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Conserved and divergent expression patterns of markers of axial development in reptilian embryos: Chinese soft-shell turtle and Madagascar ground gecko

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ABSTRACT

The processes of development leading up to gastrulation have been markedly altered during the evolution of amniotes, and it is uncertain how the mechanisms of axis formation are conserved and diverged between mouse and chick embryos. To assess the conservation and divergence of these mechanisms, this study examined gene expression patterns during the axis formation process in Chinese soft-shell turtle and Madagascar ground gecko preovipositional embryos. The data suggest that NODAL signaling, similarly to avian embryos but in contrast to eutherian embryos, does not have a role in epiblast and hypoblast development in reptilian embryos. The posterior marginal epiblast (PME) is the initial molecular landmark of axis formation in reptilian embryos prior to primitive plate development. Ontogenetically, PME may be the precursor of the primitive plate, and phylogenetically, Koller's sickle and posterior marginal zone in avian development may have been derived from the PME. Most of the genes expressed in the mouse anterior visceral endoderm (AVE genes), especially signaling antagonist genes, are not expressed in the hypoblast of turtle and gecko embryos, though they are expressed in the avian hypoblast. This study proposes that AVE gene expression in the hypoblast and the visceral endoderm could have been independently established in avian and eutherian lineages, similar to the primitive streak that has been independently acquired in these lineages.

1. Introduction

The developmental processes leading up to gastrulation have changed markedly during the evolution of vertebrates. How the mechanisms of axis and germ layer formation, the most fundamental steps in animal development, are conserved and diverged is uncertain even among amniotes. In amniotes, early embryogenesis leading to gastrulation has mostly been examined in mouse and chick embryos. Our previous study of rabbit, porcine and *Suncus* embryos (Yoshida et al., 2016) suggested that the initial expression of *Nodal* throughout the epiblast and visceral endoderm is conserved among eutherians (the extraembryonic endoderm underlying the epiblast before gastrulation is derived from inner cell mass (ICM) throughout eutherian embryos, and is termed here the visceral endoderm to distinguish it from the hypoblast in avian and reptilian embryos, although this endoderm is often called the hypoblast in non-rodent eutherian embryos). In mice, this *Nodal* expression has been demonstrated to suppress premature neural differentiation of the epiblast and to maintain

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pluripotency and proliferation of the epiblast (Camus et al., 2000). *Nodal* expression is also essential for visceral endoderm development (Camus et al., 2006; Mesnard et al., 2006; Yamamoto et al., 2009). The subsequent formation of anterior visceral endoderm (AVE) is also conserved among eutherians, although there are differences in the topology and sequence of the AVE formation (Yoshida et al., 2016). AVE expresses a series of genes (AVE genes) that include genes encoding transcriptional factors, such as *Otx2*, *Hhex*, *Gsc*, *Foxa2* and *Lhx1* (Hoshino et al., 2015; Kimura et al., 2000; Perea-Gomez et al., 1999; Thomas et al., 1998), and signaling antagonists, such as *Dkk1, Cer1*, *Lefty1*, *Sfrp1* and *Sfrp5* (Belo et al., 1997; Hoshino et al., 2015; Kemp et al., 2005; Kimura-Yoshida et al., 2007; Perea-Gomez et al., 2002; Yamamoto et al., 2004). AVE restricts *Nodal* expression to the future posterior where the expression of *Wnt3*, *Brachyury*, *Fgf8* and other genes occur to form the primitive streak and to generate mesendoderm (Barrow et al., 2007; Biechele et al., 2013; Ciruna and Rossant, 2001; Conlon et al., 1994; Liguori et al., 2003; Liu et al., 1999; Rivera-Perez and Magnuson, 2005; Sun et al., 1999; Tortelote et al., 2013). In mouse embryos, AVE has been demonstrated to suppress primitive streak formation; AVE, together with the anterior mesendoderm, has been also suggested to develop adjacent epiblast into anterior neuroectoderm (Beddington and Robertson, 1998; Camus et al., 2000; Kimura et al., 2000; Perea-Gomez et al., 2002; Rossant and Tam, 2009; Tam and Steiner, 1999; Thomas and Beddington, 1996).

In chick embryos, *cNodal* is not expressed throughout epiblast or hypoblast, implicating that NODAL signaling does not have a role in multipotency and proliferation of epiblast and development of hypoblast. The initial landmark of axis formation in chick embryos is Koller's sickle and posterior marginal zone (PMZ) that express a series of genes, such as *cGata6, cGsc, cWnt8a/Wnt8c* and c*Vg1* (Izpisua-Belmonte et al., 1993; Shah et al., 1997; Skromne and Stern, 2001; Stern, 2004). The expression of these genes is first observed during stage X. At stage XIII, prior to the primitive streak formation, the hypoblast expresses most of mouse AVE gene orthologs, such as *cOtx2, cHhex, cGsc, cLhx1, cFoxa2, cDkk1, cCer1*and *cCrescent.* The chick hypoblast also suppresses the primitive streak formation (Bertocchini and Stern, 2002; Foley et al., 2000; Voiculescu et al., 2007; Waddington, 1932, 1933) and promotes the development of anterior neuroectoderm, though not sufficient (Eyal-Giladi and Wolk, 1970; Foley et al., 1997; Streit et al., 2000). Mouse AVE and chick hypoblast are thus proposed to be a homologous amniote innovation (Stern and Downs, 2012), but this fact remains to be demonstrated. Some mouse AVE genes, such as *Lefty,* are not expressed in chick hypoblast. In eutherian embryos, the expression of AVE genes occurs within the *Otx2* positive domain in a nested and graded pattern, with the *Dkk1* expression occurring at the anterior-most region in a horseshoe shape (Hoshino et al., 2015; Yoshida et al., 2016). AVE genes are not expressed in the posterior visceral endoderm, where primitive streak is to be formed. In contrast, AVE gene expression does not occur in a nested pattern in chick embryos. All genes are widely expressed in the hypoblast. To form the primitive streak, the hypoblast is replaced with endoblast (secondary hypoblast) that arises from the posterior germ wall and does not express AVE genes (Bertocchini and Stern, 2002; Stern, 2004). Cell thickening is a common feature in eutherian AVE (Hassoun et al., 2009; Idkowiak et al., 2004; Rivera-Perez et al., 2003; Viebahn, 1999, 2004; Yoshida et al., 2016), but cell thickening has been not observed in avian hypoblast. Data from reptilian development is critical to assess how avian and eutherian patterns of the early development leading to the axis formation and primitive streak formation are related and diverged.

Among amniotes, birds are distantly related to mammals. There are four extant reptilian groups: crocodiles (Crocodilia), turtles (Testudines), snakes/lizards (Squamata) and tuataras (Sphenodontia). Taxonomically, birds and crocodiles constitute Archosauria; Archosauria and Testudines constitute Archelosauria (Fig. 11A) (Crawford et al., 2015; Wang et al., 2013) (NCBI Taxonomy browser (http://www.ncbi.nlm.nih.gov/taxonomy)). Squamata and Sphenodontia constitute Lepidosauria. Archelosauria and Lepidosauria constitute Diapsida. In contrast, Mammalia belongs to Synapsida. Reptiles undergo an avian pattern of discoidal cleavage. In eutherians, epiblast and visceral endoderm develop from ICM, but reptiles do not form ICM, similarly to avian development. Their epiblast is derived from superficial blastoderm cells, and the hypoblast is derived from the blastoderm and/or the epiblast. Koller's sickle has been not identified in reptilian embryos or in eutherian embryos. The initial morphological landmark of the axis formation in reptiles is an opaque thickening, called the primitive plate, in the future posterior (Gilland and Burke, 2004) (this study discriminate between the plate formed before blastopore formation as the primitive plate and the plate formed after its formation as the blastoporal plate). Both eutherian and avian embryos form the primitive streak for gastrulation or mesendoderm formation, but reptiles do not form the primitive streak and undergo gastrulation through a blastopore that is formed in the anterior portion of the primitive plate. The primitive streak has been independently acquired in mammalian and avian lineages.

In this study, we examined gene expression patterns during axis formation in the Chinese soft-shell turtle (*Pelodiscus sinensis*), in the order Testudines, and the Madagascar ground gecko (*Paroedura picta*), in the order Squamata, to examine whether and how similar the patterns of axis formation in reptiles are to the patterns in avians. The data suggest that NODAL signaling does not play a role in epiblast multipotency and hypoblast development in reptilian embryos, similarly to avian embryos. The posterior marginal epiblast (PME) is the initial molecular landmark of axis formation. PME may be the precursor of the primitive plate ontogenetically, and Koller's sickle and PMZ in birds may have been derived from the PME phylogenetically. Most AVE genes, especially signaling antagonist genes, are not expressed in the turtle and gecko hypoblast prior to blastopore formation. This study proposes that AVE gene expression in the hypoblast and the visceral endoderm could have been independently acquired in avian and eutherian lineages, similar to primitive streak that has been independently acquired in these lineages.

2. Results

2.1. Staging of preovipositional Chinese soft-shell turtle embryos

Preovipositional development in turtle has been reported for the wood turtle, *Clammy insculpta* (Agassiz, 1857) and the green turtle, *Chelonia mydas* (Miller, 1985). At the time of oviposition, Chinese soft-shell turtle embryos were at the late gastrulation stage. The egg was spherical and 20 mm in diameter. It took 11–14 days to lay eggs from the last laying, and intra-oviducal days were counted from the previous oviposition. A unique feature in preovipositional development of Chinese soft-shell turtle embryos is extensive epiboly. At the intra-oviducal day 5 (stage 2), the embryos were at the cleavage stage (Fig. 1Aa and Ba); the blastoderm was a size of approximately 3 mm in diameter at one pole of macrolecithal eggs and consisted of multiple layers of cells (Fig. 1Aa, Ba, Ca, and a′). Upper layers were comprised of small cells, and cells in deeper layers were large and most were still open basally to the yolk mass (Fig. 1Ba, Ca, and a′). Cells at the surface layer were weakly *PsNanog* (a marker for pluripotent epiblast cells in both avian and eutherian)-positive in the central region but not in the periphery (Fig. 1Ba and Ca). At the intra-oviducal day 6 (stage 3), all cytoplasm was now cleaved into true

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