REPRODUCTION

REVIEW

Evolution of the germ line–soma relationship in vertebrate embryos

Andrew D Johnson, Emma Richardson, Rosemary F Bachvarova[1] and Brian I Crother[2]

School of Biology, Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK, [1]Department of Cell and Developmental Biology, Weill Medical College of Cornell University, New York, New York 10065, USA and [2]Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402, USA

Correspondence should be addressed to A D Johnson; Email: andrew.d.johnson@nottingham.ac.uk

Abstract

The germ line and soma together maintain genetic lineages from generation to generation: the germ line passes genetic information between generations; the soma is the vehicle for germ line transmission, and is shaped by natural selection. The germ line and somatic lineages arise simultaneously in early embryos, but how their development is related depends on how primordial germ cells (PGC) are specified. PGCs are specified by one of two means. Epigenesis describes the induction of PGCs from pluripotent cells by signals from surrounding somatic tissues. In contrast, PGCs in many species are specified cell-autonomously by maternally derived molecules, known as germ plasm, and this is called preformation. Germ plasm inhibits signaling to PGCs; thus, they are specified cell-autonomously. Germ plasm evolved independently in many animal lineages, suggesting convergent evolution, and therefore it would be expected to convey a selective advantage. But, what this is remains unknown. We propose that the selective advantage that drives the emergence of germ plasm in vertebrates is the disengagement of germ line specification from somatic influences. This liberates the evolution of gene regulatory networks (GRNs) that govern somatic development, and thereby enhances species evolvability, a well-recognized selective advantage. We cite recent evidence showing that frog embryos, which contain germ plasm, have modified GRNs that are not conserved in axolotls, which represent more basal amphibians and employ epigenesis. We also present the correlation of preformation with enhanced species radiations, and we discuss the mutually exclusive trajectories influenced by germ plasm or pluripotency, which shaped chordate evolution.
Reproduction (2011) **141** 291–300

Introduction

Primordial germ cells (PGCs) are the precursor cells to the eggs and sperm that comprise the germ line. PGCs are specified in the early stages of metazoan development, segregating the germ line from the somatic cell lineages, or soma, that will give rise to the organ systems of the adult. The germ line and the soma play distinct roles in metazoan evolution. The sole function of the germ line is to transmit genetic information between generations, thereby maintaining a genetic lineage. The biological role of the soma, on the other hand, is to act as the vehicle that mediates germ line transmission (see Dawkins 1976). Thus, reproductive fitness is determined by the evolution of somatic traits in response to selective pressures. However, the forces driving somatic diversity are countered by constraints that resist change. So, for example, the mechanisms governing the development of organs that sustain vertebrate physiology are conserved across vast phylogenetic distances because their functions are indispensable to survival of the adult. A less obvious, but perhaps more interesting, constraint is the requirement for germ cell production, because defects in germ cell development affect species' survival across generations. Nevertheless, changes in somatic development that would compromise the germ line cannot be tolerated, and so are not maintained (Johnson *et al.* 2003*b*). This brings into focus the paradox of germ cell specification, because the mechanisms governing PGC specification are not conserved like those controlling specification of somatic tissue. Instead, two distinct mechanisms of germ cell specification are known, and these show a punctuated distribution throughout the animal kingdom (Extavour & Akam 2003, Johnson *et al.* 2003*a*, Crother *et al.* 2007). On the one hand, preformation describes a mechanism in which PGCs are specified cell-autonomously by molecules inherited from the egg, known as germ plasm. On the other hand is epigenesis, in which extracellular signals trigger PGC specification from pluripotent precursors. Remarkably, at several taxonomic levels, both epigenesis and preformation exist in different lineages, and consequently, PGC specification is governed by entirely different means in closely related species. How and why this should be the

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/REP-10-0474
Online version via www.reproduction-online.org

case is not understood, but it is appropriate to assume that it reflects a response to natural selection. Therefore, the distribution of epigenesis and preformation must result from the influence each mode of germ cell specification has on the development of the soma.

Understanding how the two modes of germ cell specification evolved requires an elucidation of their natural history, and for this purpose, vertebrate development is particularly instructive. Vertebrate embryology is, in general, highly conserved, and vertebrate evolutionary history is well established, so it is possible to draw unambiguous conclusions about the ancestry of embryological traits. Thus, mammalian embryos do not contain germ plasm (Eddy 1975); germ plasm is found in birds (Tsunekawa *et al*. 2000), but not in turtles (Bachvarova *et al*. 2009*b*); among amphibians, anurans (frogs) employ germ plasm, but urodeles (salamanders) do not (Smith 1966, Johnson *et al*. 2001); teleost embryos contain germ plasm (Olsen *et al*. 1997, Yoon *et al*. 1997), but those of actinopterygian fish probably do not (Bachvarova *et al*. 2009*a*). Viewing this pattern at a glance, two opposing hypotheses are possible. Germ plasm might be conserved, but selectively lost in individual lineages, mandating the repeated emergence of epigenesis. Alternatively, epigenesis is conserved, in which case germ plasm must have evolved in several vertebrate lineages, independently. A resolution to this problem becomes clearer when considering both modes of specification within a phylogenetic distribution of other embryological traits. For example, mammals, basal reptiles, urodeles, and actinopterygians retain basic features of primitive chordate embryology (Cooper & Virta 2007, Shook & Keller 2008), and in each of these taxa, PGCs arise by induction in the posterior lateral mesoderm (Bachvarova *et al*. 2009*a*). In contrast, PGCs form in very different locations in bird, frog, and teleost embryos, and germ plasm is inherited in each of these embryos in different ways (Johnson *et al*. 2003*b*). From comparisons such as these, it has been concluded that epigenesis is conserved and germ plasm evolved repeatedly (Johnson *et al*. 2001, 2003*a*, Extavour & Akam 2003, Crother *et al*. 2007). However, if this is true, it raises the intriguing question: why has germ plasm evolved so many times?

Though it is not conserved, the germ plasm of vertebrates and invertebrates accomplishes the same task; it inhibits the transcriptional apparatus of nascent germ cells, rendering them transcriptionally unresponsive to somatic inducing signals (Nakamura & Seydoux 2008, Venkatarama *et al*. 2010). Transcription is inhibited during the period in which lineage specification is underway, and as a consequence, nascent germ cells avoid being diverted to a somatic fate. This mechanism contrasts with the ancestral state, in which signals from somatic cells are required to induce PGCs. And, that it evolved many times suggests that germ plasm conveys a selective advantage, which would indicate an effect on somatic development. Because germ plasm functions in early development, while the somatic germ layers are being programmed, and it renders germ cell specification independent of interactions with the somatic environment, the presence of germ plasm might indirectly affect somatic gene regulatory networks (GRNs), liberating them to evolve without jeopardizing germ line development.

Below, we discuss the theory that the force driving the evolution of germ plasm is enhanced evolvability of the soma (Johnson *et al*. 2003*b*, Crother *et al*. 2007). We summarize recent studies on germ plasm, focusing on vertebrates. However, we pay special attention to amphibians. Amphibian embryos are uniquely well suited to addressing how the germ line–soma relationship evolved, because the two modes of PGC specification are found in the two major lineages, and these are represented by experimental models, axolotls and *Xenopus*, urodeles and anurans respectively. However, the recognition that epigenesis is conserved provides an interesting corollary. It suggests that the mechanisms governing pluripotency are also conserved. Consistent with this, ground state pluripotency, a cellular state from which progenitors of the germ line or somatic lineages can be derived, is conserved in the early embryos of urodele amphibians and mammals; frog embryos, in contrast, do not contain cells with equivalent potential (Dixon *et al*. 2010). We discuss the apparent conflict between predetermination and pluripotency in vertebrate evolution, and we identify the dynamic nature of the germ line–soma relationship as a major regulatory component of the forces that shaped its pattern of species diversification.

Preformation

Since Weismann (1898) proposed the Germ Plasm Theory, it has, until recently, been widely assumed that preformation is conserved in the animal kingdom. In large part, this view emerged from studies that showed that maternally inherited germ plasm was essential for PGC development in frogs and flies (Smith 1966, Illmensee & Mahowald 1974), and that the structure of germ plasm in these organisms is extraordinarily similar (Mahowald & Hennen 1971). Indeed, germ plasm in vastly divergent phyla is produced in oocytes and contains abundant mitochondria and a structural component referred to, generally, as germinal granules. Though the precise composition of these granules is unknown, several studies show that they contain or are associated with proteins involved in RNA processing, and the RNAs that encode them (Anderson & Kedersha 2006).

Typically, germ plasm is asymmetrically organized in oocytes. In oocytes of *Xenopus* (Houston & King 2000) and zebrafish (Kosaka *et al*. 2007), germinal granules and mitochondria collectively form a region known as the 'mitochondrial cloud' (MC), which translocates germ

plasm to the vegetal cortex. Germ plasm RNA localizes in *Xenopus* oocytes through an 'early' pathway, while a second 'late' pathway translocates RNAs involved in somatic patterning to the vegetal pole, for example the RNAs encoding Vg1 and VegT (King *et al.* 2005). Interestingly, neither the early nor late pathway is present in oocytes from axolotls (Johnson *et al.* 2001, Nath & Elinson 2007). Considering that urodeles retain primitive amphibian traits (Johnson *et al.* 2003*b*, Ahlberg *et al.* 2005), it can be concluded that evolution of the MC was an innovation of frogs. Importantly, its evolution resulted in a repositioning of PGCs to the endodermal compartment, away from the ancestral mesodermal origin of amphibian PGCs (Johnson *et al.* 2001, Bachvarova *et al.* 2004). Repositioning of the PGCs in early embryos has been associated with major innovations that characterize frog development, most notably the dramatic anteriorization of the trunk (Johnson *et al.* 2003*b*).

Recent work identified at least part of the mechanism of germ cell specification in *Xenopus*. Using isolated PGCs as a point of entry, Venkatarama *et al.* (2010) showed that transcriptional activity in *Xenopus* PGCs is repressed, relative to somatic cells, from mid-blastula through neurula stages, coinciding with the interval during which somatic germ layers are specified. Consequently, as in *Caenorhabditis elegans* and *Drosophila*, transcriptional repression prevents specification of the nascent germ line to a somatic fate. Remarkably, transcriptional repression is achieved by inhibiting phosphorylation of serine residue 2 (Ser2) in the C-terminal domain (CTD) of RNA polymerase II (polII), which is the same general mechanism through which PGC transcription is inhibited in flies and worms. However, the molecules responsible for this activity in flies and worms, Pgc and Pie1 (Nakamura & Seydoux 2008) respectively, are not conserved in other animal lineages, suggesting that frogs evolved a third independent approach towards tackling this problem. Presumably, the molecules that inhibit polII are found among those associated with the germ plasm, though this remains to be demonstrated. What emerges from these comparisons, nevertheless, is a common theme of transcriptional repression in PGCs by germ plasm to prevent their diversion to somatic fates.

Interestingly, *C. elegans*, *Drosophila*, and *Xenopus* are each mosaic organisms with respect to the distribution of maternal determinants that specify somatic, as well as germ line, fates. As a result, the PGC progenitors must repress the cell-autonomous effects of maternally encoded transcription factors, in addition to repressing the response to external stimuli. For example, in *Xenopus* embryos, germ plasm counters the potential effects of VegT, which is an endodermal determinant whose RNA is also localized to the vegetal hemisphere and inherited by the PGCs (Venkatarama *et al.* 2010). However, VegT is not localized in oocytes of axolotls (Nath & Elinson 2007), or even basal species of fish (H Chen, M Loose and A D Johnson, unpublished observations), raising the possibility that the evolution of germ plasm, generally, is a precondition for the evolution of a mosaic distribution of somatic determinants.

Germ plasm is also essential for the development of PGCs in zebrafish (Hashimoto *et al.* 2004). RNA encoding Vasa was the first germ plasm marker cloned from zebrafish (Olsen *et al.* 1997, Yoon *et al.* 1997), and other RNAs that localize to the germ plasm have since been cloned and characterized (Raz 2003). RNAs encoding Dazl, Nanos, and Vasa homologs from zebrafish associate with a MC and translocate to the vegetal pole (Kosaka *et al.* 2007). In almost all of these respects, zebrafish oocytes resemble those of *Xenopus* (though *Xenopus vasa* RNA is not localized; Komiya *et al.* 1994). Bucky ball expression is necessary and sufficient for the organization of zebrafish germ plasm (Bontems *et al.* 2009). Homologs of Bucky ball have been identified in other vertebrates, but it is unknown whether these also participate in germ plasm organization. Post-fertilization, germ plasm RNAs segregate to the distal ends of the cleavage furrows, away from the bottom of the yolky mass. The method of translocation of zebrafish germ plasm RNAs from the bottom of the yolk mass to the top tier of the embryo is not fully understood, and indeed individual RNA species are recruited to the animal hemisphere by different pathways (Theusch *et al.* 2006). It is important to note, in addition, that in frog embryos, germ plasm is not translocated to the animal hemisphere, so the embryological location in which germ plasm functions in *Xenopus* and zebrafish embryos is not conserved (see Fig. 1).

Understanding how germ plasm evolved in teleost fish is more confusing than in frogs because the mechanisms are not conserved across all species. For example, while

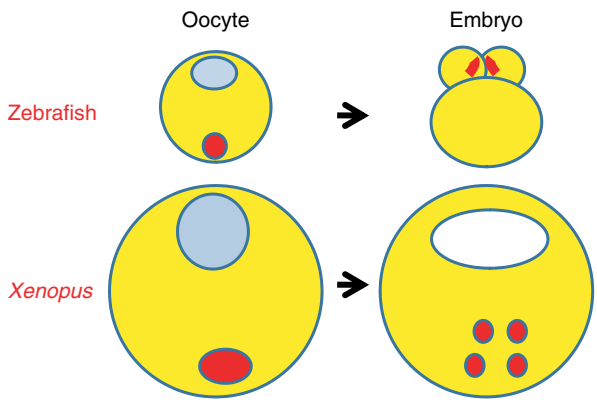

Figure 1 Germ plasm localizes to different regions of zebrafish and *Xenopus* embryos. Germ plasm (represented in red) is localized to the vegetal hemisphere in oocytes of zebrafish and *Xenopus*. In zebrafish, the germ plasm translocates from the vegetal hemisphere of oocytes to the cleavage furrows in the animal hemisphere of early embryos (see Raz 2003). In *Xenopus* embryos, germ plasm incorporates into vegetal blastomeres of early embryos, and PGCs are sister cells to the somatic endoderm.

vasa and *dazl* RNAs are localized in zebrafish, *vasa* RNA is not localized in medaka oocytes (Knaut *et al*. 2002) or embryos (Herpin *et al*. 2007), yet it is localized in the oocytes of butterfly fish, representing a more primitive species (Knaut *et al*. 2002). To clarify when the localization of germ plasm RNAs evolved in teleosts, we turned to sturgeons, which retain the embryological characteristics of ancient Actinopterygii. Among these characters is holoblastic cleavage, which was retained during amphibian evolution (Bolker 1993, Cooper & Virta 2007). We cloned the *vasa* and *dazl* orthologs from gulf sturgeons (*Acipenser oxyrhynchus*) and hybridized probes from these molecules to sections from adult ovaries containing growing as well as fully-grown oocytes. Figure 2 demonstrates that, in contrast to oocytes from zebrafish, or butterfly fish, the *vasa* ortholog from sturgeon is not localized; rather, it is expressed diffusely around the oocytes' cytoplasm (Fig. 2B and C), in a manner similar to the pattern found in the oocytes of axolotls (Bachvarova *et al*. 2004). Sturgeon *dazl* RNA (Fig. 2D and E) also resembles *dazl* expression in oocytes from axolotls (Johnson *et al*. 2001), not from zebrafish (Kosaka *et al*. 2007). In no case did we identify localized expression of either *vasa* or *dazl* RNAs in sturgeon oocytes, regardless of oocyte size. From these data, we conclude that germ plasm evolved sometime after the divergence of teleosts from more primitive lineages. We suggest that the establishment of germ plasm in the teleost lineage was a necessary precondition for the evolution of meroblastic cleavage (Cooper & Virta 2007), similar to the proposed morphological effects of germ plasm in frogs (Johnson *et al*. 2003*b*).

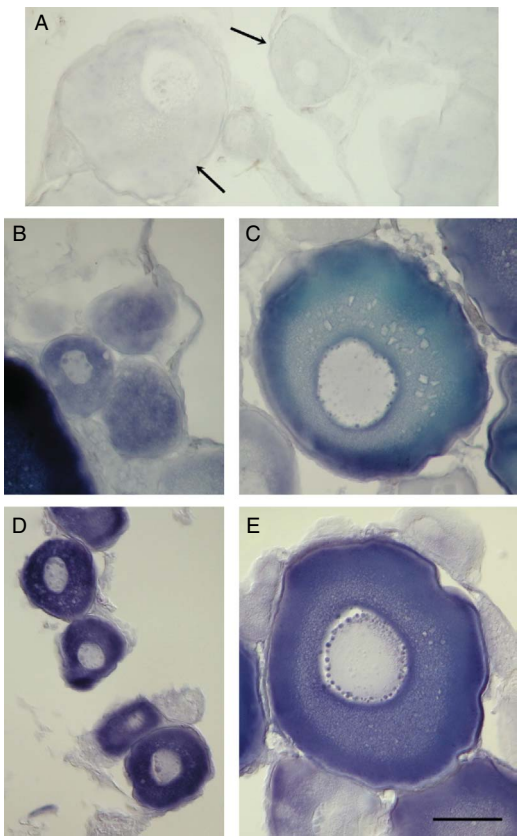

Figure 2 Germ plasm RNA localization is not conserved in sturgeon oocytes. Ovary from the gulf sturgeon (*Acipenser oxyrhynchus*) was sectioned and hybridized with sense probe corresponding to the sturgeon *dazl* gene (A) or antisense probes against *vasa* (B and C) and *dazl* (D and E). Arrows show oocytes. Staining pattern indicates the absence of RNA localization. Scale bar in panel E = 100 μm for all panels.

Epigenesis

Nieuwkoop (1969) reported that cells in the primitive ectoderm (animal cap) of axolotl embryos could be induced to form PGCs, and is thus credited with identifying regulative germ cell specification in vertebrates. He proposed that urodele PGCs are derived from 'unspecialized' cells, meaning that they do not contain germ plasm (Ikenishi & Nieuwkoop 1978). But as a concept, this hypothesis did not receive serious attention until equivalent findings were reported in mouse embryos. This was accomplished in two landmark studies. First, Lawson & Hage (1994) fate mapped the PGC precursors of mouse embryos to the proximal epiblast (PE), and showed that they also give rise to somatic mesodermal derivatives, such as blood and allantois. These studies established the principle that mouse PGCs are not predetermined, but are specified relatively late in development. Tam & Zhou (1996) later showed that cells from distal epiblast, which normally become neurectoderm, could produce PGCs when transplanted to a proximal position, demonstrating that mouse PGCs are specified by signals in their local environment, i.e. extracellular signals.

To resolve the problem that two distinct modes of germ cell specification exist in vertebrates, Johnson *et al*. (2001, 2003*a*) noted the parallels of development in axolotls and mice, and proposed that epigenesis, not predetermination, is conserved. Strong support for this hypothesis comes from studies with turtles, representing reptiles. Turtle oocytes lack germ plasm, and PGCs are generated in the primitive streak, suggesting they are specified by induction (Bachvarova *et al*. 2009*b*). From comparisons between turtles and mammals with axolotls, it is possible to conclude that epigenesis was conserved from urodele-like amphibians to the basal amniotes from which mammals evolved.

Epigenesis in mammals has been most intensely studied in mice. Mouse PGCs are specified by bone morphogenetic protein 4 (BMP4) signals emanating from the extraembryonic ectoderm (Lawson *et al*. 1999). *In vitro*, epiblasts dissected between E5.5 and 6 exhibit a uniform response to BMP4, in which they are specified to germ line development and can give rise to functional gametes (Ohinata *et al*. 2009). Importantly, epiblast cells must be primed for PGC competence by WNT3 signals

appearing at about E5.5, and therefore cells isolated at an earlier stage cannot produce germ cells. The effect of this priming step is unknown; however, normal PGC development is also dependent on BMP8B and BMP2 (Ying *et al*. 2000, Ying & Zhao 2001) signals, demonstrating the intricate involvement of embryonic patterning signals in mouse germ cell specification.

An early response to BMP4 (about E6.25) is the expression of BLIMP1, a PRDM domain transcription factor that is a master regulator of PGC specification (Ohinata *et al*. 2005, Vincent *et al*. 2005). BLIMP1 positively affects induction of a number of PGC-specific genes (Kurimoto *et al*. 2008); however, its unique function is to inhibit incipient expression of somatic mesodermal genes. As a result, specification towards mesoderm is reversed, and PGCs are redirected towards germ line development. Later, BLIMP1 targets repressive epigenetic modifications to the PGC chromatin that are thought to maintain lineage restriction of migratory PGCs (Hayashi *et al*. 2007, Surani *et al*. 2007). Superficially, these effects of BLIMP1 are reminiscent of those of germ plasm in frogs and invertebrates, i.e. they prevent somatic specification. However, BLIMP1 does not induce global transcriptional repression; rather, it accomplishes germ lineage restriction by inhibiting expression of specific genes in an actively transcribing cell (Surani *et al*. 2007). Interestingly, transcription is transiently repressed in migratory PGCs by inhibition of Ser2 CTD phosphorylation (Seki *et al*. 2007), the same modification directed by germ plasm in other species. However, polII inhibition in mouse PGCs occurs from about E8 to about E9.25, in cells that already express *Stella*, so it occurs after specification (Saitou *et al*. 2002). Furthermore, it is concomitant with arrest in G2 of the cell cycle and the epigenetic remodeling characteristics of migratory PGCs (Seki *et al*. 2007, Hajkova *et al*. 2008). Therefore, polII inhibition in the mouse germ line is unlikely to be a component of the specification process. Nevertheless, it is tempting to speculate that polII inhibition is conserved. One possibility is that polII suppression was advanced to an earlier, specification, stage of development by the evolution of germ plasm. But clarification of this issue will require investigation in more primitive species that employ epigenesis.

As yet, it is unclear whether PGC specification in mouse embryos is representative of mammals at large. BMP signaling induces PGCs from human embryonic stem cells (ESC; Kee *et al*. 2009), and BMP4 induces PGC-specific gene expression from pig epiblast stem cells (Alberio *et al*. 2010), suggesting that a role for BMP signaling in PGC specification is conserved in large mammals. However, whether or not BLIMP1 is involved in early specification of PGCs in other mammals remains to be elucidated. It is interesting that the cup-like shape of the mouse epiblast is unique to rodents (Chuva de Sousa Lopes & Roelen 2008), and it may have evolved as part of the accelerated development that makes mouse embryos attractive models to study mammals. In this regard, it is conceivable that the role of BLIMP1 evolved in rodents to achieve early germ cell segregation, thus fulfilling the role of a 'zygotic germ plasm'. However, this will only become clear from work with other experimental systems. Nonetheless, it is evident that epigenesis, as a process, is conserved in mammals.

Among lower vertebrates, regulative specification has only been investigated in detail in embryos from axolotls. Axolotls are particularly useful because they resemble the tetrapod ancestors (Ahlberg *et al*. 2005, Anderson *et al*. 2008), and therefore provide a point of reference for understanding how developmental mechanisms evolved in terrestrial vertebrates. The precursors of axolotl PGCs arise in the ventral marginal zone (VMZ), adjacent to nascent blood cells (Nieuwkoop 1947, Smith 1964). They pass over the blastopore during gastrulation, and by tailbud stage, bonafide PGCs can be detected in the posterior compartment of the dorsal–lateral mesoderm (Johnson *et al*. 2001, Bachvarova *et al*. 2004). PGC specification is completed by mid-gastrula stage (Smith 1964), and early PGCs express *Brachyury* and *Mix* (Johnson *et al*. 2003*a*; A D Johnson, unpublished observations), consistent with their mesodermal origin. However, they do not initiate expression of germ cell-specific genes (*dazl* and *vasa* homologs) until early tailbud stages. Prior to this, they express Nanog, but they do not express *Blimp1* (C Redwood and A D Johnson, unpublished observations). Thus, it is unclear how the 'germ line mesoderm' is maintained in axolotl embryos, and this remains an intriguing problem.

Nieuwkoop's original findings on PGC induction were confirmed in several follow-up studies, which concluded that PGCs are produced in response to ventral mesoderm inducing signals (Kocher-Becker & Tiedemann 1971, Boterenbrood & Nieuwkoop 1973, Sutasurja & Nieuwkoop 1974). In accordance with this, robust induction of PGCs from animal caps is achieved with a combination of fibroblast growth factor (FGF) and BMP signals, and these signals are also required in intact embryos, suggesting they are the natural inducers of the axolotl germ line (M O'Reilly, R F Bachvarona and A D Johnson, unpublished observations). The VMZ of axolotl embryos is patterned by the reciprocal effects of Nodal and FGF; Nodal induces blood in the VMZ, while FGF represses blood specification and induces PGCs. The effect of FGF on blood specification is conserved in the VMZ of *Xenopus* embryos (Walmsley *et al*. 2008), and at least in part stems from Nodal signaling inhibition. Nevertheless, excess SMAD signaling can override the effects of FGF in the axolotl VMZ (A D Johnson, unpublished observations), driving potential PGCs towards endoderm specification. Therefore, the block to somatic specification is finite in axolotls, and is not like the complete repression of somatic cell fate that is affected in *Xenopus* embryos by germ plasm.

Importantly, these studies highlight a major difference in the developmental potential of axolotl and *Xenopus* animal caps, since *Xenopus* animal caps cannot be made to produce PGCs (Michael 1984). The unrestricted developmental potential of axolotl animal caps (primitive ectoderm), which can produce any somatic cell type, or PGCs, is reminiscent of ESC, as well as the early epiblast of mouse embryos, suggesting that ground state pluripotency (Nichols & Smith 2009) is conserved. Intruigingly, the first decision made by cells in an axolotl animal cap is a commitment to either the germ line or soma, in a process regulated by Nanog activity (Z Ferjentsik and A D Johnson, unpublished observations). This initial regulatory step in development is not conserved in the cells from *Xenopus* animal caps, whose potential is restricted to the production of somatic cell types, and therefore, cells in the animal cap of frog embryos initiate development downstream of the pluripotent ground state. It is fascinating to speculate on how this somatically primed pluripotent state evolved, but it can be assumed that its evolution contributes to the more rapid development of frog embryos, and was therefore a selective advantage.

Pluripotency is conserved in chordates

The unrestricted potential of cells in the pluripotent ground state (pluripotent cells) is an essential component of epigenesis, so it would be assumed to be conserved in chordates. In mammals, pluripotency is governed by the transcription factors POU5F1 (OCT4) and NANOG, and orthologs of these molecules are co-expressed in the animal cap of axolotl embryos (Bachvarova *et al*. 2004, Dixon *et al*. 2010), indicating that the pluripotency network is conserved from urodeles to mammals. In fact, molecules with NANOG activity have been identified as far back as hemichordates (J Dixon and A D Johnson, unpublished observations), at the base of deuterostomes, suggesting that pluripotency is ancestral to chordates. However, pluripotency is not uniformly conserved. For instance, in *Xenopus* and zebrafish, the pluripotency network is not conserved.

Nanog was deleted from the frog genome some time after the anuran and urodele lineages diverged from their last common amphibian ancestor (Dixon *et al*. 2010). Whether Nanog was retained in teleosts is less certain. The synteny of mammalian Nanogs is not conserved with the putative Nanog homolog of medaka. Also, the medaka gene regulates cell cycle events, not pluripotency (Camp *et al*. 2009), so its function is not conserved, and it cannot be considered orthologous. The evolution of POU5F1 is also complex. POU5F1 is conserved from axolotls through reptiles to mammals (Bachvarova *et al*. 2004); it is not conserved, however, in *Xenopus* and in zebrafish (Frankenberg *et al*. 2010). These species contain a paralogous protein called Pou2, which is also not conserved. For example, the *Xenopus* genome encodes three tandemly arrayed variants of Pou2, known as XlPou60, XlPou25, and XLPou91 (Hellsten *et al*. 2010). Of these, XlPou91 is of particular interest because it has potent ability to rescue POU5F1 activity in mouse ES cells (Morrison & Brickman 2006), and it is expressed in PGCs after the neurula stage, presumably as part of the restoration of somatic potential that accompanies their later development (Venkatarama *et al*. 2010; see Wylie *et al*. 1985). In contrast, the *pou2* gene product from zebrafish cannot rescue ESC (Morrison & Brickman 2006), so the activity seen in XlPou91, and other *Xenopus* homologs, as well as axolotl POU5F1, is not conserved in the most related gene in zebrafish.

The apparent divergence of the vertebrate pluripotency network is surprising, given its fundamental role in the development of mammals and urodeles; however, it is consistent with the inability of frog animal caps to produce PGCs in response to inducing signals. These findings also raise the possibility that the evolution of germ plasm was a precondition for the deletion of Nanog, and other pluripotency molecules, from the genome of frogs and teleosts. This conjecture seems likely since NANOG is required for PGC development in mice (Chambers *et al*. 2007) and axolotls (A D Johnson, unpublished observations). From this view, existing data strongly support the hypothesis that the evolution of germ plasm relaxes constraints on the GRNs that govern vertebrate development (Crother *et al*. 2007, Swiers *et al*. 2010).

The convergent evolution of germ plasm

Convergent evolution implies that a similar biological trait appears *de novo* in unrelated lineages. Examples of convergence are rare, but easily recognized. For example, the camera eye evolved in both octopus and human (Ogura *et al*. 2004); dorsal fins evolved independently in cetacean mammals and fish; the forelimbs of birds and bats independently evolved wings. The selective advantage conveyed by the appearance of each of these traits is easy to comprehend, and each arises relatively late in development as a specialization of a pre-existing adult body form. So from this perspective, it is difficult to identify the selective advantage that is conferred by the evolution of germ plasm, which acts early in development, during the stages in which the somatic germ layers are being patterned. However, given the distribution of germ plasm in the animal kingdom, it is logical to assume that early segregation of the germ line presents a selective advantage. So the challenge, then, is to identify how this might affect the evolution of somatic development.

Decades of work with *Xenopus* embryos has revealed a complex GRN that governs mesoderm specification (Loose & Patient 2004). However, while the functions of many genes within the *Xenopus* mesoderm GRN (mGRN) are conserved, the mGRN itself is not. For example, in *Xenopus* embryos, mesoderm specification

involves over twenty-five functionally redundant copies of the mesoderm inducer Nodal (Takahashi *et al.* 2006), and seven copies of the transcription factor Mix (Hellsten *et al.* 2010). Teleost embryos also express multiple copies of *nodal* and *mix* (Hirata *et al.* 2000, Trinh *et al.* 2003, Fan & Dougan 2007). However, this is not the basal state for chordates, and it is not conserved in amphibians. In axolotl embryos, for instance, mesoderm specification is initiated by a single *nodal* gene, and this is the conserved state (Swiers *et al.* 2010). Furthermore, whereas the *mix* genes specify endoderm in *Xenopus* development, the single *mix* gene in axolotl embryos acts upstream of *brachyury* in the genetic cascade that regulates specification of mesoderm, and this role for *mix* is shared with mammals (Swiers *et al.* 2010). And finally, localized expression of the RNA for VegT, a T box transcription factor that regulates early mesoderm and endoderm specification, is a novelty of frog embryos, and VegT is not involved in mesoderm specification in axolotls (Y Chen, M Loose and A D Johnson, unpublished observations). As with the pluripotency network, these observations pose the question: is the evolution of novelty in the frog mGRN a cause or a consequence of germ plasm?

As discussed above, in axolotl embryos, PGCs are specified within the mesodermal germ layer by a combination of FGF and BMP signals, and are maintained by a balance of mesoderm-patterning agents. Disruptions to this balance can terminate the germ line, for example from excess SMAD2 signaling. It can be assumed, therefore, that expansion of the *Nodal* gene family, which acts through SMAD2, is under constraint in urodeles. Expansion of the *mix* genes, which act

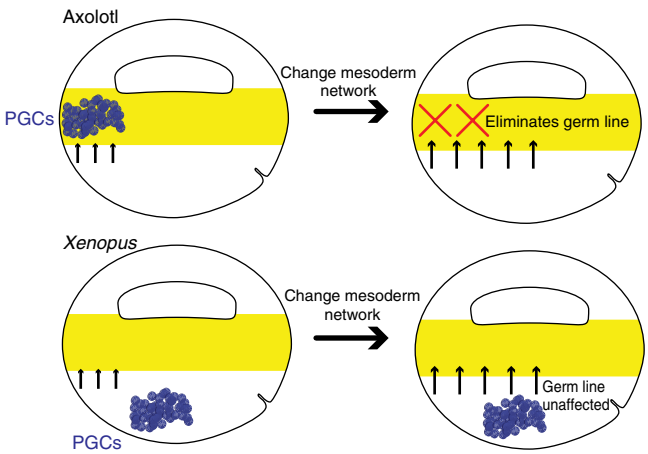

Figure 3 Germ plasm relieves constraints on evolution of the mesoderm GRN. Axolotl PGCs are specified by induction in the mesoderm (yellow) on the ventral side of the embryo, opposite the blastopore. Increased somatic mesoderm-inducing signals, resulting from gene expansion in the mesoderm GRN, would eliminate the axolotl germ line. *Xenopus* PGCs develop in the vegetal hemisphere and are refractory to somatic signals, so gene expansion in the mesoderm GRN does not affect the germ line.

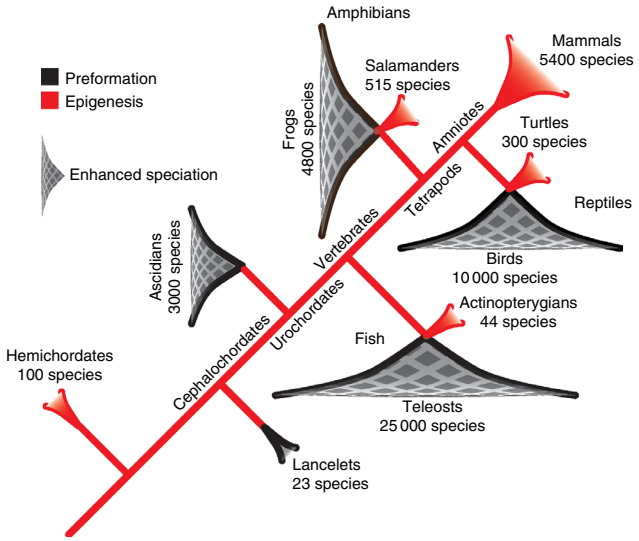

Figure 4 The evolution of germ plasm enhances speciation. In vertebrates the evolution of germ plasm is correlated with increased numbers of species within individual lineages, when compared with sister taxa that do not contain germ plasm. In contrast, pluripotency has been conserved in the major trunk of chordates, and is associated with the major transitional innovations of chordate evolution.

cell-autonomously in mesoderm specification, would also compromise the germ line. On the other hand, *Xenopus* PGCs are protected from the effects of extrinsic, and intrinsic, signals by transcriptional repression (Venkatarama *et al.* 2010), and therefore a similar constraint on *nodal* and *mix* gene expansion would not exist. In this light, it is not surprising that the mGRN of *Xenopus* was liberated to evolve novel interactions. Using amphibians as a generalized paradigm for vertebrates, we conclude that the evolution of germ plasm liberates the potential to evolve change in the GRNs that govern development of the somatic germ layers. This concept is illustrated in the model presented in Fig. 3.

Germ plasm can enhance evolvability

Ultimately, selective pressures are interpreted by reproductive fitness, operating within an environmental niche, and there exist clear examples of the contribution of embryological innovations to such a process. For example, the evolution of amniote development enabled tetrapods to exploit dry land. A less obvious example is the evolution of meroblastic cleavage in the embryos of teleost fish, which greatly accelerated the rate of development from embryo to adult, and is associated with enormous diversity of adult body size and form. However, the basic tetrapod body form was conserved as reptiles evolved from urodele-like amphibians, indicating that while the emergence of extraembryonic structure was a major innovation of amniotes, the morphogenetic movements associated with the embryo, proper, were unchanged. In contrast, the evolution of teleost embryos was accompanied by novel

morphogenetic movements, the consequence of which was a major reordering of adult body plan away from that of ancestral fish. A similar event is associated with amphibian evolution (Johnson *et al*. 2003*b*). Frog embryos evolved novel morphogenetic movements, and adult frogs possess a body plan that is unique in nature. Counter-intuitively, then, the body plan of salamanders more closely resembles that of modern lizards than it does frogs, even though salamanders and frogs share a much closer phylogenetic relationship.

Retention of the body plan through amniote evolution is a clear indication of a constraint on the mechanisms governing development of the soma. Conversely, adult frog and teleost morphology provide evidence for the absence of a similar constraint. Birds also do not resemble basal reptiles, and therefore provide a third example of the absence of morphological constraint. We propose that the absence of constraint is a manifestation of the altered germ line–soma relationship that results from the evolution of germ plasm. Thus, in the presence of a fixed germ line that is refractory to any change in somatic development, the zygotic mechanisms that govern somatic development are free to change, the net result of which is the evolution of specific lineages with enhanced evolvability (Crother *et al*. 2007).

In Fig. 4, chordate evolution is diagrammed with a consideration of species that employ epigenesis, or species that employ germ plasm, as a mechanism to derive PGCs. While epigenesis is conserved in the major trunk of chordate evolution, the evolution of germ plasm in individual branches is associated with enhanced species radiations. If germ plasm liberates change in somatic GRNs, then selective pressures would be expected to direct these towards networks that result in accelerated development, an obvious advantage for organisms that develop from free-swimming larvae. And indeed, the novel mGRNs of *Xenopus* and zebrafish promote accelerated development compared to their sister species that employ epigenesis. This is an example of how the freedom to evolve change, which is afforded by germ plasm, can lead directly to a competitive advantage, and minor perturbations of a robust genetic network can then promote micro-evolutionary changes that lead to enhanced speciation within a lineage. On the other hand, germ plasm evolved in amphioxus (Wu *et al*. 2011), yet there is no evidence for enhanced speciation in cephalochordates, and they retain a primitive body plan, indicating that germ plasm is permissive, not instructive, for the evolution of radical embryological innovation. Nevertheless, over evolutionary time, the germ line–soma relationship has been dynamic, and it reflects a balance between preformation and epigenesis. Thus, where epigenesis is conserved, so is pluripotency, since it is required for the derivation of PGCs. The conservation of pluripotency has important ramifications. Pluripotent cells provide raw embryological material whose potential can be co-opted to evolve novel structures. Furthermore, the development of embryos that pass through the pluripotent ground state is slower than that of mosaic embryos, or embryos that initiate development from cells primed for somatic development, like those of *Xenopus*. Thus, this form of embryogenesis presides over the more slowly evolving changes that result in macroevolution: for example, fins evolved into legs; the amnion evolved, and vertebrates could develop on land; trophectoderm evolved, which lead to mammals. These innovations illustrate the selective advantage of embryos that employ epigenesis, and they result in 'vertical evolution'. Yet these innovations emerge amidst the constraints imposed on somatic GRNs by epigenesis, so the early embryology is constrained, and the basic body plan of adult vertebrates has, therefore, been conserved through the millennia by the ancestral germ line–soma relationship.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work.

Funding

Original work presented or cited in the text was funded by the Medical Research Council, and EvoCell Ltd (UK).

Acknowledgements

A D Johnson dedicates this paper to Prof. Bob Lloyd, who taught me an appreciation for the molecular mechanisms that underlie the astonishing ability of eukaryotic genomes to evolve change. A D Johnson is also very grateful to Azim Surani for helpful suggestions, particularly as they relate to mouse embryos. The suggestions of Garry Morgan and Fred Sablitzky are also very much appreciated. The concepts presented in this paper could not have developed without the immeasurable intellectual contributions of Ramiro Alberio and Matt Loose. Finally, we thank our dedicated colleagues who over the years have worked so hard to develop the axolotl model system and our concepts of the evolution of pluripotency.

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Received 22 November 2010
First decision 20 December 2010
Accepted 12 January 2011