MECHANISMS OF ENDODERM FORMATION IN A CARTILAGINOUS FISH REVEAL ANCESTRAL AND HOMOPLASTIC TRAITS IN JAWED VERTEBRATES

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ABSTRACT

In order to gain insight into the impact of yolk increase on endoderm development, we have analyzed the mechanisms of endoderm formation in the cartilaginous fish S. canicula, a species exhibiting teolecithal eggs and a distinct yolk mass. We show that in this species, endoderm markers are expressed in two distinct tissues, the deep mesenchyme, a mesenchymal population of deep blastomeres lying beneath the epithelial-like superficial layer, already specified at early blastula stages, and the involuting mesendoderm layer, which appears at the blastoderm posterior margin at the onset of gastrulation. Formation of the deep mesenchyme involves cell internalizations from the superficial layer prior to gastrulation, by a movement suggestive of invaginations. These cell movements were observed not only at the posterior margin, where massive internalizations take place prior to the start of involution, but also in the center of the blastoderm, where internalizations of single cells prevail. Like the adjacent involuting mesendoderm, the posterior deep mesenchyme expresses anterior mesendoderm markers under the control of Nodal/activin signaling. Comparisons across vertebrates support the conclusion that endoderm is specified in two distinct temporal phases in the blastoderm posterior margin of the cartilaginous fishes and myxinoids. These adaptations have gone together with transitions from holoblastic to meroblastic cleavage modes and major changes in the early embryo architecture (Arendt and Nübler-Jung, 1999). One of the most visible examples of such changes is the presence of endodermal components morphologically distinct from the embryonic gut and traditionally referred to as extraembryonic, such as the early lower layer of birds (hypoblast and endoblast), primitive endoderm of mammals or yolk syncytial layer (YSL) of teleosts. The cellular organization and mode of formation of these tissues have been extensively studied in the mouse, chick and zebrafish and they appear highly divergent between these species. For instance, in the mouse, the primitive endoderm forms a morphologically distinct polarized epithelium, which arises from the blastocyst inner cell mass by a cell sorting mechanism, shortly after blastocoel formation. It later subdivides into two components, the parietal endoderm and visceral endoderm, which contrary to the traditional view of an absolute early segregation between embryonic and extraembryonic tissues, is now known to contribute to the gut (Rossant and Tam, 2009; Kwon et al., 2008). In the chick, hypoblast formation has long been thought to involve the merging of cell clusters, originating from the early epiblast by poly-ingression, and the occurrence of single cell invasions from the epiblast prior to gastrulation has recently been confirmed at stages preceding primitive streak formation (Stern and Downs, 2012; Voiculescu et al., 2014). Finally, the zebrafish YSL forms by a collapse of marginal blastomeres with the yolk cell cytoplasmic cortex between the 512- to 1024-cell stage (Carvalho and Heisenberg, 2010).

INTRODUCTION

Spectacular expansions of the egg yolk mass have taken place several times during vertebrate evolution, extreme examples of this evolutionary trend being observed in amniotes, cartilaginous fishes and myxinoids. These adaptations have gone together with transitions from holoblastic to meroblastic cleavage modes and major changes in the early embryo architecture (Arendt and Nübler-Jung, 1999). One of the most visible examples of such changes is the presence of endodermal components morphologically distinct from the embryonic gut and traditionally referred to as extraembryonic, such as the early lower layer of birds (hypoblast and endoblast), primitive endoderm of mammals or yolk syncytial layer (YSL) of teleosts. The cellular organization and mode of formation of these tissues have been extensively studied in the mouse, chick and zebrafish and they appear highly divergent between these species. For instance, in the mouse, the primitive endoderm forms a morphologically distinct polarized epithelium, which arises from the blastocyst inner cell mass by a cell sorting mechanism, shortly after blastocoel formation. It later subdivides into two components, the parietal endoderm and visceral endoderm, which contrary to the traditional view of an absolute early segregation between embryonic and extraembryonic tissues, is now known to contribute to the gut (Rossant and Tam, 2009; Kwon et al., 2008). In the chick, hypoblast formation has long been thought to involve the merging of cell clusters, originating from the early epiblast by poly-ingression, and the occurrence of single cell invasions from the epiblast prior to gastrulation has recently been confirmed at stages preceding primitive streak formation (Stern and Downs, 2012; Voiculescu et al., 2014). Finally, the zebrafish YSL forms by a collapse of marginal blastomeres with the yolk cell cytoplasmic cortex between the 512- to 1024-cell stage (Carvalho and Heisenberg, 2010). Despite these differences in morphogenesis, the amniote hypoblast/AVE (anterior visceral endoderm) and teleost dorsal YSL share expression of signaling molecules and transcription factors known as components of AME (anterior mesendoderm) genetic programs, a similarity proposed to be related to independent recruitments in the amniote and actinopterygian lineages (Stern and Downs, 2012).

How the increase in egg yolk amount may affect early endoderm formation and patterning is poorly known outside osteichthyans (bony fishes and their descendants, including tetrapods), which comprise all established vertebrate model organisms. Cartilaginous fishes or chondrichthyans, which form one of the three major vertebrate phyla and comprise about 1100 extant species, are of interest to address this issue for two reasons. First, as the closest outgroup to osteichthyans, the other major
ptilium of gnathostomes (jawed vertebrates), they are essential to reconstruct gnathostome ancestral characteristics, through comparisons with other vertebrate models (Coolen et al., 2008a). Second, a lecithotrophic mode of embryonic nutrition is likely to be ancestral in chondrichthyans and most elasmobranchs develop from large telolecithal eggs, endowed with a distinct yolk sac (Blackburn, 2014). This is in particular the case of the catshark *Scyliorhinus canicula*, one of the most extensively studied representatives of chondrichthyans (Coolen et al., 2008a).

This species develops from large, telolecithal eggs, which undergo a discoidal mero blastogenic cleavage and are laid at early stages of blastocoel formation (Ballard et al., 1993). Following egg deposition, the blastoderm consists of two cell layers, a superficial one, exhibiting an epithelial-like morphology, and an inner cell population of dispersed blastomeres, referred to as deep mesenchyme (Ballard et al., 1993; Coolen et al., 2007). This bilayered structure persists for about seven days, a period characterized morphologically by a size expansion of the blastoderm (Ballard et al., 1993). A marked change is observed at stage 11, which is considered as the start of gastrulation. At this stage, a novel cell population identified as mesendoderm, based both on molecular characterization and histology, starts to involute along the blastoderm posterior margin, adjacent to the deep mesenchyme (Ballard et al., 1993; Coolen et al., 2007). This involution movement results in the formation of a posterior overhang, which initially elongates over the yolk from anterior to posterior and is later found lining the developing archenteron (Coolen et al., 2007). Concomitantly, lateral and anterior regions of the blastoderm become thinner and spread over the yolk to later form a distinct yolk sac, connected to the embryo via a vascularized stalk. From the morphological appearance of the embryonic axis (stage 12) and until neural tube closure, the developing embryo thus appears strictly restricted to the posterior part of a growing, flattened disc (Ballard et al., 1993; Coolen et al., 2007). This posterior restriction of embryo formation is a major difference with teleosts and was previously suggested to correspond to the orientation of the future elongating embryonic axis, and corresponds to the ventral to dorsal, or ab-organizer to organizer polarity of amphibians and teleosts.

**MATERIALS AND METHODS**

**Embryo production and maintenance, staging and nomenclature**

*S. canicula* eggs were produced by the Biological Marine Resources facility of Roscoff Marine Station and kept in 17°C oxygenated sea water until the desired stages were obtained. This study was performed on catshark embryos prior to formation of the nervous system and of any other organ and is therefore exempt from a special license under the terms of institutional and national regulations. Embryos were staged after Ballard et al. (Ballard et al., 1993) and a description of the stages studied is provided in supplementary material Movies 1 and 2. Stage 11 is considered as the start of gastrulation, based on two criteria (1) the appearance of a distinct mesendoderm layer (Ballard et al., 1993) and (2) the onset of *Brachyury* expression (Coolen et al., 2007). Prior to stage 11, the anterior to posterior polarity of the blastoderm refers to the orientation of the future elongating embryonic axis, and corresponds to the ventral to dorsal, or ab-organizer to organizer polarity of amphibians and teleosts.

**Probe isolation and characterization**

The *S. canicula Dkk1* probe was amplified by degenerate RT-PCR from stage 9–15 cDNA by a nested PCR, successively using the following pairs of primers: 5’-GAYGCGNAATGGTGYGTYCC and 3’-ATTYTT-RCTCCARAAARTG, respectively encoding the conserved DAMCCP and HFWSKI amino acid motifs of the Dkk1 peptide of other vertebrates, and 5’-GCAGATGGTGTYGCGNGG and 3’-ARRCACTRTCNCCYTC, respectively encoding the conserved motifs AMCCPG and EGDMCL. The amplified cDNA fragments were subcloned in the pGEM-T easy vector and sequenced. *ScSox17, ScHex, ScLeftyB*, Sc*Fgf17, ScShh* and *ScChd* probes were obtained from a large-scale cDNA sequencing project described (Coolen et al., 2007). Novel sequences were included in molecular phylogenetic trees to confirm their identity (supplementary material Fig. S1). *ScT, ScOtx5, ScGata6* and *ScLim1* probes were reported in previous studies (Coolen et al., 2007; Plouhinec et al., 2005; Sauka-Spengler et al., 2003).

**In situ hybridization and histological analyses**

Whole-mount in situ hybridizations were conducted using standard protocols adapted to the catshark and followed by embryo embedding in paraaffin and sectioning, as described previously (Dérobert et al., 2002). For semi-thin sections, embryos were fixed in 4% glutaraldehyde, 0.25 M sucrose in 0.2 M cacodylate buffer pH 7.4, post-fixed in 1% OsO4 and embedded in Epon. 0.5 μm sections were cut and stained with toluidine blue.

**Dil cell labeling**

Stage 8 to 10 embryos were removed from the shell and transferred to 0.45 μm filtered sea water. CellTracker CM-Dil (Invitrogen) was diluted (1/10) in 0.3M sucrose from a 5 mg/ml stock solution in ethanol and applied to embryo territories by ejection from a capillary tube. A control was also performed after one hour of culture, in order to check the absence of internal labeling due to tissue disruption related to the process of dye application. Labeled embryos were cultured in filtered sea water for 24 hours, prior to fixation, paraaffin embedding and sectioning (12 μm). Sections were stained with DAPI, mounted and photographed using a Leica SP5 confocal microscope. The presence or absence of labeled cells was assessed in the deep mesenchyme or involved mesendoderm, taking into account heavily labeled cells.

**Pharmacological treatments**

Pharmacological treatments were conducted by in ovo injection of 200 μl of a 500 μM dilution of the Alk4/5/7 inhibitor SB-505124 in 0.01% DMSO in stage 8/9 catshark embryos. This solution was replaced by the same volume of 0.01% DMSO in control embryos. Following injections, eggs were maintained for 3 days in oxygenated sea water at 17°C, with viabilities higher than 90%. They were then dissected, fixed in PFA 4%, dehydrated and stored at −20°C in methanol 100% prior to in situ hybridization.

**RESULTS**

**Two distinct phases of ScSox17, ScGata6 and ScHex expressions in the early catshark embryo**

In order to unambiguously identify endodermal cell populations in the catshark, we analyzed expression of homologues of three genes known to be expressed in extraembryonic endoderm in amniotes and additional mesendoderm territories, *ScSox17, ScGata6, and ScHex*. Expression was observed at the earliest blastula stages studied (stages 4–6), less than 48 hours following egg deposition (supplementary material Fig. S2A). At stages 7 to 10, all three were expressed in the deep mesenchyme in contact with the yolk, as well as in and around large, subjacent yolk syncytial nuclei (Fig. 1A,B,F,G,K,L; sections in Fig. 1B1,G1,G2,L1). The deep mesenchyme persists in blastoderm territories, which lie adjacent to the elevating embryonic axis and spread anteriorly and laterally over the yolk at subsequent stages (supplementary material Movies 1 and 2).
1 and 2; Fig. S2F,G). Expressions of ScSox17, ScGata6, and ScHex in this tissue were maintained from stage 11 to at least stage 14 (Fig. 1C–E,H–J,M–Q; sections in Fig. 1D1,E1,J1,O1,Q1–Q3; see also supplementary material Fig. S2B–G). Starting from stage 11, expression of all three genes was also observed in different territories of the involuting AME layer. ScSox17 transcripts accumulated in the involuted mesendoderm along a 60° crescent of the posterior margin (Fig. 1C,D,D1; supplementary material Fig. S2D). ScGata6 territory largely overlapped with ScSox17, the signal extending further laterally in marginal territories known to express lateral mesoderm markers (Fig. 1H,I; supplementary material Fig. S2E). ScHex expression appeared in the involuting AME at stage 11, initially confined to the midline of the posterior margin (Fig. 1M) and progressively displaced to the anterior aspect of the involuting layer and adjacent deep mesenchyme as involution proceeded (Fig. 1N–P,O1). An additional signal was also transiently observed more posteriorly, in the prospective margin (Fig. 1C–E,H–J,M–Q; sections in Fig. 1D1,E1,J1,O1,Q1–Q3; see also supplementary material Fig. S2E).

**Deep mesenchyme formation in the catshark**

In order to gain insight into the mode of formation of the deep mesenchyme, we next conducted a histological description, based on analysis of semi-thin sections from stage 9 to stage 11. This analysis highlighted the presence of several populations of inner cells, differing by their morphology. At stage 9, all cells appeared round shaped, with thin randomly oriented protrusions (Fig. 2A,B). At stages 10–10+, this cell morphology persisted in the anterior part of the blastoderm, lying underneath the superficial layer (Fig. 2C,D,G; see also Fig. 2K at stage 11) but cells showing an altered morphology appeared in the posterior part of the blastoderm, close to the posterior margin (Fig. 2F,I,I'). At these stages, the superficial layer of the posterior part of the blastoderm displayed a columnar morphology (Fig. 2E,H), as previously reported (Coolen et al., 2007) and cells exhibiting apical constrictions suggestive of internalizations could frequently be observed (Fig. 2E). At stage 11, two novel cell morphologies could be observed in the deep mesenchyme, (i) flattened cells lying beneath the superficial layer at the extreme anterior part of the blastoderm (Fig. 2J) and (ii) elongated cells located adjacent to, and anterior to the involuting layer, close to the yolk cell (Fig. 2L,M). The latter exhibited protrusions oriented along the AP (antero-posterior) axis, perpendicular to those of the adjacent AME involuting layer (compare Fig. 2M,N). These cell morphologies suggest that the formation of the deep mesenchyme may involve single cell internalizations and migrations from the superficial layer and posterior margin. To directly address this possibility, we used DiI cell labeling to track cells originating from these locations from mid-blastula to early gastrula stages (Fig. 3, Fig. 4). After one hour of culture following local applications of the DiI solution either at the posterior margin or at the center of the blastoderm (Fig. 3A, Fig. 4A; supplementary material Tables S1 and S2), labeled cells...
formed a single, superficial territory comprising 5 to 20 fluorescent cells (Fig. 3B, Fig. 4B) and were never observed either in the deep mesenchyme or involuting mesendoderm (supplementary material Tables S1 and S2). Labeled embryos were then cultured for 24 hours after DiI application and the location of fluorescent cells was examined on histological sections. In the youngest embryos injected at the posterior margin (stage 8–9; stage 10 after culture; n=4), all fluorescent cells were found internalized as a cluster of mesenchymal cells, close to the site of injection (Fig. 3C,D; supplementary material Table S1). A marked change in the organization of fluorescent cells was observed when DiI was applied at the posterior margin at subsequent stages (stages 10/11). In these embryos, labeled cells were found displaced within the involuting mesendoderm layer as a highly coherent group but never observed in the deep mesenchyme (Fig. 3F,H; supplementary material Table S1; n=4). DiI application at the center of the blastoderm at stages 8 (mid-blastula) to 10 (late blastula) also led to the presence of labeled cells in the deep mesenchyme in all embryos studied after culture (Fig. 4C–E,C′–E′; supplementary material Table S2; n=6). In this case however, the superficial layer remained heavily labeled at the site of dye application, which was not observed when the dye was applied at the level of the posterior margin. Of note is that during early gastrulation, application of the dye at lateral levels of the margin resulted in an organization of fluorescent daughter cells similar to the one observed at the posterior midline at earlier stages (Fig. 3E,G). Thus, as development proceeds, cell internalizations progress laterally along the posterior margin, following the same succession of distinct cell behaviors as observed in the midline (Fig. S1).

The posterior deep mesenchyme and AME share expression of the same signaling molecules

In line with their roles in embryo patterning or germ layer specification, the amniote AVE or hyoblast are a source of secreted signals, such as Fgf8 or the Nodal and Wnt antagonists Lefty and Dkk1. These signals differ from those later secreted by the organizer, which expresses the BMP antagonist Chordin. In order to address the signaling properties of the deep mesenchyme, we analyzed expression of catshark orthologues of Lefty, Dkk1 and of Fgf17, a member of the Fgf8/Fgf17/Fgf18 class, from early blastula stages to 14. No expression of a Lefty orthologue (referred to hereafter as ScLeftyA) was previously detected in this tissue (Coolen et al., 2007). We reassessed this conclusion by analyzing a second Lefty orthologue, termed ScLeftyB, isolated from additional EST sequencing (supplementary material Fig. S1). ScLeftyB and ScFgf17 expression were already detectable at the posterior margin less than 48 hours after egg deposition (stage 4 to 6, supplementary material Fig. S2A). From stages 9 to 11, they showed expression characteristics very similar to ScDkk1. At stages 9–10+, all three shared a prominent positive territory at the posterior margin and the adjacent deep mesenchyme (Fig. S5A,B,F,G,K,L and corresponding sections 5B1,F1,G1,K1). At stage 11, the signal also disappeared for all three genes from the medial-most part of the margin, persisting more laterally (Fig. S5C,H,M, sections in Fig. S5C1,H1). Their territories withdrew from the deep mesenchyme and segregated at subsequent stages. At stages 12–13, expression of all three genes persisted in the mesendoderm at the posterior arms level (Fig. 5D,I,N) and in the case of ScDkk1, its anteriormost component at the midline level (Fig. 5N1,N2). These broad expression characteristics were maintained in the elongating embryonic axis except for ScLeftyB, restricted at stage 14 to a small midline territory of the forming trunk (Fig. 5E,J,O). In order to compare these profiles with those of an organizer specific marker, we next analyzed ScChd, the catshark orthologue of Chordin. ScChd expression remained undetectable at stage 10 but a strong signal was observed at stage 11 in a medial domain of the involuting posterior margin (Fig. 6A,A1), where a morphological structure referred to as the notochordal triangle later becomes visible (Sauka-Spengler et al., 2003). At later stages, expression was restricted to this structure, excluding the ScT positive posterior arms (Fig. 6B,C). ScShh, the catshark orthologue of Shh, was also expressed in the notochordal triangle starting from stage 12 (Fig. 6D,E). At later stages, as in other vertebrates, ScChd and ScShh were expressed in the forming notochord, visible in the catshark from stage 13 (Fig. 6C,E). Unlike ScLeftyB, ScFgf17, ScDkk1 and ScLim1 (Coolen et al., 2007), ScChd expression was never observed in the deep mesenchyme.
and it never reached the anteriormost region of the involuting layer (Fig. 6A1; summary in Fig. 6F). At later stages, it was also excluded from the foregut diverticulum, the signal intensity exhibiting a gradual decrease from chordal to prechordal levels of the axial mesendoderm (compare Fig. 6B1 and Fig. 6B2).

Nodal/activin signaling is essential for the regionalization of the deep mesenchyme and the specification of the posterior margin

Nodal/activin signaling is essential for the specification of mesoderm and gut endoderm in all vertebrates studied and also

Fig. 3. Temporal regulation of cell behaviors at the posterior margin of S. canicula embryos from late blastula to early gastrula stages. (A) Scheme showing the experimental procedure used, and the plane and location of the sections shown in panels B–H (red dotted lines). (B–H) DAPI staining (blue) and Dil fluorescence detection (red) on sections of embryos labeled as in panel A. (B) Example of a control stage 10 labeled embryo, cultured for one hour following Dil application. (C,D) Mid-sagittal sections of two embryos labeled in the midline at stage 9 and cultured for 24 hours after Dil application. (E,F) Respectively para-sagittal and mid-sagittal sections of an embryo labeled at lateral and medial levels at stage 10 and cultured for 24 hours after Dil application. Same in panels G,H, with Dil application at stage 10+. White and yellow arrowheads point to Dil labeled cells in the deep mesenchyme or the involuting mesendoderm respectively. Panel I schematizes the types of movements observed for cells derived from the posterior margin depending on both stage and location along the margin (see Results). Whether internalizations by an ingestion-like process take place in early gastrulae at the transition zone between the involuting mesoderm and deep mesenchyme (i.e. the anterior-most aspect of the involuting layer) could not be addressed, due to the inaccessibility of this territory and limitations in embryo culture times (orange question mark). Same abbreviations as in Fig. 1.

Fig. 4. Cell internalizations from the superficial layer at the center of the blastoderm from stages 9 to 10 in S. canicula. (A) Scheme showing the experimental procedure and plane and location of the sections shown in panels C–E (red dotted lines). (B–E) DAPI staining (blue) and Dil fluorescence detection (red) on sections of embryos labeled as in panel A. (B) Example of a control stage 10 labeled embryo, cultured for one hour following Dil application. (C) Mid-sagittal section of an embryo labeled in the center of the blastoderm at stage 9 and cultured for 24 hours after Dil application. (C′) Higher magnification of the territory boxed in panel C showing labeled internalized cells. (D,E) Respectively para-sagittal and mid-sagittal sections of an embryo labeled in the center of the blastoderm at stage 9 and cultured for 24 hours after Dil application. (D′,E′) Higher magnification of the territories boxed in panels D and E showing labeled internalized cells. White arrowheads point to Dil labeled cells in the deep mesenchyme.
required for AVE specification in the mouse (Mesnard et al., 2006). In order to analyze the role of Nodal/activin signaling in endoderm development in the catshark, we conducted an in vivo pharmacological approach, using SB-505124, a selective inhibitor of activin Alk4/5/7 receptors. The drug or control DMSO was injected inside the eggshell at stages 8 to 9, reached about 5 days following egg deposition. Eggs were then maintained for three days in 17°C oxygenated sea water prior to embryo fixation and dissection. DMSO injected control embryos appeared normal and their stages ranged between 11 (n=21) and 12 (n=7), as expected for uninjected embryos at this temperature. SB-505124 treated embryos could be classified into two classes based on their general morphology (supplementary material Table S3). The majority, referred to as class 1 embryos, appeared as flattened blastodiscs, without evidence of posterior fold formation (Fig. 7B,D,F,H,J,P). A minority of treated embryos, referred to as class 2 embryos, showed similarities to stage 12 embryos, in that they exhibited distinct posterior arms on each side of the forming embryonic axis (Fig. 7L,N). In order to assess the loss of Nodal/activin signaling in the experimental conditions tested, we first focused on expression of the feedback antagonist ScLeftyB in SB-505124-treated and control embryos (supplementary material Table S3; Fig. 7A,B). While present at the posterior margin in all control embryos tested (n=3, Fig. 7A), ScLeftyB expression remained undetectable in all treated embryos (class 1, n=3, Fig. 7B), in line with a loss of Nodal/activin signaling. We next analyzed expression of the general mesoderm marker ScT (Fig. 7C,D) and of the notochordal triangle marker ScChd (Fig. 7E,F). In both cases, control embryos exhibited the expected signals (n=5 for ScT, n=2 for ScChd) around the whole margin (ScT, Fig. 7C) or in the involuting axial mesoderm (ScChd, Fig. 7E). In contrast, ScT and ScChd expressions were abolished in all treated embryos analyzed (n=4 and n=3 respectively, Fig. 7D,F). Similarly, ScOtx5 and ScLim1 signals were observed, in the involuting mesendoderm and adjacent deep mesenchyme of control embryos (n=4 for ScOtx5, same for ScLim1, Fig. 7G,I), but lost in all treated embryos (n=2 and n=4 for ScOtx5 and ScLim1 respectively; Fig. 7H,J). Finally, we analyzed the effect of the drug on three genes characterized by an early expression in the deep mesenchyme and syncytial nuclei, and a later expression phase at different levels of the involuting margin as shown above, ScSox17, ScGata6 and ScHex (Fig. 7K–P). In all cases (total of 11 embryos tested), the signal in the deep mesenchyme and yolk syncytial nuclei was maintained in control and SB-505124 treated embryos (compare Fig. 7K and Fig. 7L, Fig. 7M and Fig. 7N, Fig. 7O and Fig. 7P; see also supplementary material Fig. S3A,B). In contrast, no marginal expression was observed in any of the treated embryos (n=13, Fig. 7L,N,P), while it was present in control embryos (Fig. 7K,M,O; compare Fig. 7K and Fig. 7L, Fig. 7J and Fig. 7P; see also supplementary material Fig. S3A,B). In contrast, no marginal expression was observed in any of the treated embryos (n=13, Fig. 7L,N,P), while it was present in control embryos (Fig. 7K,M,O; compare Fig. 7K and Fig. 7L, Fig. 7J and Fig. 7P; see also supplementary material Fig. S3A,B). In contrast, no marginal expression was observed in any of the treated embryos (n=13, Fig. 7L,N,P), while it was present in control embryos (Fig. 7K,M,O; compare Fig. 7K and Fig. 7L, Fig. 7J and Fig. 7P; see also supplementary material Fig. S3A,B).
DISCUSSION
Endoderm is specified in two phases in the catshark as in osteichthyan

Analysis of Hex, Gata6 and Sox17 orthologues in the catshark show that not only the anteriormost part of the involuting layer but also the deep mesenchyme is endowed with an endodermal identity. The timing of their specification appears as a major difference between these two tissues, since the latter already expresses Sox17, Hex and Gata6 at the earliest stages accessible, which shortly follow blastocoel formation and precede gastrulation by more than seven days (Ballard et al., 1993; Coolen et al., 2007; Sauka-Spengler et al., 2003). As previously noted (Godard and Mazan, 2013), two phases of endoderm specification are most obvious in mammals, birds and teleosts, all endowed with distinct extraembryonic tissues, but have also been reported in some amphibians exhibiting no evidence for two morphologically distinct endoderm components, including Xenopus. While a key role of Nodal in mesendoderm formation has been demonstrated in amniotes, amphibians and teleosts (Conlon et al., 1994; Erter et al., 1998; Feldman et al., 1998; Schier, 2000; Schier and Shen, 2000; Steiner et al., 2006), the mechanisms controlling the earliest phase of endoderm specification appear to vary extensively across vertebrates. In the catshark, formation of the involuting layer and expression of all mesoderm and mesendoderm markers were abolished following SB-505124 treatments, in line with a conservation of the role of Nodal/activin signaling in mesendoderm specification. In contrast, no evidence for a loss of the endodermal identity was observed in the deep mesenchyme following abrogation of Nodal signaling activity. This observation cannot rule out a role of Nodal signaling in the initial steps of deep mesenchyme specification, as ScSox17, ScHex and ScGata6 expressions were already established at the time of egg laying, making it difficult to assess the effect of the drug prior to the onset of their expression. However, it argues against a major role of the pathway in the maintenance of the deep mesenchyme endodermal identity. This conclusion is also supported by the localized deep mesenchyme expression of the catshark orthologue of Lefty, a feedback antagonist of Nodal signaling (Chen and Schier, 2002; Juan and Hamada, 2001; Meno et al., 1999), which suggests a posterior restriction of Nodal signaling activity at all stages studied.

From an evolutionary standpoint, the reiteration of a biphasic mode of endoderm specification now found in chondrichthyans as in all major osteichthyan lineages supports the hypothesis that it may be an ancestral characteristic of jawed vertebrates (Godard and Mazan, 2013). However, in the absence of mechanistic arguments, it remains difficult to formally exclude independent rises of the earliest specification event in the different vertebrate phyla. Finally it should be noted that a partitioning of nutritive tissues into a vegetal mass without contribution to the gut and an embryonic component derived from the blastopore margin has also been proposed in the lamprey (Takeuchi et al., 2009). However, in this species, this distinction primarily relies on the absence of endoderm marker expression in the vegetal mass, a criterion, which argues against homology with the early specified tissue identified in the catshark.

Cell internalizations suggestive of ingestion movements precede gastrulation in the catshark

We had previously observed that the physical continuity between the posterior deep mesenchyme and the adjacent AME goes together with a molecular continuity, both expressing not only endoderm but also AME regional markers, such as Lim1 or Gsc (Coolen et al., 2007). This study extends this conclusion to signaling molecules such as the catshark orthologues of Lefty, Fgf17, a member of the Fgf8/17/18 family, or Dkk1, known to be specifically expressed in AME as well as in endodermal extraembryonic components of amniotes and teleosts (Stern and Downs, 2012). We further show that this regional identity is lost following abrogation of Nodal/activin signaling, which as reviewed previously (Godard and Mazan, 2013) is reminiscent of the molecular phenotype observed in the mouse embryonic visceral endoderm (Mesnard et al., 2006). Finally, we find that massive cell internalizations from the posterior margin take place prior to gastrulation and contribute to posterior deep mesenchyme formation. The posterior deep mesenchyme and involving mesendoderm thus lie adjacent to each other, exhibit the same, Nodal-dependent, AME regional identity (Fig. 8A,B), and are also related by their embryonic origin. However, the cell internalizations from the posterior margin, which contribute to their formation, differ by their timing and the cell movements involved. The low cohesion of cells internalized at the posterior margin from stages 8 to 10, prior to the appearance of a bilayered overhang, contrasts with the tightly clustered cell organization.
observed at later stages, and suggests that massive ingestion-like movements precede involution at the posterior margin (Fig. 8C). In addition to these cell movements taking place at the posterior margin prior to gastrulation, we also obtained evidence for cell internalizations in the center of the blastoderm. These internalizations differed from the former in that a prominent labeling persisted in the superficial cell layer following DiI application and embryo culture, suggesting that they may only concern small clusters of cells or individuals, as suggested by histological analyses. Taken together, these data provide evidence that early development in the catshark involves cell internalizations from the superficial layer, with a shift in cell orientation at the transition zone. Abbreviations used: AME, anterior mesendoderm; PME posterior mesendoderm. (D) Comparisons of the endoderm regional pattern between the catshark, zebrafish, xenopus and turtle at the onset of gastrulation. The color code is as follows: yellow for territories exhibiting an endodermal identity, but express neither AME markers, nor Brachyury, blue for endoderm of anterior regional identity, red for mesendoderm of posterior regional identity.

Evolutionary implications: homoplastic traits between chondrichthians and amniotes
From an evolutionary standpoint, the cell movements, shown here to occur during catshark early development, strikingly recall some aspects of amniote development. Firstly, as already noted (Godard and Mazan, 2013) and confirmed in this study, the catshark posterior deep mesenchyme and involuting AME appear to differ by their cell organization rather than their regional identity. The establishment of fate maps in the catshark is currently hampered by difficulties to maintain embryo viability during extended durations following cell marking procedures but based on its cell organization and location relative to the developing embryonic axis, the deep mesenchyme is likely to have a major contribution to extraembryonic structures, such as the yolk sac, syncytiotrophoblast (Lechenault and Mellinger, 1993) or stalk connecting the embryo to the yolk sac. Deep mesenchymal cells thus persist until at least somite stages in blastoderm territories spreading over the yolk, at increasing distances from the site where involution and embryo formation take place (Ballard et al., 1993; Coolen et al., 2007; this study)
EST sequences were obtained with the support of Genoscope, Evry, France. We are grateful to Sébastien Henry, Ronan Garnier, Régis Lasbleiz and Sébastien Patruno for their help in embryo maintenance. Diane Schausi for excellent technical help. J. Andrew Gillis for sharing his expertise in DII labeling, Altana Perea-Gomez, Anne Camus, Jérôme Collignon, De-Li Shi, Hélène Mayeur and William Norton for helpful comments on the manuscript.

Competing interests
All authors declare that they have no financial and competing interests.

Author contributions
SM designed the study; BGG, MC, SLP, AG, SF-G, LL, RL performed experiments; PW, JP, CDS; SK and WC contributed to sequences and analyses; BGG, AB and SM analyzed the results and wrote the manuscript.

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References


Supplementary Material
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Fig. S1. See next page for legend.
Fig. S1. Molecular phylogeny of the *S. canicula* genes identified in this study. Maximum-likelihood (ML) trees were inferred using the program PhyML version 3.0 (Guindon and Gascuel, 2003) for *Sox7/17/18* (A), *Gata6* (B), *Hex* (C), *Lefty* (D), *Fgf8/17/18* (E), *Dkk1* (F) and *Chordin* (G). The sequence alignments were constructed with the alignment editor XCed in which MAFFT is implemented (Katoh et al., 2005) including the deduced amino acid sequences of the *S. canicula* cDNA identified in this study as well as peptide sequences of other species retrieved from the Ensembl and GenBank databases. The JTT model of amino acid substitutions was assumed with the among-site rate heterogeneity based on the gamma distribution taken into account. The shape parameter for the gamma distribution that maximizes the likelihood of a preliminary neighbor-joining tree was computed for each gene. The numbers of the amino acid residues in the input alignments were: 82 amino acids (aa) (A); 155 aa (B); 128 aa (C); 51 aa (E); 108 aa (F); 611 aa (G). Accession numbers for the catshark sequences are the following: *ScSox17*, KJ190304; *ScLeftyB*, KJ190305; *ScChd*, KJ190306; *ScShh*, KJ190307; *ScGata6*, KJ190308; *ScFgf17*, KJ190309; *ScHex*, KJ190310; *ScDkk1*, KJ190311.

Fig. S2. Additional sections of embryos hybridized with *ScHex*, *ScSox17* and *ScGata6* probes. (A) Views of catshark embryos less than 48 hours after egg deposition (stages 4–6) after whole-mount in situ hybridizations with *ScSox17* (A1,A2), *ScGata6* (A3,A4), *ScHex* (A5,A6), *ScLeftyB* (A7) and *ScFgf17* (A8) probes. (A1,A3,A5,A7,A8) Dorsal views. (A2,A4,A6) Ventral views. (B) Scheme of the posterior part of a stage 12 catshark blastoderm, showing the level of the transverse sections shown in panels D and E. (C) Mid-sagittal section of a stage 12+ embryo following whole-mount hybridization with the *ScHex* probe. (D,E) Transverse sections following whole-mount hybridization with *ScSox17* and *ScGata6* probes respectively. (F,G) Ventral view (F) and transverse sections (G) of a stage 13+ catshark embryo following whole-mount in situ hybridization with a *ScSox17* probe. The red and yellow arrowheads in panel C show *ScHex* signals respectively in the presumptive prechordal mesendoderm derived from the early notochordal triangle and in the anterior-most region of the involuting mesendoderm where the foregut diverticulum forms. White arrowheads point to labeled cells in the deep mesenchyme, black arrowheads point to the labeled part of the involuting mesendoderm, thin arrows point to labeled yolk syncytial nuclei. Dotted boxes in the sections presented in panels D1, D2, E1 and E2 show the lateral blastoderm margins, where differences between *ScGata6* and *ScSox17* are observed. Red dotted lines indicate section planes and level. The antero-posterior axis is indicated at the right of the pictures. White arrowheads point to labeled deep mesenchymal cells, which persist at this stage. Scale bars: 500 μm.
Fig. S3. Sagittal sections of control and SB-505124 type 1 and type 2 catshark embryos, following hybridizations with respectively ScSox17 and ScGata6 probes. (A1,A2,C1,C2) Control embryos, (B1,B2,D1,D2) SB-505124 treated embryos, (A1,B1,C1,D1) mid-sagittal sections, (A2,B2,C2,D2) para-sagittal sections (section levels indicated by dotted red lines for panels A1, A2, B1 and B2. The posterior overhang is either absent (Type 1 embryos, (B)) or present but less extended with a thinner IME (involuting mesendoderm) layer (Type 2 embryos, (D1,D2)) in SB-505124 embryos. Deep mesenchymal cells (white arrowheads) express ScSox17 and ScGata6 in SB-50124 treated embryos as in controls but the signal is lost in the IME layer (black arrowheads; magnifications of panels C1 and D1 at the level of the posterior margin are shown in Fig. 7M1,N1).
Table S1. Location of labeled cells following DiI application at the posterior margin of stage 9 to 11 catshark embryos

<table>
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<th>Culture time</th>
<th>1 h</th>
<th>24 h</th>
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<tr>
<td>Injection stage</td>
<td>St. 9–11</td>
<td>St. 9</td>
</tr>
<tr>
<td>Resulting stage</td>
<td>St. 10–12</td>
<td>St. 10</td>
</tr>
<tr>
<td>Number of embryos analyzed</td>
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<td>4</td>
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<tr>
<td>Number of embryos showing labeled cells in the location indicated</td>
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<td>DM: 4/4</td>
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PM, posterior margin superficial cells; DM, deep mesenchyme; IME, involuting mesendoderm.

Table S2. Presence of labeled cells within the deep mesenchyme (DM) following DiI application in the center of the blastoderm

<table>
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</thead>
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<td>Injection stage</td>
<td>St. 9–11</td>
<td>St. 9–9+</td>
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<tr>
<td>Resulting stage</td>
<td>St. 10–11</td>
<td>St. 10–10+</td>
</tr>
<tr>
<td>Number of embryo analyzed</td>
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<td>2</td>
</tr>
<tr>
<td>Number of embryos showing labeled cells in the DM</td>
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<td>IME: 2/2</td>
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Table S3. Number of control and SB-505124 treated embryos analyzed

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<tbody>
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<tr>
<td>LeftyB</td>
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<td>3</td>
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<tr>
<td>Hex</td>
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<td>Lim1</td>
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