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Expression of *Dazl* and *Vasa* in turtle embryos and ovaries: evidence for inductive specification of germ cells

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SUMMARY In bilaterian animals, germ cells are specified by the inductive/regulative mode or the predetermined (germ plasm) mode. Among tetrapods, mammals and urodeles use the inductive mode, whereas birds and anurans use the predetermined mode. From histological data it has been predicted that some reptiles including turtles use the inductive mode. Examining turtle oocytes, we find that *Dazl* RNA, *Vasa* RNA, and Vasa protein are not localized, suggesting that germ plasm is not present. In turtle embryos at somite stages, primordial germ cells (PGCs) expressing *Dazl* lie on a path from the lateral posterior extraembryonic endoderm through the gut to the gonad as previously described. In gastrulating embryos, cells expressing *Dazl* are found in the blastoporal plate and subsequently below the blastoporal plate, indicating that PGCs are generated at the equivalent of the early posterior primitive streak of mammals. *Vasa* RNA is expressed in somatic cells of gastrula to early somite stages, and *Vasa* RNA and protein are expressed in PGCs of later embryos. Taken together the evidence strongly suggests that turtles, and other reptiles (lacertoid lizards) with the same location of PGCs in embryos, use the inductive mode of germ cell specification. Phylogenetic analysis of the available evidence supports the following hypotheses: (1) the inductive mode is basal among reptiles, indicating that this mode was maintained as basal tetrapods evolved to amniotes, (2) the predetermined mode arose twice within reptiles, and (3) the induced mode may be used in several lepidosaurs whose PGCs are located in an unusual pattern distributed around the embryo.

INTRODUCTION

Two modes of germ cell specification are found among vertebrates, and indeed throughout most of the animal kingdom. From phylogenetic analysis of a variety of species it has been concluded that the inductive mode, in which germ cells are induced from a wide region of competent cells within the embryo, is the basal mode, whereas the predetermined mode, in which inheritance of a specific plasm specifies the germ cells in the early embryo, is a derived mode (Johnson et al. 2001, 2003a, b; Extavour and Akam 2003; Crother et al. 2007). Among vertebrates, definitive evidence indicates that birds, anurans, and zebrafish use the predetermined mode, whereas mammals and urodeles use the inductive mode. Moreover, the evidence suggests that lungfish and sturgeon are in the latter group (Johnson et al. 2003b; Zelazowska et al. 2007;A. D. Johnson et al., unpublished data). Among tetrapods, the study of reptiles may provide key information on the evolution of germ cell development in amniotes, including primitive amniotes that gave rise to mammals and reptiles. With respect to position of early germ cells and mode of arrival in the gonad, reptiles are a diverse group. The histological evidence is difficult to interpret for some species, and for others suggests they may use one mode or the other or even both (Tribe and Brambell 1932; Pasteels 1953; Hubert 1969, 1976, 1985; Nieuwkoop and Sutasurya 1979).

The early development of germ cells in turtles has been studied in several species, identifying primordial germ cells (PGCs) by their spherical shape, euchromatic nucleus, distinct nucleolus, retention of yolk granules, distinct cell outlines, and/or PAS staining material (Allen 1906; Dustin 1910; Jordan 1917; Risley 1933; Fujimoto et al. 1979; Merchant-Larios et al. 1989). When first detected at early somite stages, these cells are in the junctional endoderm just outside the embryo in the lateral posterior region. By the 14 somite stage they are found more medially, progressing through the hindgut/

midgut endoderm, splanchnic mesoderm, and finally to the gonadal ridge. As these results are similar to those found for mammals, and different from that for birds, it has been provisionally concluded that turtles (and lizards with a similar location of PGCs in the embryo) use the inductive mode of germ cell determination (Johnson et al. 2003b; Crother et al. 2007).

We have prepared probes for the germ cell-specific markers *Dazl* and *Vasa*. *Dazl* encodes an RNA binding protein and *Vasa* encodes a DDX4 type of ATP-dependent RNA helicase. These proteins are expressed in germ cells of most organisms (see Extavour and Akam 2003; Johnson et al. 2003b) and are involved in regulating translation of germ line mRNAs (Reynolds et al. 2005; Thompson et al. 2008). Using these probes we have analyzed the position of PGCs in turtle embryos and have been able to confirm and expand the details observed by earlier workers for somite and later stages. We have examined oocytes in adult ovaries with these molecular markers for evidence of the presence of germ plasm. Most important, we have been able to describe the position of presumptive PGC precursors in embryos at earlier stages than previously possible. The results of these observations strongly suggest that turtles use the inductive mode of germ cell determination.

MATERIALS AND METHODS

Embryos

Laid eggs of *Trachemys scripta* (red-eared sliders) were shipped overnight or collected from Robert Clark's Turtle Farm in Hammond, LA. Freshly laid embryos were dissected from the yolk, and later embryos loosened from the shell. Eggs were incubated at 24–30°C and staged according to Yntema (1968) for *Chelydra serpentina* embryos, fixed in 4% paraformaldehyde in phosphate-buffered saline, transferred to 100% methanol, and stored at −20°C.

Cloning of *T. scripta* cDNAs

Probes derived from the coding region of axolotl *Dazl* (Johnson et al. 2001) and *Vasa* (Bachvarova et al. 2004) sequences were used to screen a lambda ZAP (Stratagene, La Jolla, CA, USA) cDNA library prepared from ovarian *T. scripta* RNA, using conventional methods. Two identical *Dazl* and *Vasa* clones were isolated. No other sequence with any relation to these genes was identified. Plasmid DNA was produced by in vivo excision.

In situ hybridization (ISH)

RNA probes were transcribed from plasmid DNA in the antisense orientation, using digoxigenin-labeled UTP as described by Johnson et al. (2001). Embryos and ovaries were embedded in paraffin and sectioned at 10–15 μm; in some cases alternate sections were stained with hematoxylin and eosin (H&E). ISH was carried out on sections or whole embryos essentially as described by Nagy et al. (2003) with the following changes: proteinase K treatment ranged from 1 to 8 min at 3 μg/ml in PBST at 37°C, the hybridization solution contained 1% SDS and 4 × SSC, temperature of hybridization was 63°C, and after hybridization samples were washed twice in 50% formamide, 2 × SSC, 0.5% SDS at 63°C. Sections were mounted in 80% glycerol.

Immunohistochemistry

Immunohistochemical detection was performed with an automated processor Discovery XT (Ventana Medical Systems, Tucson, AZ, USA). Paraffin sections were hydrated, blocked with 10% normal goat serum in 2% BSA, and incubated with the polyclonal rabbit anti-Vasa antibody (DDX4/MVH, Abcam, Cambridge, MA, USA) or normal rabbit IgG for 30 min at 37°C. A streptavidin-HRP and DAB detection kit with inhibitors (Ventana Medical Systems) was used according to the manufacturer's instructions.

RESULTS

Sequence of turtle (*T. scripta*) *Dazl* and *Vasa*

The amino acid sequence encoded by turtle *Dazl* cDNA was very similar to that of chick, mammalian, and urodele Dazl proteins (Table 1). The sequence encoded by turtle *Vasa* cDNA included a 150 amino acid conserved region with strong similarity to mammalian DDX4/Vasa (Table 1). Note that the turtle Vasa sequence is more similar to mammalian Vasa than to that of its nearest available relative, the chick.

Table 1. Comparison of turtle *Dazl* and *Vasa* amino acid sequences with those of various vertebrates

	Dazl		Vasa	
Organism	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
Chick	82	89	77	88
Mammal[1]	79	86	88	95
Urodele[2]	76	84	80	89
Anuran[3]	57	71	76	88
Zebrafish	48	62	75	85

The *Trachemys scripta* Dazl sequence (accession number FJ665612) encodes 258 amino acids, starting at amino acid 38 and running to the C terminus of the mouse Dazl protein. It includes the 90 amino acid RNA recognition motif.

The *T. scripta* Vasa sequence (accession number FJ665613) encodes 357 amino acids starting at the N terminus. The analysis covered the most conserved 150 amino acids corresponding to amino acids 213–362 of the mouse DDX4/vasa protein. This sequence includes the conserved ATP binding motifs PTREL and AQTGSGKT, but not the DEAD box.

[1]Values are averages for human, mouse, and dog.

[2]Values for Dazl are averages of axolotl (*Ambystoma mexicanum*) and *Cynops pyrrhogaster*, and for Vasa are averages of axolotl and *Pleurodeles waltl*.

[3]Values are averages of *Xenopus laevis* and *Rana lessonae*.

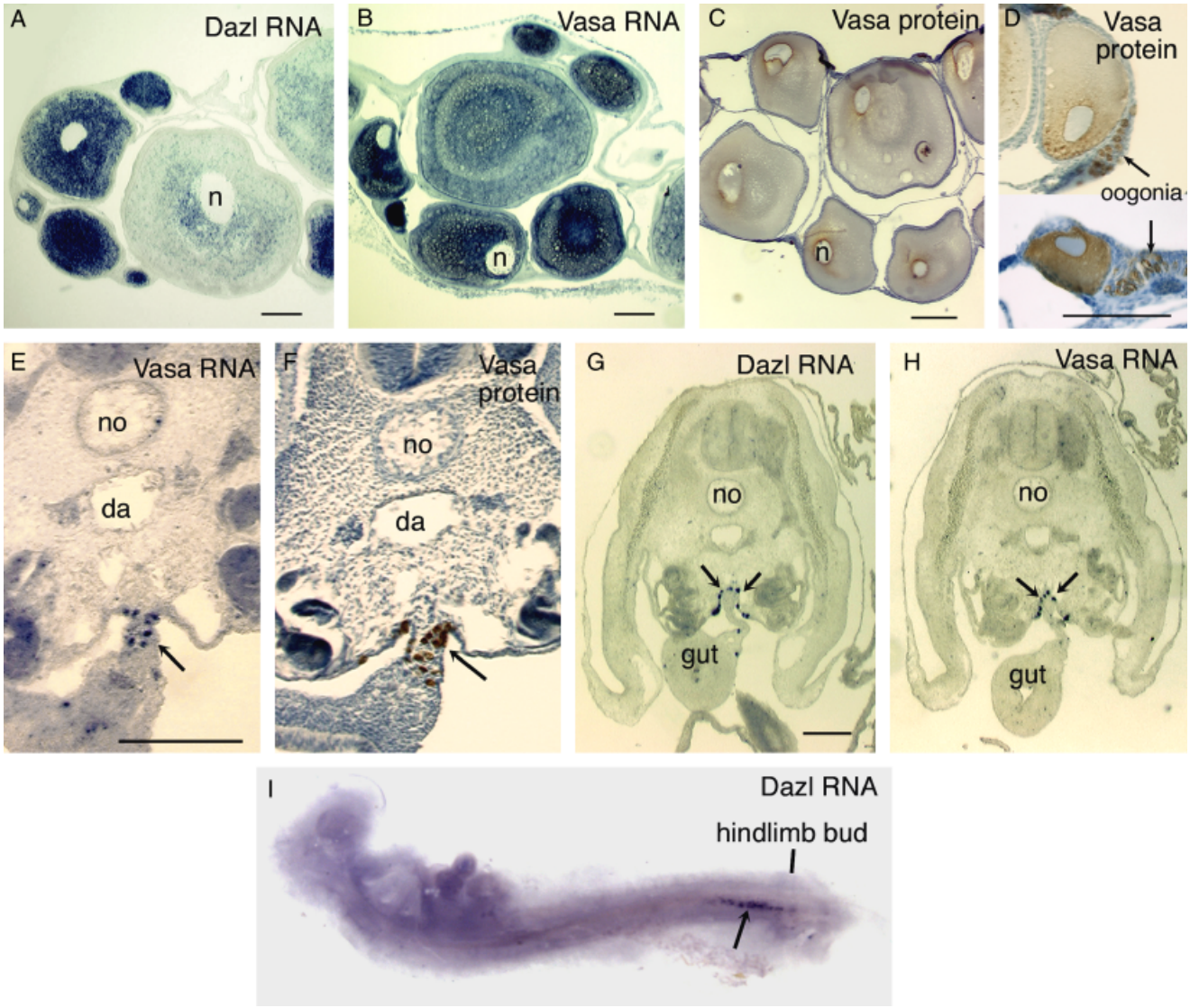

Fig. 1. Expression of *Dazl* and *Vasa* RNAs and Vasa protein in ovary and late embryos. (A – D) Sections of adult ovary showing oocytes of various sizes. (A) ISH, *Dazl* probe. (B) *Vasa* probe. (C) Vasa protein detected by immunohistochemistry showing oocytes 400–600 μm in diameter with a thin rim of Vasa protein (brown) around the nucleus (n). Counterstained with hematoxylin. (D) Vasa protein in small oocytes. In the lower oocyte (about 100 μm in diameter) it is distributed homogeneously. In the upper oocyte (about 200 μm in diameter) Vasa protein forms a gradient toward the eccentrically located nucleus. Vasa protein is present in the very small oogonia. (E, F) Sections of a stage 12 early limb bud embryo. Arrows indicate presumed PGCs in the dorsal mesentery. (E) ISH, *Vasa* probe. (F) Vasa protein (brown) detected by immunohistochemistry. (G, H) Sections of a stage 13 limb bud embryo. Arrows indicate presumptive PGCs arriving in the coelomic epithelium of the gonadal ridge. (G) ISH, *Dazl* probe. (H) *Vasa* probe. (I) Whole mount ISH, *Dazl* probe, stage 11 embryo about 7 mm in length, showing the position of the PGCs in the posterior trunk. Arrow indicates PGCs near the midline. Background stain is seen throughout the embryo. Scale bars = 200 μm. da, dorsal aorta; n, nucleus; no, notochord; ISH, in situ hybridization; PGCs, primordial germ cells.

Verification of the germ cell specificity of the probes

To verify that our probes for germ cells are expressed in definitive germ cells, we carried out ISH and immunohistochemistry on sections of adult ovaries. Small growing oocytes reacted positively with the probes for *Dazl* and *Vasa* RNA (Fig. 1, A and B). Vasa protein was present in growing oocytes (Fig. 1, C and D) and in oogonia (Fig. 1D). Sections hybridized to a control probe or analyzed by immunohistochemistry using normal rabbit IgG rather than anti-Vasa antibody gave no signal (not shown). In addition, all three probes-labeled cells distributed in a pattern typical of that of definitive germ cells near or at the gonad in limb bud stage embryos (Fig. 1, E–I). Although *Dazl* RNA and Vasa protein

were expressed exclusively in germ cells, *Vasa* RNA was also found in somatic cells of the embryo.

Expression of *Dazl* and *Vasa* in adult turtle ovaries

Oocytes up to 1 mm in diameter were examined for evidence of localization of *Dazl* or *Vasa* RNA, or Vasa protein. In anuran oocytes germ plasm is found at the vegetal pole of growing oocytes and eggs. However, because turtle oocytes grow to a relatively large size (*T. scripta* egg yolks are about 1.9 cm in diameter by 2.7 cm in length), it was more likely that, if present, germ plasm would take the form found in hen oocytes. In these, Vasa protein is localized in small cortical bodies in oocytes of about 300 μm in diameter (Tsunekawa et al. 2000).

In growing turtle oocytes up to 800 μm in diameter both RNAs were distributed evenly throughout the cytoplasm, with no evidence of a region of concentration of RNA (Fig. 1, A and B). Staining decreased to approximately background levels in oocytes of 1 mm diameter.

Vasa protein was found in a rim around the nucleus in oocytes larger than 300 μm in diameter (Fig. 1C). In the smallest growing oocytes it was found homogenously distributed in the cytoplasm, and in slightly larger oocytes in a gradient in the cytoplasm as it moves toward the eccentrically located nucleus (Fig. 1D). No evidence for a concentration of the protein at one pole or in multiple small peripheral bodies (as in hen oocytes) was found, including cases in which all sections of oocytes up to 700 μm were examined.

Expression of *Dazl* and *Vasa* RNA in early somite to limb bud stage embryos

In a very early somite embryo, *Dazl*-expressing cells were found in a horseshoe-shaped region around the posterior end of the embryo, lying under the peripheral blastoporal (primitive) plate (Fig. 2, A–A″). These cells are in a position similar to that described for germ cells identified by histological criteria at early somite stages (Allen 1906; Jordan 1917; Risley 1933). The PGCs lie in the junctional extraembryonic endoderm between the yolky endoderm of the area opaca and the embryonic endoderm.

At this stage *Vasa* RNA was expressed in the thick mesodermal mass of the blastoporal plate (Fig. 2B). More anteriorly, it was present in all layers, extending out toward but apparently not overlapping with the *Dazl*-expressing cells in the lower layer (Fig. 2, B′–B″). No Vasa protein was found by immunohistochemistry at these stages (not shown).

At later stages our results confirmed those of earlier workers. In embryos with 5–10 pairs of somites the PGCs continue to form a horseshoe around the posterior end. In cross sections they appear as two clusters of *Dazl*-expressing cells on either side of the embryo, lying in the junctional endoderm (Fig. 2, E and G–G″). These clusters could be identified as eosinophilic in H&E-stained sections (Fig. 2F), lying in the splanchnopleure of the extraembryonic coelom. Hybridization to a control probe showed little expression (Fig. 2, G′,H′, inset).

During these stages *Vasa* RNA expression declined in the neural tube and axial mesoderm, was strong in mesoderm at the angle of the forming coelom (Fig. 2, C and H-H″) and extended anteriorly well beyond the germ cell region (not shown). It was moderate in endoderm extending laterally to overlap with the PGCs (Fig. 2, H-H″). *Vasa* RNA was also seen in bilateral clumps of cells in the mesoderm of the area opaca, tentatively identified as blood island precursors (Fig. 2C). Hybridization at higher stringency did not reduce the signal in somatic cells, suggesting this signal was not due to a less related RNA. However, anti-Vasa antibody did not detect Vasa protein in somatic cells or in germ cells (Fig. 2D), indicating little translation of *Vasa* RNA. In several other species *Vasa* RNA, probably maternal, is found in somatic cells of the early embryo, but translation is more restricted (see Ikenishi and Tanaka 2000; Knaut et al. 2002; Voronina et al. 2008). Because Dazl regulates translation of *Vasa* RNA (Reynolds et al. 2005), its absence could explain the lack of Vasa protein in somatic cells.

In embryos with 14–19 pairs of somites the bilateral cords of *Dazl*-expressing PGCs have lost their connection in the posterior region. As the trunk folds ventrally and the gut elongates and narrows, the PGCs appear more medially, now located within the posterior gut (Fig. 2I). By very early limb bud stages they have reached the forming dorsal mesentery and germinal epithelium (Fig. 2K), and at an early limb bud stage they are in their definitive position (Fig. 1I).

In embryos with 14–19 pairs of somites, expression of *Vasa* RNA continued especially in the coelomic angle and intermediate mesoderm, and became distinct in individual PGCs (Fig. 2J). At limb bud stages, Vasa protein became detectable in individual PGCs (Fig. 1F) whereas expression of *Vasa* RNA in somatic cells declined (Figs. 2, L and 1, E and H).

Throughout the embryonic period, the number of germ cells at a given stage was variable (see Risley 1933; Fujimoto et al. 1979 for earlier data), and some PGCs appeared to be located further anterior than expected if destined for the gonads.

Expression of *Dazl* and *Vasa* RNA from gastrula to neural plate stages

The position of germ cells has not been described during gastrulation and neural plate stages of turtle embryos. At oviposition gastrulation is already underway. In stage 0–1 embryos (Yntema 1968; Coolen et al. 2008) a prominent blastopore is located in the posterior half of the embryonic disc and a blastoporal canal of varying length extends ante-

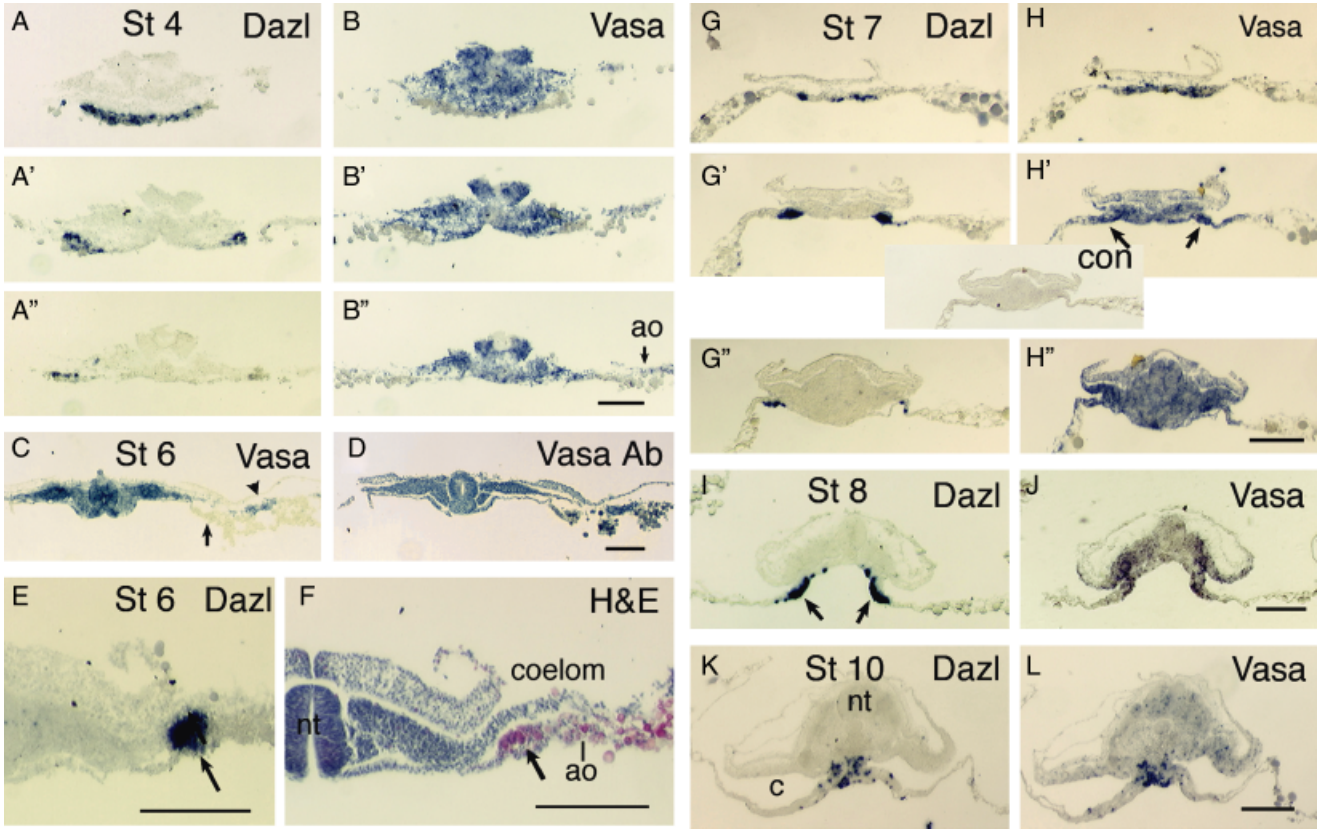

Fig. 2. *Dazl* and *Vasa* expression in cross sections of the posterior region of somite stage embryos. (A–A″, B–B″) Successively more anterior regions of a very early somite stage embryo (stage 4). (A–A″) ISH, *Dazl* probe. (B–B″) *Vasa* probe on nearby sections. (A) The posterior region of the blastoporal plate where the PGCs are continuous across the midline. (A′) Just anterior to the neurenteric canal. (A″) Most anterior extent of the PGCs at the level of the posterior closing neural tube. (C, D) An embryo with five to six pairs of somites (stage 5). (C) ISH, *Vasa* probe. Arrow indicates position of *Dazl* expression in PGC in a nearby section. Arrowhead indicates clump of *Vasa*-expressing cells in the area opaca lateral to the PGCs. (D) Anti-Vasa antibody on a nearby section, stained with hematoxylin. (E, F) High magnification views of one side of a stage 6 embryo. (E) ISH, *Dazl* probe. Arrow indicates a cluster of PGCs. (F) A nearby section stained with H&E, showing a larger region at the same magnification. Arrow indicates a cluster of PGCs (pink) within the endodermal layer but not bordering the yolk sac cavity. Yolk platelets (pink) to the right of the PGCs are in the extraembryonic endoderm of the area opaca. The forming neural tube is cut by the neurenteric canal. The extraembryonic coelom is indicated. (G–G″, H–H″) Successively more anterior regions of a 10 somite embryo (stage 7). (G–G″) ISH, *Dazl* probe, (H–H″) *Vasa* probe on nearby sections. (G) The posterior edge of the blastoporal plate. A few stained PGCs are present. (G′) Two clusters of stained PGCs are seen (G″) just anterior to the neuropore with closed neural tube. Only a few stained PGCs are present at this level. (H′, H″) Arrows indicate the approximate position of PGCs. Inset in (G′, H′): nearby section hybridized to a control probe, axolotl sense *Wnt 8*. (I, J) A 14 somite embryo (stage 8). (I) ISH, *Dazl* probe, revealing PGCs (arrows) in the walls of the forming gut. (J) *Vasa* probe on a nearby section, expression is apparent in PGCs (arrows). (K, L) A 24 somite embryo (stage 10), sections through the midgut region with open gut wall. (K) ISH, *Dazl* probe. (L) *Vasa* probe. Both demonstrate PGCs in the gut and forming dorsal mesentery. Scale bars = 200 μm. ao, area opaca; c, coelom; nt, neural tube; ISH, in situ hybridization; H&E, hematoxylin and eosin; PGCs, primordial germ cells.

riorly from the blastopore under the blastopore lip (Fig. 3, see diagrams). The blastoporal plate lies initially posterior to the blastopore and later projects anteriorly to form the floor of the blastoporal canal (Fig. 3, B and C).

The pattern of *Dazl*-expressing cells was somewhat variable during this period. In five embryos, scattered cells expressing *Dazl* at a low level were located in the blastoporal plate including in the floor of the canal (Fig. 4, A, A′ and G, I). Hybridization to a control probe resulted in no significant label (Fig. 4H). In two embryos, a significant number of labeled cells were also seen in the lower layer (the lower layer will be referred to as endoderm, without attempting to distinguish between hypoblast, endoblast, and embryonic endoderm) (Fig. 4C). In five embryos, presumptive PGCs expressed *Dazl* more strongly and were seen predominantly in or adjacent to the endodermal layer (Fig. 4, E and K). We assume that these three patterns represent a developmental sequence, suggesting that *Dazl*-expressing cells (PGCs) orig-

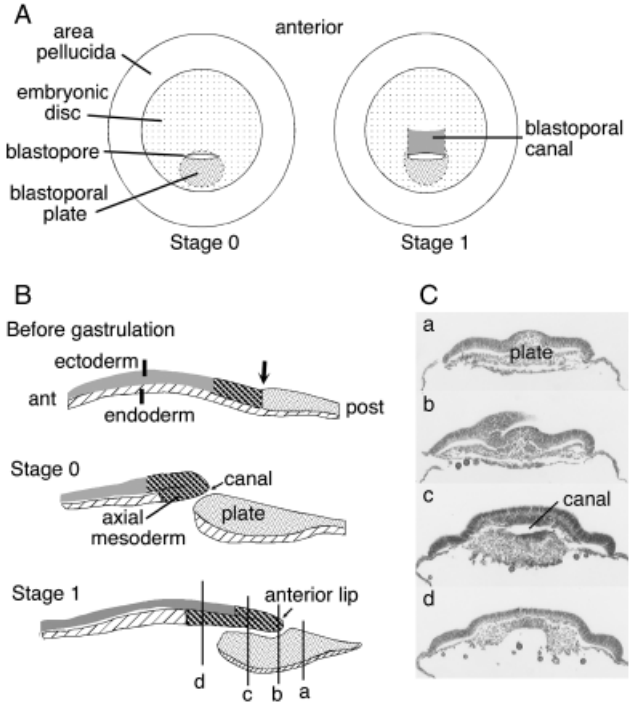

Fig. 3. Overview of turtle gastrulation. Gastrulation in the turtle and other reptiles involves two major processes (see Gilland and Burke 2004): involution of axial and paraxial mesoderm at the anterior lip of the blastopore (reminiscent of the dorsal lip of the amphibian gastrula), and internalization of lateral and posterior mesoderm at the lateral lips and in the blastoporal plate (a region with some resemblance to the early posterior primitive streak of mammals). (A) Diagrams of the embryonic disc lying within the area pellucida, as seen from above. Left: stage 0, early gastrulation. The blastopore has appeared in the anterior region of the blastoporal plate. Right: Stage 1, gastrulation underway. The entrance to the blastoporal canal, and the extent of the underlying canal are indicated. (B) Diagrams of sagittal sections. Before gastrulation. Arrow indicates the site where the blastopore will form. Stage 0. Involution at the blastopore is underway and the blastoporal canal has formed with the blastoporal plate as its floor. The axial/paraxial mesoderm has fused with the endoderm to form the ventral opening of the canal. Stage 1. During further involution at the blastopore, the canal has elongated. (C) Cross sections of an embryo at the levels indicated by vertical lines in the diagram of (B), stage 1. Hematoxylin and eosin stain.

inate from the posterior epiblast, are internalized in the region of the blastoporal plate into the mesodermal layer, and soon move to the endoderm layer. During these stages, as seen from above, the *Dazl*-expressing cells lie in an oval region, centered on the blastopore (Fig. 4O) or located posterior to the blastopore under the blastoporal plate (Fig. 4P).

In gastrulation stage embryos, *Vasa* RNA is expressed in the blastoporal plate and at a lower level in the epiblast (Fig. 4, D and F). Expression extends to cells in the endoderm, which intermingle or overlap with *Dazl*-expressing cells (Fig. 4, D and F). No Vasa protein was detected (Fig. 4J).

In embryos with forming notochord and neural plate, the germ cell region has moved posteriorly and laterally, bending to form a crescent around the blastopore (Fig. 4, M and Q), similar to that described above for the early somite stage.

At these stages *Vasa* RNA continues to be expressed as before, but it does not appear to overlap the region of *Dazl*-expressing cells (Fig. 4N).

DISCUSSION

The evidence strongly suggests that turtles use the inductive mode of germ cell specification

Our probes for the expression of *Dazl* RNA and for *Vasa* RNA and protein clearly reacted with the appropriate RNA or protein in small oocytes in ovaries and in definitive PGCs in embryos. However, in oocytes up to 1 mm in diameter, well beyond the size at which the Vasa-containing bodies were visible in hen oocytes (Tsunekawa et al. 2000), we found no localization of *Dazl* or *Vasa* RNA. Vasa protein was spread throughout the cytoplasm of the smallest growing oocytes and later localized around the nucleus as expected for a nuage protein; no evidence was found for its localization in peripheral bodies. In vertebrates with the predetermined mode of germ cell specification one or more of these is localized to the germ plasm: *Dazl* RNA in *Xenopus* and zebrafish (Houston and King 2000; Kosaka et al. 2007), *Vasa* RNA in zebrafish (Knaut et al. 2000; Kosaka et al. 2007), Vasa protein in chick (Tsunekawa et al. 2000) and the Vasa XVLG1 protein in *Xenopus* (Komiya et al. 1994; Bilinski et al. 2004). We tentatively conclude that turtle oocytes do not have germ plasm.

In addition, in our key finding, we detected cells expressing *Dazl* in the blastoporal plate of the gastrulating turtle embryo. This is known as a region of nascent mesoderm formation, confirmed by its expression of *Brachyury* (Coolen et al. 2008). Because *Dazl* expression is specific to germ cells (see Extavour and Akam 2003; Johnson et al. 2003b) and cells expressing *Dazl* more strongly are found at later stages moving away from the center of the blastoporal plate and toward known PGC sites, we conclude that these are PGCs or their precursors. Their low level of expression when in nascent mesoderm, just below the surface, may indicate they are in the process of specification. Unexpectedly we found that *Dazl* expression begins distinctly earlier than in mouse, in which upregulation occurs as germ cells enter the gonad (Lin and Page 2005).

Little is known about cell movements in the blastoporal plate during gastrulation of the turtle (Gilland and Burke 2004). Pasteels (1937) has described cells as moving anteriorly in the plate, then ventrally in the floor of the canal, then posteriorly and laterally in the blastoporal plate. Our observations are consistent with this path for PGC precursors. The demonstration that the germ cells of the turtle originate in the blastoporal plate supports the conclusion that they are in-

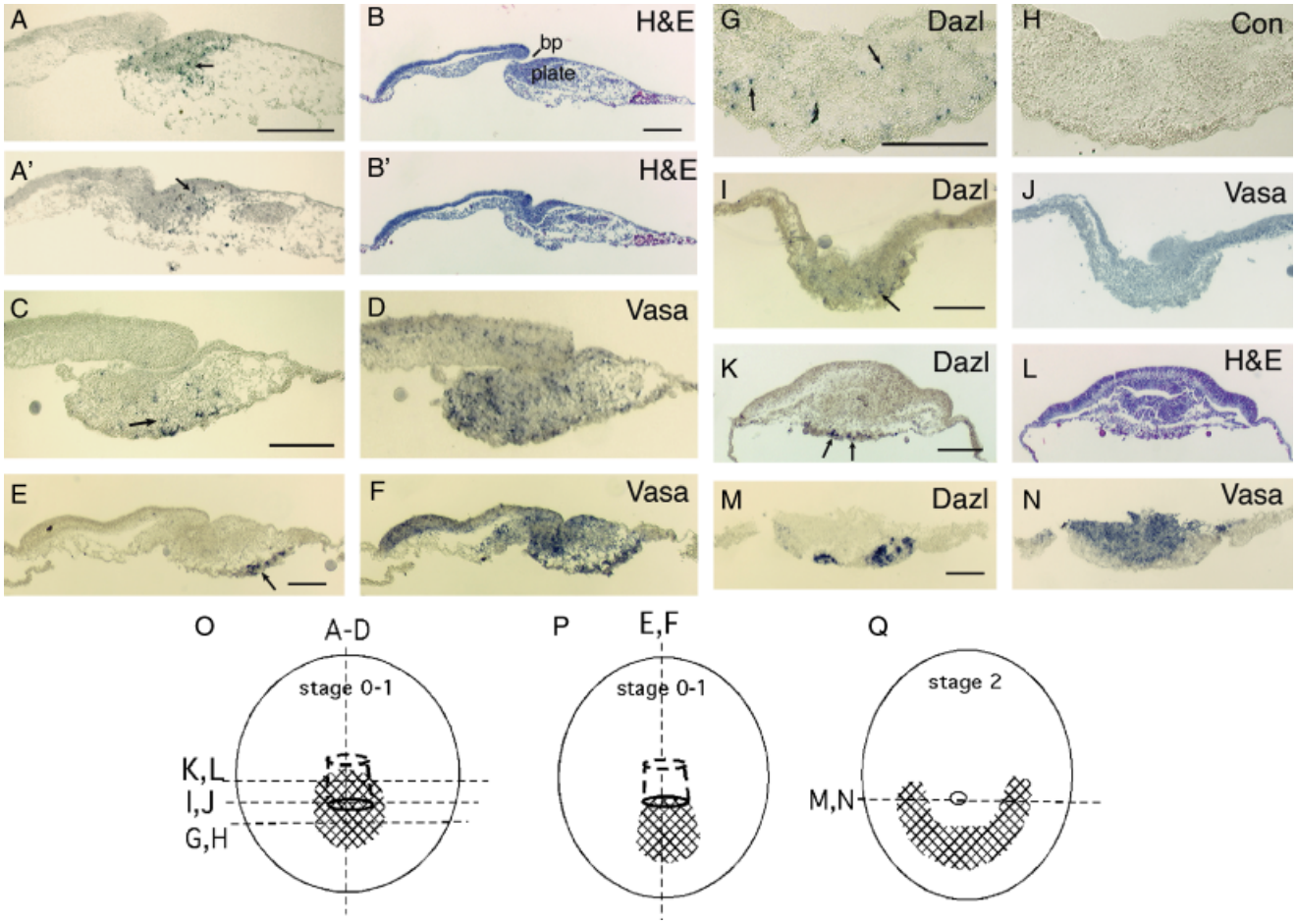

Fig. 4. *Dazl* and *Vasa* expression in early turtle embryos. The location of the sections is shown in the diagrams (O – Q). In sagittal sections anterior is to the left. (A–L) Gastrula, stage 0–1. (M, N) Neural plate, stage 2. (A–A′, B–B′) Sagittal sections of one embryo. (A, B) pass through the blastoporal canal. (A′, B′) pass lateral to the canal. (A, A′) ISH, *Dazl* probe. Expression is seen in cells scattered in the mesodermal layer. (B, B′) Nearby H&E-stained sections at lower magnification showing the whole embryonic disc. (C, D) Sagittal sections. (C) ISH, *Dazl* probe. Expression is seen in cells scattered in the mesodermal layer and in the endoderm. (D) *Vasa* probe on a nearby section. (E, F) Sagittal sections. (E) ISH, *Dazl* probe. Labeled cells are located in the endoderm under the posterior region of the blastoporal plate. (F) *Vasa* probe on a nearby section. (G, H) Cross sections of the blastoporal plate. (G) ISH, *Dazl* probe. Small dots of *Dazl* expression are seen in the mesodermal layer and in the endoderm. (H) Control probe (*Axkit*) on a nearby section. (I, J) Cross section of the blastoporal plate. Lateral regions of the embryonic disc are bent upwards. (I) ISH, *Dazl* probe. Small dots of expression are seen in the mesoderm. (J) Anti-Vasa antibody on a nearby section, demonstrating the absence of Vasa protein. Stained with hematoxylin. Vasa protein would appear brown. (K, L) Cross sections through the blastoporal canal. (K) ISH, *Dazl* probe. Labeled cells are predominantly in the endoderm. (L) H&E stained nearby section. (M, N) Cross sections through the blastoporal plate of a stage 2 embryo with neural plate. (M) ISH, *Dazl* probe. The section passes through the arms of the developing crescent of PGCs located in a thick endodermal layer. (N) *Vasa* probe. Expression is seen in the blastoporal plate, but is mainly absent in the region of the germ cells. (O – Q) Diagrams of surface views of embryos analyzed by ISH. Cross hatched regions show the position of *Dazl*-expressing cells, based on data compiled from sections. Dashed lines indicate the approximate positions of the sections in (A)–(N). (O) Stage 0–1 gastrula. In some embryos the PGCs of the blastoporal plate extend throughout the floor of the canal. (P) Stage 0–1 gastrula. In some embryos PGCs are located posterior to the blastopore. (Q) Stage 2 embryo with neural plate. The region containing the PGCs has formed a crescent. Diameter of the embryonic disc is about 1.2 mm. Arrows indicate examples of presumptive PGCs. bp, blastopore leading into blastoporal canal; plate, blastoporal plate. Scale bars = 200 μm. ISH, in situ hybridization; H&E, hematoxylin and eosin; PGCs, primordial germ cells.

duced. In vertebrates with the inductive mode of germ cell formation (urodele, mouse), the germ cells pass through the primitive streak or its equivalent, and conversely in those with the predetermined mode of germ cell determination (anurans, zebrafish, and chick) they do not pass through this structure.

Although it is theoretically possible that germ plasm lacking *Dazl* RNA and lacking *Vasa* RNA and protein could be present, and that *Dazl* RNA expression in cells of the blastoporal plate is stimulated by germ plasm components, the combined data strongly suggest that turtles use the in-

ductive mode of germ cell specification and lack germ plasm, and that the basic embryology of PGC formation has been conserved during evolution from basal tetrapods through early amniotes to reptiles and mammals. Some changes in germ cell development that occurred as amniotes evolved are discussed in Bachvarova et al. (2009).

Germ cell specification mode in reptiles, based on location of germ cells in embryos

In birds, it has long been known that PGCs are found at early somite stages in a germinal crescent *anterior* to the forming head and later move into the blood, arriving at the gonad at about the 25 somite stage (Swift 1914; Nieuwkoop and Sutasurya 1979). In blastoderm stage embryos, the PGCs delaminate from the epiblast into the hypoblast and then move anteriorly with the hypoblast to the germinal crescent in gastrula stage embryos (Dubois 1967; Nieuwkoop and Sutasurya 1979; Karagenc et al. 1996). Using an anti-Vasa antibody to follow PGCs, it has been shown that germ plasm islands containing Vasa protein are found in chicken oocytes, in cleavage stage embryos, and in a few cells of the pregastrula epiblast, as well as in later PGCs (Tsunekawa et al. 2000), indicating that germ cells form by the predetermined mode. Thus, the history of PGCs in chick embryos contrasts distinctly with that in turtles.

Provisional analysis of the distribution of the modes of germ cell specification in other reptiles can be made based on histological data on the position of germ cells, which is available for some lepidosaurs (lizards, snakes, and *Sphenodon*). We have selected the species with the most reliable information, revising the list of reptiles with different patterns provided by Hubert (1985). Names of species and additional references on germ cells in reptiles are included in the legend of Fig. 5.

PGCs are located in three major patterns at early somite stages (Pasteels 1953; Nieuwkoop and Sutasurya 1979; Hubert 1985).

(i) The turtle pattern: posterior crescent, inductive mode. In the turtle pattern, germ cells are located early in a posterior crescent or horseshoe-shaped region, followed later by interstitial movement through the gut and dorsal mesentery to the gonad. This pattern has been found in several turtle species (see Fig. 5, legend), and is also seen in several species of lacertoids (Hubert 1969). The position of the PGCs has been confirmed experimentally (Hubert 1971a). We assume that, as in the turtle, these lacertoids use the inductive mode to specify their germ cells.

(ii) The snake pattern: anterior crescent, predetermined mode. In snakes (Hubert 1969), the PGCs are seen at the onset of gastrulation in the lower hypoblast layer anterior to the blastopore, are carried forward to an anterior crescent, and from there to the gonad via the blood. Because of this similarity to birds, they are believed to use the predetermined mode.

(iii) The circumferential pattern: circumferential germ cells, mode unknown. In this pattern, the PGCs are located in the junctional zone circumferentially all around the early somite embryo, often in a gradient with somewhat more in the anterior region. They then migrate in the blood to the gonad. This pattern is found in lygosomine skinks (Pasteels 1953), a chameleon (Pasteels 1953), and an anguid (Hubert 1969, 1971b). The mode of germ cell determination is unknown in these groups. It should be noted that in snakes and birds a small minority of PGCs are found in the posterior at early somite stages (Hubert 1969) showing there may not be a sharp distinction between the location of germ cells in the predetermined mode and in the circumferential pattern. In *Sphenodon* although some PGCs are located posteriorly, the bulk of the PGCs are found in an anterior crescent at early somite stages (Tribe and Brambell 1932). We tentatively place *Sphenodon* in the circumferential group although it may be closer to the snake pattern.

The circumferential pattern is widespread among lepidosaurs, but it is difficult to deduce the mode of germ cell specification when PGCs are located all around the embryo at early somite stages, and then transported in the blood. Possibly both modes could be used in any given case. It can plausibly be argued that either mode could be operative as follows. If PGCs are specified by germ plasm and delaminate from the central epiblast into the hypoblast, they could be pushed both anteriorly and laterally (and some posteriorly as seen to some degree in birds and snakes) by the notochordal process and other movements in the lower layer. Alternatively, a case can be made that the PGCs are specified by induction in the blastoporal plate, migrating with blood island forming mesoderm first posteriorly, and then lateriorly and anteriorly around the embryo. Unfortunately, data from gastrula stages that could resolve this issue are not available.

Phylogeny and evolution of PGC specification

When the expanded data set is optimized (Swofford and Maddison 1987) on a tetrapod phylogeny (Fig. 5) (Meyer and Zardoya 2003; Lee et al. 2004), clear hypotheses about the evolution of PGC specification mode emerge.

First, we have corroborated earlier hypotheses (Extavour and Akam 2003; Johnson et al. 2003b; Crother et al. 2007) that the induced mode is primitive among tetrapods.

Second, the induced mode was maintained in early amniotes that gave rise to mammals and reptiles, and in early reptiles as well.

The third hypothesis supported by Fig. 5 is that the predetermined mode is derived and convergently evolved in two lineages of reptiles, the avians and the snakes. This is sur-

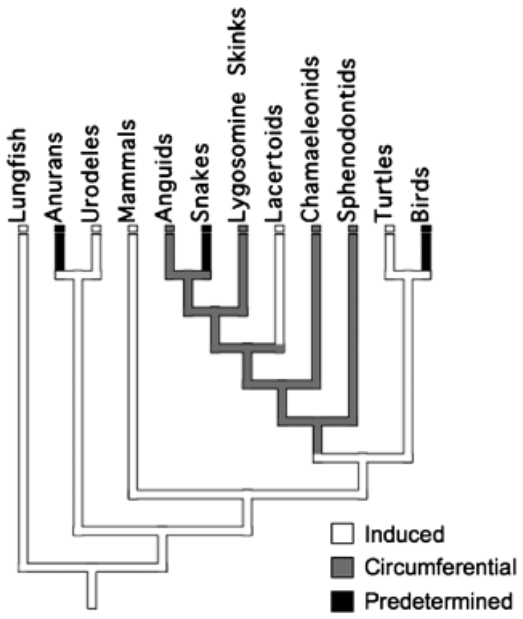

Fig. 5. Optimization analysis of modes of primordial germ cell (PGC) specification on a phylogeny of tetrapods showing the currently deemed most likely position of turtles as sisters to archosaurs. The optimization analyses were conducted with the software MacClade (Maddison and Maddison 2000). Branch lengths do not reflect genetic distances. Two other possible positions of turtles, as a sister group to lepidosaurs or as a sister group to reptiles (Meyer and Zardoya 2003; Lee et al. 2004), do not affect the results. The reptile species used for this analysis are as follows: anguid: *Anguis fragilis* (Hubert 1969, 1971b); snake (*Vipera aspis*, Hubert 1969), lygosomine skinks: *Trachylepis megalura* and *Trachylepis striata*, as *Mabuya* (Pasteels 1953); lacertoids: *Zootoca vivipara* as *Lacerta vivipara*, *Podarcis muralis*, *Lacerta agilis*, and *L.viridis* (Hubert 1969); chamaeleonid: *Chamaeleo bitaeniatus* (Pasteels 1953); sphenodontid: *Sphenodon punctatus* (Tribe and Brambell 1932); turtles: *Chrysemys picta*, as *C. marginata* (Allen 1906; Dustin 1910), *Caretta caretta* (Jordan 1917; Fujimoto et al. 1979), *Sternotherus odoratus* (Risley 1933), *Lepidochelys olivacea* (Merchant-Larios et al. 1989); and birds: see Nieuwkoop and Sutasurya (1979), *Gallus gallus* (Tsunekawa et al. 2000). Limited data are available for germ cells in a scincine skink (*Chalcides ocellatus* as *Gongylus ocellatus*, Gasparro 1908) and an iguanian (*Phrynosoma cornutum*, Jarvis 1908). A cordylid (*Cordylus anguina*) and an agamid (*Acanthocercus atricollis*) are listed as circumferential in Hubert (1985).

prising in light of the remarkable similarity in development of PGCs in birds and snakes.

We have previously proposed that germ plasm evolved independently in several vertebrate lineages, and consequently its molecular makeup and mechanism of action differs between individual taxa (Johnson et al. 2003a, b). Interestingly, the data in Table 1 suggest that the turtle Vasa amino acid sequence is more similar to the mammalian sequence than to that of its nearest relative the chick. Also the turtle Dazl sequence is quite similar to the urodele and mammalian sequences, whereas quite different from the anuran sequence. It appears that the chick Vasa and anuran Dazl may be more divergent than expected. We have previously commented on the tendency for germ cell-specific genes to be highly conserved in species that produce germ cells by induction, whereas in species that contain germ plasm they may be more divergent and may even depart from the expected phylogenetic relationships (Johnson et al. 2001; Johnson et al. 2003a).

The repeated evolution of predetermined germ cells suggests that the mechanisms involved in the evolution of germ plasm are relatively easily acquired (Johnson et al. 2003b, see Extavour 2007). Furthermore, we have suggested that the predetermined germ line is fixed in individual lineages by positive selection driven by the liberation of developmental constraints and the enhancement of evolvability (Crother et al. 2007). The resulting morphogenetic changes may be incompatible with maintenance of the inductive signals necessary for germ cell specification. These considerations help to explain the finding that there is no clear case in the animal kingdom in which the germ plasm mode has reverted to the inductive mode (Extavour and Akam 2003; Johnson et al. 2003b; Crother et al. 2007; Extavour 2007).

The fourth hypothesis revealed by the optimization analysis (with parsimony as the optimality criterion) is that the circumferential pattern, in which germ cells are located all around the embryo at early somite stages, evolved once at the hypothetical ancestor of lepidosaurs (Fig. 5). This analysis also suggests the possibility that in lacertoids the circumferential pattern reverted to the turtle pattern (Fig. 5). Considering that reversion from the germ plasm mode to the inductive mode is unlikely, we predict that most or all of those lepidosaurs with the circumferential pattern use the inductive mode, or possibly both the inductive and the predetermined modes.

In conclusion, among reptiles, the data support the hypothesis that the inductive mode is primitive, and suggest there are at least two cases of independent derivation of the predetermined mode. The data also suggest an unusual diversity in the patterns of early germ cell development, but how these relate to specification mode has yet to be determined.

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