizing a conditional allele, we show that a brief period of Dazl expression is sufficient for this commitment to gametogenesis.

This model is further corroborated by our findings in DAZL-deficient pigs, where the germline similarly retains the capacity for teratoma formation after gonadal colonization. These findings—in two species whose most recent common ancestor lived about 95 million years ago—strongly suggest that DAZL-dependent commitment, occurring after gonadal colonization, operated in the last common ancestor of all eutherian mammals. Moreover, in fish (22, 23), frogs (24, 25), and birds (26), orthologs of DAZL are essential constituents of germ plasm, which functions in segregating germline from soma. Thus, DAZL is a key factor in germline commitment, regardless of whether germline segregation occurs by induction or germl plasm.

**Distinguishing Gonadal Germ Cells from Their Migratory Germline Precursors.** If the germline’s broad developmental potential is extinguished only after PGC colonization of the gonads, then the roles of key regulators expressed in PGCs prior to gonadal entry must also be considered. Here we will incorporate findings from both mammals and nonmammalian vertebrates to suggest that these critical regulators of migratory PGCs repress the cells’ capacity for somatic differentiation, insulating them from inductive cues.

Consider Nanos and Dnd1, which are expressed in migratory PGCs of many vertebrates and encode repressors of cellular differentiation. When dnd1 is knocked down in fish, PGCs adopt...
A brief period of Dazl expression is sufficient for germ cell commitment, and for completion of oogenesis. (A) Time course of Ddx4-Cre (MtvCre^{mOrange}) recombinase activity in embryonic germline using a fluorescent Cre reporter mouse line, LSL-tdTomato. Cre-mediated recombination (resulting in tdTomato expression) in Oct4:EGFP-positive cells is assayed by flow cytometry. Numbers of embryos tested are listed in each column, mean ± SD. (B) Breeding scheme for Dazl conditional knockout (Dazl cKO) mice. (C) Histology of control (Upper) and B6.Dazl cKO ovary (Lower) stained with PAS at 20 d of age, with primary (arrow head) and secondary (chevron) follicles marked. (D) Immunofluorescence of control (Upper) and B6.Dazl cKO tests.

Somatic cell fates have been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this
Implications for the Pathogenesis of Germ Cell Tumors. Our insights into germ cell commitment in the embryo have ramifications for our understanding of germ cell tumors (GCTs), the most common cancer in young men (83). Specifically, our present studies in embryos converge in striking fashion with recent epidemiological and genome-wide association (GWA) studies of GCTs. For example, GWA studies implicate pluripotency factors (e.g., PRDM14, SALL4, TFCP2L1, and ZFP42), as well as genomic sites of binding for transcription factors of pluripotency (e.g., KLF4, NANOG, POU5F1, and SOX2), in the pathogenesis of testis cancer (84, 85). Most importantly, DAZL has been identified as a susceptibility locus, implicating DAZL as a key factor in both the pathogenesis of GCTs in humans (86) and germ cell commitment. Consistent with a critical role for apoptosis, GWAS has implicated BAK1 in the heritability of GCTs (87), akin to our observations in Bax-deficient mice. Along similar lines, the receptor:ligand pair KIT and KIT ligand (KITLG) function in several cellular contexts to protect cells from apoptosis (88). Like BAK1, polymorphism at KITLG is implicated in the heritability of human GCTs, and teratoma incidence in mice (87, 89, 90). Human GWA studies also implicate GATA4 (84), which is required in mice for gonad development and induction of Dazl (81). Reinforcing the view that somatic gonadal development is central to germ cell determination, young children with disorders of gonadal development are at markedly increased risk of germ-line neoplasia (91). These many connections lead us to suggest that germine neoplasms arise from embryonic cells that, having failed to complete germ cell determination on their arrival at the gonad, remain uncommitted and susceptible to tumor formation. Accordingly, our revised understanding of germ cell commitment will help clarify the developmental origin of GCTs, and inform efforts to account for their dramatically increased incidence in recent decades (92).

In conclusion, we demonstrate that germ cell determination in mammals occurs late in embryonic development—after the body plan has been established, and organogenesis begun—through an ancient germ cell program induced as PGCs colonize the nascent gonads (Fig. 7). This model has deep implications for the genesis of germine neoplasms in humans, and for the stepwise commitment and determination of germ cells in mammals and across the vertebrates.

Materials and Methods

Further details can be found in SI Appendix, SI Materials and Methods.

Animals. All experiments involving mice or pigs conformed to principles and guidelines approved by the Committee on Animal Care at the Massachusetts Institute of Technology, Cincinnati Children’s Hospital Medical Center, or by International Center for Biotechnology, respectively. Further details can be found in SI Appendix, SI Materials and Methods.

Cell Isolation. Embryos carrying fluorescent reporter alleles were dissected, and gonadal cells were subjected to flow cytometry, followed by pluripotent cell line derivation, or to RNA isolation for transcriptional analysis, as described in SI Appendix, SI Materials and Methods.

Histology. Gonads were removed and fixed in 4% paraformaldehyde, or Bouin’s solution, embedded in paraffin, sectioned, and stained for immunohistology, or stained with hematoxylin and periodic acid-Schiff. Teratoma formation was confirmed by the presence of cells from each somatic germ layer. Full details are available in SI Appendix, SI Materials and Methods.
Transcriptional Analyses. Sequence data were aligned to the appropriate reference genome, and differential expression was calculated as outlined in SI Appendix, SI Materials and Methods.

Data Availability. Data generated by array and RNA-seq have been deposited at Gene Expression Omnibus (accession no. GSE87771) and Sequence Read Archive (accession no. PRJNA434733), respectively.

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