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Commentary

Extragonadal primordial germ cells or placental progenitor cells?

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A B S T R A C T

Primordial germ cells (PGCs), the precursors of the gametes, are now claimed to segregate within the extra-embryonic tissues of three species of placental mammals. In this brief Commentary, I raise the question of whether the so-called PGCs are not PGCs at all, but rather, progenitor cells that build the fetal–placental interface in Placentalia.

Introduction

Biologists have long been preoccupied with the origin of the mammalian germ line. At present the general consensus, based on studies in the mouse model, is that its antecedents arise within the pluripotent epiblast, move to the extra-embryonic region where they segregate from the soma, and from there, primordial germ cells (PGCs) re-enter the embryo to migrate to the gonads via the hindgut. This conclusion is based on over a century of description, beginning with morphology (Simkins, 1923), and followed by biochemical activity for alkaline phosphatase (AlkP) (Chiquoine, 1954; Witschi, 1948). Then, once molecular methods became available for sorting small cell populations, AlkP-positive cells were probed for genes 'uniquely' expressed at the embryonic–extra-embryonic interface (Ohinata et al., 2005; Saitou et al., 2002). At present, gene expression is the *status quo* by which PGCs are identified and studied in the posterior region of Placentalia.

Despite current efforts directed toward establishing higher orders of gene expression within AlkP-positive cells (e.g. Sasaki et al., 2016), germ cell biologists have overlooked a fundamental principle of developmental biology: gene expression and cell lineage do not form an obligate ancestral relationship (Beddington, 1988). In the case of the germ line, not a single study has demonstrated that extra-embryonically 'segregating' PGCs end up in the gonads (reviewed in Mikedis and Downs, 2014). A similar lament was published nearly a century ago when PGCs were claimed to be recognizable by morphology (Simkins, 1923):

> "Many investigators have considered the problem solved in favour of a morphological continuity of germ cells when certain cells, designated as primordial germ cells, have been followed from their place of origin over long distances of intervening structures to the site of the germ-gland fundament. Some workers have been so sure that these cells were the true fore-runners of the reproductive cells that they have not followed the history of them through the critical period of gonogenesis, although such study is essential for determining whether or not these cells actually become transformed into definitive ova and spermatozoa; it was assumed that they were, because of certain resemblances which they bore to the early oogonia and spermatogonia."

In this brief Commentary, I point out that fate mapping presumptive PGCs from the extra-embryonic region of the mammalian conceptus to the gonads remains elusive. Further, based on new insights into the properties of the posterior embryonic–extra-embryonic region, where PGCs are thought to segregate from the soma, I ask whether the so-called extra-embryonic antecedents to the germ line are not PGCs at all, but rather progenitor cells that build the fetal–placental interface.

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https://doi.org/10.1016/j.rbmo.2017.09.013

I conclude with current – and equally controversial – thoughts about where mammalian germ line progenitors might originate.

Origin of the germ line

Pluripotency is expected to be a major feature of PGCs which, after maturation into the gametes and their subsequent union, recapitulate the entire organism. During cleavage stages, all blastomeres appear capable of contributing to the germ line (Tarkowski et al., 2010). Upon formation of the blastocyst, two major cell types appear: the inner cell mass (ICM) and the trophoblast ('trophectoderm' in mice). Trophectoderm does not contribute to tissues other than the placentae (Gardner et al., 1973; Papioannou, 1982; Tanaka et al., 1998), while individual cells of the ICM are pluripotent (Gardner and Lyon, 1971). At implantation, the ICM differentiates into the epiblast and primitive endoderm. Pluripotent epiblast cells contribute to soma, both embryonic and extra-embryonic, and to the germ line (Gardner et al., 1985). By contrast, primitive endoderm has not historically demonstrated such widespread potential (Gardner, 1972; Gardner and Rossant, 1979; Kunath et al., 2005). Thus, the germ line resides somewhere within the epiblast.

During gastrulation, the embryo becomes apparent as a result of formation of the primitive streak, or antero-posterior body axis (Corner, 1944); the embryo's associated extra-embryonic tissues, amnion, allantois, yolk sac and chorion, coalesce into two major placentae, the chorio-allantoic placenta and the chorio-vitelline (yolk sac) placenta, which collaborate to create a patterned and efficient vascular conduit from the fetus to its mother for exchange of nutrients, wastes and gases within the womb.

Epiblast-derived cells enter the posterior extra-embryonic space and there, segregate from the soma: yolk sac visceral endoderm in humans (Witschi, 1948), the base of the allantois in the mouse (Lawson and Hage, 1994; Ozdzenski, 1967), and the amnion in a non-human primate (Sasaki et al., 2016). Is there a pattern? Yes, it would seem so: major tissue components of the placentae segregate PGCs.

Why would the mammalian germ line come into being at such great distances from the eventual site of gonad formation? The most cited rationale is that pressures of determination and differentiation in the embryo during gastrulation threaten PGC potency that must be preserved (McLaren, 1992). As a result, germ cell antecedents take refuge in extra-embryonic tissues, segregating there and then re-entering the embryo when the coast is clear. However, not only is a formal demonstration of continuity between putative PGCs and the gonads lacking, but this proposition is wholly embryo-centric: it fails to acknowledge that extra-embryonic tissues have properties, too. For example, by contrast with the epiblast, extra-embryonic tissues can sustain higher orders of ploidy (Tarkowski et al., 1977). Thus, the extra-embryonic environment is more tolerant than the embryonic one in accommodating cells that accumulate changes in chromosomal number. On that basis, an extra-embryonic site would hardly seem the place to invite formation of the future germ line, whose chromosomal integrity must be upheld.

As far as concerns McLaren's claim that gastrulation is confined to the embryo, this long-held belief has recently been challenged. Gastrulation is defined by the activity of the primitive streak (Snell and Stevens, 1966). Contrary to prevailing notions (Downs, 2009), the mouse primitive streak is not limited to the embryo proper (Sobotta, 1911) but rather, extends into the allantois, where its posterior terminus expands into a dense cellular core, defined by Brachyury, and is provisionally named the 'allantoic core domain' (ACD) (Downs et al., 2009). Fate mapping showed that this extra-embryonic ACD, like the embryonic primitive streak (Tam and Beddington, 1987), is a pluripotent progenitor cell pool that contributes to derivatives of all three primary germ layers at the fetal–placental interface, both embryonic and extra-embryonic (Mikedis and Downs, 2012). Thus, gastrulation encompasses the extra-embryonic region, too.

The discovery of the ACD within the base of the allantois represented a paradigm shift in understanding the biology of Mammalia, because a primitive streak that extends into the allantois possesses not only progenitor cells that build this vital organ, but also requisite spatial coordinates to organize the vascular connection between the fetus and its placenta. Indeed, via Brachyury, the primitive streak regulates placement of a unique but hitherto undocumented blood vessel, the 'vessel of confluence' (Daane et al., 2011; Downs et al., 1998; Inman and Downs, 2006), within the allantois (Rodriguez et al., 2017). As a result, the axially positioned vessel of confluence becomes a fixed branchpoint that, through patterning involving the fibroblast growth factor family, unites the umbilical, omphalomesenteric and fetal cardiovascular systems (Rodriguez et al., 2017). Mis-patterning the fetal–placental connection leads to severe birth defects (Rodriguez et al., 2017; Schreiner and Hoornbeek, 1973). The vessel of confluence and dense allantoic core are conserved across all Placentalia thus far examined, including humans (Rodriguez et al., 2017); conservation underscores the collective importance of these newly unearthed features in the fetal–placental connection.

Such fresh insights into the biology of the posterior embryonic–extra-embryonic region highlight the principle that, in Placentalia, the fetus does not make contact with its mother via its own devices. Rather, embryonic and extra-embryonic regions are unified through a common axial midline that organizes this relationship, and whose progenitor cells contribute to the fetal–placental interface.

Gene expression, potency and placental tissues

Not only does the allantois possess a source of progenitor cells used to build the placentae but so, too, do the amnion (Miki and Strom, 2006), chorion (Uy et al., 2002) and allantois-associated yolk sac (Rodriguez and Downs, 2017). All of these extra-embryonic tissues exhibit AlkP activity (Hahnel et al., 1990; MacGregor et al., 1995) which is found in most, if not all, stem cells (Benham et al., 1983; Bernstine et al., 1973). Despite its preponderance in the extra-embryonic region and throughout the epiblast, to the present day, AlkP activity is claimed to selectively identify PGCs within the base of the allantois, associated visceral endoderm and the ventral component of the hindgut (reviewed in Mikedis and Downs, 2014). In addition to AlkP, other factors involved in the biology of stem cells have been found in extra-embryonic tissues. For example, *c-myc* is present in the derivatives of trophectoderm and in the base of the allantois (Downs et al., 1989) while OCT-3/4 has been identified in the ACD and adjacent yolk sac (Downs, 2008; Scholer et al., 1990).

Transcriptome analysis of posterior AlkP-positive cells led to the identification of BLIMP1 (PRDM1) and STELLA (DPPA3, PGC7) (Ohinata et al., 2005; Saitou et al., 2002), whose initial localization heralded these proteins as unique to the germ line antecedents (Ohinata et al., 2005; Saitou et al., 2002). Thus, on the basis of localization that was limited to a few stages and a few tissues in whole mount analyses,

BLIMP1 and STELLA became the gold standard, in common use, for identifying PGCs. However, more thorough immunohistochemical analysis in histological sections at numerous developmental timepoints separated by 2–4 hour intervals from just before the time of appearance of the allantois through hindgut formation contradicts claims of restriction, showing rather that BLIMP1 and STELLA localize, both alone and together, to tissues that encompass the entire posterior embryonic–extra-embryonic interface (Mikedis and Downs, 2012, 2017). Such widespread localization precludes calling any single cell a PGC; further, localization is consistent with the presence of placental progenitor cells that build the fetal–placental connection.

Within the PGC trajectory, at least five STELLA sub-populations have now been documented (Wolfe et al., 2017). These include co-localization with RUNX1, which is associated with hemangioblasts (North et al., 1999, 2002), and with FOXa2, a protein generally associated with endoderm (Kubo et al., 2004). Cells bearing 'markers' diagnostic of PGCs have been proposed as progenitors of hematopoietic cells (Scaldaferri et al., 2015), and STELLA facilitates differentiation into endoderm in human cells (Wongtrakoongate et al., 2013). Thus, STELLA is not found in a single cell type, but rather collaborates in distinct mesendodermal cell sub-populations at the fetal–placental interface in the mouse gastrula. Further, fate mapping showed that the STELLA-positive distal component of the ACD contributes only to the placenta and not to the fetus (Mikedis and Downs, 2012), making it more unlikely that STELLA is a unique marker of allantoic PGCs. Thus, while STELLA and BLIMP1 may be key players of a generalized pluripotent state (Nakamura et al., 2007; Payer et al., 2003; Surani et al., 2007), they cannot identify a segregated PGC population at the embryonic–extra-embryonic interface.

PGCs and the hindgut

From their site of segregation in extra-embryonic tissues, the PGCs translocate into the hindgut (Chiquoine, 1954; Clarke and Eddy, 1975; Lawson and Hage, 1994). Given the lack of fate mapping, this claim may be based on observations that AlkP activity is attenuated in the base of the allantois at the same time that it goes up in the hindgut (Chiquoine, 1954). However, as shown by results of more recent studies, this period of AlkP down/up-regulation coincides with differentiation of the allantois into the nascent umbilical cord and the appearance of the hindgut. Specifically, as the allantois fuses with the chorion (Downs and Gardner, 1995), its mitotic index decreases (Downs and Bertler, 2000), and prospective allantoic angioblasts differentiate into patent blood vessels (Downs et al., 1998). The ACD down-regulates Brachyury, retracting toward the presumptive hindgut (Downs et al., 2009).

Fate mapping the ACD revealed that some of its descendant cells colonized the hindgut (Mikedis and Downs, 2012) where both STELLA and BLIMP1 have been identified (Mikedis and Downs, 2012, 2017). While there appears to be developmental continuity between the allantois and hindgut, are any of the ACD-derived hindgut cells really PGCs? Perhaps they are progenitor populations for building the gut. Without being able to trace hindgut 'PGCs' to the gonads, there is no proof for or against. To add further ambiguity to this question, the gut is composed, in part, of extra-embryonic visceral endoderm (Kwon et al., 2008) which also exhibits STELLA, BLIMP1 and OCT-3/4 (Downs, 2008; Mikedis and Downs, 2012, 2017). Visceral endoderm might be a progenitor population that contributes to the dazzling array of gut cell types, or to the enteric nervous system, whose origin is almost wholly obscure (Mikedis and Downs, 2017). Gene expression cannot tell us this. Only fate mapping can.

Induction of PGCs *in vitro*

As a counterpoint to the thesis presented here, mouse cells bearing the BLIMP1 and STELLA molecular signature have been induced *in vitro* to become eggs (Hayashi et al., 2012) and/or sperm (Hayashi et al., 2011). However, even peripheral blood can be transformed into the germ line of mice (Kamimura et al., 2013). As differentiation is operationally defined (Gardner, 1993), investigators should have introduced induced PGCs into the base of the mouse allantois, and tracked them as far as the limitations of whole embryo culture would allow. If, according to conventional wisdom, they were functionally segregated PGCs, they should have been restricted to the early PGC trajectory, i.e. the base of the allantois, visceral endoderm and hindgut over time. Unfortunately, this experiment, which had the potential to provide support for an extragonadal origin of PGCs at the posterior interface, while feasible, was not performed. Thus, whether 'induced' PGCs are really functionally segregated germ line antecedents remains to be seen.

The germinal epithelium as a source of PGCs

In both vertebrate amniotes and anamniotes, male and female gonads differentiate from indifferent genital ridges during embryogenesis (Grier et al., 2016). As discussed in this Commentary, it is believed that the PGCs migrate toward the genital ridges through the hindgut after which they enter its dorsal mesentery. Then, together with the coelom-derived somatic epithelial cells, PGCs form a 'germinal epithelium' at the nascent gonadal surface. Depending upon hormonal activity dictated by the sex chromosomes, the PGCs will become the spermatogonia of the testis and the oogonia of the ovary, while the somatic epithelial cells will become the supporting Sertoli cells in the testes and the follicular cells in the ovaries. Although the germinal epithelium produces the supporting somatic cells in both sexes, and males produce spermatogonia throughout their lifetime ('lifetime indeterminate fecundity'), a long-standing but controversial view is that the ovarian germinal epithelium does not produce new oogonia throughout the female's lifetime ('lifetime determinate fecundity') (reviewed in Tilly et al., 2009). This view goes back to the latter part of the 19th century (Waldeyer, 1870). Despite an array of subsequent challenges, beginning in 1923 and continuing to the present (Adsell, 1964; Allen, 1923; Allen and Creadick, 1937; Bukovsky et al., 2004, 2005; Butcher, 1927; Duke, 1941, 1944; Hargitt, 1930; Johnson et al., 2004; Latta and Pederson, 1944; Nikura et al., 2009; Pliske, 1938; Risley, 1934; White et al., 2012), Waldeyer's view has predominated. However, in recent years, the possibility that both male and female germ cells arise from a common origin in the germinal epithelia has finally gained traction (Grier et al., 2016; Tilly et al., 2009). Moreover, across the vertebrate kingdom, the germinal epithelium has maintained a constant form and function throughout 500 million years of vertebrate evolution (Grier et al., 2016). By contrast, the South American plains vizcacha, *Lagostomus maximus*, a rodent and close relative of the guinea pig, shows no signs of the 'classical' molecular PGC network

in the posterior region (Leopardo and Vitullo, 2017); likewise, the rabbit and the pig show no similarity to the mouse (Hassan and Viebahn, 2017). Given broad conservation of the germinal epithelium across vertebrate species, formation of germ cells at the actual site of gonad formation makes more biological – indeed parsimonious – sense than the status quo of extra-embryonic segregation from the soma based on indiscriminate morphology and gene expression and a long spatiotemporal voyage to the gonads.

Conclusions

An extra-embryonic origin of the germ line ignores the obvious and far simpler possibility that PGCs are not segregated germ cells, but rather, extra-embryonic progenitor cells that build the fetal–placental interface. To exhort an old phrase: if it smells like an extra-embryonic placental progenitor cell, then perhaps it is a placental progenitor cell. The possibility that 'PGCs' are placental progenitor cells is entirely consistent with current data. Moreover, loss of so-called 'PGC markers', including AlkP (MacGregor et al., 1995), does not cause infertility, suggesting either that 'PGC' proteins carry out redundant roles in germ cell antecedents or they are not truly required for PGC formation (Mikedis and Downs, 2014). While BLIMP1 has been claimed to reduce the population of PGCs (Ohinata et al., 2005; Vincent et al., 2005), the readout for this conclusion was AlkP activity.

Evoking PGCs time and again as a specified and segregated population bearing a particular molecular signature goes against fundamental principles of embryology, and further ignores the properties of extra-embryonic tissues. Over time, good science should be self-correcting: in the presence of new data, even cherished beliefs deserve to be questioned. As long as scientists, editors and peer reviewers accept that gene expression is equivalent to cell lineage, and fail to acknowledge new evidence concerning the nature of the fetal–placental interface, progress in germ cell biology and indeed, mammalian development as a whole, will continue, at best, to be painfully slow, and at worst, misleading and wasteful of valuable funds.

Given the lack of evidence for germ line continuity between extra-embryonic tissues and the gonads, the field is ripe for new theories or, as described in the previous section, serious reconsideration of old ones. In addition, an earlier challenge that the mammalian germ line is set aside during pre-implantation development (Soriano and Jaenisch, 1986) might be revisited. While this conclusion was discounted in its day on the grounds that the extra-embryonic tissues had not been examined (Gardner and Beddington, 1988) (although based on arguments in this Commentary, the objection may be moot), as presented here, there is no *bona fide* support for segregation of PGCs within extra-embryonic tissues. With increasing evidence that antero-posterior patterning occurs in advance of streak formation (Gardner, 2010), a similarly heretical theory, the possibility that the mammalian germ line segregates during pre-implantation stages, becomes less remote.

Acknowledgements

K.M.D. is funded by the National Institutes of Child Health and Development (RO1 HD079481); she has no competing financial interests. K.M.D. gratefully acknowledges Dr Adriana Rodriguez, Dr Adam Wolfe and Professor Richard Gardner for comments on an earlier version of this manuscript.

ARTICLE INFO

Article history:
Received 20 December 2016
Received in revised form 22 September 2017
Accepted 26 September 2017
Declaration: The authors report no financial or commercial conflicts of interest.

Keywords:
Allantois
Amnion
Placenta
Primitive streak
Primordial germ cells
Yolk sac

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