

Regulative germ cell specification in axolotl embryos: a primitive trait conserved in the mammalian lineage

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How germ cells are specified in the embryos of animals has been a mystery for decades. Unlike most developmental processes, which are highly conserved, embryos specify germ cells in very different ways. Curiously, in mouse embryos germ cells are specified by extracellular signals; they are not autonomously specified by maternal germ cell determinants (germ plasm), as are the germ cells in most animal model systems. We have developed the axolotl (*Ambystoma mexicanum*), a salamander, as an experimental system, because classic experiments have shown that the germ cells in this species are induced by extracellular signals in the absence of germ plasm. Here, we provide evidence that the germ cells in axolotls arise from naive mesoderm in response to simple inducing agents. In addition, by analysing the sequences of axolotl germ-cell-specific genes, we provide evidence that mice and urodele amphibians share a common mechanism of germ cell development that is ancestral to tetrapods. Our results imply that germ plasm, as found in species such as frogs and teleosts, is the result of convergent evolution. We discuss the evolutionary implications of our findings.

Keywords: axolotl; mouse embryo; primordial germ cell; phylogeny; regulative specification; determinative specification

1. INTRODUCTION

Over 100 years ago, Weismann (1885) proposed the concept that germ plasm, a specialized region of egg cytoplasm, would contain sufficient material to direct the establishment of the germ cell lineage during embryonic development. Since that time, germ plasm has been identified in the eggs of many species from both the protostomal and deuterostomal lineages (Kloc *et al.* 2001; Mahowald 2001). As suggested by Weismann, germ plasm contains molecules that act as determinants to specify germ cells.

During early development germ plasm segregates asymmetrically to only one of the two blastomeres produced at each cell division. The progeny of cells inheriting the germ plasm eventually give rise to PGCs, the founder cells of the germline, which later move to the gonad and develop into gametes (Houston & King 2000a; Kloc *et al.* 2001). Interestingly, the eggs of many of the non-mammalian model systems used to study animal development contain germ plasm (known by various different names: e.g. pole plasm in *Drosophila*, or P granules in *C. elegans*). For this reason it has long been assumed that germ plasm and the

process of cell-autonomous germ cell specification that it governs, is a mechanism that has been evolutionarily conserved as part of the developmental programme in all animal embryos. However, experiments in recent years with mouse embryos suggest otherwise.

In mouse embryos germ plasm has never been identified (Eddy 1974, 1975), and the presumptive germ cells are clonally related to somatic cells, such as allantois and primitive blood (Lawson & Hage 1994). Indeed, cells that would not ordinarily become germ cells, i.e. presumptive somatic cells, can be made to enter the germline if they are transplanted to appropriate locations in the embryo (Tam & Zhou 1996), indicating that mouse PGCs are not specified cell-autonomously. Several studies have demonstrated that BMPs (members of the TGF- β family of signalling molecules) secreted from adjacent extraembryonic tissues are required for the production of PGCs in mouse embryos (Lawson *et al.* 1999; Ying & Zhao 2001; Ying *et al.* 2001). Moreover, recent work suggests that interferon-related signals play a significant role in governing the final allocation to the germline (Saitou *et al.* 2002). From these studies it is clear that mouse PGCs are not specified by maternal germ cell determinants, and in this way they differ from other animal models. In addition, extracellular signalling is clearly responsible for allocating cells to the germline during mouse development.

One of the major concepts fostered by modern developmental biology is that most events occurring during animal

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development are evolutionarily conserved across vast phylogenetic distances. (For example, consider conservation of the role of *Hox* genes in axial patterning.) However, the mechanism of germ cell specification in mouse embryos is clearly not a process that has been conserved with the embryos of more primitive model systems, whose germ cells are predetermined by germ plasm. Therefore, either: (i) mammalian embryos evolved a novel mode of germ cell determination that does not involve germ plasm; or (ii) mammals inherited a pre-existing mode of germ cell specification that has not been characterized in more primitive model systems.

To understand the evolution of developmental mechanisms in mammals, it is important to recognize that all terrestrial vertebrates share a common amphibian ancestor (Cannatella & Hillis 1992; Milner 1992). Therefore, all developmental processes in tetrapod embryos have either diverged or been conserved from a single ancestral mechanism. From the ancestral tetrapod two major lineages of modern amphibians have evolved: anurans (frogs) and urodeles (salamanders). The embryos of each of these lineages have been extensively used in experimental studies dating back over a century, and basic aspects of the process of PGC development have been elucidated. Interestingly, anurans and urodeles develop PGCs in very different ways.

Much is known about the development of PGCs in anuran embryos, primarily from experiments on *Xenopus laevis*, the species that has come to dominate amphibian embryology in recent years. *Xenopus* oocytes produce germ plasm early in oogenesis, and transport it to the vegetal cortex, where it is found in fully-grown oocytes (Houston & King 2000a; Kloc *et al.* 2001). Thus, *Xenopus* eggs, and those of all other anuran species that have been studied (Blackler 1958), contain germ plasm in a region of cytoplasm that later contributes to the endodermal germ layer. Anuran germ plasm has a well-defined structure, including ribosomes, mitochondria and electron-dense particles called germinal granules. Recent studies with *Xenopus* show that germ plasm also contains a diverse population of both coding and non-coding RNAs. Among the messenger RNAs found in *Xenopus* germ plasm are sequences encoding RNA binding proteins. The role of these RNAs in germ cell specification is unknown. However, oocytes depleted of one germ plasm specific message, *Xdazl*, encoding the *Xenopus dazl*-like RNA binding protein (Houston *et al.* 1998), produced tadpoles depleted in or devoid of PGCs (Houston & King 2002b). These observations complement more classical studies, in *Xenopus* and other anuran species, in which germ plasm has been disrupted by surgical removal or UV irradiation (Smith 1966; Zust & Dixon 1975; Ikenishi & Kotani 1979). These treatments result in embryos devoid of PGCs, indicating that germ plasm, and the individual components therein, acts as a determinant necessary for germ cell development in anuran embryos.

The possible existence of a germ plasm in the embryos of urodele amphibians has been discussed for many years (Nieuwkoop & Sutasurya 1979; Smith *et al.* 1983). However, working at the ultrastructural level, Ikenishi & Nieuwkoop (1978) examined embryos from the axolotl (*Ambystoma mexicanum*), a representative urodele species, and were unable to find material resembling germ plasm

in the PGCs during the early stages of development. More recently, Johnson *et al.* (2001) cloned the *dazl* sequence from axolotls, *Axdazl*, and used it to probe axolotl oocytes and early embryos. *Xdazl* RNA can be used to mark the location of germ plasm, hence it shows localized expression in *Xenopus* oocytes and early embryos. However, *Axdazl* RNA is uniformly distributed in axolotl oocytes and early embryos, only showing germline restricted expression late in development, in PGCs that are approaching the genital ridges. These results support the view that anuran-type germ plasm does not exist in urodele amphibians.

Urodele PGCs also have a different embryological origin than those of anurans. In urodele embryos PGCs develop in the lateral plate mesoderm (Humphrey 1925, 1929; Ikenishi & Nieuwkoop 1978) from cells mapped to the ventral marginal zone of gastrula stage embryos (Nieuwkoop 1947). In the absence of germ plasm, it is unclear how presumptive germ cells are distinguished from somatic cells developing in adjacent domains, such as the primitive blood. However, in his classic experiments to show the induction of mesoderm in amphibian embryos, Nieuwkoop (1969) showed that mesoderm-inducing signals produced from vegetal blastula stage cells (presumptive endoderm) could induce ectopic PGCs from presumptive ectoderm (animal caps). Later work further distinguished the signals needed to produce PGCs, showing that they are derived from ventral vegetal cells (Boterenbrood & Nieuwkoop 1973; Sutasurya & Nieuwkoop 1974). Yet, these same signals also produce a wide variety of somatic ventral and lateral cell types, including blood, mesonephros and mesothelium. From these studies it can be concluded that, unlike anurans, in urodele embryos PGCs do not develop from predetermined cells. Rather, urodele PGCs appear to develop from unspecialized mesodermal tissue that has responded to appropriate extracellular influences.

Therefore the embryos of urodele and anuran amphibians have diverged in the way that they produce PGCs. However, clear parallels exist in the way that PGCs are produced in the embryos of urodeles and mammals. This raises two possibilities. First: (i) cell-autonomous germ cell specification (i.e. the anuran mechanism) may be ancestral to tetrapods, in which case mammals and urodeles must have converged on common aspects of germ cell development. Alternatively: (ii) regulative germ cell specification may be ancestral to tetrapods and retained in urodeles and mammals. In this case anurans must have evolved a novel mechanism on land. Because cell-autonomous germ cell specification has been identified in zebrafish embryos (Knaut *et al.* 2000) and those of other teleosts (Knaut *et al.* 2002), as well as in a variety of protostomal species, it would appear that this mechanism might be ancestral to tetrapods, supporting the first hypothesis. However, we have recently proposed that regulative germ cell specification is ancestral to tetrapods, and that germ plasm has evolved independently several different times, in several different lineages (Johnson *et al.* 2003). Here, we present data to support this view.

We show that axolotl PGCs are produced from naive mesoderm in response to simple mesoderm-inducing agents, consistent with the prevailing hypotheses concerning the development of mouse PGCs. In addition, we have

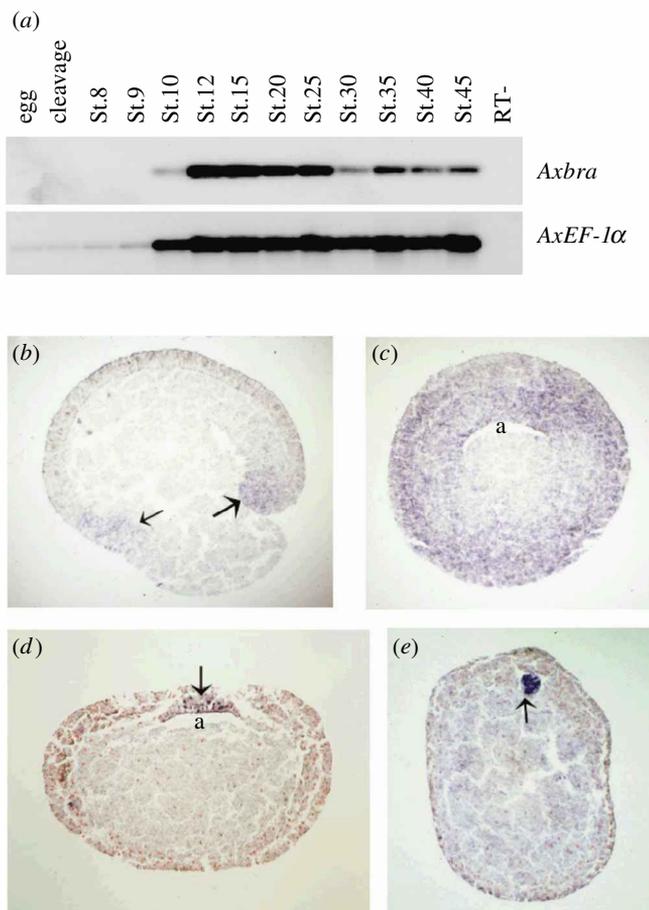


Figure 1. Expression of the axolotl brachyury gene, *Axbra*. (a) RT-PCR analysis of *Axbra* transcriptional activation. RNA was extracted from embryos staged according to Bordzilovskaya *et al.* (1989), as indicated on the figure. cDNA was amplified using primers specific to *Axbra* or *AxEF-1 α* , as indicated, using previously published methods (see Bachvarova *et al.* 2001). (b–e) Expression of *Axbra* RNA as seen by *in situ* hybridization to sections of axolotl embryos. (b) Sagittal section at mid gastrula (stage 11), demonstrating *Axbra* RNA at the dorsal lip (large arrow) and the ventral lip (small arrow) of the blastopore. (c). Cross-section at late gastrula (stage 12) near the blastopore showing expression throughout the marginal zone and newly invaginated mesoderm. (d) Cross-section at early neurula (stage 15) showing expression in the notochordal plate (arrow) forming the roof of the archenteron. (e) Cross-section at stage 22 with labelled notochord (arrow). Staining in the yolk represents background. In sections in (c) and (d), nuclei were counterstained red with safranin. a: archenteron.

isolated from axolotls genes involved in the development of the germline, that were identified in other species. These include the *vasa* and *dazl* genes, both of which encode RNA-binding proteins, in addition to a homologue of the mouse *Oct-4* gene, encoding a transcription factor expressed in mouse PGCs. We included each of these genes in independent phylogenetic analyses, revealing the following surprising relationships. In each of the three analyses the axolotl sequence was more closely aligned with the mouse sequence than it was with the homologous sequence from *Xenopus*. The relationships reflect conservation of genes that govern a shared developmental process; they do not reflect the known species phylogeny. Further analyses suggest that frog sequences have diverged

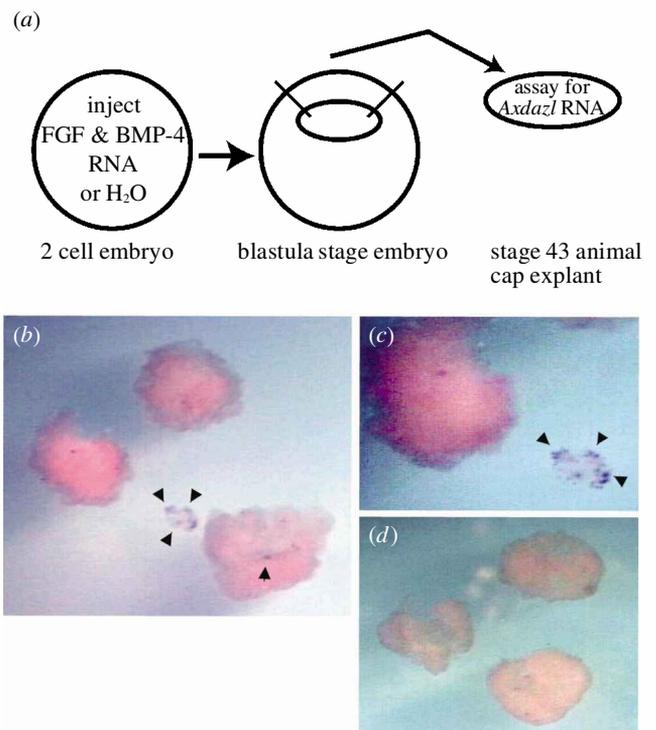


Figure 2. Induction of PGCs in isolated axolotl animal caps. (a) Schematic diagram showing experimental protocol. (b) Animal caps injected with eFGF and BMP-4 RNAs, probed for *Axdazl* expression by whole-mount *in situ* hybridization, using digoxigenin labelled *Axdazl* antisense RNA as a probe (see Johnson *et al.* 2001). Arrows indicate *Axdazl* positive cells. *Axdazl* expressing cells are always produced in clusters in these experiments. (c) Same figure as in (b), magnified. (d) Animal cap from embryo injected with H₂O. *Axdazl* expressing cells are absent.

from a conserved sequence that has been retained by mouse and axolotl. The results suggest that the similarity of germ cell determination in mice and axolotls results from conservation of a shared ancestral mechanism, not convergence on a similar process. The data are discussed with respect to how and why different modes of germ cell determination have evolved in the vertebrates.

2. BRACHYURY EXPRESSION IS UNIFORM WITHIN THE MESODERM WITH GERM-CELL-FORMING POTENTIAL

Previously we presented results supporting the view that axolotl embryos do not contain germ plasm (Johnson *et al.* 2001). As germ cells are then unlikely to be predetermined, this raises the issue of the character of the mesoderm from which the germ cells arise during axolotl development.

PGCs have never been identified in axolotl embryos prior to the early tailbud stage when they have been observed by Ikenishi & Nieuwkoop (1978) in the posterior lateral plate mesoderm. However, deletion and transplantation studies performed on embryos from earlier stages have defined the regions from which these cells arise. Deletions of invaginating ventral marginal zone, adjacent to the domain containing the presumptive posterior blood islands, resulted in elimination of gonadal PGCs in later stage embryos (Nieuwkoop 1947). Smith (1964) dissected

genetically marked tissue from the involuting lateral lips of late gastrulae, transplanted this into the same region of a recipient embryo, and produced fertile adults harbouring germ cells derived from donor tissue. Finally, Maufroid & Capuron (1972) performed a series of extirpation experiments that demonstrate that at the early tailbud stage the PGCs are located postero-ventrally, lateral to the cloaca. Together, these studies demonstrate that in urodeles PGCs derive from posterior ventrolateral mesoderm.

In a previous report, we probed staged axolotl embryos to detect expression of the axolotl *wnt-8* gene, *Axwnt-8* (Bachvarova *et al.* 2001). We found that from mid-gastrula stages onward, *Axwnt-8* was expressed in newly invaginated mesoderm and early lateral plate mesoderm in the posterior region, the region known to include precursors of PGCs. Since *Axwnt-8* expression was uniform within the ventral and lateral tissue, we assume that it was expressed within the presumptive germ cells. This suggests that germ cells in axolotls arise from mesoderm equivalent to that which forms somatic derivatives. To extend these observations we cloned the axolotl *brachyury* gene, *Axbra*, to probe its expression in developing embryos. In vertebrate embryos *brachyury* is a pan-mesodermal marker (Smith *et al.* 1991), and recent studies with mouse embryos have indicated that the *brachyury* gene is expressed in presumptive PGCs (Saitou *et al.* 2002). We asked a similar question of axolotl embryos.

We isolated *Axbra* by screening a neurula stage axolotl cDNA library (Busse & Seguin 1993) at high stringency with a *brachyury* sequence isolated by RT-PCR. The predicted protein sequence was aligned with *brachyury* sequences from other vertebrates. Axolotl *brachyury* shares 61% identity and 75% similarity with the *Xenopus brachyury* sequence (data not shown). RT-PCR analysis (figure 1a) performed on RNA from staged embryos shows that the gene becomes transcriptionally active at the early gastrula stage (stage 10), as expected from results with *Xenopus*.

We next examined the spatial expression profile of *Axbra* in early embryos, paying particular attention to those tissues that are known to harbour the germ cell precursors during early development. We sought to determine if there are cell populations within the fields of mesoderm known to produce PGCs, which do not express the *brachyury* gene. By *in situ* hybridization to sectioned embryos, we found that *brachyury* is expressed in a pattern very similar to that in *Xenopus*. *Axbra* RNA is found all around the marginal zone from stage 11 and throughout the newly internalized posterior and lateral mesoderm through the neurula stage (figure 1b–d), with no obvious non-expressing cells. (Interestingly, at stage 15 *Axbra* is expressed in the archenteron roof, where the developing notochord is positioned; Brun & Garson 1984.) Since germ cell precursors pass through this region we conclude that, as in mice, these cells actively transcribe the *brachyury* gene. This suggests that in axolotls PGCs are produced from unspecialized naive mesoderm.

3. INDUCTION OF PGCs FROM AXOLOTL ANIMAL CAPS

Our results suggest that in axolotls PGCs are produced from unspecialized mesodermal cells. In his classic work

to demonstrate the induction of mesoderm, Nieuwkoop (1969) cultured presumptive animal cap ectoderm from axolotl embryos with cells from the vegetal hemisphere. He showed that inducing signals from the vegetal hemisphere were sufficient to convert the presumptive ectodermal tissue to a complex array of mesodermal cell types. Among the cell types induced within the animal moiety of the embryo were PGCs. Later work demonstrated that the PGCs were produced in response to signals from the ventral vegetal cells, which induce ventral mesoderm (Boterenbrood & Nieuwkoop 1973; Sutasurya & Nieuwkoop 1974).

Since the time of Nieuwkoop's experiments, work with *Xenopus* embryos has established that the inducing agents secreted from the cells of the vegetal hemisphere in amphibians are growth factors of surprisingly few major families. These can be introduced into embryos as exogenous agents, by microinjection of synthetic RNAs from cloned templates, and they are capable of inducing mesoderm in isolated animal cap explants (Jones & Smith 1999). The character of the mesoderm produced in these experiments can be controlled either by introducing specific agents or by altering the levels of these agents. Moreover, factors can be used alone or in combination to produce mesoderm of dorsal, ventral or intermediate type.

Among the endogenous growth factors identified in *Xenopus* embryos is eFGF. FGF signalling is required to produce posterior mesoderm in normal embryos (Amaya *et al.* 1991). eFGF maintains the expression of the *brachyury* gene (Isaacs *et al.* 1994), and it can induce mesoderm in animal cap explants. BMPs represent another growth factor family expressed in *Xenopus* (Dale & Jones 1999). BMPs are expressed across the dorsal–ventral (D/V) axis of *Xenopus* embryos. However, BMP antagonists secreted from the dorsal organizer (Spemann organizer) establish a gradient of BMP activity, with high levels of BMP activity on the ventral side and low levels on the dorsal side (Jones & Smith 1998). These, in turn, pattern the mesodermal layer. High levels of BMP activity induce the production of ventral mesoderm, and dorsal mesoderm is produced in response to the low BMP activity on the dorsal side. In animal cap assays, the introduction of exogenous BMP-4 causes ventral mesoderm to be produced (Dale *et al.* 1992; Jones *et al.* 1992). Animal caps isolated from *Xenopus* embryos that are injected with RNA encoding eFGF and BMP-4 express high levels of the embryonic globin gene, α -globin, a marker of primitive blood (Mead *et al.* 1998). Because in axolotls primitive blood develops adjacent to the germ cell precursors (Nieuwkoop 1947), we asked whether the combination of eFGF and BMP-4 is sufficient to direct the induction of PGCs in isolated axolotl animal caps.

Axolotl embryos were injected at the one-cell stage with 5 ng each of RNA encoding *Xenopus* eFGF and BMP-4 (axolotl embryos are 10-fold larger than *Xenopus* embryos, accordingly we inject them with 10 times the amount of RNA; see figure 2). Control embryos were injected with an equal volume of water. The embryos were cultured in amphibian saline media until the early blastula stage (stage 8; Bordzilovskaya *et al.* 1989); then animal caps were cultured in isolation as explants. Animal caps from both experimental and control samples were harvested at an early larval stage (stage 43). This stage was chosen

because of previous reports that PGCs become identifiable in animal caps cultured until about this stage. Also, the PGC specific molecular marker *Axdazl* is strongly expressed by this stage (Johnson *et al.* 2001). Harvested animal caps were probed for cell-specific *Axdazl* expression by whole-mount *in situ* hybridization. In two experiments we detected *Axdazl* expression (arrows) in 100% of those animal caps isolated from embryos injected with eFGF and BMP-4 RNAs (Experiment 1: 7/7; Experiment 2: 5/5). An example of a representative sample is shown in figure 2. By contrast, we have not detected *Axdazl* expression in any of the caps isolated from control embryos (Experiment 1: 0/5; Experiment 2: 0/5). Other experiments have shown that *Axdazl* expressing cells can also be induced in animal caps from uninjected embryos treated with purified growth factors (R. Bachvarova, unpublished data). These preliminary results indicate that PGCs can be induced from axolotl animal caps by simple combinations of defined growth factors, which are also known to produce somatic cell types. Given that axolotl PGCs are not predetermined by germ plasm, this suggests that they are a normal product of mesoderm formation.

4. THE EVOLUTION OF REGULATIVE GERM CELL SPECIFICATION

(a) *Is regulative germ cell specification primitive or derived?*

McLaren (1983) suggested that the PGCs in mouse embryos are derived from uncommitted totipotent cells that arise from the epiblast during early development. In this way mouse PGCs were proposed to differ from those of most other animal model systems in which PGCs are known to be specified cell-autonomously through the differential inheritance of maternally deposited germ cell determinants, i.e. germ plasm. However, only recently, through a combination of transplantation (Tam & Zhou 1996), fate mapping (Lawson & Hage 1994), gene knockout (Lawson *et al.* 1999; Ying & Zhao 2001; Ying *et al.* 2001), tissue explant (Pesce *et al.* 2002) and differential screening (Saitou *et al.* 2002) experiments, has it become clearly established that mouse germ cells are not predetermined, and that they arise in response to appropriate cell-signalling regimes during development. Given the absence of regulative germ cell development in the embryos of non-mammalian model systems, it has been assumed that mammals evolved a novel mode of germ cell specification, perhaps as a part of the evolution of the process of mammalian development in general. Indeed, in mouse embryos, the POU domain transcription factor Oct-4 is required for development of the early embryo (Nichols *et al.* 1998) and its expression is correlated with totipotent cell lineages (Pesce & Scholer 2001). It is expressed throughout the early epiblast, is eventually restricted to PGCs (Scholer *et al.* 1990), and is assumed to be required for germ cell development *per se*. That true homologues of Oct-4 have not been identified in non-mammalian model systems has supported the view that the regulative mode of germ cell specification observed in mouse embryos is a mammalian innovation.

However, Nieuwkoop & Sutasurya (1979) proposed that germ cells are produced by a regulative mechanism in urodele embryos. Indeed, a significant number of stud-

ies have demonstrated that it is possible to induce PGCs from the prospective ectoderm of various urodele species using as inducing agents the vegetal blastomeres of urodele embryos (Nieuwkoop 1969; Boterenbrood & Nieuwkoop 1973; Sutasurya & Nieuwkoop 1974; Michael 1984), the vegetal blastomeres of anuran embryos (Michael 1984) or even soluble inducing agents present in extracts made from chick embryos (Kocher-Becker & Tiedemann 1971). These studies indicate that tissue that normally does not participate in germ cell production can be made to do so if triggered by the appropriate inducing agents, and that these agents are not specialized. Furthermore, Ikenishi & Nieuwkoop (1978) were unable to detect germ plasm in the PGCs of axolotl embryos, suggesting that during normal development PGCs are induced from unspecialized tissue. Recently, Johnson *et al.* (2001) reached a similar conclusion using the axolotl *daz-like* gene as a molecular marker. Thus, regulative germ cell specification appears to be a property of the embryos of at least two lineages of terrestrial vertebrates, mammals and urodele amphibians.

The production of germ cells is among the most critical events in early development, since in the absence of germ cells an organism's lineage will cease to exist. Thus, the existence of highly variable mechanisms of germ cell development appears to present a paradox, since most events of early development have been highly conserved throughout the animal kingdom. To consider a unifying paradigm to explain the divergence of germ cell determining mechanisms, Johnson *et al.* (2003) examined the distribution of species with and without predetermined germ cells in deuterostomes. They concluded that species with predetermined germ cells are associated with derived cell movements during gastrulation, leading to derived adult morphologies (e.g. adult frog morphology). Conversely, species in which germ cells form in response to inducing signals develop from highly conserved gastrulation movements and have more primitive adult morphologies (e.g. adult salamander morphology). (A summary of their conclusions is presented in figure 3.) They concluded that the most likely hypothesis is that germ plasm evolved independently in several different lineages of deuterostomes. They propose (see figure 3) that regulative germ cell specification is primitive, whereas the predetermined mode is derived. As such, among terrestrial vertebrates, anurans as well as avians (Tsunekawa *et al.* 2000) independently evolved germ plasm and determinative germ cell specification. Mammals and urodeles, on the other hand, have retained the primitive mechanism.

(b) *PGC gene phylogeny*

Fundamentally, for any newly acquired trait to evolve it requires that the trait itself is genetically transmissible. In this regard, the sequences of the genes that govern PGC determination provide intriguing insights into how germ cell-determining mechanisms evolved.

The usefulness of DNA sequence analysis as a method for gauging species relatedness is widely recognized. In general, the relatedness of sequence between genes of any given species is a reflection of the order of species divergence. Thus, because urodeles and anurans are sister taxa (i.e. they are both amphibians), any gene would be expected to show greater phylogenetic relationship

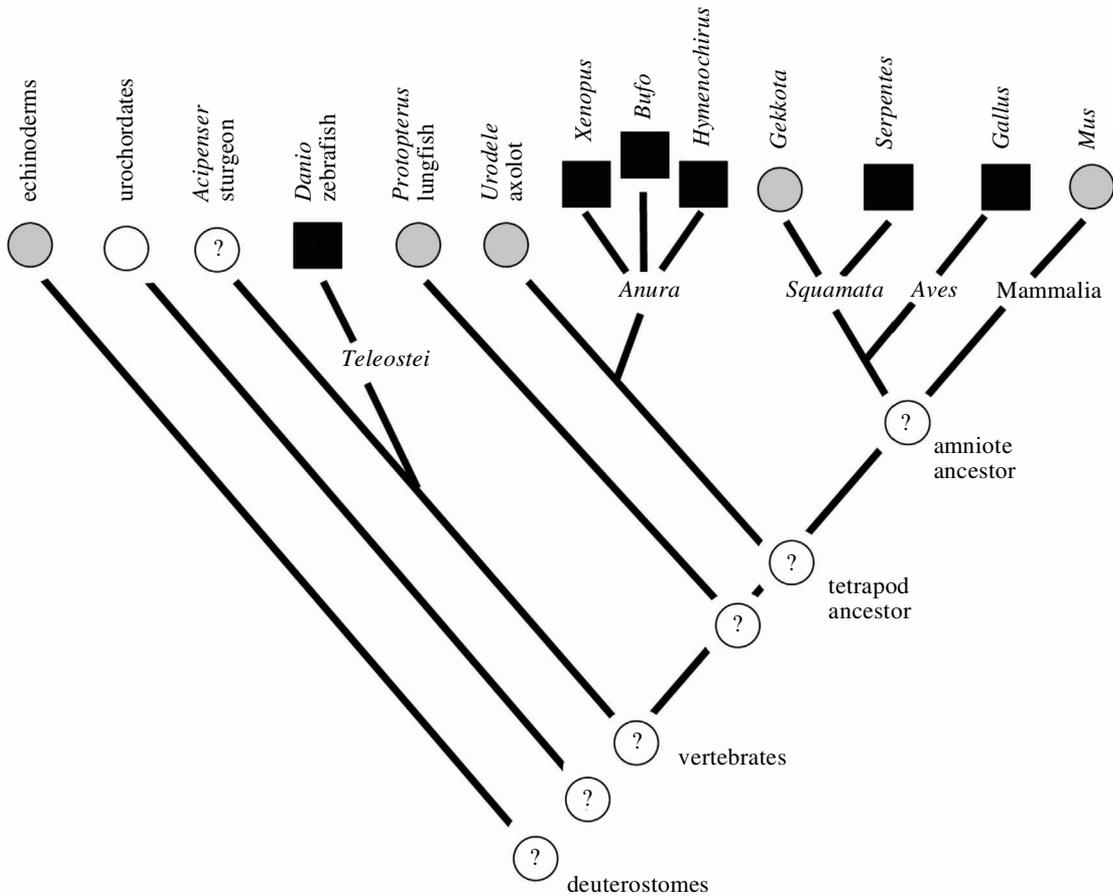


Figure 3. The distribution of germ plasm in animals with either primitive or derived modes of gastrulation. Gastrulation movements are defined as either being primitive to the vertebrate lineage, or a derived mode, as judged by the position of the notochord at the completion of gastrulation (see Johnson *et al.* (2003) for details). Embryos with derived movements tend to give rise to adults with derived adult features. Gastrulation movements are correlated with whether or not available evidence indicates that the embryos in question contain germ plasm, as indicated. Key: circles, conserved embryology; squares, derived embryology; black symbols, evidence for predetermined germ cells; grey circles, evidence for regulative germ cells; open symbols, evidence for mechanism of germ cell determination is inconclusive.

between these groups than with less related groups, such as mammals. We have tested this hypothesis with several genes known to regulate early embryogenesis, *GATA-2*, *Brachyury* and *gooseoid*, and in each case the gene reflects the species phylogeny, with axolotl and *Xenopus* being highly related (data not shown). However, we performed similar analyses on genes that are known to regulate germ cell determination and obtained quite different results.

Several genes that regulate germ cell development in diverse species have now been identified. As discussed above, the gene encoding Oct-4 is expressed in mouse PGCs. In addition, the germ-cell-specific RNA-binding proteins *dazl* and *vasa* have been found in many model organisms. We isolated homologues of each of these genes from axolotls (for *Axdazl*, see Johnson *et al.* 2001; for *AxOct-4*, T. Masi and A. D. Johnson, in preparation; for axolotl *vasa* (*Axvh*), M. Drum and A. D. Johnson, in preparation). In addition, we isolated homologues of each of these genes from the African lungfish, *Protopterus annectans* (M. Drum and A. D. Johnson, in preparation). *Protopterus* was chosen because it has primitive adult and embryonic features, and because phylogenetic information indicates that it is likely to be among the extant species that is most closely related to the ancestral tetrapod (Zardoya *et al.* 1998). Thus, by analysing the sequences

of germ cell specific genes from lungfish, within a phylogenetic context that included homologous genes from terrestrial vertebrates, we hoped to reveal the patterns of sequence conservation and divergence that evolved in vertebrates on land. These data could be used to infer how mechanisms of germ cell determination evolved in the tetrapods.

Figure 4 shows the general results of phylogenetic analysis of germ cell-specific genes from mouse, axolotl, *Xenopus* and lungfish. Superimposed on the same figure is the known phylogeny of these species. The results demonstrate that the sequence of germ cell specific genes does not track with species phylogeny, as other genes do (see above). Thus, rather than recover the standard frog–salamander species relationship, the analysis of germ cell specific genes recovers a close lungfish–salamander–mammal relationship, indicating that germ cell-specific genes have diverged in frogs. When these data are considered with respect to the correlation of regulative germ cell development in animals with primitive embryological development (see figure 3), they strongly support the likelihood that determinative germ cell development in frogs is a divergent trait. Moreover, since determinative germ cell development is known to occur in various species of teleost fishes, as well as avians, the data suggest that germ

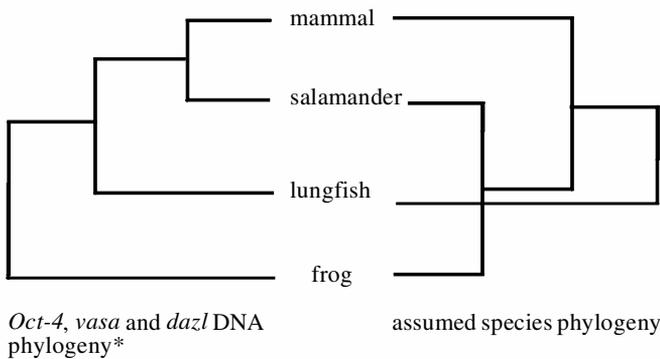


Figure 4. A comparison of the general PGC determination gene phylogeny and the assumed species tree. As described in the text, the gene phylogeny reflects the similarity of the PGC determination mechanism and is incongruent with the expected species phylogeny. The gene phylogeny results were recovered under both parsimony and likelihood analytical frameworks. The asterisk denotes that the *dazl* tree did not place lungfish in the position shown here, but *dazl* still reflected the mechanism relationships by recovering the mammal–salamander clade.

plasm has evolved independently in several vertebrate lineages.

Among the major results of this study was the isolation of cDNAs encoding Oct-4 from axolotl and lungfish. As discussed above, sequences homologous to Oct-4 have been unobtainable from conventional model organisms. However, the embryos of model organisms such as zebrafish, *Xenopus* and chick, contain germ plasm, and their germ cells are specified cell-autonomously. We suggest that Oct-4 is necessary for the process of regulative germ cell specification, and is therefore present in the genomes of animals whose embryos retain primitive embryological features. As a demonstration of this, we have also isolated an Oct-4 sequence from the Gulf sturgeon, *Acipenser oxyrinchus* (M. Drum and A. D. Johnson, in preparation), a species with the predicted characteristics (Ballard & Ginsburg 1980; Bolker 1993; Johnson *et al.* 2003).

5. DISCUSSION AND CONCLUSIONS

(a) Why did divergent germ cell specifying mechanisms evolve?

Above and elsewhere (Johnson *et al.* 2003), we have proposed that regulative germ cell specification is primitive to the vertebrate lineage. This implies directly that determinative germ cell specification, as observed in zebrafish, anuran amphibians and chicks, evolved independently in each of the respective lineages. This hypothesis refutes the long-standing belief that determinative germ cell specification is an ancestral conserved process, shared within the deuterostomal and protostomal lineages. We consider the appearance of germ plasm in the embryos of divergent lineages of animals to be an example of convergent evolution.

To suggest convergent evolution of a process or a structure demands that it must be acted upon favourably by the pressures of natural selection. The end result of natural selection is to select for evolutionary events that enhance an organism's ability to reproduce, i.e. to pass genetic

material to a new generation through the germline. It then follows that events that stabilize the germline will undergo positive selection, whereas events that compromise maintenance of the germline will encounter negative selective pressure. In keeping with this, while mutations that would prevent the development of germ cells would clearly terminate an animal's lineage and be eliminated from a population, events that guaranteed the production of germ cells might be favourably selected. Within this context, we assume that the emergence of germ plasm within an animal lineage would stabilize the process of germ cell formation in the embryos of that lineage, and be favourably selected.

Conventionally, natural selection is thought to act on adult traits. However, it is important to recognize that any event that would enhance the survival of embryos would also encounter positive selection. For example, any event that would speed the development of a free-swimming aquatic embryo (such as an amphibian embryo) would be favoured by natural selection. In this regard, it is reasonable to assume that the specification of germ cells by pre-existing maternal molecules, i.e. germ plasm, occurs much earlier in development than when PGCs are specified by extracellular signals, and this would probably allow accelerated embryogenesis. In addition, germ cells specified by maternal molecules are likely to be less sensitive to changes in developmental signalling, which could create conditions permissive for embryos to evolve novel signalling mechanisms that further accelerate development. In this regard, it should be noted that the embryos of anuran amphibians and teleost fishes, both of which contain germ plasm, develop considerably faster than their sister taxa (urodeles and chondrosteian fish) with regulative germ cells.

(b) Mechanistic versus species phylogeny

Phylogenetic reconstructions are used to understand a species' evolutionary history. Rarely are developmental mechanisms used as characters to construct phylogenies, in part because it is often assumed that most aspects of development are highly conserved within specific groups. However, germ cell specification is a process that is not conserved, and this is particularly evident in the amphibians. Through phylogenetic analysis of genes that govern germ cell development, we have demonstrated that mammals, urodeles and lungfish can be grouped into a class of animals whose embryos retain an ancestral mode of germ cell development in addition to retaining primitive embryological features (see Johnson *et al.* 2003). This phylogeny of developmental mechanisms or 'mechanistic phylogeny', does not emerge from the analysis of the adult traits more conventionally used as characters for phylogenetic reconstructions. However, in some cases, understanding the evolution of developmental traits may provide more relevant insight into how a species evolved. Within this framework, on the basis of the mechanistic phylogeny that we present here, we propose that the amphibian template from which mammals evolved was one resembling urodele but not anuran amphibians.

We suggest that it is important to recognize processes in development that are representative of vertebrates at large, and to distinguish these from processes that are exclusive to specific lineages. Presently, much effort is

being put towards evolutionary genomics in the hope of understanding how the human genome evolved. Within the present context, we suggest that attention should be focused on the genomes of animals with primitive vertebrate features, since the genomes of these animals are more likely to approximate the ancestral genome from which the mammalian genome evolved.

(c) *Axolotls as a model system*

We have developed axolotl embryos as a model system to study regulative germ cell development. Our initial focus was to isolate markers that would enable us to characterize the development of PGCs, related cell types and the tissues from which they arise. We present data indicating that it is possible to induce ectopic PGCs using controlled conditions that include cloned growth factors. We hope to use this system to dissect the molecular pathways that lead to the production of PGCs using conventional technologies developed for *Xenopus* embryos. As no other non-mammalian model system produces PGCs by regulative mechanisms, axolotl embryos provide unique advantages towards understanding how maintenance of the totipotent condition is governed during vertebrate development.

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GLOSSARY

- BMP: bone-morphogenetic protein
 eFGF: embryonic fibroblast growth factor
 PGC: primordial germ cell