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Review Article

A little winning streak: The reptilian-eye view of gastrulation in birds

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The primitive streak is where the mesoderm and definitive endoderm precursor cells ingress from the epiblast during gastrulation. It is often described as an embryological feature common to all amniotes. But such a feature has not been associated with gastrulation in any reptilian species. A parsimonious model would be that the primitive streak evolved independently in the avian and mammalian lineages. Looking beyond the primitive streak, can one find shared features of mesoderm and endoderm formation during amniote gastrulation? Here, we survey the literature on reptilian gastrulation and provide new data on *Brachyury* RNA and laminin protein expression in gastrula-stage turtle (*Pelodiscus sinensis*) embryos. We propose a model to reconcile the primitive streak-associated gastrulation in birds and the blastopore-associated gastrulation in extant reptiles.

Introduction

As a successful group with 10 000 living species, birds occupy a unique position among the amniotes. Modern birds evolved from theropod dinosaurs (Chiappe 2004), and molecular phylogeny studies revealed that their closest living relatives are the crocodiles (Chiari *et al.* 2012). Birds therefore should be considered as representing the reptiles when compared to mammals. But how representative are they of the reptiles? Extant non-avian reptiles (referred to as reptiles hereafter) consist of five major groups: the crocodiles, turtles, snakes, lizards and tuatara (Fig. 1A). Pioneers of comparative embryology like Kupffer, Balfour, Will, Mitsukuri, Peter and Pasteels had studied reptilian embryos in great detail (Balfour 1879; Kupffer 1882; Will 1892, 1896; Mitsukuri 1894; Peter 1935, 1939 Pasteels 1937, 1957b). But concerning their early development, very few papers have been published in the last two decades (Arendt & Nubler-Jung 1999; El Mouden *et al.* 2000; Gilland & Burke 2004; Coolen *et al.* 2008; Bachvarova *et al.* 2009). Molecular studies on amniote gastrulation have been focused predominantly on two species, the chick, representing birds, and the mouse, representing mammals. Can we have a "reptilian-eye" view of amniote early development? And if we consider the chick as representing reptiles, can we reconcile the differences between the birds on one hand and the rest of the reptiles on the other? In this article, findings on reptilian gastrulation from old literature will be discussed together with recent molecular data. We will emphasize the lack of a phylogenetically conserved primitive streak in reptiles. We will also highlight their shared features (with birds) in mesoderm/endoderm formation, and variable contributions of involution and ingression to the gastrulation process. Finally, we will propose a model to explain the evolutionary transformation from a circumblastoporal mode of mesendoderm formation in amphibians to the primitive streak-associated mode seen in birds and mammals, possibly with an intermediate mode exemplified in reptiles.

Gastrulation and primitive streak

Gastrulation in amniotes is traditionally described as a process involving formation of the primitive streak. The primitive streak manifests itself as a thick patch of cells at the posterior edge of the area pellucida. Initially of a triangular morphology and soon taking a rod-like shape, which elongates towards the center of the blastodisc, the primitive streak is where epiblast cells ingress to form the endoderm and mesoderm layers

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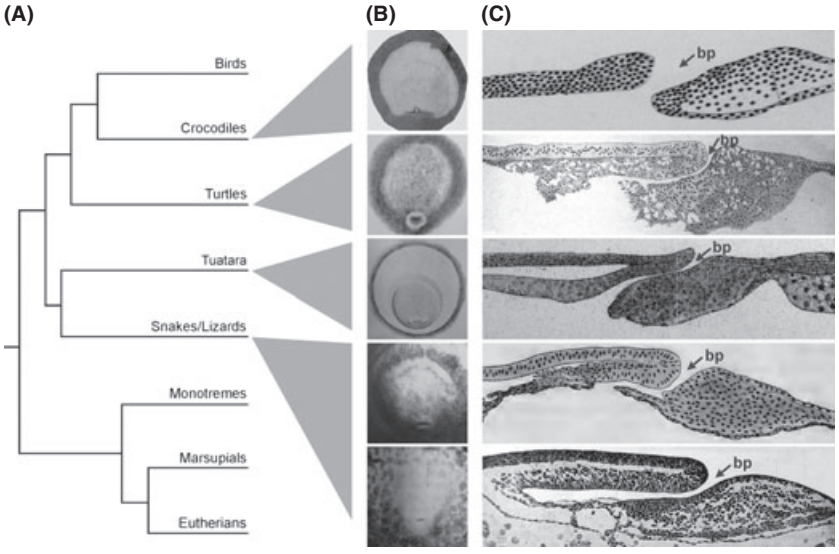

Fig. 1. Amniote phylogenetic tree and reptilian gastrulation. (A) Phylogenetic tree of extant amniotes. Although the position of the turtles is still controversial, we have adopted this tree based on the most recent molecular evidence (Chiari *et al.* 2012; Hedges 2012). (B) External morphology (dorsal view) of gastrula-stage reptilian embryos. An avian-like primitive streak is absent, and a blastopore-like structure is present in all species examined. In the snakes/lizards group, upper panel: lizard; lower panel: snake. (C) Sagittal sections through the blastopore of embryos slightly older than shown in (B). bp, blastopore. All images in (B) and (C) are reproduced with permission from respective publishers. Images for crocodile *Alligator mississippiensis* are from (Reese 1908), for turtle *Caretta caretta* from (Mitsukuri 1894), for tuatara *Sphenodon punctatus* from (Schauinsland 1899); for lizard *Lacerta agilis* from (Wenckebach 1891) and *Zootoca vivipara* from (Dufaure & Hubert 1961); for snake *Tropidonotus natrix* from (Gerhardt 1901). The same observation, that reptilian gastrulation is associated with a blastopore instead of a primitive streak, has been reported in many other species, including the turtles [*Pelodiscus sinensis* (Mitsukuri & Ishikawa 1887); *Clemmys japonica* (Mitsukuri 1891; Will 1892); *Chrysemys picta* (Davenport 1896; Brachet 1914); *Chelopus insculptus* (Davenport 1896); *Chelydra serpentina* (Davenport 1896; Yntema 1968); *Clemmys leprosa* (Pasteels 1937); *Pseudemys virginica* (Pasteels 1957b); *Geoemyda trijuga and Lissemys punctata* (Nayar 1959, 1966); *Chelonia mydas* (Miller 1985); *Emys orbicularis* (Mehnert 1892; Coolen *et al.* 2008) and *Trachemys scripta* (Bachvarova *et al.* 2009)]; the lizards [*Chamaeleo vulgaris* (Peter 1935); *Chamaesaura anguinea*, *Mabuia megalura* and *Chamaeleo bitaeniatus* (Pasteels 1957a,b); *Lacerta vivipara* (Hubert 1970); *Agama impalearis* (El Mouden *et al.* 2000); *Platydactylus mauritanicus* (Will 1890); and *Lacerta muralis*, *Lacerta lilfordi* and *Lacerta viridis* (Will 1896)], the snakes [*Tropidonotus natrix* (Ballowitz 1901); *Thamnophis sirtalis* (Zehr 1962)]; the tuatara [*Sphenodon punctatus* (Dendy 1899; Tribe & Brambell 1932)] and the crocodiles [*Alligator mississippiensis* (Clarke 1891); and *Crocodylus niloticus* (Voeltzkow 1899)].

(Stern 2004). This mode of endoderm and mesoderm formation in amniotes is fundamentally different from that seen in their anamniote ancestors, that is, via involution through the blastopore. This classical, primitive streak-centered view of amniote gastrulation is derived from analyses on eutherian mammals and birds. The chick system, the main avian model system, has been paramount to the understanding of morphogenetic rearrangement events that accompany primitive streak formation and elongation, and of the cellular ingression movement through the primitive streak that is characteristic of amniote gastrulation. Epiblast cell intercalation in the middle posterior portion of the blastodisc results in the formation of the primitive streak (Voiculescu *et al.* 2007); coordinated cell ingression through the anterior part of the early primitive streak generates the endoderm (Kirby *et al.* 2003; Lawson & Schoenwolf 2003; Kimura *et al.* 2006); and mesoderm precursor cells ingress through the length of the primitive streak and interpose between the epiblast and the endoderm (Nicolet 1965; Tam & Beddington 1987; Selleck & Stern 1991; Tam *et al.* 1993; Psychoyos & Stern 1996; Nakaya *et al.* 2008; Nakaya & Sheng 2009). The original anterior-posterior positional difference along the primitive streak translates into the final dorsal-ventral position in the embryo. However, in addition to eutherian mammals and birds, the amniote world includes two other mammalian groups, the Prototheria and Metatheria, which will not be discussed here, and the Reptilia. So how do reptilian embryos gastrulate?

Reptilian blastopore and primitive plate

Similar to birds, early embryogenesis in reptiles goes through meroblastic cleavage, cellularization and blastoderm formation processes, resulting in a two-layered structure in the area pellucida: an epiblast layer of

single-cell thickness and epithelial morphology and a hypoblast layer of variable thickness and morphology (Agassiz 1857; Balfour 1879; Will 1896; Ballowitz 1901; Peter 1934; Pasteels 1937, 1957a; Dufaure & Hubert 1961; Hubert 1962; Miller 1985; El Mouden *et al.* 2000). In avian embryos, initiation of gastrulation is linked to primitive streak formation. But an avian-like primitive streak has not been reported in any of the reptilian species studied. What appears to be conserved among the reptiles is a blastopore-like structure where epiblast cells are internalized to become endoderm and mesoderm cells. Representative images of the blastopore in each reptilian group are shown in Figure 1B (surface views) and Figure 1C (sagittal section views), and similar observations have been reported in many other reptilian species (Fig. 1 legend). The blastopore is the external opening of invaginated epiblast epithelium. The space enclosed by this invagination is the archenteron. Located posterior to the blastopore, partially constituting the posterior wall (and the ventral-posterior wall later on) of the archenteron, is the primitive plate (also called blastoporal plate). The primitive plate is another conserved feature in reptilian embryos, and has been variably likened to the primitive streak in birds, the yolk plug in amphibians, or the ventral lip of the blastopore in amphibians. The reptilian blastopore itself (more precisely the anterior and lateral rims of the blastopore) has been compared to the dorsal blastopore lip or the entire circumblastoporal lip in amphibians, or the Hensen's node or the entire primitive streak in birds. These comparisons were based primarily on histological analyses of fixed reptilian embryos from above-mentioned studies (Fig. 1 legend). Before we offer our opinions on the structural homology of these features, let us first take a look at how the endoderm and mesoderm are generated in reptiles.

Endoderm

Mechanistic understanding of mesendoderm formation in reptiles is scanty, and relies heavily on a handful of descriptive studies of histological sections. The few exceptions are studies by two authors, Pasteels (1937) and Nayar (1959, 1966), who applied dye on the epiblast of *Chelonia* embryos and registered the position of the marker after a short period of time. The results of these histological and labeling studies pointed to a mechanism based mainly on involution as the mode of mesendoderm internalization at the site of gastrulation. While involution of epiblast cells through the blastopore lip is clear, ingression also appears to play a role. These two modes combined would more satisfactorily account for the presence of the mass of cells positioned in the anterior/lateral and posterior regions of the gastrulating reptile embryo (Coolen *et al.* 2008; Bachvarova *et al.* 2009) (Fig. 4A). Characterization of the lower layers in reptiles (the hypoblast or extra-embryonic endoderm and the definitive endoderm) is far from being accomplished. In chick embryos the hypoblast forms a flat layer of cells underlying the pre-gastrulating epiblast. The canonical view of hypoblast formation in amniotes (mostly derived from chick studies) is based upon delamination of cells from the epiblast and their aggregation into groups called the island of the hypoblast, followed by coalescence and spreading over the lower surface of the epiblast (Stern & Downs 2012). In reptiles, the germ cell marker *Dazl* is expressed in cells embedded in the lower layer of an early gastrulaing embryo (Bachvarova *et al.* 2009), suggesting the presence of a chick-like hypoblast layer. Histologically, the development of the lower layer in gastrulating reptile embryos has been analyzed and debated by a number of researchers including Will, Kupffer, Mitsukuri, Schauinsland, Ballowitz, Peter and Pasteels. Peter, for example, argued that the first endodermal layer forms under the embryonic shield *in loco* from the "deeper blastomeres" (Peter 1934), while Pasteels proposed that in turtles and lizards the hypoblast (endophylle) forms by delamination from the upper epiblast (ectoblast) (Pasteels 1937, 1957a), like in chick. With regard to the definitive endoderm (the entoblast), Pasteels (1957a), summarizing his own work on turtles and that of others before him, suggested that these cells involute at the blastopore lip and account for the formation of a thin layer of endoderm cells covering the ventral surface of the epiblast. In the midline, the axial mesendoderm called chordahypoblast is continuous with this thin layer of cells (Mitsukuri 1891; Pasteels 1957a). The hypoblast is then pushed into extra-embryonic areas by the endoderm. The idea of endoderm pushing the pre-existing lower layer had been the accepted view in the amniote experimental models, chick and mouse. However, recent experiments suggested a certain degree of intercalation between the hypoblast and the forming endoderm, with hypoblast cells contributing to definitive endoderm (Bertocchini & Stern 2008; Kwon *et al.* 2008; Burtscher & Lickert 2009). A possible partial contribution of the hypoblast to the definitive gut epithelium in reptiles was brought up in the old literature (Mehnert 1892; Will 1892), but it is still unclear whether and to what extent this hypoblast/endoderm mixing occurs in reptiles. In addition to the hypoblast and the involuting endoderm, a third population of lower layer cells is recognized in the primitive plate. These endoderm cells ingress from the epiblast, and, together with mesoderm cells (see below), form a uniform mass

of not yet characterized tissue. The lack of lower-layer molecular markers, together with the paucity of information from histological sections, makes it difficult to speculate on the positioning and movements of the different dorsal and ventral components of the endoderm.

Mesoderm

Vertebrate mesoderm can be divided into four lineages: the axial, paraxial, intermediate and lateral plate. In amniotes, extraembryonic mesoderm is added lateral to the lateral plate mesoderm (Nakazawa *et al.* 2006; Shin *et al.* 2009). These mesoderm lineages are patterned initially along the dorso-ventral body axis of a vertebrate embryo, so that the axial mesoderm is generated from the dorsal-most territory of an early gastrula and the lateral plate from the ventral-most (Psychoyos & Stern 1996; Alev *et al.* 2010). Reptilian embryos generate mesoderm from two populations of cells (Coolen *et al.* 2008) (Figs 2, 3, 4A,B). The first population is internalized through the blastopore lip. The blastopore takes the shape of an anteriorward bending narrow crescent, which changes gradually into a posteriorward bending horseshoe shape (Fig. 4A, left). Mesoderm internalization takes place throughout the entire width of the blastopore (*Brachyury* staining in Fig. 2, schematized in Fig. 4A and summarized in Fig. 4B). Similar to what is known in the anamniotes, the most axial, chordal mesoderm is internalized through the center part of the blastopore, and progressively more lateral mesoderm types (paraxial, intermediate and lateral plate mesoderm) are internalized through more lateral territories of the blastopore lip (Coolen *et al.* 2008) (Fig. 4B). The internalized dorsal mesoderm is initially in direct contact with the

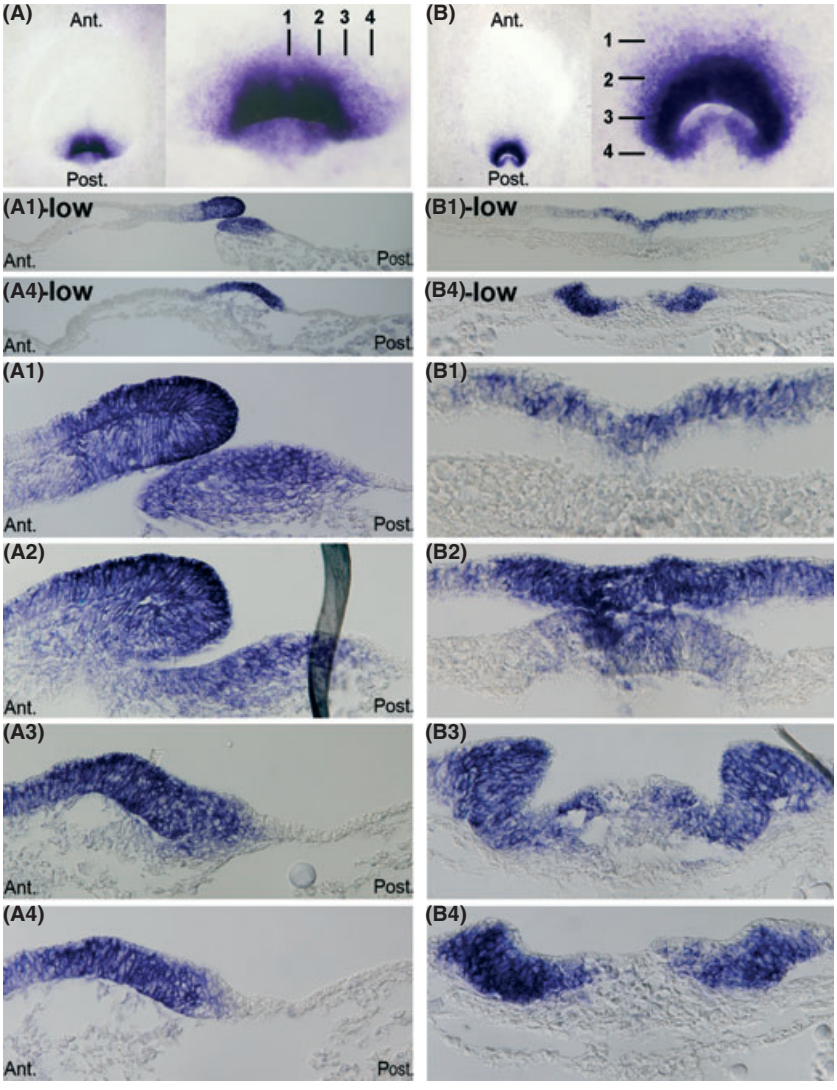

Fig. 2. *Brachyury* gene expression in late gastrula-stage turtle (*Pelodiscus sinensis*) embryos. *Brachyury* is used here as a mesendoderm precursor marker. This gene marks the blastopore in amphibians and the primitive streak in mammals and birds. (A) A stained embryo (whole-mount, epiblast-side view) used for sagittal sections. Sections through the middle and the edge of the blastopore are shown at low magnification, and high-magnification panels (A1–A4) depict gradual changes in blastopore morphology. (B) A stained embryo (whole-mount, epiblast-side view) used for transverse sections. (B1)-low and (B4) low: low magnification. (B1–B4): high magnification.

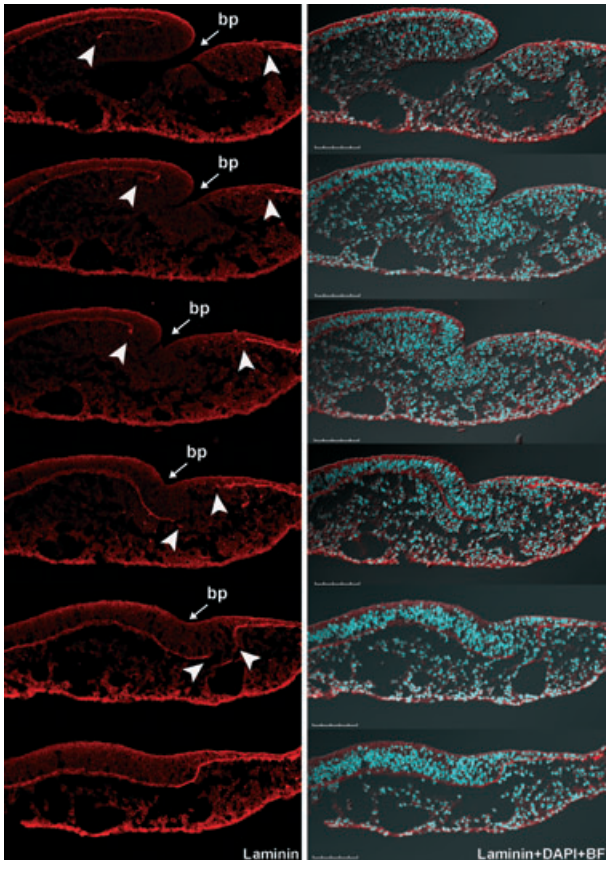

Fig. 3. Laminin protein localization in a gastrula-stage turtle (*Pelodiscus sinensis*) embryo. All sagittal sections. This embryo is of a stage slightly younger than in Figure 2. The archenteron is not yet connected to the subgerminal cavity. Top→Bottom panels: Medial→Lateral sagittal sections of the blastopore. Left panels: laminin (red). Apical surface signals are due to background staining. bp, blastopore. Arrowhead: limit of laminin expression, indicating the starting point of loss of epithelial integrity. Mesoderm cells leaving the epiblast after losing epithelial integrity are considered to be generated by ingression. Mesoderm cells leaving the epiblast while still retaining epithelial polarity are considered to be generated by involution. Right panels: laminin (red) + 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) (cyan) + bright field. Scale bar, 100 μm (20 μm for each smaller unit).

elongated archenteron as its superior wall and is only later covered underneath by an inward/medialward moving endoderm layer. The mesoderm of more ventral fate appears to be internalized by moving between the epiblast and endoderm layers (Fig. 2). Thus the dorso-ventral axis of mesoderm precursors in reptiles is reflected in the medio-lateral positional difference of the blastopore lip through which these cells are internalized. Along the same axis, involution (i.e., internalized cells keep their epithelial polarity and move collectively) is observed more prominently in dorsal/axial mesoderm (laminin retention in involuted cells seen in Fig. 3), and ingression (i.e., internalized cells lose their epithelial polarity and engage in mesenchymal like cell migration) more prominently in ventral mesoderm (laminin breakdown in internalized cells seen in Fig. 3). The second population of mesoderm cells is generated from the primitive plate (Figs 2–4). Although the boundary of the primitive plate has not been defined clearly in any literature and it has been used sometimes to denote the entire area with active gastrulation movements, we refer to the primitive plate as an area including the ventral-posterior aspect of the invaginated archenteron and its extension into the epiblast situated posterior to the blastopore and bound laterally by the lateral wings of the blastopore lip (when it has adopted a horseshoe shape) (gray area in Fig. 4B,C). In the primitive plate, mesoderm cells are internalized primarily through an ingression process (Fig. 3). The active zone of ingression can be visualized by the status of laminin breakdown. Cell labeling experiments (Pasteels 1937; Nayar 1966) suggested that epiblast cells in the primitive plate move towards the blastopore (likely towards the active ingression zone, which occupies a narrower territory) followed by internalization and lateral/posterior-directed dispersal (summarized in Fig. 4B) (Pasteels 1937). Generation of mesoderm from two spatially distinct populations of mesodermal cells, the primitive plate derived "prostomial" mesoderm and the blastoporal invagination associated "gastral" mesoderm was already suggested towards the end of the nineteenth century by scientists including Wenckebach, Will and Mitsukuri (Wenckebach 1891; Will 1892; Mitsukuri 1894). The exact fate of mesoderm cells internalized through the primitive plate is not clear. They most likely contribute to the majority of extraembryonic mesoderm and possibly also to some parts of the lateral plate mesoderm, as was suggested also by Mehnert, Schauinsland and Peter (Mehnert 1892; Schauinsland 1899; Peter 1934).

Model

So how can we compare the reptilian mode of gastrulation with what we know of in birds? It appears that the key difference is largely morphogenetic, whereas the cellular sources for the endoderm and mesoderm, the types of mesoderm generated, and the dorsoventral patterning of the mesoderm are all very similar in birds and reptiles (Fig. 4B). The morphogenetic difference is manifested in two aspects. In the first, the blastopore formation and archenteron invagination seen in reptiles do not take place in birds. The embryological equivalent of the blastopore in chick, the epiblast above and anterior to the Koller's sickle, marks a topologically and functionally orthologous territory. But

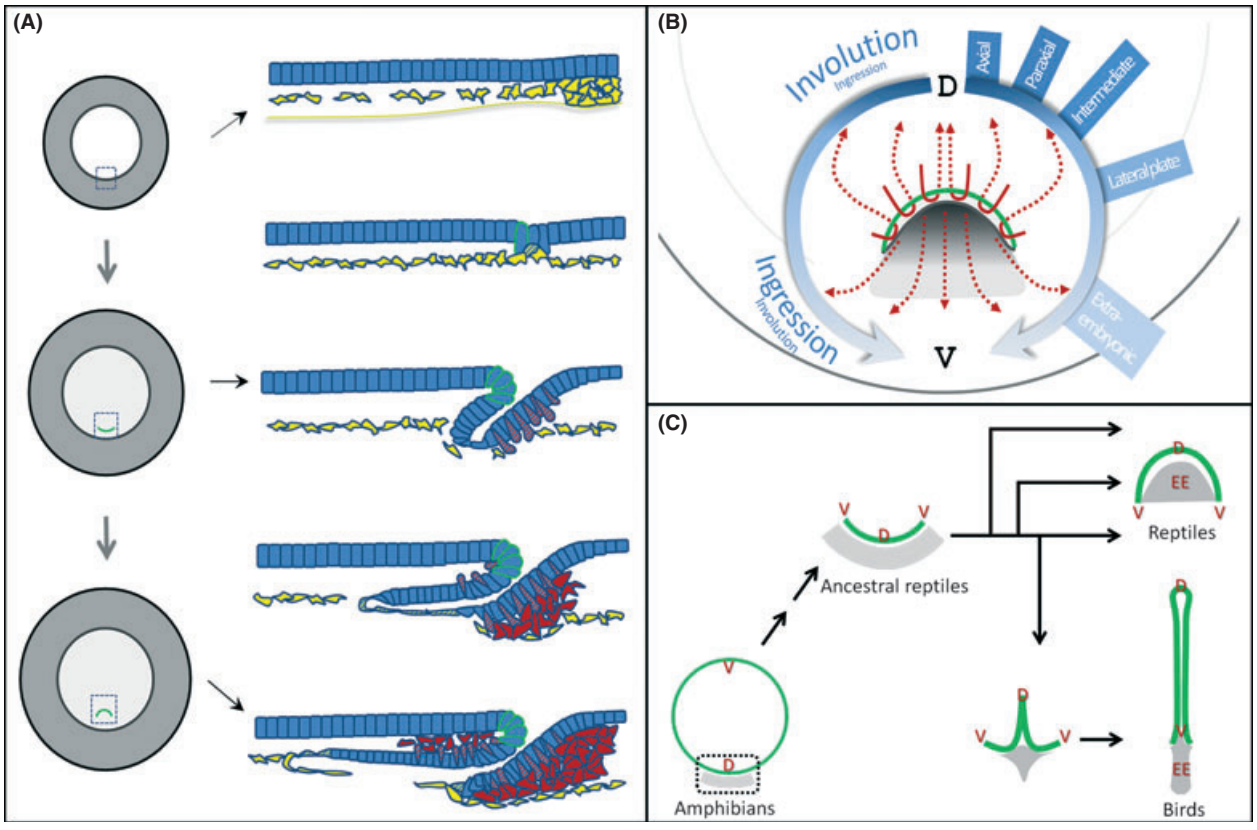

Fig. 4. Models for gastrulation in the reptiles. (A) Blastopore initiation, archenteron invagination, and mesoderm and endoderm formation. Left: schematic views of early-stage embryos. Not to scale (the area opaca covers a much greater area than depicted here). Right: sagittal-section views of the boxed regions on the left. Posterior side oriented towards the right. Hypoblast/endoderm: yellow; mesoderm: red; epiblast and involuted epiblast cells: blue. Top-right panel has also the yolk syncytial cell membrane depicted. Green outlined cells: blastopore lip at the anterior and anterior-lateral rims of the invaginated archenteron. Cells from the ventral and ventral-anterior archenteron will join the hypoblast layer. Definitive endoderm cells (still depicted blue in the diagram) will form from the epithelial portion of cells involuted from the blastopore lip. Mesoderm cells form from the primitive plate by ingression and from the blastopore lip by involution plus ingression or collective dissolution of epithelial characters. (B) Dorsal-ventral patterning of the mesoderm cells, which form by involution/ingression from the blastopore (green), is reflected along the medial-lateral axis and the blastopore. The axial mesoderm is internalized through the midline, and progressively more ventral types (paraxial, intermediate and lateral plate mesoderm) are internalized from more lateral regions of the blastopore lip. The lateral-most regions of the blastopore lip may also generate the extraembryonic mesoderm. Mesoderm cells ingressing through the primitive plate will give rise predominantly to the extraembryonic type, but may also contribute to the lateral plate. D, dorsal; V, ventral. (C) Model for how to reconcile the difference in mesoderm and endoderm formation between the reptiles and the birds. Green, marginal zone or blastopore lip. Gray, primitive plate/nieuwkoop center-like structures. D, dorsal; EE, major source of the extraembryonic mesoderm; V, ventral.

instead of a blastopore, avian embryos initiate and elongate a primitive streak (Fig. 4C). This is achieved through morphogenetic rearrangement of epiblast epithelial cells located anterior to the prospective "blastopore" prior to and during mesendoderm internalization. In reptiles, epithelial cells in the epiblast appear also to undergo rearrangement, reflected in the dramatically changing morphology of the blastopore at early stages of gastrulation (Fig. 4A,C). But this is a separate, and likely more ancestral among the amniotes, form of epiblast cell rearrangement. The second aspect of morphogenetic difference is seen in the relative contributions of involution and ingression to mesoderm formation (Fig. 4B). Involution through the blastopore is the main mode of mesendoderm internalization in amphibians. Ancestral amniotes, evolving from the amphibians, may have inherited this to a large extent. Although in birds, ingression is used as the primary mode of mesendoderm formation, it is interesting to see that extant reptiles use a mixture of both, with involution being more prominent during dorsal mesendoderm formation, and ingression more prominent during the formation of more ventral mesoderm types.

These two aspects of morphogenetic difference between the birds and the reptiles are likely connected causally. One may speculate that an initial

small difference in the ancestral avian group, for instance in the signals regulating planar cell polarity of the epiblast epithelium, can initiate changes leading to both aspects of avian-specific morphogenesis. The fact that mammalian gastrulation likewise involves a primitive streak instead of a blastopore suggests that such changes occurred at least twice independently during amniote evolution. It is probably premature at this moment to speculate on whether similar changes underlie the loss of the blastopore and the gain of the primitive streak in the avian and mammalian lineages. But the conservation in germ layer formation and patterning, as mentioned above, suggests that such variations in morphogenesis do not significantly alter the hard-wired regulatory logic of amniote gastrulation.

Finally, based on morphogenetic, cell fate and cell biological comparisons, we propose a structural orthology between the blastopore and primitive plate on one hand, and the primitive streak on the other (Fig. 4C). By this we mean that the reptilian blastopore lip (dorsal and lateral rims of the horseshoe-shaped blastopore) is evolutionarily homologous to the anterior two-thirds or three-quarters of the avian primitive streak (including the Hensen's node), and both structures are homologous to the blastopore in amphibians. The blastoporal plate is evolutionarily homologous to the posterior third or fourth of the primitive streak, and both are dedicated mainly to generating the extraembryonic mesoderm (an amniote invention) and are probably derived from a Nieuwkoop center-like structure in the amphibian ancestor of the amniotes (Fig. 4C).

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