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Perspective

On the fate of primordial germ cells injected into early mouse embryos

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ABSTRACT

Primordial germ cells (PGCs) are the founder cells of the germline. Via gametogenesis and fertilisation this lineage generates a new embryo in the next generation. PGCs are also the cell of origin of multilineage teratocarcinomas. In vitro, mouse PGCs can give rise to embryonic germ (EG) cells – pluripotent stem cells that can contribute to primary chimaeras when introduced into pre-implantation embryos. Thus, PGCs can give rise to pluripotent cells in the course of the developmental cycle, during teratocarcinogenesis and by in vitro culture. However, there is no evidence that PGCs can differentiate directly into somatic cell types. Furthermore, it is generally assumed that PGCs do not contribute to chimaeras following injection into the early mouse embryo. However, these data have never been formally published. Here, we present the primary data from the original PGC-injection experiments performed 40 years ago, alongside results from more recent studies in three separate laboratories. These results have informed and influenced current models of the relationship between pluripotency and the germline cycle. Current technologies allow further experiments to confirm and expand upon these findings and allow definitive conclusions as to the developmental potency of PGCs.

Introduction

In mammals, cells of both the early embryo and the germline may be considered to maintain full developmental potency and can be incorporated into an exclusive cycle that is exited upon somatic differentiation (Leitch and Smith, 2013; Leitch et al., 2013c; Monk, 1990, 1981; Pesce et al., 1998). Although competence for pluripotency is maintained throughout, cells at different stages of the germline cycle have different functional properties. In the mouse embryo, cells of the inner cell mass, specifically the pre-implantation epiblast, can contribute to chimaeras including the germline, following blastocyst injection (Gardner, 1985, 1968; Gardner and Rossant, 1979). Furthermore, embryonic stem (ES) cell lines derived from the pre-implantation epiblast maintain this capacity following in vitro culture (Bradley et al., 1984). Such proven capacity to reintegrate into the embryo and contribute functionally into development of all somatic lineages and the germline are attributes associated with the term naïve pluripotency (Nichols and Smith, 2009). Strikingly, naïve pluripotent stem cell lines, called embryonic germ (EG) cells, can also be derived from primordial germ cells (PGCs) at later stages of embryogenesis (Matsui et al., 1992; Resnick et al., 1992). Indeed, recent studies have demonstrated that early PGCs can give rise to pluripotent stem cell lines with an efficiency comparable to that of the pre-implantation epiblast (Leitch et al., 2013b; Nichols et al., 2009; Rugg-Gunn et al., 2012). Once established, EG cells may be indistinguishable from ES cells functionally and at the molecular level (Leitch et al., 2010, 2013a; Sharova et al., 2007). Thus, EG cells can only be definitively distinguished from ES cells by their origin. Although PGCs can give rise to EG cells in vitro and are the cell of origin of multilineage testicular teratocarcinomas (Stevens, 1967), during normal development they appear to give rise exclusively to the gametes. The presumption in the field has been that PGCs do

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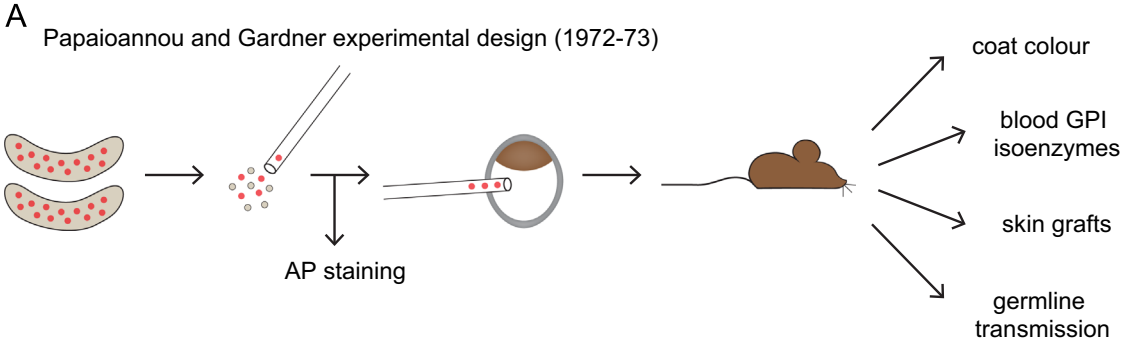
not contribute to primary chimaeras, consistent with not being in an equivalent pluripotent state to the cells of the pre-implantation epiblast. However, the primary blastocyst injection data, although informally communicated to the scientific community (Leitch and Smith, 2013; Matsui, 1998; Rossant, 1993), has never been published. Here we present the original experimental data obtained by two of us (V.E.P. and R.L.G.) alongside unpublished findings from subsequent studies by Colin Stewart (C.L.S) and Matsui (D.O. and Y. M.) and McLaren (G.D.) laboratories. We discuss the impact of these experiments on current perception of the mammalian germline and pluripotency, and highlight unanswered questions.

Results – PGC blastocyst injections across four decades

The technique for producing mouse chimaeras by blastocyst injection rather than via morula aggregation was devised more than four decades ago (Gardner, 1998, 1968). This advance enabled the Gardner laboratory to test rigorously the ability of different cell populations to reintegrate into mouse embryogenesis. These studies demonstrated that cells of the pre-implantation epiblast but not the hypoblast (primitive endoderm) could contribute extensively to chimaeras and produce functional gametes (Gardner, 1985; Gardner and Rossant, 1979). They also found that the capacity for functional incorporation into the blastocyst is lost after implantation (Rossant et al., 1978). Remarkably, tumour-derived teratocarcinoma cells and embryonal carcinoma (EC) cell lines could also contribute to blastocyst injection chimaeras, albeit they frequently induced embryonic lethality or produced tumours in the offspring, and did not give rise to germline transmission (Papaioannou et al., 1978, 1975). Thus, pluripotency appears to be compromised during the cellular transformation associated with teratocarcinogenesis. As PGCs are the cell of origin of testicular teratocarcinoma (Stevens, 1967) there was an obvious rationale to test PGCs in the blastocyst injection assay. However, in 1972, without modern molecular labelling techniques, this was not a facile undertaking (Fig. 1A). To isolate PGCs, the genital ridges of male embryos at either E10.5 or E11.5 were dissected, enzymatically digested and PGCs manually picked based on morphology. The purity of the separated PGCs was assessed by alkaline phosphatase staining of a small aliquot, and was found to be between 40% and 100% (median 72%) across five different experiments. Two or more PGCs were then injected into the blastocoel cavity and the injected blastocysts transferred to pseudopregnant hosts. The pregnant females were left to term resulting in 44 live births (20 females and 24 males) in total, 15 of which were derived from blastocysts injected with E10.5 PGCs and the remainder from injection of E11.5 PGCs (Fig. 1B). The donor PGCs were isolated from *extreme non-agouti* (a^e/a^e) embryos of the AG/Cam strain or F1 embryos from AG/Cam x CBA mice and injected into random bred albino CFLP host blastocysts (Anglia Laboratory Animals) allowing assessment for coat colour chimaerism. No coat colour chimaeras were obtained. Chimaerism was further assessed by measuring the GPI-1 isozyme type of blood samples but this indicated that the blood was also of host embryo origin in all 44 cases. Furthermore, 13 of the animals were subjected to skin grafts from mice of the donor PGC genotype – if chimaerism was present then these would have been recognised as 'self'; however no grafts survived. Finally, the potential chimaeras were test-bred and the 36 fertile animals (20 male, 16 female) produced 2695 pups, all of which were albino. Thus, despite these exhaustive analyses no evidence could be found that the injected PGCs contributed to the somatic tissues or colonise the germline of the adult mice (Fig. 1B).

A subsequent attempt to introduce PGCs to early embryos used a different method, aggregation with 8-cell embryos (Nagy et al., 1990; Stewart, 1980). These experiments were reported in the doctoral thesis of C.L.S (referenced in Stewart et al., 1994), although the experimental details were not formally published.

A



B

	PGC stage	Embryonic stage injected	Number of embryos transferred	Stage asssessed for chimaerism	Number recovered	Chimaeras
Gardner	E10.5/E11.5	blastocyst	?	adult	44 (15/29)*	0
McLaren	E11.5	blastocyst	39	E8.5/E9.5	38 (29/9)†	0
Matsui	E7.5	8-cell	22	E6.25	4	0

*injected PGCs isolated at (E10.5/E11.5)

†host embryos assessed at (E8.5/E9.5)

Fig. 1. Blastocyst injection of PGCs across the decades. (A) Schematic representation of the experimental design for the original PGC blastocyst injection experiments by Papaioannou and Gardner. AP=alkaline phosphatase. (B) Comparison of PGC injection experiments. No evidence of chimaerism was detected at any stage.

PGCs were isolated from E11.5 gonads by the pricking method (de Felici and McLaren, 1982) prior to aggregation with early 8-cell stage embryos. After overnight culture embryos were fixed and sectioned. In 11 out of 57 cases, cells with the morphological characteristics of PGCs were observed in the late morula/early blastocyst. The observed cells had not divided. No embryos were transferred to uteri to assess for chimaerism and further experiments were not undertaken due to the poor adherence of PGCs to early blastomeres. Very similar experiments were performed by G. D. in the laboratory of Anne McLaren. Developments in transgenic technologies allowed PGCs, and their progeny, to be tracked by using donor mice with a β-geo transgene integrated into the constitutively active ROSA26 locus (Friedrich and Soriano, 1991). However, using the same experimental protocol as C.L.S, no PGCs could be detected in the developed blastocysts by whole-mount X-gal staining after overnight culture (data not shown). Blastocyst injection experiments were also performed. Male and female PGCs were kept separate and 10–15 male or 8–10 female PGCs were injected into each blastocyst. After injection blastocysts were cultured overnight and then transferred to recipient females. Rather than waiting to term, chimaerism was assessed at E8.5 or E9.5 using X-gal staining. Again no contribution of PGC-derived cells was observed to either soma or germline (Fig. 1B).

More recent transgenic technologies have allowed PGCs to be isolated at earlier stages in their development. Using a *mil*-1-GFP transgenic line (Tanaka et al., 2004) D.O. and Y.M. were able to isolate newly specified PGCs at E7.5 (Fig. 2A). They injected 5–10 PGCs into blastocysts, but, after 2 h of culture these PGCs were excluded from the embryos and showed no sign of incorporation (Fig. 2B). They then injected PGCs into 8-cell embryos (Poueymirou et al., 2006) which were cultured for 24 h to form blastocysts. In contrast to the results obtained in the McLaren laboratory using E11.5 PGCs, the presence of PGCs, or PGC-derived cells, could be observed in the cultured embryos by the continued expression of a constitutive *DsRed* transgene (Fig. 2C). Furthermore, the expression of *mil*-1 (also known as *Ifitm* and *fragilis*) was apparently downregulated in the surviving cells (Fig. 2B). Those embryos which had apparently incorporated PGCs were transferred to pseudopregnant hosts. However, when embryos were assessed at E6.25 no DsRed expressing cells remained, indicating a lack of chimaerism (Fig. 1B). Notably, in these experiments injection of PGCs appeared to have a negative impact on embryogenesis with only 18% of transferred blastocysts developing to E6.25 (Fig. 1B), compared to 58% of control embryos injected with somatic cells (which also showed no chimaeric contribution).

Discussion

Here, for the first time, are detailed the experimental findings which lie behind the assertion that PGCs do not contribute to chimaeras when introduced to the pre-implantation mouse embryo. The studies encompass work from four independent laboratories. The observations continue to inform current models of the germline cycle. They have lent support to the view that PGCs have a restricted developmental potency and cannot directly differentiate to non-germline lineages. Thus, PGCs have been considered unipotent and their transition to pluripotency as a reprogramming phenomenon (Durcova-Hills et al., 2008; Kimura and Nakano, 2011). In keeping with this there are differences in gene expression between PGCs and pluripotent stem cells (Leitch et al., 2013a, 2013b; Sabour et al., 2010), and epigenetic changes which are unique to the germline (Hajkova, 2011; Ng et al., 2013) highlighting that PGCs are a distinct cell type. However, PGCs express pluripotency factors and can be converted into pluripotent stem cells in vitro with remarkably high efficiency (Leitch et al., 2013b). Therefore, PGCs may rather be considered to harbour a latent or dormant form of pluripotency which is revealed during EG cell derivation or teratocarcinogenesis (Leitch and Smith, 2013). In either model, the distinction between the potency attributed to PGCs, and the naïve pluripotency present in the cells of the pre-implantation epiblast (as well as in ES and EG cells), has its experimental underpinnings in the findings presented here.

That these results have remained unpublished reflects the negative findings. It may be objected that none represents a definitive dataset, accompanied by all the experimental controls. However, it has to be noted that each of the laboratories involved have extensive experience and success in generating mouse chimaeras. For example, a contemporaneous study in the Gardner laboratory reported that 28% of embryos injected with single ICM cells produced live-born chimaeras (Gardner and Lyon, 1971). Therefore, the negative results are highly unlikely to reflect trivial technical failures.

So what conclusions can we draw from the data? A consistent finding is that genital ridge stage PGCs do not contribute to chimaeric animals. Indeed, the more recent data from G.D. indicates that even prior to midgestation no contribution to embryonic development is evident. Furthermore, the same authors found no PGC derivatives in blastocyst outgrowths initiated from embryos which had been aggregated with PGCs at the 8-cell stage (Durcova-Hills et al., 2006). However, these investigators did not attempt blastocyst injection with early stage PGCs. This is an important consideration, because the properties of PGCs are known to change dramatically as development progresses (Matsui, 1998). Furthermore, PGCs isolated at E7.5 or E8.5 can give rise to pluripotent EG cells with very high efficiency, but this capacity diminishes greatly by E11.5 (Labosky et al., 1994; Leitch et al., 2013b). Thus, newly specified, pre-migratory PGCs might be considered the most likely stage to demonstrate pluripotency following introduction to the pre-implantation embryo. Therefore the experiments completed in the Matsui laboratory are particularly noteworthy.

The use of dual fluorescent reporter mice allowed not only the earliest specified PGCs to be isolated but also their in vivo behaviour to be tracked. It is surprising that E7.5 PGCs were excluded from the embryo following injection into the blastocoel cavity. This was not reported for genital ridge stage PGCs. This may be due to differential adhesive properties, which are known to change during PGC development (de Felici and Dolci, 1989; García-Castro et al., 1997), or to loss of cell adhesion molecules during isolation. Further studies will be required to see if this expulsion can be prevented, perhaps by adjusting the number of injected cells. In contrast, the 8-cell injection experiments provide the first indication that PGCs, or PGC-derived cells, can continue to intermingle with cells of the embryo after 24 h of culture. This contrasts somewhat with the findings of the McLaren laboratory published here and previously (Durcova-Hills et al., 2006). This may be due to the injection of E7.5 PGCs, as opposed to aggregation with later genital ridge stages, or it is possible that PGCs were lost during the subsequent blastocyst outgrowths performed in the published study (Durcova-Hills et al., 2006). The degree to which the PGCs are actually integrated into the host ICM remains to be determined. Future studies should attempt to assess this in greater detail, for instance by examining whether the cellular properties of the injected PGCs have changed (beyond the apparent downregulation of *mil*-1) or indeed whether apoptosis pathways might have been activated. Why most of the PGC-injected embryos are subsequently lost also merits further investigation. It is also possible that injection of fewer PGCs may allow better survival – whether this would result in chimaeric contribution still remains an open question. However, the preliminary data presented here suggests that like later stage PGCs, newly specified

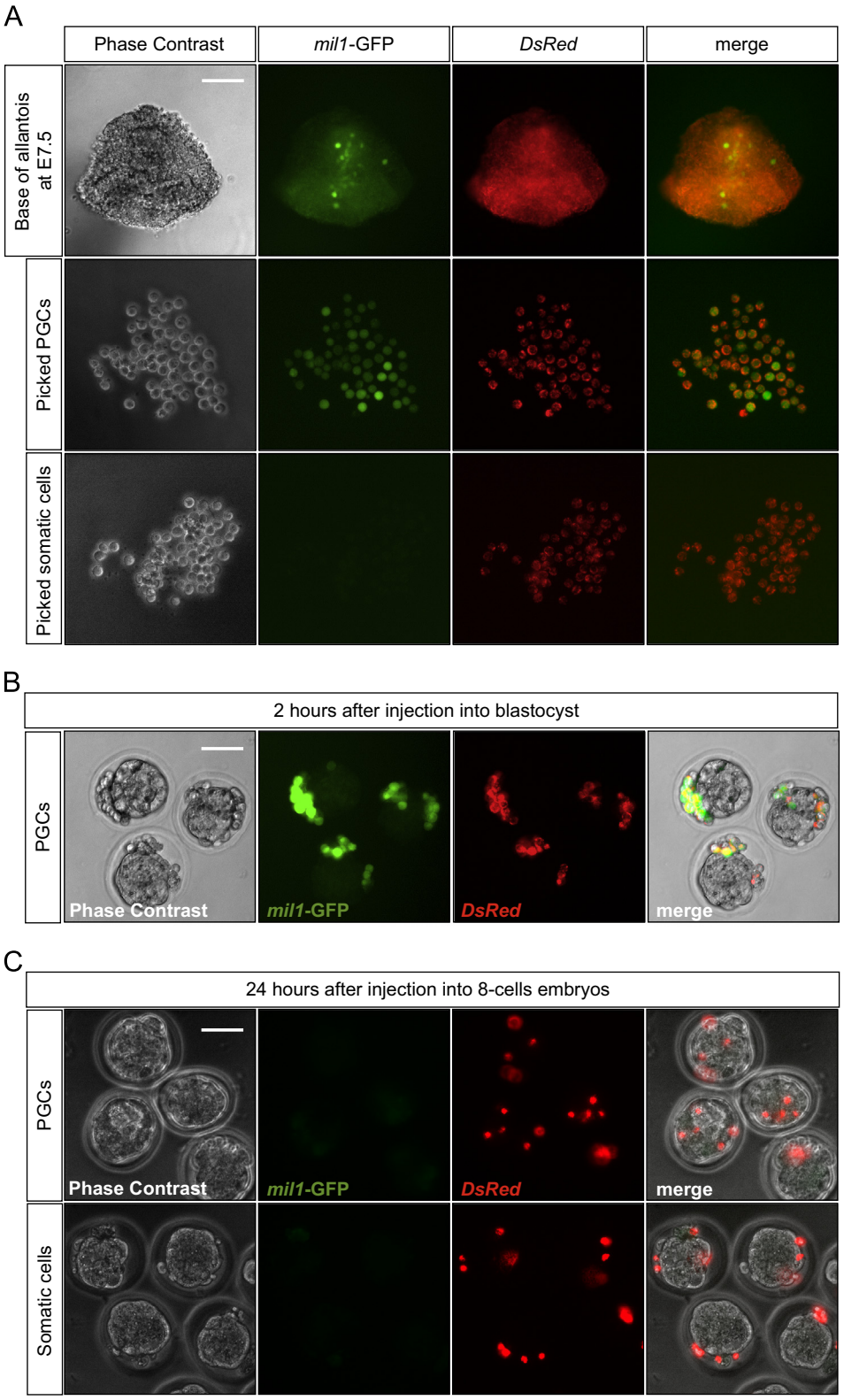


Fig. 2. A modern approach to assessing the pluripotency of PGCs. (A) Phase contrast and fluorescence images showing the isolation of PGCs and somatic cells from E7.5 embryos expressing both *mil*1-GFP, expressed in PGCs, and a constitutively active CAG-*DsRed* transgene, expressed in all cells. Scale bar, 100 μm (top row) and 50 μm (bottom two rows). (B) Phase contrast and fluorescence images of blastocysts 2 h after injection with *mil*1-GFP positive PGCs. Scale bar, 50 μm. (C) Phase contrast and fluorescence images of embryos 24 h after injection of either PGCs or control somatic cells. *Mil*1-GFP-positive PGCs or GFP-negative somatic cells were injected under the zona pellucida of 8-cell stage embryos. Injected cells can still be visualised by DsRed fluorescence 24 h later. Notably *mil*1-GFP expression was lost in the injected PGCs. Some fluorescent foci with the appearance of apoptotic fragments are also present. Scale bar, 50 μm.

PGCs cannot take part in embryogenesis. It would be valuable to confirm this finding in a larger cohort of embryos, and also for PGCs isolated at E8.0–E9.5. Given the potent effect of the cytokine leukaemia inhibitory factor (LIF) on acquisition of pluripotency by PGCs in culture (Leitch et al., 2013b) and in the early embryo (Do et al., 2013) it would be interesting to test whether exposure

of injected 8-cell embryos to LIF might facilitate integration. An alternative approach that may be worth considering is heterotopic grafting into post-implantation egg cylinder embryos, a developmental stage closer to PGCs. This technique was recently used to explore the in vivo developmental potential of post-implantation epiblast stem cells (EpiSCs) (Huang et al., 2012).

The experiments presented here provide a historical account of work that although never before published has undoubtedly shaped our thinking about germ cell biology and pluripotency. The advent of transgenic technologies has now improved our ability to isolate cell populations and to track their fate after transplantation. This highlights the opportunity for further experiments to determine conclusively the developmental potency of PGCs

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