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Decoupling of amniote gastrulation and streak formation reveals a morphogenetic unity in vertebrate mesoderm induction

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SUMMARY

Mesoderm is formed during gastrulation. This process takes place at the blastopore in lower vertebrates and in the primitive streak (streak) in amniotes. The evolutionary relationship between the blastopore and the streak is unresolved, and the morphogenetic and molecular changes leading to this shift in mesoderm formation during early amniote evolution are not well understood. Using the chick model, we present evidence that the streak is dispensable for mesoderm formation in amniotes. An anamniote-like circumblastoporal mode of gastrulation can be induced in chick and three other amniote species. The induction requires cooperative activation of the FGF and Wnt pathways, and the induced mesoderm field retains anamniote-like dorsoventral patterning. We propose that the amniote streak is homologous to the blastopore in lower vertebrates and evolved from the latter in two distinct steps: an initial pan-amniote posterior restriction of mesoderm-inducing signals; and a subsequent lineage-specific morphogenetic modification of the pre-ingression epiblast.

KEY WORDS: Amniotes, Avian, Blastopore, Gastrulation, Mesoderm, Primitive streak

INTRODUCTION

The mesoderm is one of the three principle germ layers and is formed during gastrulation. Mesoderm induction is regulated by evolutionarily conserved molecular mechanisms (Harvey et al., 2010; Kimelman, 2006), yet morphogenetic events during its formation vary in different vertebrate clades (Arendt and Nübler-Jung, 1999; Meinhardt, 2006; Shook and Keller, 2008; Solnica-Krezel, 2005). In lower vertebrates (anamniotes), mesoderm cells are induced radially at the blastopore. This circumblastoporal mode of mesoderm formation was lost during early amniote evolution. In birds and mammals, mesoderm cells are generated exclusively from the streak, a transitory structure unique to the amniotes. The evolutionary origin of the streak has long been debated and its relationship to the blastopore remains unresolved. The reptiles, the stem amniote lineage from which both the mammals and birds evolved, do not have a streak (Bertocchini et al., 2013). Furthermore, mammals and birds have been hypothesized to employ different cellular mechanisms to generate a streak (Arnold and Robertson, 2009; Chuai and Weijer, 2008; Stern, 2004; Voiculescu et al., 2007; Williams et al., 2012).

In this work, we used the chick model and examined the relationship between streak formation and mesoderm induction and that between the streak and blastopore. We found that irrespective of whether a streak forms or not, mesoderm cells can be induced radially (‘circumblastoporally’) in the marginal zone. These ectopically formed mesoderm cells undergo migration and initiate terminal differentiation. This capacity to generate mesoderm radially was revealed by manipulating the availability of mesoderm inducer FGF and requires cooperative activation of the FGF and Wnt signaling pathways. Dorsoventral patterning in the induced mesoderm mimics that in anamniote blastopore. Using additional avian and reptilian species, we demonstrated that the ability to uncouple mesoderm and streak formation, and to generate mesoderm in an ancestral ‘circumblastoporal’ mode, is conserved among amniotes. Finally, we presented a model to explain the evolutionary shift from a radial to a dorsally restricted mode of mesoderm formation.

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MATERIALS AND METHODS

Subgerminal cavity injection

Fertilized chicken, quail, emu and turtle eggs were purchased from local farms. The following growth factors were used: FGF4 (#235-F4, R&D Systems; final concentration 50 ng/μl), FGF2 (#03-0002, Stemgent; 50 ng/μl), FGF8 (#F6926, Sigma; 50 ng/μl), activin A (#338-AC, R&D Systems; 50 ng/μl) and Wnt3a (#1324-WN, R&D Systems; 10 ng/μl). SB431542 was from Sigma (#S4317-5MG; 25 μM). Growth factors/chemical inhibitors were diluted in Pannett-Compton to their final concentrations, together with Fast-Green for visualization (#061-00031, Wako; final 0.1%). After brief warm-up (30 minutes at 38.5°C), chicken eggs were opened and their content transferred to pre-cut foster shells. For quail, emu and turtle eggs, host shells were cut with a drill or small scissors to leave the cut-edges smooth. A volume of 1-2 μl was injected by mouth-pipetting using a pulled glass capillary (#2-000-050, Drummond). The subgerminal cavity can hold up to 5 μl (Petitte et al., 1990; Sang, 2004; van de Lavoir et al., 2006) and control-injected embryos developed normally. Afterwards, shells were filled up with thin albumen, covered with thin cling-film (from Nippon Paper-Pak), and assembled using plastic rings (Showa Furanki Company) and rubber bands (supplementary material Fig. S1). Eggs were incubated horizontally at 38.5°C in a humidified incubator. Turtle eggs were incubated at 32°C vertically after covering injected embryos with cling-film.

Embryology and imaging

In situ hybridization for chicken, quail, emu and turtle embryos followed standard chicken protocol (Nagai et al., 2011; Stern, 1998), as with paraffin-embedded sectioning (10 μm, Microm-HM325 microtome), whole-mount embryo imaging (Olympus-SZX12; Olympus-DP70) and section imaging (Olympus-BX51; Olympus-DP70). For immunofluorescence (Fig. 1; supplementary material Fig. S1), unincubated embryos were fixed *in ovo* (4% paraformaldehyde), collected and stained with phalloidin (AlexaFluor-

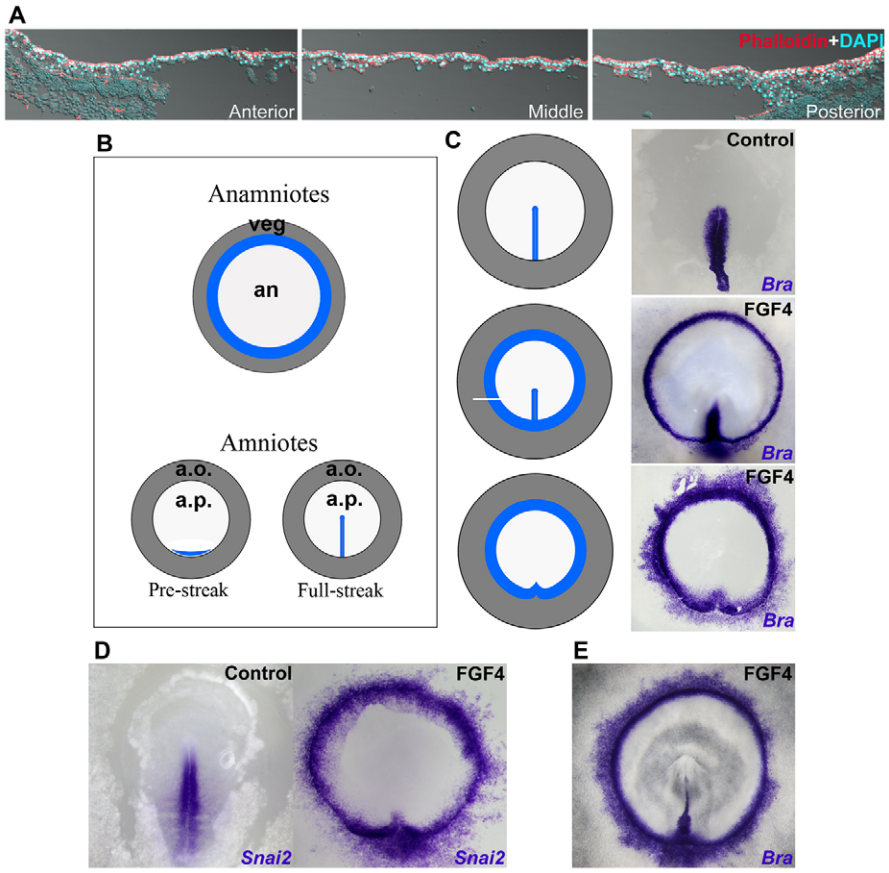

Fig. 1. FGF induces a mesoderm ring in the marginal zone. (**A**) Sagittal section of an unincubated chick embryo (supplementary material Fig. S1C) stained with phalloidin (red) and DAPI (cyan), showing tissue organization in anterior, middle and posterior regions. (**B**) Schematic comparison of mesoderm formation (blue) in anamniotes and amniotes. an, animal; veg, vegetal. a.o., area opaca; a.p., area pellucida. (**C**) FGF4-induced mesoderm rings shown schematically (left) and stained for Brachyury (right). Top, control injected; middle and bottom, FGF4 injected. White line indicates section level shown in supplementary material Fig. S3A. (**D**) *Snai2* expression in control-injected (left) and FGF4-injected (right) embryos. (**E**) *Brachyury* expression in an embryo grown for 36 hours after FGF4 injection (equivalent to HH7).

568 phalloidin, #A12380, Invitrogen), cryosectioned (14 μm, LEICA-CM3050S), mounted with ProLongGold-Antifade with DAPI (#P36931, Invitrogen) and imaged using Olympus FV1000 microscope. Electroporation of GFP (Momose et al., 1999), CA-FGFR2 (Nakazawa et al., 2006) and CA-β-catenin (Shin et al., 2009) was performed *ex ovo* using TSS20-Ovodyne electroporator (Intracel, UK; 3×50 ms pulses of 5 V with 250 ms intervals). Heparin acrylic beads (#H5263, Sigma) were used for graft experiments. Time-lapse movie (supplementary material Movie 1) was taken using NIKON AZ-C1 macro laser confocal microscope.

In situ hybridization probes

Chicken *Brachyury* probe corresponds to GGU67086 nucleotides 301-602, used also for quail *Brachyury in situ* hybridization. Emu *Brachyury* probe corresponds to HQ336494 nucleotides 43-409 (Nagai et al., 2011). Turtle *Brachyury* fragment (420 bp) was cloned by PCR and the sequence has been deposited in the NCBI database (Accession Number JN022614). Other *in situ* probes used here correspond to the following sequences: *FGF4* (nucleotides 285-585, NM_001031546), *FGF8* (nucleotides 1-650, U41467.1; for both chicken and quail), *Wnt8C* (nucleotides 747-1704, NM_205531), *Cdx1* (nucleotides 1779-2295, NM_204676), *Chordin* (2385-3191, AF031230), *Snai2* (nucleotides 138-945, XM_419196), *Eomes* (nucleotides 630-1089, XM_426003), *Mixl1* (nucleotides 173-788, NM_204990), *Sprouty2* (nucleotides 902-1869, NM_204800), *HoxB1* (nucleotides 954-1426, X68353), *Gsc* (nucleotides 282-936, NM_205331), *FoxA2* (nucleotides 1367-1834, NM_204770), *Vg1* (nucleotides 54-683, GGU73003), *Nodal* (nucleotides 233-702, XM_424385), *Lmo2* (nucleotides 7-962, NM_204271) and *dHAND* (nucleotides 167-1135, BBSRC chickEST #333817.4).

RESULTS

The streak is not essential for amniote mesoderm formation

Using the chick model, we asked whether it was possible to uncouple mesoderm formation from streak formation and generate mesoderm precursors radially in the marginal zone. *Brachyury*, a pan-mesoderm-precursor marker, starts its expression in the posterior marginal zone at late stage HH1 (~6-8 hours of incubation) (Knezevic et al., 1997) and *Brachyury*$^{+}$ cells ingress through the streak from HH3 (~12 hours) (Fig. 1A,B; supplementary material Fig. S1A-D). We devised a new protocol for *in ovo* subgerminal-cavity injection (supplementary material Fig. S1E-I) to modulate globally the activity of Wnt, TGFβ or FGF pathway before *Brachyury* expression. These three pathways are known to regulate both mesoderm induction and streak formation (Bertocchini et al., 2004; Harvey et al., 2010; Kimelman, 2006; Stern, 2004; Voiculescu et al., 2007). Control injection did not affect embryo morphology, growth rate or *Brachyury* expression (Fig. 1C, top). Wnt3a did not elicit strong effect on either streak morphology or *Brachyury* expression (supplementary material Fig. S2A). Activin A produced a drastic dorsalizing phenotype, with the axial marker *Gsc* expressed in the central epiblast as a pocket and weak *Brachyury* at its rim (supplementary material Fig. S2B-D). Activation of the FGF pathway (FGF4, Fig. 1C, middle and bottom) generated a ring of *Brachyury*-positive cells in the marginal zone. Induction by FGF4 was highly efficient (70/72; 97%), with the streak recognizable in 42% of the cases (Fig. 1C, middle) and absent in the remainder (Fig. 1C, bottom). Regardless of whether the streak formed or not, induced mesoderm precursors expressed the epithelial-mesenchymal transition (EMT) marker *Snai2* (Fig. 1D) (Nieto et al., 1994) and gave rise to migratory mesoderm cells (supplementary material Fig. S3; Movie 1) as in normal gatrulation EMT (Nakaya et al., 2008). Growth of FGF4-injected embryos did not exceed HH7 (Fig. 1E), possibly owing to its pleiotropic effect on somitogenesis and axial elongation (Bénazéraf et al., 2010). Taken together, FGF injections (FGF4 in Fig. 1C, and FGF2 and

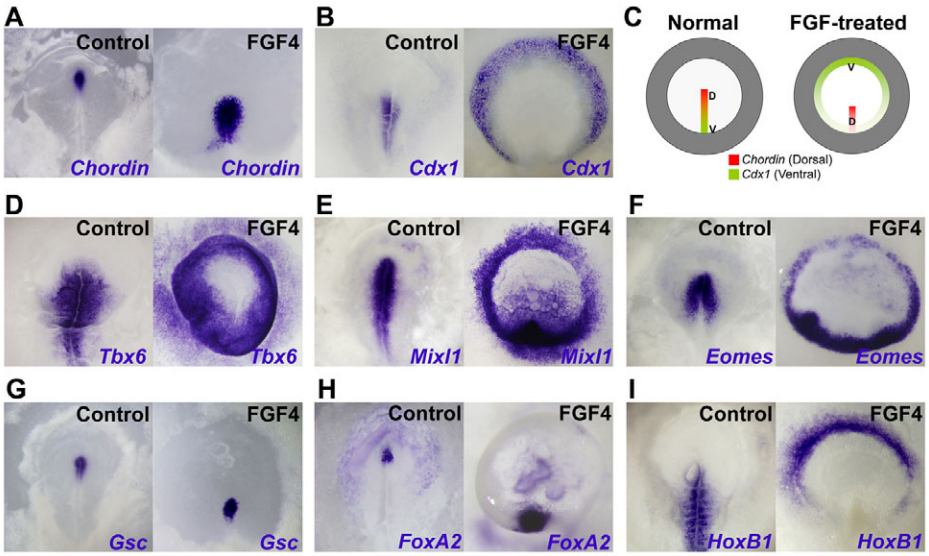

Fig. 2. Induced mesoderm ring exhibits anamniote-like dorsoventral patterning. (**A**) *Chordin* expression. (**B**) *Cdx1* expression. (**C**) Mesoderm dorsoventral patterning in control-injected (left) and FGF-injected (right) embryos. Red, dorsal; green, ventral. (**D**) *Tbx6* expression. (**E**) *Mixl1* expression. (**F**) *Eomes* expression. (**G**) *Gsc* expression. (**H**) *FoxA2* expression. (**I**) *HoxB1* expression. Control injected on the left and FGF4 injected on the right.

FGF8 in supplementary material Fig. S2E,F) led to potent mesoderm induction throughout the marginal zone and to the disruption of streak formation, suggesting that chick mesoderm can be generated irrespective of streak morphogenesis.

The induced mesoderm ring shows an anamniote-like dorsoventral polarity

We next investigated the dorsoventral patterning in ectopically generated mesoderm cells. Expression analysis of FGF4-treated embryos for *Chordin* (Fig. 2A) and *Cdx1* (Fig. 2B) revealed that the induced mesoderm field had a dorsoventral polarity similar to that seen in anamniotes (Fig. 2C), with its dorsal side at the posterior where the rudimentary streak forms and the ventral side at the anterior. This observation was further supported by the analyses using additional pan-mesoderm (*Tbx6*) (Fig. 2D), mesendoderm (*Mixl1*, *Eomes*) (Fig. 2E,F), dorsal (*Gsc*, *FoxA2*) (Fig. 2G,H) and ventral (*HoxB1*) (Fig. 2I) markers.

Wnt and FGF pathways act cooperatively during mesoderm induction

FGFs are known to be potent mesoderm inducers (Amaya et al., 1991; Böttcher and Niehrs, 2005; Dorey and Amaya, 2010; Kimelman et al., 1988; Slack et al., 1987), and their essential role in avian mesoderm formation has also been reported (Chuai et al., 2006; Mitrani et al., 1990; Stern, 1992). In our assay, central epiblast cells exposed to FGFs did not express *Brachyury*, suggesting that FGF activation alone is insufficient. *Sprouty2*, an early response gene of the FGF pathway (Chambers and Mason, 2000; Nutt et al., 2001), was induced as a ring after overnight culture (Fig. 3A), similar to *Brachyury* (Fig. 1C). Progressive shortening of post-injection incubation time revealed a pan-epiblast induction of *Sprouty2* after 4 hours (Fig. 3B), indicating that the central epiblast is capable of rapid response to FGF signaling. Similar time-series experiments with *Brachyury* showed no induction after 4 hours (Fig. 3C), and robust induction was seen only in the marginal zone

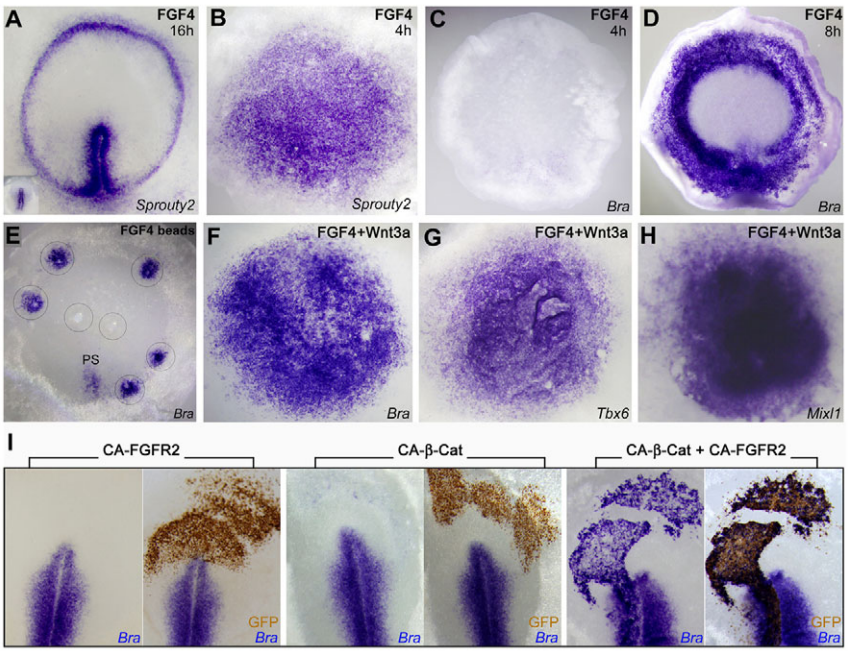

Fig. 3. Induction requires both FGF and Wnt activities. (**A**) *Sprouty2* expression 16 hours after FGF4 injection. Inset shows *Sprouty2* expression 16 hours after control injection. (**B**) *Sprouty2* expression 4 hours after FGF4 injection. (**C**) *Brachyury* expression 4 hours after FGF4 injection. (**D**) *Brachyury* expression 8 hours after FGF4 injection. (**E**) FGF4-soaked beads (circles) induce *Brachyury* only in the marginal zone. (**F-H**) Pan-epiblast induction of *Brachyury* (F), *Tbx6* (G) and *Mixl1* (H) after co-injection of FGF4 and Wnt3a. (**I**) Constitutively active FGFR or β-catenin does not induce *Brachyury* expression in central epiblast; co-expression does.

and after 6-8 hours (Fig. 3D), suggesting that the FGF pathway acts indirectly in mesoderm induction.

Among all the major pathways, only canonical Wnt signaling was reported to be active radially (Roeser et al., 1999; Skromne and Stern, 2001). We confirmed *Wnt8C* radial expression in unincubated embryos (supplementary material Fig. S4A), which quickly becomes restricted to the posterior marginal zone by late HH1 (supplementary material Fig. S4B). To test whether Wnt and FGF pathways act cooperatively in mesoderm induction, we grafted FGF4-soaked beads either in the marginal zone, which induced *Brachyury* potently, or in the central epiblast, which did not (Fig. 3E). Co-electroporation of constructs expressing constitutively active-FGFR (CA-FGFR2) and CA-β-catenin in central epiblast cells activated *Brachyury* expression strongly, whereas either CA-FGFR2 or CA-β-catenin alone did not (Fig. 3I). Co-injection of FGF4 and Wnt3a into the subgerminal cavity transformed the entire epiblast into mesoderm precursors (*Brachyury*, Fig. 3F; *Tbx6*, Fig. 3G; *Mixl1*, Fig. 3H). These data showed that co-activation of the FGF and Wnt pathways is sufficient for the anamniote-like radial and pan-epiblast mesoderm induction.

TGFβ signaling contributes to mesoderm dorsalization and EMT

We next asked what role the TGFβ pathway plays in this induction. Chicken Vg1 and Nodal, two endogenous TGFβ molecules, regulate mesoderm dorsalization and streak formation (Bertocchini and Stern, 2002; Bertocchini and Stern, 2012; Roeser et al., 1999; Skromne and Stern, 2001). The dorsalizing effort was supported by our Activin A injection data (supplementary material Fig. S2C,D). Interestingly, both *Vg1* and *Nodal* were induced as a ring by FGF4 injection (supplementary material Fig. S4C,D). Most of the *Vg1*- and *Nodal*-positive cells were located in the ventral territory (Fig. 2B,C), reflecting their roles in pan-mesoderm EMT after initial dorsoventral patterning (Acloque et al., 2009; Skromne and Stern, 2001). Supporting this notion, co-injection of FGF4 and a strong TGFβ pathway inhibitor SB431542 still led to radial expression of *Brachyury* (supplementary material Fig. S4E). In this case, however, *Brachyury*$^{+}$ cells were located only in the ectoderm (supplementary material Fig. S4F,G, arrowheads), and those few ingressed mesoderm cells failed to migrate away from the ingression site (supplementary material Fig. S4F,G, arrows). Collectively, these data suggest that mesoderm induction requires active FGF and Wnt signaling, whereas the TGFβ pathway contributes mainly to dorsalization and internalization; and each of these three pathways is required for proper mesoderm formation.

Ability to uncouple mesoderm generation and streak formation is evolutionary conserved

We next asked whether the potential to uncouple mesoderm generation from streak formation is unique to the chick model or evolutionarily conserved. All extant birds form a streak in their early development, whereas reptiles have a blastopore and primitive plate together acting as a 'primitive' primitive streak. To test whether the phenomena observed in chick can be extrapolated to amniotes in general, we performed similar experiments in two other avian species, the quail (*Coturnix japonica*) and emu (*Dromaius novehollandiae*). In both cases, a mesoderm ring was induced in the marginal zone by FGF4 (Fig. 4A,B). Embryos of the Chinese soft-shelled turtle (*Pelodiscus sinensis*), a reptilian species that does not form a streak in its early development (Bertocchini et al., 2013;

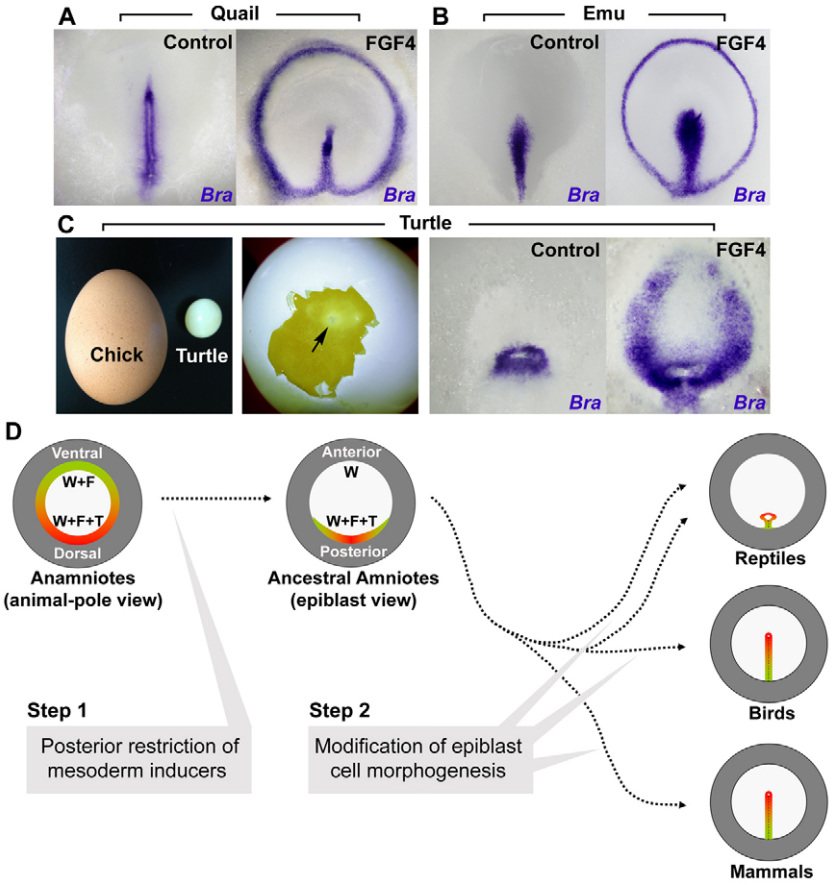

Fig. 4. The potential to generate circumblastoporal mesoderm is conserved among amniotes. (**A**) Quail embryo with control (left) or FGF4 (right) injection and stained for *Brachyury*. (**B**) Emu embryo with control (left) or FGF4 (right) injection and stained for *Brachyury*. (**C**) Turtle embryo with control (third panel) or FGF4 (right panel) injection stained for *Brachyury*. First panel shows turtle and chicken eggs; second panel shows unincubated turtle egg with open shell (arrow indicates embryo). (**D**) Anamniote-to-amniote transition in mesoderm formation. W, Wnt; F, FGF; T, TGFβ. Red, dorsal; green, ventral. Anamniotes have Wnt and FGF activities radially and mesoderm is induced circumblastoporally. A shift in FGF signals results in posterior marginal zone-restricted mesoderm induction in ancestral amniotes. Secondary and independent modifications in epiblast morphogenetic movement lead to formation of a streak in birds and mammals, and a blastopore/primitive plate in reptiles.

Coolen et al., 2008), were also able to generate an anamniote-like 'circumblastoporal' *Brachyury* ring after FGF4 injection (Fig. 4C).

DISCUSSION

The streak in birds and mammals is generally seen as the anatomical correlate of gastrulation and germ layer formation. However, the ontogenetic relationship between the streak and mesoderm, and its phylogenetic association with the anamniote blastopore are not well understood. Mesoderm precursors in birds and mammals are known to be specified in the posterior marginal zone prior to streak formation. Their ingression/internalization from the epiblast is considered to occur only after the streak has appeared. For this reason, streak formation has often been equated with mesoderm formation. In this work, we demonstrated that the streak is not a prerequisite for gastrulation or mesoderm formation in amniotes. The potent radial induction of mesoderm throughout the marginal zone following FGF injection, in the absence of a properly formed streak, revealed that mesoderm and streak formation can be uncoupled, and that the streak is dispensable for the generation of mesoderm.

Cells in the induced mesoderm ring could not only express mesoderm marker genes, but undergo EMT and migration, establish dorsoventral patterning (Figs 1, 2; supplementary material Fig. S3) and initiate terminal differentiation (supplementary material Fig. S5A,B). This supports the hypothesis that the marginal zone in amniotes is evolutionarily homologous to the blastopore in anamniotes and that the streak is a morphogenetic adaptation of the posterior marginal zone (Fig. 4D). Molecular and morphological similarities observed in a few experimental species, however, should not be taken as direct evidence for a common evolutionary origin. Further support for this model would require comparative molecular studies in diverse non-model vertebrate groups (e.g. urodeles, caecilians, reptiles and prototherians). Moreover, the observations that cell movement leading to streak formation differs in mammals and birds, and that extant reptiles do not have a streak, suggest that this morphogenetic adaptation is not a conserved feature in amniotes. We propose that during amniote diversification, the streak evolved secondarily and independently in mammalian and avian lineages. We also propose that an evolutionarily conserved feature of amniote mesoderm formation is the restriction of the mesoderm precursor fate to the posterior marginal zone (Fig. 4D).

Tendency for such posterior marginal zone-restricted mesoderm formation is not limited to anamniote ancestors of amniotes. Mesoderm precursors, defined by *Brachyury* expression, are induced radially in most anamniote species examined so far, but a strong dorsal marginal zone preference has been reported for mesoderm precursor internalization in dogfish (Sauka-Spengler et al., 2003), axolotl (Shook and Keller, 2008; Swiers et al., 2010) and lamprey (Takeuchi et al., 2009), and to a certain extent such preference is also seen in most other anamniote species. Therefore, the stereotypic, circumblastoporal mode of anamniote mesoderm formation is evolutionarily prone to morphogenetic modification. Embryological causes for such modification likely vary in different vertebrate lineages. In ancestral amniotes, additional lineage-specific adaptations, such as the increase in yolk amount and the need to form extra-embryonic tissues, may be causally linked to the posterior marginal zone-restriction of mesoderm formation.

Molecular cause for such a restriction is unclear. In the avian lineage, this can be partially accounted for by a restriction in mesoderm inducers like FGFs. The FGF molecules, expressed radially in anamniotes, are secreted in chick from asymmetrically distributed hypoblast cells at HH1 (FGF8 shown in supplementary material Fig. S5C,D) (Streit et al., 2000). The initial radial Wnt expression in germ wall deep-layer cells is restricted quickly to the posterior marginal zone (supplementary material Fig. S4A,B). Mutual positive regulation of these two pathways and *Brachyury* leads to posterior marginal zone-restricted mesoderm induction. The fact that co-activation of Wnt and FGF pathways resulted in widespread *Brachyury* expression in the epiblast suggests that these two pathways play a major and cooperative role in mesoderm induction. Interestingly, our data also indicate that the TGFβ signaling pathway appears to be mainly involved in dorsalization and EMT. However, it is possible that low levels of TGFβ pathway activity acts as a permissive signal for mesoderm induction by the other two pathways, and precise molecular mechanism for this posterior restriction of mesoderm induction may vary among different amniote lineages.

Although our model predicts that a similar radial mesoderm induction is possible for mammalian embryos, this has not been tested owing to technical difficulties. Embryo topography of eutherian mammals has undergone dramatic changes as a consequence of yolk reduction and placentation. Sources for mesoderm-inducing signals may have undergone likewise changes. Prototherians, the primitive mammals with yolky embryos more similar to birds and reptiles than to eutherians, would be an ideal model for such tests in the future. Despite these differences, however, an avian-like *Brachyury* expression has been reported in several mammalian species (Guillomot et al., 2004; Hassoun et al., 2009; Tam and Loebel, 2007; Viebahn et al., 2002). Interestingly, mouse embryos in normal development exhibit weak and transitory radial expression of *Brachyury* (Perea-Gomez et al., 2004; Rivera-Pérez and Magnuson, 2005) and mesoderm fate can be induced radially in the proximal epiblast margin in mouse mutants with an AVE migration defect (Ding et al., 1998).

In summary, we have shown that the streak is not a prerequisite for mesoderm formation in amniotes. Our data reveal a hidden unity in vertebrate mesoderm induction and suggest that the streak is not a defining feature of amniote mesoderm formation. Because the streak is an important criterion in ethical debates surrounding the use and *in vitro* culture of human stem cells (Daley et al., 2007), the embryological definition and evolutionary significance of the streak deserve re-evaluation.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

C.A. and G.S. designed the experiments; C.A., Y.W., Y.N. and G.S. performed the experiments; C.A., Y.W., Y.N. and G.S. analyzed the data; C.A. and G.S. wrote the paper.

Supplementary material

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