Matrix-filled microcavities in the emerging avian left-right organizer

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Abstract

Background: Hensen node of the amniote embryo plays a central role in multiple developmental processes, especially in induction and formation of axial organs. In the chick, it is asymmetrical in shape and has recently been considered to represent the left-right organizer. As mechanisms of breaking the initial left-right symmetry of the embryo are still ill-understood, analyzing the node’s microarchitecture may provide insights into functional links between symmetry breaking and asymmetric morphology.

Results: In the course of a light- and electron-microscopic study addressing this issue we discovered novel intercellular matrix-filled cavities in the node of the chick during gastrulation and during early neurulation stages; measuring up to 45 μm, they are surrounded by densely packed cells and filled with nanoscale fibrils, which immunostaining suggests to consist of the basement membrane-related proteins fibronectin and perlecan. The cavities emerge immediately prior to node formation in the epiblast layer adjacent to the tip of the primitive streak and later, with emerging node asymmetry, they are predominantly located in the right part of the node. Almost identical morphological features of microcavities were found in the duck node.

Conclusions: We address these cavities as “nodal microcavities” and propose their content to be involved in the function of the avian node by mediating morphogen signaling and storage.

KEYWORDS

chicken, duck, ECM, gastrulation, Hensen node, left-right symmetry breaking

INTRODUCTION

In most studied vertebrates the initial left-right symmetry breaking event occurs at the beginning of somitogenesis and is—in many model organisms—believed to be initiated by a cilia-driven leftward fluid flow on the ventral surface of the posterior notochord area or equivalent structures. In the chick, however, breaking of bilateral symmetry occurs even prior to notochord formation, namely at advanced stage 4: at this stage the primitive streak (as the major mesoderm forming domain in the embryo) has reached its full length and at its anterior tip a mature primitive node (as the amniote organizer) starts to give rise to the prechordal mesoderm. At the same
time, the node shows asymmetrical morphogenesis whereby its right dorsal surface appears higher comparable to the left one. This emerging asymmetry is temporarily correlated with counterclockwise leftward cell movement in the epiblast, the dorsal cell layer of the embryo anterior to the node. Subsequently, the asymmetry of the node leads to asymmetrical morphogenesis of axial organs in that the notochord emerges from the dense right shoulder of the node whereas the floorplate of the future neural tube emerges from the left node shoulder. Intriguingly, the morphological asymmetry of the node precedes molecular asymmetry as shown by the expression of several key genes involved in left-right symmetry breaking such as the morphogens nodal, FGF8 and shh as well as the motor protein Kif5c.

However, mechanisms responsible for the initial left-right symmetry breaking event or, specifically, for the steps leading to the asymmetric node morphogenesis in the chick have not been elucidated yet, while a ciliated area functionally corresponding to the posterior notochord of mammals or the gastrocoel roof plate (GRP) in Xenopus does not form in the chick. The cells of the notochord are completely covered by endoderm and so called subchordal mesoderm, leaving no space for possible motile cilia to create the leftward flow of fluid. This fits morphological and molecular studies in other amniotes such as pig and cattle which make flow-based symmetry breaking mechanisms an unlikely proposition in these species, too. We, therefore, decided to focus on the cellular microarchitecture of the emerging node in the chick to obtain new insights for a potential functional link between asymmetric morphology and left-right symmetry breaking. To our surprise, we found that the left-right organizer (LRO) region contains small cavities filled with extracellular matrix (ECM) components during node formation and early notochord stages.

2 RESULTS

2.1 Emerging and regressing node morphology

The chick node forms at stage 4 as a horse-shoe shaped density at the anterior tip of the primitive streak (Figure 1B,B'). Immediately prior to node formation, at stage 3+, the primitive streak has a line of reduced density, the primitive groove, between two bilaterally symmetrical narrow bands of high density, the primitive streak ridges (Figure 1A,A'). Focused illumination shows at stage 4 the node density to be higher than the one of the primitive streak ridges (Figure 1B'). Corresponding sections show that in the midline the node density (Figure 2B) and the reduced density immediately posterior to it, that is, the primitive pit (Figure 2C), consist of densely packed cells with no clear separation between epiblast and the emerging mesoderm (cf. Figure 2B). This tissue arrangement matches the original definition of the node area by Hensen in the rabbit and guinea pig embryos. Together with a further thickening of the node at the late stage 4 (Figure 1C), the primitive streak continues to elongate anteriorly (Figure 1B",C",E".). With the primitive streak reaching its maximal length (stage 4+), the node reaches its most anterior position and displays the first morphological asymmetry in its horse-shoe shape (see below). The horse-shoe shaped density is now distinguishable from the dense area anterior to it which corresponds to the prechordal mesoderm (Figure 1B",C") and consists of several layers of polymorphous cells (Figure 1F). Scanning electron microscopy (SEM) of a transverse fracture surface running perpendicularly through the anterior node density reveals that the axes of columnar epiblast cells in the node bend apically towards the pit and thus display a curved morphology: the basal parts of cells are located slightly peripherally whereas the apical parts are located more centrally (Figure 1F,F'). As a result, cells from the left and right half of the node density taken together form a dome-shaped structure.

A relative shift of the node towards the posterior pole, also called node regression, sets in at stage 5— with the emergence of the narrowing notochord density between the prechordal mesoderm and the increasingly asymmetrical node. Node regression becomes particularly obvious if embryos of closely spaced stages are mounted next to each other with the anterior margin of the area pellucida arranged at the same level (Figure 1C",D",E",E'.). Sections show that the right node density (also defined as the right node shoulder, cf. Reference 7) corresponds to the elevated right side of node tissues and consists of epiblast and axial mesoderm (Figure 2B,C). With further regression and concomitant elongation of the notochord the node undergoes counterclockwise tilting. As a result its shoulders form angle of up to 45° with the anterior-posterior axis and the primitive pit, a less dense area between the node shoulders, is now distinguishable from the primitive groove (Figure 1E,G,G'). Sections at the node level in an oblique plane confirm this arrangement revealing a deep indentation between the left and the right node shoulders (Figure 2C,D). Similarly to the situation seen in transverse sections (cf. Reference 7), the epiblast basement membrane within the left (posterior) node shoulder extends closer to the ventral surface of the embryonic disc reaching the level close to the node hypoblast. At the same time the basement membrane of the right (anterior) node shoulder is elevated towards the dorsal surface due to a thick and dense bulk of emerging...
FIGURE 1  Dorsal views of whole Araldite-embedded chicken embryonic discs at stages 3+ to 5 (A-E) and detailed views on their node area under focused illumination (A'-E'). Node areas in A'-E' are arranged in relation to the relative distance to the anterior margin of the area pellucida to show the elongation of the primitive streak towards the anterior margin during progression from stage 3+ to 4+ (A'-C') and regression of Hensen node towards the posterior margin during further development to stage 5 (C'-E'). Oblique lines in E' indicate the position of the semithin sections shown in Figure 2. F-G'—frontal (F, G) and dorsal (F', G') scanning electron microscopic views of specimens at stages 4 and 5 fractured in the epiblast layer at a level immediately anterior to the node. Asterisks point to the primitive pit filled with debris. Inset in G' shows the dorsal mesodermal surface at high magnification. Scale bar in G' is 700 μm for A-E, 250 μm for A'-E', 16 μm for F and G, 20 μm for F' and 80 μm for G'. Intersecting arrows in A indicate the body axes for (A-E', F', and G'): a, anterior; p, posterior; l, left; r, right
axial mesoderm below the epiblast. Oblique sectioning through the asymmetrically forming axial mesoderm leads to transverse sections of the emerging posterior notochord. The mesoderm anterior to the right node shoulder can thus be seen to consist of (a) an epithelial dorsal part which corresponds to the emerging posterior notochord and (b) a mesenchymal ventral part corresponding to the subchordal mesoderm (Figure 2A, B). Laterally positioned cells of the posterior notochord display a curved morphology where the apical pole of the cell is bent towards the midline of the embryo. In dorsal SEM views of a fractured surface exposing the basal side of the emerging notochord, cells form a flat sheet with a convexity towards the dorsal surface. Those cells are more closely positioned to each other than the cells of the neighboring antero-lateral, nonaxial mesoderm (Figure 1G and inset in G'). SEM also shows that the notochord is attached to the right (and not to the left) node shoulder (Figure 1E,G').

2.2 | Microcavities in the node at early notochord stages

A closer look at the cellular arrangement of the asymmetric node (ie, HH stages 5−, 5, and 6) reveals the presence of previously uncharacterized extracellular structures, here called nodal microcavities (NMCs). In semithin sections stained with methylene blue/azur II solution NMCs can be distinguished from unstained intercellular gaps as well as from yellow-gray intracellular yolk droplets on the basis of their weak light blue staining (Figures 2 and 3). In addition and in contrast to the unstained intercellular gaps, NMCs are distinctly enclosed by surrounding cells. Topographically, NMCs are mostly related to the densely packed tissue of the right node shoulder. Some of them lie close to the medial edges of the epiblast basement membrane (Figures 2A and 3B') whereas others lie close to the primitive pit, that is, adjacent to the asymmetric node shoulders. NMCs display irregular shapes and measure 10-15 μm in height (along the dorso-ventral axis) and 8-13 μm in width (along the transverse axis) and, at stage 5, can be followed through 15 semithin (1 μm) sections. Similarly sized NMCs are also seen at early somite stages 7 and 8; here they are primarily located in the dense tissue of right node shoulder and in the notochord forming from it (Figure 3D'). Preliminary evidence for the presence of NMCs in another avian species was obtained in a first small series of duck embryos (n = 3) where extracellular structures almost identical to the ones described above for the chick were found in the

**FIGURE 2** Oblique semithin sections of a stage 5+ chick embryo stained with methylene blue/azur II. Orientation and positions of sections are marked in the whole-mount view of this specimen shown in Figure 1E'. Square brackets in A and B embrace columnar epithelial cells of the posterior notochord covering the subchordal mesoderm below it (ventrally); curved arrow in C surrounds the edge of the basement membrane and indicates the involution movements of epiblast cells around this edge. Inset in C shows the NMC in the right node shoulder of this section at high magnification. Arrows in C indicate the position of further NMCs in this section. Arrowheads point to unstained intercellular gaps and hashes to yellow-gray lipid droplets of different sizes. Scale bar in E is 50 μm for A-E. Intersecting arrows indicate the body axes: d, dorsal; v, ventral; l, left; r, right
dense area of the node (but not in any other parts of the embryo) at stages HH5 and HH6 (Figure 6).

2.3 | Nodal microcavities prior to node and notochord formation

At stage HH3+, NMCs can be found in a majority of specimens (2/3) and they lie exclusively between densely packed epiblast cells in the midline immediately cranial to the anterior tip of the primitive groove; with their long axes, NMCs are mainly oriented along the dorso-ventral axis (Figure 3A′, A″) and can be followed through up to 45 serial sections. At stage HH3, NMCs are not present (0/3) in any of the serial sections (data not shown).

At stage 4 and 4+, NMCs are found in all studied specimens (4/4 and 2/2, respectively) and are located mainly within the node density, that is, either in the mesodermal area close to the epiblast or at the level of, and medial to, the epiblast basement membrane (Figure 3B′, B″). In the former position, a typical NMC measures between 14 and 17 μm in the transverse and 2-5 μm in the dorso-ventral axis and can be followed through 23 consecutive transverse sections. In the latter position (close to the basement membrane), a typical microcavity has a width of up to 40 μm, a height of 8 μm, and an extension along the craniocaudal axis of 12 μm.

2.4 | Ultrastructure and basement membrane components in nodal microcavities

Ultrathin sections show individual NMCs with a topographic distribution relative to the node described above which are filled with an irregular matrix ranging from fine fibrillar to homogeneously fine granular (Figure 4A, A′, A″). Some of the contents display similarities to the matrix of the basement membrane underlying the epiblast (Figure 4C). Some sections through NMCs display

![Figure 3](image-url)
cellular processes defined as membrane-enclosed cytoplasmic areas of higher electron density; however, microtubular structures (as possible signs of monocilia) were not found. Cells forming the boundary of the cavities display immature cell-to-cell contacts: membranes of neighboring cells are in close proximity to each other and form “kiss-like” arrangement (Figure 4A″; cf. Reference 19) and the space between cell membranes may reveal some electron-dense material but signs of membrane fusion were not found.

SEM of embryos fractured in the area of the node (Figure 4B,B′,B″) resemble the cellular structures of the

**FIGURE 4** Transverse semithin (A) and ultrathin (A′-A″″) sections of the same NMC in the node of a stage HH4+ embryo. Arrowheads in A′ point to membrane enclosed cellular processes within the NMC. Black rectangle in A′ indicates the region magnified in A″″. A″—cell-cell contacts at high magnification enclosing unstained “empty” expansions of the intercellular space; inset in A″ shows the upper contact zone at high magnification. A″″—fibrillar NMC matrix structure at high magnification. B-B″—SEM views of a stage HH4+ embryo fractured through the epiblast in the node area. Black rectangle in B indicates the region magnified in B′. Black rectangle in B′ points to the NMC magnified in B″. Arrowhead in B″ points to the cell process of a cell forming the roof of a cavity. C and D—TEM (C) and SEM (D) view of fibrous basement membrane between epiblast and mesoderm lateral to the node area in a stage 4 embryo. Scale bar in D is 20 μm for A, 2.9 μm for A′, 0.66 μm for A″ and A″″, 25 μm for B, 5 μm for B″ and D, 0.2 μm for C and 0.75 μm for B″. Intersecting arrows indicate the body axes: d, dorsal; v, ventral; l, left; r, right
germ layers found in semithin sections and confirm the presence of many intercellular gaps of different sizes and shapes in the mesoderm. Dorsally, a sheet of columnar cells forms the epiblast layer separated from the layer below, the mesoderm, by a continuous plane (Figure 4D), which is equivalent to the basement membrane. Apart from this, both mesodermal and epiblast cells display spherical depressions corresponding to the yolk-filled spaces found in semithin sections (Figure 4B). The node appears as an area of high cell density with no clear separation between mesoderm and epiblast and the primitive pit is covered dorsally by irregular structures of subcellular sizes, here addressed as cellular debris (Figure 4B and 2C). The mesodermal cells lateral to the node show variable shapes and form numerous cell processes reaching out to neighboring cells across intercellular spaces that appear to be empty (Figure 4B, B', B''). Within the dense node area, in contrast, intercellular areas were found in positions and with extension similar to NMCs in semithin sections which were filled with fine fibrillary

**FIGURE 5**  A-C, Whole-mount immunofluorescence staining of a stage HH4+ embryo for the extracellular matrix protein fibronectin (A) counterstained with phalloidin for the presence of actin (B) in separate (A, B) and merged images (C). D-F, Whole-mount immunofluorescence staining of a stage HH5 embryo for basement membrane component perlecan (D), counterstained with phalloidin (E) in separate (D, E) and merged images (F). Asterisk indicates the position of the primitive pit. G-J, Technovit sections of a stage HH5 embryo immunohistochemically stained for fibronectin (G, H) and perlecan (I, J). Insets in G and I show the position of the sections in the dorsal view on the node region. Gamma-tubulin localization in the node (K) prior to counterstaining the same section with methylene blue/azur II (L). Note the presence of NMCs in (typical) positions near the basal aspects of epiblast cells in the node region. Inset in L shows magnification of NMC at position indicated by black rectangle. Scale bar in K is 55 μm for A-F, 60 μm for G-J, and 50 μm for K and L. Intersecting arrows in A indicate the body axes for A-F. Intersecting arrows in G indicate the body axes for G-L: a, anterior; p, posterior; d, dorsal; v, ventral; l, left; r, right.
material. These irregular fibers measure between 20 and 30 nm in diameter (which includes the sputter coating material, cf. experimental procedures) and are thus significantly smaller than the adjacent cell processes which measure about 150 nm in diameter (Figure 4B). Fibers with similar size and structural characteristics are also seen in areas of the epiblast basement membrane (Figure 4D).

Immunofluorescent analysis for fibronectin on whole-mount preparations of embryos at stages HH4-HH7 revealed a variety of strong immunoreactions in the area of the node (Figure 5A-C). The strongest staining is seen anterior to the primitive pit (in which the cellular debris is marked by phalloidin counterstaining in the shape of a large, irregular fluorescent dot) and thus colocalizes with the typical position of most of the NMCs found in semithin sections. Posterior to the level of the pit, smaller fibronectin-positive dots can be seen distributed across the width of the primitive streak. Immunohistochemical staining for fibronectin in whole-mount embryos at stages HH4 and HH5 shows a strong staining reaction also in the region anterior to the primitive pit (inset in Figure 5D). Technovit-sections revealed a continuous immunoreaction below the epithelial sheet of epiblast and multiple patches medial to the edges of the basement membrane along both the primitive streak and the node. Within the node perlecan displays a localization pattern similar to that of fibronectin. In contrast, staining for gamma-tubulin as typical component of the apical part of epiblast cells was strongly positive in the apical parts of node cells (Figure 5K), that is, at the cellular poles opposite to the region where NMCs can be found. Interestingly, counterstaining of 5 μm Technovit sections with methylene blue/azur II revealed NMCs as matrix filled intercellular spaces at typical positions and distinct from empty intercellular spaces between mesoderm cells, for example Figure 5L.

3 | DISCUSSION

Using high-resolution light microscopy as well as transmission and SEM, the present report reveals small matrix filled extracellular spaces in Hensen node of gastrulating chick and duck embryos, which we suggest to address as NMCs. Highly irregular in shape, NMCs are different from apparently empty intercellular gaps, spherical yolk droplets and cellular debris, frequently found near the primitive node. Prior to development of the node and with the emergence of the primitive groove, NMCs appear in the anterior part of the primitive streak in some specimens. Later, when the node has formed, they are found in all specimens. As a regular feature of asymmetrical node microarchitecture NMCs are preferentially
found in the right part of the node which gives rise to notochord and subchordal mesoderm as the main components of the axial mesoderm. Initial immunofluorescent and immunohistochemical characterizations using fibronectin and perlecan antibodies are in line with the NMCs’ close association with the basal parts of epiblast cells. The term “nodal microcavity” was chosen with regard to position, size and contents: NMCs are reminiscent of both apical microlumina recently described in zebrafish embryo and larger embryonic cavities such as the chorionic cavity and the cavity containing the cardiac jelly, which are not internally lined by a bona fide epithelium. Closely connected to the morphological asymmetry of the avian node and its central role during left-right symmetry breaking NMCs may be promising targets for experimental embryology.

3.1 Nodal microcavities as a novel structure of Hensen node

The discovery of novel extracellular spaces in the avian node comes as a surprise in the face of the long history of studies describing and using the morphology of the gastrulating chick embryo and its node as the archetypal amniote organizer. Specifically, microcavities were not mentioned in previous studies using semithin sections of the chicken node although they can be recognized in some of the published figures (Reference 4: fig. 12, 25, and 26; Reference 7: fig. 2I, G). Likewise, NMCs were missed in previous ultrastructural studies using transmission and scanning electron microscopy (which include fractured specimens after hypoblast removal) possibly because NMCs are relatively small and the node region was not the focus of these studies. Apart from the consistent and exclusive presence at certain stages and locations, the reproducible differences between regular NMCs and the variable appearance of extracellular spaces after different fixation (using paraformaldehyde, glutaraldehyde, and the mixture of both) and embedding protocols (water-soluble and -insoluble epoxy resins) argue against preparation artifacts as the basis for the NMCs’ occurrence. Fibronectin and perlecan had been shown in the gastrulating chick embryo in a colocalization with the basement membrane and in “interstitial bodies” at the periphery of the zona pel lucida. However, a detailed analysis of either fibronectin or perlecan localization in the node region, again, had not been published to date. Laminin and fibrillin as further obvious candidates of the “immature” ECM of the early embryo have produced negative (unpublished) or NMC-unrelated results, respectively, and will in the case of laminin be subject of a reanalysis using variations in fixation and antigen retrieval.

3.2 Potential homologous structures

The central position of NMCs during node and notochord formation raises the question as to homologies with known embryonic structures related to the organizer and axial mesoderm in other model vertebrates. A possible candidate—in view of the node as the LRO—is the notochordal canal which emerges as a wide cavity anterior to the primitive node and ventral to the posterior notochord in a subgroup of mammals, temporally precedes the exposure of the apical, cilia-bearing surface of the notochordal plate to the yolk sack cavity and, hence, provides the structural basis for ciliary flow and its involvement in left-right symmetry breaking. However, the close topographical relationship of NMCs to the medial edges of the basement membrane, the presence of basement membrane constituents and gamma-tubulin localization at the opposite pole of cells surrounding NMCs all suggest that NMCs face the basal side of the node epiblast. NMCs may, indeed, be homologous to the Brachet cleft in the gastrulating Xenopus which is located at the basal side of ectoderm, filled with a lose network of fibronectin fibrils and is devoid of laminin.

3.3 Hypothetical functions of NMCs

Since NMCs are spatially related to the basement membrane the question arises as to whether this structure has a specific function connected to the ECM of node cells or whether they simply represent remnants of the basement membrane material brought to the node area by the extensive morphogenetic movements of epiblast cells towards the node prior and during gastrulation and their rapid ingress and involution which produces axial and paraxial mesoderm, for example, and leaves behind the cellular debris found on the dorsal side in the primitive pit. Interestingly, however, chordin is secreted from the organizer and forms a gradient along the fibronectin-rich Brachet cleft below the ectoderm. The NMCs fibronectin content may thus reveal the NMCs’ involvement in molecular signaling from the chick organizer, too. This is supported by the fact that ECM proteins are known to display a multi-domain structure and to bind different secreted signaling molecules including morphogens from FGF, hedgehog, BMP, and Wnt families influencing their function in different ways. Proteins from the TGF-beta family, for example, were shown to be stored in ECM by formation of large latent complexes (LLCs), which may include fibronectin, collagens, and fibrillin. LLC formation enables spatio-temporal regulation of interaction of TGF-beta with cell surface receptors which is prevented by LLCs and is enabled by local proteolytic cleavage of ECM. Furthermore, proteins of ECM possess elements which are
released by proteolysis and activate matrikines signaling by binding to cell surface receptors (as shown for fibronectin producing a rise of matrikines which in turn promote cell motility\textsuperscript{57}). Testing this hypothesis would require focused perinodal knockdown of ECM-related NMC components to minimize global deleterious effects even if this may be challenging in view of the dynamic character of the node.\textsuperscript{58} However, the intriguing presences and composition of the NMCs described here in the avian organizer calls for functional studies and could provide clues as to the evolutionary plasticity of vertebrate gastrulation.\textsuperscript{11}

4 | EXPERIMENTAL PROCEDURES

4.1 | Embryos and staging

Fertilized chicken eggs of the White Leghorn or Vorwerk strains were incubated in a humidified incubator at 38°C for 12-24 h to obtain embryos between stages 3 and 7.\textsuperscript{2,59} Fertilized duck (Anas domestica) eggs were incubated at 38°C for 30-36 h to obtain stages 5 and 6.\textsuperscript{60} After opening the egg shell most of the albumen was decanted and the intact yolk was transferred to a 100 mL glass bowl filled with Locke solution. After excision of the yolk membrane carrying the embryo, the embryo was gently detached from the yolk membrane and transferred to the fixatives described below with an inverted Pasteur pipette (cf. References 61 and 62). Staging criteria were based on classical morphological descriptions\textsuperscript{2} and further refinements for stages 3-5.\textsuperscript{7,63} In short, a parallel-sided primitive streak without a groove characterizes Hamburger and Hamilton stage 3 (HH3) while emergence of the primitive groove characterizes stage HH3+; these two substages correspond to stages 3a and 3b/c defined by Schoenwolf,\textsuperscript{22} respectively (cf. Reference 63). Appearance of the primitive pit and a node distinguishable by its width from the primitive streak characterizes stages HH4– and HH4, respectively; these substages correspond to stage 3d.\textsuperscript{22} A small (triangular) density anterior to the node defines stage 4+; first signs of notochord formation (a short dense stripe protruding from the node) caudal to the prechordal mesoderm (now appearing as inverted triangular density), define stage 5– (cf. Reference 63). The notochord, as a distinct narrow and long density extending from the node, defines stage 5.\textsuperscript{63}

4.2 | Morphological analysis

For light- and transmission electron microscopic analysis embryos (n = 3 for stage 3, n = 3 for stage 3+, n = 4 for stage 4, n = 2 for stage 4+, n = 3 for stage 5–, n = 3 for stage 5, n = 1 for stage 6, n = 2 for stage 7, n = 1 for stage 8) were fixed in a reduced concentration of Karnovsky fixative (64; 1.5% glutaraldehyde and 1.5% paraformaldehyde in phosphate buffered saline [PBS]) for 2 h at room temperature, post-fixed in 1% OsO\textsubscript{4} in PBS for 2 h at room temperature and embedded in Araldite (Serva, Heidelberg) as previously described.\textsuperscript{55,66} Prior to serial sectioning dorsal view photographs were taken of the whole area pellucida for staging purposes and of the node area using focused (“spot-light”) illumination as previously described.\textsuperscript{7} Serial semithin (1 μm) sections covering the complete area pellucida and a wide margin of the adjacent chorion were obtained in the transversal and sagittal planes and, additionally at stage 5–, in an oblique plane which mirrors the leftward tilt of the asymmetrical node by forming a 45° angle with the transverse plane and opening to right side of the embryo (s. labeling in Figure 1E\textsuperscript{4} for stages 3b/c). To evaluate the morphology, semithin sections were stained with Richardson stain\textsuperscript{18} consisting of equal volumes of 1% methylene blue solution in 1% borax and 1% azur II in water. The position of sections was double-checked by (1) morphological landmarks such as the border between (a) the prenodal epiblast with its continuous basement membrane and (b) the node with its signs of epithelial to mesenchymal transition (s.\textsuperscript{4,18} and by (2) a calculation based on the total length of the fixed specimen divided by the number of serial semithin (1 μm) sections.\textsuperscript{18,45} For ultrastructural analysis, semithin sections of interest were re-embedded in Araldite and cut using a diamond knife to obtain ultrathin (50 nm) sections.\textsuperscript{18,45} For immunofluorescence staining and immunohistochemistry the embryos were fixed in 4% PFA in PBS for 1 h at room temperature and stored at 4°C in PBS. For

4.3 | Immunofluorescence staining and immunohistochemistry

For immunofluorescence staining and immunohistochemistry the embryos were fixed in 4% PFA in PBS for 1 h at room temperature and stored at 4°C in PBS. For
whole-mount immunofluorescent staining the embryos were washed 3 times for 5 min in phosphate buffer containing 0.1% Triton X-100 (PBT). Blocking was performed in 1% normal goat serum (NGS) in PBT. Incubation with the primary antibodies against Fibronectin (VA1(3) or Perlecan (5C9; both from Developmental Studies Hybridoma Bank, Iowa) or against gamma-tubulin (GTU-88, Sigma Aldrich, Oberkochen) diluted to 1:50 in PBT containing 0.1% NGS was performed over night at 4°C. After washing three times in PBT the secondary antibody (Cy3-conjugated AffiniPure Goat Anti-Mouse IgG [H + L], Dianova, Hamburg) was used at a dilution of 1:200 in PBT containing 0.1% NGS and applied for 1 h at room temperature followed by washing in PBT. Counterstaining with Phalloidin iFluor 488 Reagent (Abcam, Cambridge, UK) was performed according to the manufacturer’s manual. After embedding in Mowiol (Hoechst, Frankfurt) photos were taken using an Axioplan 2 light microscope (Carl Zeiss, Jena).

For whole-mount immunohistochemistry fixed embryos were incubated in 1 mM citrate buffer (pH = 6.0) for 4 min at 96°C. After cooling off at room temperature (RT) for 10 min they were washed three times for 10 min in phosphate buffer containing 0.1% Triton X-100 (PBT). Endogenous peroxidase activity was quenched in PBT containing 0.3% H2O2 for 2 h at RT followed by three times washing in PBT for 10 min and three times for 30 min. Blocking was performed in PBT containing 1% NGS for 1 h at RT. Incubation with the primary anti-Fibronectin (VA1(3)), anti-Perlecan (5C9), and anti-gamma-tubulin (GTU-88) antibodies diluted to 1:50 in PBT containing 0.1% NGS was carried out over 72 h at RT on a rocking platform. After washing embryos were incubated with goat-anti-mouse secondary antibody (BA-9200 Biotinylated Goat Anti-Mouse IgG Antibody, Vector Laboratories, California) at a dilution of 1:200 in PBT containing 0.1% NGS over night at 4°C on a rocking platform. After washing three times for 10 min and three times for 30 min the embryos were incubated with avidin-biotin-peroxidase complex (ABC) for 1 h at RT. After washing three times for 10 min the embryos were incubated with 3,3’-Diaminobenzidine (DAB, Sigma, Taufkirchen) at a concentration of 500 ng/μL in 0.1 M Tris-buffer containing 0.015% H2O2 until sufficient staining was achieved (3-10 min); the reaction was stopped by incubating the embryos in PBS. Finally the embryos were embedded in Technovit 8100 V (Kulzer, Wehrheim, Germany) according to the supplier’s instructions and cut into 5-μm serial sections as previously described. To visualize cytological details which remained unstained after the immunoreaction selected sections of interest were counterstained by covering slides briefly (1-2 s) with methylene blue/azur II solution at RT immediately followed by washing in distilled water to avoid overstaining or covering up the immunohistochemical staining reaction.

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**REFERENCES**

1. Blum M, Feistel K, Thumberger T, Schweickert A. The evolution and conservation of left-right patterning mechanisms. *Development*. 2014;141:1603-1613.

2. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol*. 1951;88:49-92.

3. Cooke J. The evolutionary origins and significance of vertebrate left-right organisation. *Bioessays*. 2004;26:413-421.

4. Dathe V, Gamel A, Männer J, Brand-Saberi B, Christ B. Morphological left-right asymmetry of Hensen’s node precedes the asymmetric expression of Shh and Fgf8 in the chick embryo. * Anat Embryol (Berl)*. 2002;205:343-354.

5. Kölliker A. Entwicklungsgeschichte des Menschen und höheren Thiere. Leipzig: Wilhelm Engelmann; 1887.

6. Rahl C. *Theorie des Mesoderms*. Leipzig: Engelmann; 1897.

7. Tsikolia N, Schröder S, Schwartz P, Viebahn C. Paraxial left-sided nodal expression and the start of left-right patterning in the early chick embryo. *Differentiation*. 2012;84:380-391.

8. Wetzel R. Untersuchungen am Huhnchen. *Die Entwicklung des Keims wahrend der ersten beiden Bruttage. Dev Genes Evol*. 1929;119:188-321.

9. Cui C, Little CD, Rongish BJ. Rotation of organizer tissue contributes to left-right asymmetry. *Anat Rec (Hoboken)*, 2009;292:557-561.

10. Gros J, Feistel K, Viebahn C, Blum M, Tabin CJ. Cell movements at Hensen’s node establish left/right asymmetric gene expression in the chick. *Science*. 2009;324:941-944.

11. Kremnyov S, Henningfeld K, Viebahn C, Tsikolia N. Divergent axial morphogenesis and early shh expression in vertebrate prospective floor plate. *Evodevo*. 2018;9:4.

12. Otto A, Pieper T, Viebahn C, Tsikolia N. Early left-right asymmetries during axial morphogenesis in the chick embryo. *Genesis*. 2014;52:614-625.

13. Dathe V, Prols F, Brand-Saberi B. Expression of kinesin kif5c during chick development. *Anat Embryol (Berl)*. 2004;207:475-480.

14. Männer J. Does an equivalent of the “ventral node” exist in chick embryos? A scanning electron microscopic study. *Anat Embryol (Berl)*. 2001;203:481-490.

15. Schröder SS, Tsikolia N, Weizbauer A, Hue I, Viebahn C. Paraxial nodal expression reveals a novel conserved structure of the left-right organizer in four mammalian species. *Cells Tissues Organs*. 2016;201:77-87.
16. Richardson KC, Jarett L, Finke EH. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* 1960;35:313-323.

17. Hensen V. Beobachtungen uber die Befruchtung und Entwicklung des Kaninchens und Meerschweinchen. *Z Anat Entwicklungsgesch.* 1876;1:213-273. and 353–423.

18. Viebahn C. Hensen’s node. *Genesis.* 2001;29:96-103.

19.Forge A, Jagger DJ, Kelly II, Taylor RR. Connexin30-mediated intercellular communication plays an essential role in epithelial repair in the cochlea. *J Cell Sci.* 2013;126:1703-1712.

20. Durdu S, Iskar M, Revenu C, et al. Luminal signalling links cell communication to tissue architecture during organogenesis. *Nature.* 2014;515:120-124.

21. Pasteels J. Etudes sur la gastrulation des vertébrés méroblastiques. III. Oiseaux. IV. Conclusions générales. *Arch Biol (Liège).* 1936;48:381-488.

22. Schoenwolf GC, Garcia-Martinez V, Dias MS. Mesoderm movement and fate during avian gastrulation and neurulation. *Dev Dyn.* 1992;193:235-248.

23. Spratt NT Jr. Regression and shortening of the primitive streak in the explanted chick blastoderm. *J Exp Zool.* 1947;104:69-100.

24. Stern CD. Gastrulation in the chick. In: Stern CD, ed. Gastrulation: from cells to embryo. New York: Cold Spring Harbor Press; 2004:219-232.

25. Izipisa-Belmonte JC, De Robertis EM, Storey KG, Stern CD. The homeobox gene goosecoid and the origin of organizer cells in the early chick blastoderm. *Cell.* 1993;74:645-659.

26. Johnson RL, Riddle RD, Lauffer E, Tabin C. Sonic hedgehog: a key mediator of anterior-posterior patterning of the limb and dorso-ventral patterning of axial embryonic structures. *Biochem Soc Trans.* 1994;22:569-574.

27. Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C. A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell.* 1995;82:803-814.

28. Ruiz i Altaba A, Placzek M, Baldassare M, Dodd J, Jessell TM. Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF-3 beta. *Dev Biol.* 1995;170:299-313.

29. Waddington CH. Beobachtungen über die Befruchtung und Entwicklung des Kaninchens und Meerschweinchen. *Z Anat Entwicklungsgesch.* 1876;1:213-273.

30. Lopez-Sanchez C, Puelles L, Garcia-Martinez V, Rodriguez-Gallardo L. Morphological and molecular analysis of the early developing chick requires an expanded series of primitive streak stages. *J Morphol.* 2005;264:105-116.

31. Bancroft M, Bellairs R. The development of the notochord in the chick embryo, studied by scanning and transmission electron microscopy. *J Embryol Exp Morphol.* 1976;35:383-401.

32. England MA, Wakely J. Scanning electron microscopy of the development of the mesoderm layer in chick embryos. *Anat Embryol (Berl).* 1977;150:291-300.

33. Jacob HJ, Christ B, Jacob M, Bijvank GJ. Scanning electron-microscope (Sem) studies on epiblast of young chick-embryos. *Z Anat Entwicklungsgesch.* 1974;143:205-214.

34. Meier S. Development of the chick-embryo mesoblast - formation of the embryonic axis and establishment of the metameric pattern. *Dev Biol.* 1979;73:25-45.

35. Wakely J, England MA. Development of the chick embryo endoderm studied by S.E.M. *Anat Embryol (Berl).* 1978;153:167-178.

36. Czirok A, Zamir EA, Filla MB, Little CD, Rongish BJ. Extracellular matrix macroassembly dynamics in early vertebrate embryos. *Curr Top Dev Biol.* 2006;73:237-258.

37. Critchley DR, England MA, Wakely J, Hynes RO. Distribution of fibronectin in the ectoderm of gastrulating chick embryos. *Nature.* 1979;280:498-500.

38. Jacob M, Christ B, Jacob HJ, Poelmann RE. The role of fibronectin and laminin in development and migration of the avian Wolffian duct with reference to somitogenesis. *Anat Embryol.* 1991;183:385-395.

39. Nakaya Y, Sukowati EW, Wu Y, Sheng G. RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. *Nat Cell Biol.* 2008;10:765-775.

40. Wakely J, England MA. Scanning electron microscopic and histochemical study of the structure and function of basement membranes in the early chick embryo. *Proc R Soc Lond B Biol Sci.* 1979;206:329-352.

41. Harrisson F, Vanroelen C, Vakaet L. Fibronectin and its relation to the basal lamina and to the cell surface in the chicken blastoderm. *Cell Tissue Res.* 1985;241:391-397.

42. Low FN. Interstitial bodies in the early chick embryo. *Am J Anat.* 1970;128:45-55.

43. Sanders EJ. Ultrastructural immunoocytochemical localization of fibronectin in the early chick embryo. *J Embryol Exp Morphol.* 1982;71:155-170.

44. Gallagher BC, Sakai LY, Little CD. Fibrillin delineates the primary axis of the early avian embryo. *Dev Dyn.* 1993;196:70-78.

45. Blum M, Andre P, Mudas K, et al. Ciliation and gene expression distinguish between node and posterior notochord in the mammalian embryo. *Differentiation.* 2007;75:133-146.

46. Rulle A, Tsikolia N, de Bakker B, Drummer C, Behr R, Viebahn C. On the enigma of the human neuroenteric canal. *Cells Tissues Organs.* 2018;205:256-278.

47. Blum M, Schweickert A, Vick P, Wright CV, Danilchik MV. Symmetry breakage in the vertebrate embryo: when does it happen and how does it work? *Dev Biol.* 2014;393:109-123.

48. Davidson LA, Keller R, DeSimone DW. Assembly and remodeling of the fibrillar fibronectin extracellular matrix during gastrulation and neurulation in *Xenopus laevis*. *Dev Dyn.* 2004;231:888-895.

49. Fey J, Hausen P. Appearance and distribution of laminin during development of *Xenopus laevis*. *Differentiation.* 1990;42:144-152.

50. Plohuinec JL, Zakin L, Moriyama Y, De Robertis EM. Chordin forms a self-organizing morphogen gradient in the extracellular space between ectoderm and mesoderm in the Xenopus embryo. *Proc Natl Acad Sci U S A.* 2013;110:20372-20379.

51. Asada M, Shinomiya M, Suzuki M, et al. Glycosaminoglycan affinity of the complete fibroblast growth factor family. *Biochim Biophys Acta.* 2009;1790:40-48.

52. Smith SM, West LA, Govindraj P, Zhang X, Ornitz DM. Drosophila perlecan mediate binding and delivery of FGF-2 to FGF receptors. *Matrix Biol.* 2007;26:175-184.

53. Park Y, Rangel C, Govindraj P, Zhang X, Ornitz DM. Heparan sulfate-binding affinity reside at opposite ends
in BMP2/4 versus BMP5/6/7: implications for function. J Biol Chem. 2018;293:14371-14383.

55. Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of wingless. Development. 2001;128:87-94.

56. Taipale J, Saharinen J, Hedman K, Kessi-Oja J. Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. J Histochem Cytochem. 1996;44:875-889.

57. Joshi R, Goliher E, Ren W, Pilichowska M, Mathew P. Proteolytic fragments of fibronectin function as matrikines driving the chemotactic affinity of prostate cancer cells to human bone marrow mesenchymal stromal cells via the alpha5beta1 integrin. Cell Adh Migr. 2017;11:305-315.

58. Joubin K, Stern CD. Molecular interactions continuously define the organizer during the cell movements of gastrulation. Cell. 1999;98:559-571.

59. Selleck MA, Stern CD. Fate mapping and cell lineage analysis of Hensen’s node in the chick embryo. Development. 1991;112:615-626.

60. Sellier N, Brillard JP, Dupuy V, Bakst MR. Comparative staging of embryo development in chicken, Turkey, duck, goose, Guinea fowl, and Japanese quail assessed from five hours after fertilization through seventy-two hours of incubation. J Appl Poultry Res. 2006;15:219-228.

61. Sydow HG, Pieper T, Viebahn C, Tsikolia N. An early Chick embryo culture device for extended continuous observation. Methods Mol Biol. 2017;1650:309-317.

62. Voiculescu O, Papanayotou C, Stern CD. Spatially and temporally controlled electroporation of early chick embryos. Nat Protoc. 2008;3:419-426.

63. Streit A, Stern CD. Operations on Primitive Streak Stage Avian Embryos. Vol 87. Methods Cell Biol; San-Diego: Academic Press; 2008;87:3-17.

64. Karnovsky MJ. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol. 1965;27:A137.

65. Hassoun R, Schwartz P, Feistel K, Blum M, Viebahn C. Axial differentiation and early gastrulation stages of the pig embryo. Differentiation. 2009;78:301-311.

66. Schwartz P, Piper HM, Spahr R, Spieckermann PG. Ultrastructure of cultured adult myocardial cells during anoxia and reoxygenation. Am J Pathol. 1984;115:349-361.

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