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Formation and function of the amniotic collar, a connection between embryo and anterior amnion.

胚体と前方羊膜をつなぐ羊膜襟の形成と機能（英文）

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本文

鳥類を含む有羊膜類では、陸上生活へ適応するため、発生中に胚体外膜が発達する。その一つである羊膜は、胚体外外胚葉と胚体外中胚葉の二層で構成され、胚を包み保護する役割を持つ。ニワトリ胚では st.10 で胚頭部腹側にある胚体外組織から羊膜褶と呼ばれるひだが形成され、st.19 にかけてそれが胚の背側を徐々に覆っていくことで、羊膜が形成される。羊膜褶の形成がどのように開始されるかを調べるため、st.10 のニワトリ胚の切片を作成し観察した。すると、咽頭付近の腹側で胚体の外・中胚葉と将来羊膜褶を形成する胚体外外・中胚葉が、側方でそれぞれ結合した特徴的な構造が形成されることを発見した。この構造を羊膜襟と名付け、その形成過程と機能を調べた。

st.10 の胚では、羊膜襟が咽頭から心臓付近にかけて V 字状に形成されており、それによって胚腹側と胚体外組織との間に外胚葉で囲まれた頭部下腔と呼ばれる空間が生じている。これらの構造がいつ形成されるかを調べるため、他のステージの胚の切片を作成し観察した。すると、st.7+まではこの羊膜襟と頭部下腔は存在せず、st.8-で形成されることが確認できた。また、st.8-では、羊膜襟は浅い U 字の形状をしており、st.9 付近にかけて深い V 字状に変化していくことが分かった。このことから、羊膜褶の形成に先立ち、羊膜襟は st.8-で形成され、st.9 付近にかけその形態を変化させることが分かった。羊膜襟の形成と形態変化がどのような細胞挙動により起こっているのかを調べるため、胚体と胚体外の境界に位置する外胚葉の細胞のラベル実験を行った。st.6 の胚を用い、前腸門のすぐ前方にある外胚葉を DiO で、前腸門の側方にある外胚葉を DiI でラベルし、タイムラプスで観察を行った。すると、まず胚体が細くなると同時に DiO ラベルされた細胞が頭尾軸に沿って放射状に広がった。次に前腸門が狭まるに伴い内胚葉が後方へ伸長するが、ラベルされた外胚葉はほぼ同じ位置に留まっていた。その後、DiO ラベルされた細胞が放射状から頭尾軸に平行な方向へと伸長方向を変えるとともに DiI ラベルされた細胞が前方へ移動することで、V 字状の羊膜襟の形態が形成されていた。st.9 まで発生させると、DiO ラベルされた細胞は羊膜襟の後端と頭部下腔全体に、DiI ラベルされた細胞は羊膜

襟の前端に寄与した。以上の結果から、羊膜襟は、胚体が細長く伸長する中で、腹側外胚葉は st.8 から後方へ伸長せず、後方へ伸長する内胚葉から離れること、さらに頭部下腔の正中付近での細胞の再配置が起こることによって形成されることが分かった。

次に、羊膜襟の機能を調べるため、st.9 前後の胚の羊膜襟を切断して 24 時間培養し、その後の胚の形態を観察した。その結果、コントロールと比較して心臓が小さい胚や背側の羊膜形成が遅れる胚、羊膜襟付近で異常な屈曲がある胚が見られた。有羊膜類は脳や心臓が大きく発達する。また、先行研究により羊膜褶が胚を覆うためには頭部が胚体外の組織に沈み込むことが重要であるとされている。そのためこれらの表現型は、羊膜襟が切断されたことにより、胚前方を支え、沈み込みを補助する構造がなくなったことによるものであると考えた。このことから、羊膜襟は胚と胚体を結合することで、心臓や頭部の立体構造の形成が行われても、胚前方が正常な位置に留まるよう維持していると考えている。

Formation and function of the amniotic collar, a connection between embryo and anterior amnion.

A thesis

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Contents

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Summary

In amniotes, extra-embryonic (exe) membranes develop during embryogenesis to adapt life on the land. The early chicken embryo has no definitive border between exe tissues and embryonic (em) tissues. As development proceeds, the embryo makes body fold at the anterior, posterior and lateral and the embryo is segregated from exe tissues. However, few studies have focused on the morphological change of border between exe/em tissues. I observed how the shape of the border changes. At stage 7, the border was an arched shape along the anterior intestinal portal. After that, the border gradually changed to a deep V shape while being separated from the anterior intestinal portal. The V-shaped border is formed around pharynx and connected to tissues that will become the amnion. I designated this V-shaped border amniotic collar from its shape and position. At the amniotic collar, exe- and em-ectoderm, exe- and em-mesoderm were respectively connected. The space called subcephalic pocket, which is surrounded by exe- and em-ectoderm, was formed in the ventral side.

First, I labeled ectoderm which locates the border to investigate how the amniotic collar is formed. The embryo at stage 6 which had no amniotic collar was labeled and traced. Near the midline of the embryo, labeled cells were gradually elongated along with anterior-posterior axis

and located the subcephalic pocket and the posterior end of the amniotic collar at stage 9. In contrast, labeled lateral cells didn't spread and located the anterior end of the amniotic collar at stage 9. These results suggest that the amniotic collar is formed by the difference in the rate of posterior extension of the ectoderm in the lateral and midline.

Next, I analyzed the function of the amniotic collar. The amniotic collar was cut around stage 9 in ovo and observed after one day of incubation. Some operated embryos had an abnormal bent and heart formation. Additionally, the area covered by the amnion in operated embryo was smaller than control at the same stage. Amniotes have developed brain and active heart. The previous study has reported that transient depression of the head in the exe tissue is important for the formation of amnion (Bernardo et al., 2014). Taken together, it is possible that the amniotic collar physically supports the embryo and keeps it in a normal position, thereby contributing to the formation of amnion on the dorsal side.

In conclusion, the present study has demonstrated that the novel extraembryonic structure, amniotic collar, supporting the normal development of the embryo in a manner different from the known extraembryonic structure.

Introduction

Amniotes including reptiles, birds and mammals reproduce on the land. Amniotes embryos have become possible to develop on land due to the acquisition of extraembryonic membranes, such as the chorion, the allantois, the yolk sac and the amnion. They play important roles in respiration, nutrient uptake and prevention of desiccation (Mess., 2003; Ferner et al., 2011).

In the chicken embryo, the formation of the extraembryonic membranes starts after gastrulation with the formation of the extraembryonic (exe) coelomic cavity in the exe-mesoderm (Patten., 1920). This cavity is formed by two exe-tissue layers: the splanchnopleure composed of the endoderm and the exe-mesoderm, and the somatopleure composed of the ectoderm and the exe-mesoderm. The splanchnopleure develops into the allantois and chorion, whereas the somatopleure develops into the chorion and amnion. The allantois, the chorion and the amnion are formed as structures continuous with the embryonic tissues (Patten., 1920).

The early chicken embryo has a sheet-like structure composed of three germ layers, and there is no apparent border between exe and embryonic (em) tissues. As development goes on, the border between exe- and em-tissues is first defined morphologically by the formation of the

body fold. The body fold at the anterior, lateral and posterior are sequentially established and the embryo becomes separated by body folds (Patten., 1920). In stage 6 chicken embryo, the head fold is formed at the anterior region of the embryo (Hamburger et al., 1951; Patten., 1920). Simultaneously, a sac-like structure called the foregut is formed on the ventral side (Bellairs., 1953). The foregut extends posteriorly afterward and the heart will develop around this anterior border between exe-em tissue. The structure and shape of this border are unknown during foregut and heart development. In this study, I focused on the anterior exe/em border in the chicken embryo.

First, I found that the anterior boundary between exe-em tissues situated along the anterior intestinal portal (AIP) in the head fold stage (stage 6) separated from AIP. The shape of the border changed from arch-like to a V-shape. This V-shaped border was designated “amniotic collar.” based on the position; near pharynx and side of the future amnion, and shape. From the observation of the transversal sections of the embryo at stage 9, in the amniotic collar exe- and em-ectoderm, exe- and em-mesoderm are connected, respectively, at the lateral side of the embryo. From labeling of the border ectoderm, the amniotic collar is formed into V-shape by the posterior elongation near the midline. Also, posterior elongation of AIP, the border between exe- and em-endoderm was faster than that of the amniotic collar. Ablation experiments showed

that the amniotic collar is involved in the shape of the embryo, heart development and amnion formation. The present study has demonstrated the novel extraembryonic structure, the amniotic collar, involved in the normal development of the embryo in the way different from the known extraembryonic structure.

Materials and Methods

Chicken embryos

Fertilized chicken (*Gallus gallus*) eggs were incubated at 38℃ for the appropriate time to obtain embryos of the required stage. In this study, staging was conducted according to Hamburger and Hamilton (1951).

New culture

Embryos were cultured with the modified New method (Stern and Ireland, 1981; Chapman et al, 2001).

Observation of the shape of exe/em border

0.1% fast green FCF (Wako) dye in 0.7M sucrose was injected with microcapillary pipettes into the ventral space between the embryo and extraembryonic tissues.

Histology

Embryos were fixed overnight with 4% paraformaldehyde in PBS. Fixed embryos were

embedded with paraffin and sliced transversally in 7μm or 10μm sections. Vectabond-coated glass slides were used. Sections were deparaffinized with xylene, hydrated in decreasing concentrations of ethanol and stained by hematoxylin solution. Finally, sections were dehydrated by increasing concentrations of ethanol, cleared in xylene and enclosed by Mount-Quick (Daido Sangyo).

3D reconstruction

Images of serial sections were binary thresholded using Adobe Photoshop. These images were aligned by using StackReg plugin in imageJ and reconstructed using 3D viewer plugin in Fiji.

Cell labeling with DiI and DiO

0.05% Carbocyanine dye DiI, (1,1-dioctadecyl-3,3,3’,3’- tetramethyl indocarbocyanine perchlorate) (DiI-C18; Molecular Probes) or 0.05% Carbocyanine dye DiO, (3,3'-Dioctadecyloxacarbocyanine Perchlorate) (DiO-C18; Molecular Probes) in 30mM sucrose was used to label cells with microcapillary pipettes.

In ovo ablation of the amniotic collar

To visualize the whole embryo, a small amount of fast green FCF (Wako) dye solution was injected with microcapillary pipettes into the subgerminal cavity at stage 9- to 9+. Then more dye solution was injected into the space between the embryo and extraembryonic tissues to highlight the amniotic collar. Make small holes in the vitelline membrane on both sides of the embryonic head. Small scissors were inserted through the hole and the amniotic collar was ablated. In the control embryo dye injection and punching in the vitelline membrane were carried out. After eggs were sealed with tape and incubated for 24 hours at 38 ℃, the embryos were collected and observed.

Results

The border of em- and exe-ectoderm composed the amniotic collar, V-shape connection in the pharynx region.

First, to analyze the position and the shape of exe/em border during development, 0.1% fast green solution in PBS was injected into the ventral space between the head and exe-tissues (Fig. 1A). The posterior end highlighted by the blue solution is the border between em- and exe-ectoderm. In the stage 7 embryo, an arched border following the shape of the anterior intestinal portal (AIP) (Fig. 1B and C) was found. At stage 7+, there was a little space between the border and AIP on both side of the embryo, and therefore, the shape of the border became almost straight (Fig. 1D and E). After that, it changed markedly, from a shallow U shape in stage 8 (Fig. 1F and G) to deep V shape in stage 9 and 10 (Fig. 1H, I, J and K). Also, em/exe border and AIP were separated not only in the side but the midline from stage 8 (Fig. 1F and G), and space became bigger and bigger in stage 9 and 10 (Fig. 1H, I, J and K). This V-shaped border was formed at pharynx region and ex-tissues just outside of the border will give rise to the lateral amniotic folds. From its shape and position, I designated this V-shaped border “the amniotic collar.”

To investigate the structure of the amniotic collar, a serial section of stage 7+, 8- and 9 embryos were analyzed. Transverse sections of each embryo were stained by Hematoxylin. In stage 7+ embryo, there was a space between the embryonic head and the exe-ectoderm and endoderm, spread in the ventral of the embryo (Fig. 2B and C). No connection between em/exe tissues was found in the head (Fig. 2B and C). At 189 μm from the anterior-most embryo, exe- and em- ectoderm (Fig. 2D and E, arrows), then exe- and em-endoderm (Fig. 2F and G, arrows) were linked in the midline (Fig. 2F and G, arrows). At the posterior part of AIP (position H in Fig. 2A), each of the three germ layers was continuous (Fig. 2H and I).

In stage 8- embryo (Fig. 3A), the em-tissue and exe-tissue were completely separated in the anterior head (Fig. 3B and C). At 329μm posterior to the anterior tip (position D in the fig. 3A), exe- and em-ectoderm were connected on the right side of the embryo (Fig. 3D and E, arrowheads). Exe-mesoderm was also joined with em-mesoderm in this site (Fig. 3D and E, asterisk). At the anterior end of AIP, in which 77 μm posterior to the front end of exe/em border (position F in the Figure 3A), exe-endoderm and em-endoderm were connected at the midline of the AIP (Fig. 3F and G, arrows). In the posterior part of AIP (position H in Fig. 3A), the three exe/em germ layers were continuous (Fig 3H and I), respectively.

In stage 9, there was no connection between the embryo and extraembryonic tissue in the

anterior head (Fig.4B and C) as stage 7 and 8. The front end of exe/em border were found on the right side (Fig. 4D and E, arrowheads) and on the left side (Fig. 4F and G, arrowheads) at 400μm (position D in Fig. 4A) and 460μm (position F in Fig. 4A) from the top of the head In both side, there was a connection of exe/em mesoderm (Fig. 4D, E, F and G asterisk). At AIP, 290 μm posterior to Figure 4D (or 4F) (position H in Figure 4A), an exe- and em- endoderm connection was found in the midline (Fig. 4H and I). It the posterior AIP, exe- and em- all the three germ layers were connected, respectively (Fig. 4J and K).

To help to understand the structure of the amniotic collar, 3D reconstructions were built from serial transverse-sections of stage 9 embryo (Fig. 5). In the anterior head, there is a space at the ventral embryo, between exe- and em- tissues (Fig. 5B and C). In the pharynx region, em- and exe-ectoderm were connected in the ventral (red arrows) and the lateral (red arrowheads) on both side of the embryo (Fig 5D and E). The ventral connection made a narrow lumen, subcephalic pocket composed of dorsal em- and ventral exe-ectoderm at the ventral embryo (Fig. 5E, white arrow). This subcephalic pocket is V-shaped structure from ventral view (Fig. 5F) because the ventral connection got close towards posterior. This is consistent with the result of fast green injection (Fig.1).

From these dates, at the amniotic collar em- and exe-ectoderm and mesoderm are

connected, respectively on the lateral side of the embryo.

The amniotic collar is formed by the difference of extension speed between lateral and midline.

To elucidate how exe/em border at AIP changes into V-shape amniotic collar, cells in exe/em border was labeled at stage 6 (Fig. 6). Cells at both side of the border in the lateral most of AIP were labeled with DiI (red spots in Fig. 6), and in more middle of AIP were labeled with DiO (green spots in Fig. 6). The embryo was observed every 30 minutes in 37 degrees incubator for 6 hours until stage 9. Throughout the incubation, DiI labeled cells were hardly spread (Fig. 6A-E). There was no significant change in both DiI and DiO labeled cells after 2 hours incubation, but space gradually expanded between the DiI labeled boundary and AIP in the lateral side (Fig. 6B, C, G and H). After 4-hours incubation, DiO labeled cells spread along with anterior-posterior axis and gathering towards the midline (Fig. 6D). At this time, the DiI labeled border and side of AIP had more distant, while DiO labeled midline border was still close to the AIP. This results in the whole shape of the border straight (Fig. 6D) from arch shape (Fig. 6C). After 6 hours incubation, DiO labeled cells spread further and were filled into the amniotic

collar and whole ventral subcephalic pocket (Fig. 6E and J). DiI labeled cells were located at the anterior end of the amniotic collar (Fig. 6E and J). At that time the whole border became completely separated from AIP (Fig. 6E and J).

From these results, it was revealed that the connection between exe/em ectoderm established at stage 6 in AIP deformed and became the V-shape amniotic collar without *de novo* connection after stage 6. In addition, the space between the boundary and AIP is gradually formed from the side to the middle due to elongation only in the median.

To confirm whether the border extends posteriorly or head extends anteriorly, the labeled embryo was recorded by time-lapse imaging (Fig. S1). The embryo which the exe-em ectoderm border at the lateral most AIP is labeled with DiI at stage 6 was incubated for 6.5 hours until stage 8+ (Fig. S1A-E). DiI labeled cells in the lateral border hardly moved (Fig. S1A-E, arrowheads), and the midline of the border and AIP extend posteriorly (Fig. S1A-E). Taken together, it is suggested that the V-shape amniotic collar is formed by posterior elongation near the midline of the border, while the side of the amniotic collar was stuck. Also, posterior movement of AIP is faster than those of the amniotic collar.

The amniotic collar is required for making the normal shape of the embryo, heart formation and amnion formation.

To investigate the function of the amniotic collar in embryogenesis, I cut the amniotic collar of the embryo from stage 9- to 9+ in ovo and cultured for 24 hours (Fig. 7A). Before the operation, fast green solution was injected into the ventral space of the embryo to highlight the amniotic collar (Fig. 7A). When amniotic collar was cut using small scissors from a hole of vitelline membrane, blue dye was invaded into the pericardiac cavity (Fig. 7A). To confirm the amniotic collar was cut completely, I made a serial section of embryos just after operation. In the sections, the amniotic collar was cut but other parts of the embryo were intact (supplemental figure 2). In the control embryo, fast green injection and a partial vitelline membrane removal were carried out.

Embryos without obvious malformation were subjected to analysis of phenotype (Fig. 7F). At stage 15, 11 out of 21 operated embryos showed abnormal bent of the embryo near AIP (Fig. 7B, B', C, C' and G), 8 out of 21 showed a smaller heart (Fig. 7B, B', D, D' and H) and 10 out of 21 showed abnormal formation of the amnion (Fig. 7B, B', E, E' and I). In the normal chicken embryo, the head gradually turns right from stage 11 and the body bent smoothly (Fig. 7B) around AIP. While, some operated embryos show a sharp bent near AIP (Fig. 7C', arrow).

Although at stage 15, the chicken embryo has developed heart with active beating bigger than the head, operated embryo shows much smaller and inactive heart (Fig. 7D and D', arrows). Interestingly, some operated embryos showed the abnormal formation of the amnion on the dorsal side despite cutting the amniotic collar which is the ventral structure. In control, the amnion covered the entire heart and posterior border of the amnion is posterior than AIP (Fig. 7B', dashed line). However, the amnion barely covered the heart in operated embryos, posterior border of the amnion is much anterior than those of control embryo (Fig. 7E', dashed line). These phenotypes were shown alone or combined in the operated embryos.

From these results, it is possible that the amniotic collar is involved in normal development of the anterior structure of embryos on both ventral and dorsal.

Discussion

Exe-membranes with various functions develop during embryogenesis in amniotes. The early chicken embryo has a sheet-like structure composed of three germ layers without an obvious border between em- and exe-tissues. Then, the embryo is segregated from exe-tissues by the formation of the body fold (Romanoff, 1960). In this study, I focused on the morphological change of the anterior exe/em border and found a new structure, the amniotic collar, supporting anterior development including formation of the heart and the amnion.

Structure and formation process of the amniotic collar

The amniotic collar was found by dye injection into anterior ventral space between embryo and exe-membrane. Just after formation of the head fold in stage 7, dye covered the whole head to AIP, the border between em- and exe-tissue (Fig. 1B and C). As embryos develop, posterior border of spreading dye was separated from AIP. Also, its shape changed from arch to V. At stage 9-10 the heart developed between dye-marked extra embryonic space and AIP. The V-shaped extraembryonic space is called the subcepharic pocket (Fig. 5E). The subcepharic

pocket is in the pharyngeal region surrounded by future lateral amniotic folds. I am interested how this space made by em/exe border is established and designate this V-shaped border between em- and exe- tissues "the amniotic collar" based on its position and shape.

To investigate the formation process of the amniotic collar, the structure of the amniotic collar was analyzed by a serial section of the embryo in the various stages. It is found that amniotic collar consisted in the border of exe-/em- ectoderm, and mesoderm, while the border of exe-/em- endoderm in AIP (Fig. 3D, E, 4D, E, F, and G). In the early stage, stage 7, exe-/em- border of all three germ layers are at AIP (Fig. 2D-G). Taken together, exe-/em- border of each germ layer acts differently during development.

Next, labeling experiments revealed a morphological change in the border of exe-/em- ectoderm. Its border cells in the lateral side of AIP labeled at the onset of head formation, stage 6, kept their position and showed no extension (Fig. 6 and S1). While border cells in the middle of AIP spread along with the anteroposterior axis (Fig. 6). The border cells spread more towards the midline of AIP and just cover whole ventral exe-ectoderm of the subcephalic pocket when the amniotic collar is established (Fig. 6E). Also, space between AIP, the exe/em border of endoderm, and future amniotic collar, the border of exe-/em- ectoderm was found on the side in the beginning and then spread into the midline (Fig. 6).

Taken together it is suggested that the amniotic collar is formed by the difference speed of posterior extension between the posterior extension of ectoderm and endoderm, and in the midline and lateral side within the border of exe-/em- ectoderm as follows.

In my model, the formation of the amniotic collar is divided into two phases (Fig. 8). Before the formation of the amniotic collar, the ventral ectoderm and endoderm extend at the same speed sharing arched shape border at AIP. At the onset of amniotic collar formation, only lateral ectoderm stops posterior extension while the midline ectoderm and whole ventral endoderm keep extending posteriorly at the same speed (phase I). As a result, the exe/em border of ectoderm becomes horizontal and there is space on the side between its border and AIP. In the second phase, the ventral midline ectoderm still extends posteriorly but the speed of the extension becomes gradually slower than that of the ventral endoderm. Therefore, exe/em border becomes V- shape and space is formed between its border and AIP, and the amniotic collar is established.

It is unknown how the extension of the ventral ectoderm changes during the formation of the amniotic collar. One possibility is the inflow of heart forming mesoderm to make the heart between the amniotic collar and AIP. The heart forming mesoderm is developed heart crescent in the anterior to the head fold at stage 5 (Rudnick, 1938; Rawles, 1936; Rawles, 1943). Those

mesoderms move to the lateral side of the embryo till stage 6-8 (Stalsberg et al., 1969). Then, they enter the anterior space near AIP from the side of the embryo (Redkar et al., 2001). From our observation, the side of AIP in stage 6 and 7 barely included mesodermal cells (Fig. 2H and I). On the other hand, stage 8 when the heart mesoderm starts to migrate, many mesodermal cells were found in the side space between exe/em border of ectoderm and AIP (Fig. 3H and I). It is possible that this mesodermal migration is involved in the formation of the amniotic collar by stopping the posterior extension of the ectoderm. To prove this hypothesis, it is needed to observe the formation of the amniotic collar in the embryo without the heart forming mesoderm in stage 6.

In addition to the posterior extension of the embryo, convergent movement of labeled cells was found. The lateral space between DiO labeled cells were getting narrower toward the midline as the embryo elongated (Fig. 6). This suggests that convergent extension, which drives the diverse developmental process, occurs actively in the midline. Since the posterior end of labeled cells at the border in stage 6 were still located on the amniotic collar in stage 9 (Fig. 6), it is suggested that the amniotic collar formed by deformation of the existent border but not by *de novo* connection between exe-/em- ectoderm.

The function of the amniotic collar in embryogenesis

In this study, the amniotic collar ablation suggests that it is involved in the curvature of the embryo, heart formation and amnion formation (Fig.7).

In amniotes, the head occupies a large volume in the whole embryo as the brain develops (Nelsen, 1953). In addition, the heart is developed in the very early stage and its beating is very active in order to establish blood circulation in the vast extraembryonic membranes for gas exchange, nutrition and buildup wastes. This suggests that the anterior body of the amniote embryo easily dislocates and the amniotic collar located just anterior to the heart can more the embryo to the underneath extraembryonic membrane for maintaining the embryonic position. For this function, V-shape connection may be important because it is physically stronger than the horizontal connection.

For normal development of the heart of amniotes, the pericardial cavity covering and protecting the heart is important (Shabetai et al.,1979). The amniotic collar is located at the anterior end of the pericardial sac that forms the pericardial cavity. It is possible that the formation of the amniotic collar keeps enough space to form the heart by stopping the posterior extension of ectoderm.

It is showed that the mesoderm in the somatopleural region on the side of the amniotic collar differentiates not only the amnion but also the heart (Asai et al., 2017). From this data, it is expected that the migration of the heart mesoderm through the amniotic collar is indispensable for heart development.

It is interesting that ablation of the amniotic collar in the ventral embryo affected amnion formation in the dorsal embryo. Formation of the amnion begins with the formation of the extraembryonic fold called amniotic fold at stage 10. First, the anterior amniotic fold covers the head, followed by subsequent formation of the lateral and posterior amniotic folds. Amniotic fold covers the dorsal side of the embryo from the anterior, and finally the entire dorsal embryo is covered with the amnion (Shore et al., 1889; Hamburger et al., 1951; Overton., 1989; Miller et al., 1994; Tipping et al., 2011). It is reported that the growth of the anterior amniotic fold would create sufficient tension to elevate the lateral amniotic fold, and the proamnion is important for the formation of the amniotic fold (Lillie, 1903; Adamstone., 1948; Bernardo et al., 2014). The proamnion is a thin exe-tissue composed of two layers, the ectoderm and the endoderm, and spread ventrally under the head at stage 10 (Shore et al., 1889). In the previous study, to depress the head into the proamnion is important for the formation of the amniotic fold (Bernardo et al., 2014). This depression may be controlled by the vitelline membrane covering

whole eggs (Shore et al., 1889) because without the vitelline membrane many embryos develop with abnormal amnion (Fukuda, personal communication). The tension of the vitelline membrane may resist against raising head by the developed heart. The amniotic collar is formed near the heart during the heart develops, then the amniotic fold is formed. It is suggested that the amniotic collar supports the formation of the amnion by inhibiting the lifting of the head during heart development. In this experiment, to eliminate the possibility that the lateral holes in the vitelline membrane for operation affect development of the amnion, phenotype of control embryo with a similar lateral hole in the vitelline membrane were analyzed.

In this study, I found a new structure, the amniotic collar formed at exe/em boundary. I have investigated the structure, formation and function of the amniotic collar. The amniotic collar physically connects the anterior embryo with exe-tissue to support normal development such as the shape of the embryo, heart development and amnion formation. Currently, this structure and the subcepharic pocket is found only in chicken embryo but it is possible that another amniote embryo has a similar structure. In other amniotes, the formation of the amnion is different. For example, in rabbit embryos, amnion is covered from mainly posterior end of the embryo (Hassan and Viebahn, 2017). I am interested in searching whether the amniotic

collar-like structure is common among the amniotes and analysis of its functional diversity.

Figure 1. Exe/em boundary changes from an arch shape to V shape.

(A) A schematic figure showing injection of fast green solution into the ventral space between the head and extraembryonic tissues in the chicken embryo. Left figure; ventral view. Right figure; lateral view. (B, D, F, H and J) Bright field images of anterior part of embryos at stage7, 7+, 8, 9 and 10, respectively. (C, E, G, I and K) Bright field images of fast green injected embryos of B, D, F, H and J, respectively. The exe/em boundary, which is highlighted by posterior end of dye solution, gradually became V shape (black dashed lines) from arch-shape and separated from AIP (magenta dashed lines). All photos in B-K are ventral view. A, anterior; P, posterior, R, right; L, left; D, dorsal; V, ventral. Scale bars = 500μm.

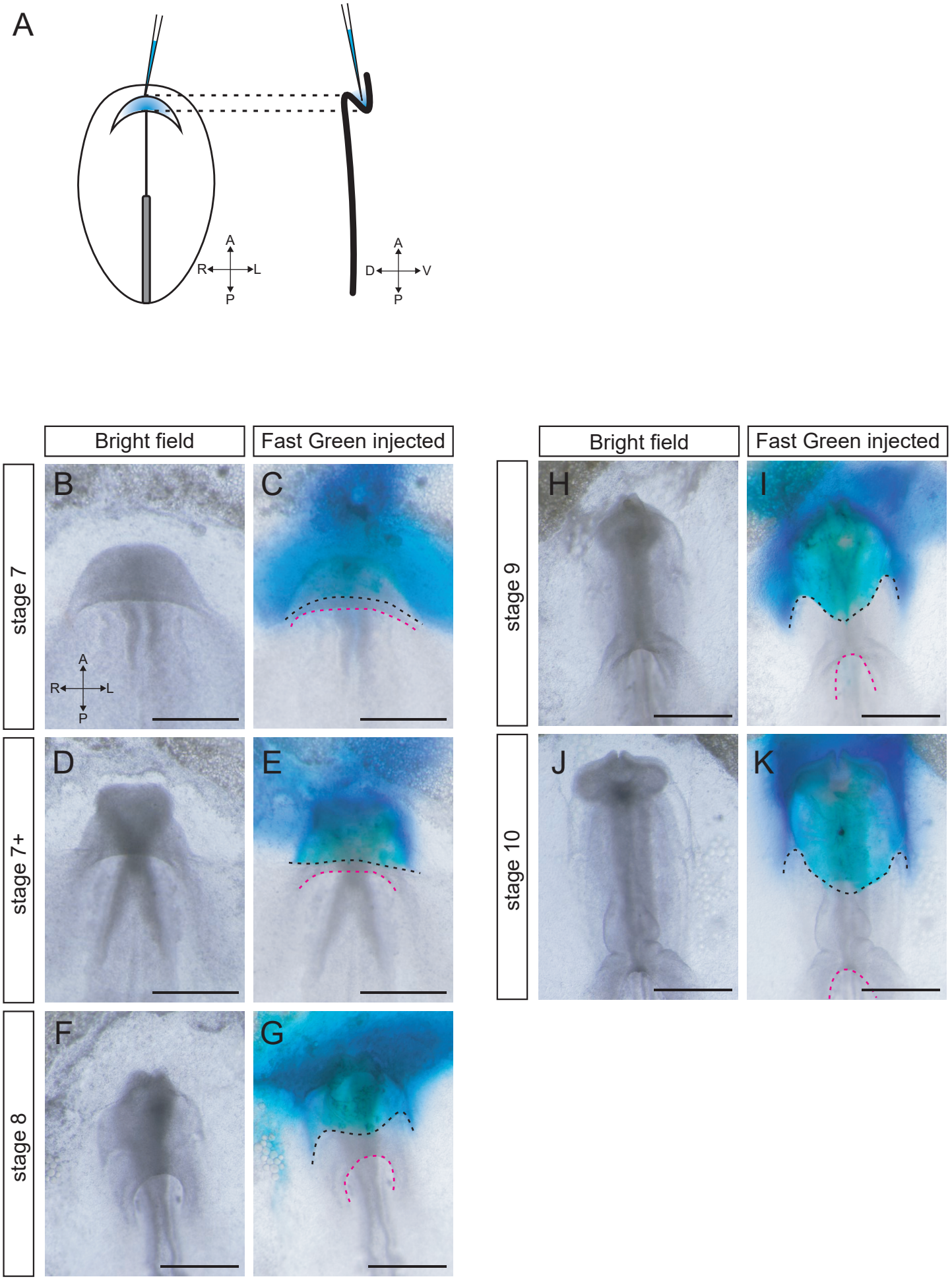
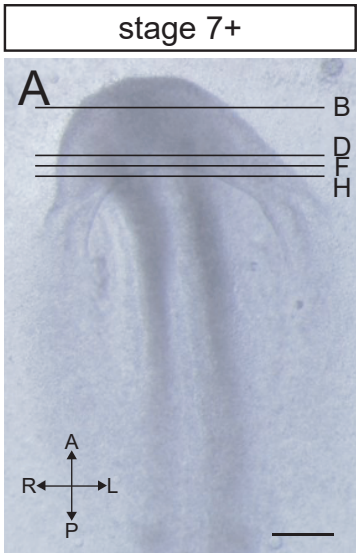
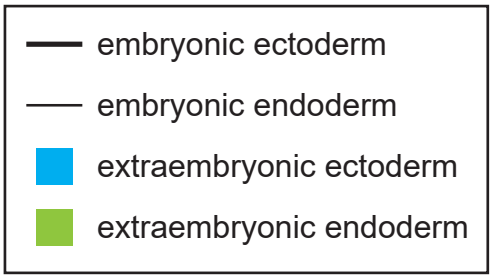
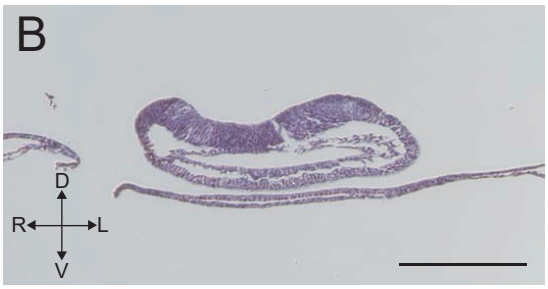

A
A
R
L
P
A
D
V
P
Bright field
Fast Green injected
stage 7
B
C
A
R
L
P
stage 7+
D
E
stage 8
F
G
Bright field
Fast Green injected
stage 9
H
I
stage 10
J
K

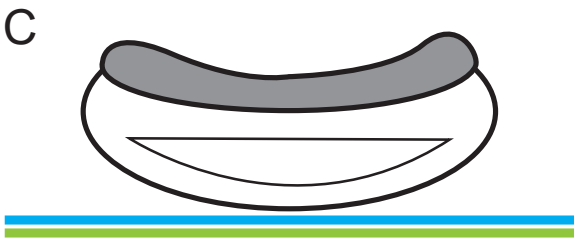
Figure 2. The anterior end of the exe/em border exists at AIP in stage 7+.

(A) Bright field image of an anterior part of chicken embryo at stage 7+ from ventral. (B, D, F and H) Transverse sections stained by hematoxylin through the embryo in panel A. Each section is 175μm (B), 189μm (D), 224μm (F) and 245μm (H) posterior from the anterior end of embryo. (C, E, G and I) Schematic figure of B, D, F and H, respectively. (D and E) The anterior end of exe/em ectoderm border located in the ventral midline (arrowheads). (F and G) The anterior end of exe/em endoderm border in the ventral midline (arrows) at AIP. A, anterior; P, posterior, R, right; L, left; D, dorsal; V, ventral. Scale bars = 200μm.


stage 7+
A
B
D
F
H
A
R
L
P


embryonic ectoderm
embryonic endoderm
extraembryonic ectoderm
extraembryonic endoderm


B
D
R
L
V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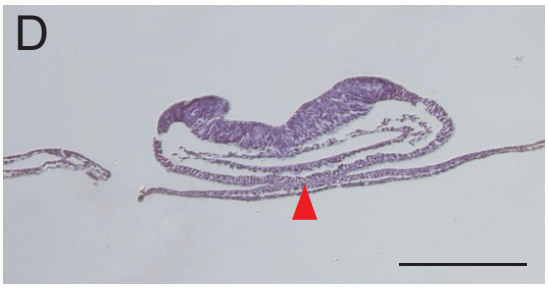
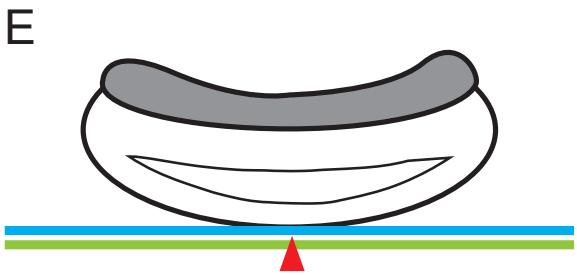

D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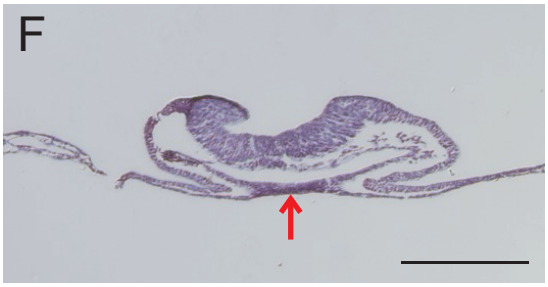
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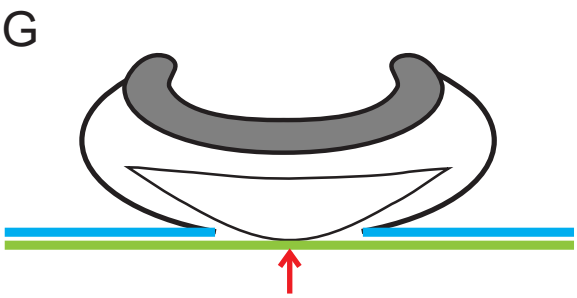
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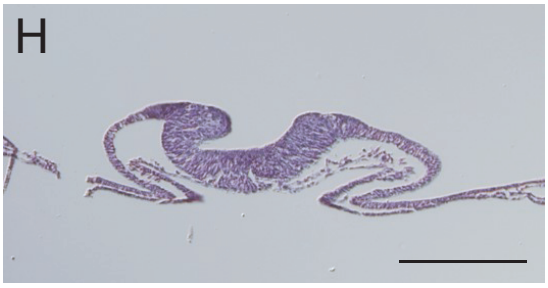
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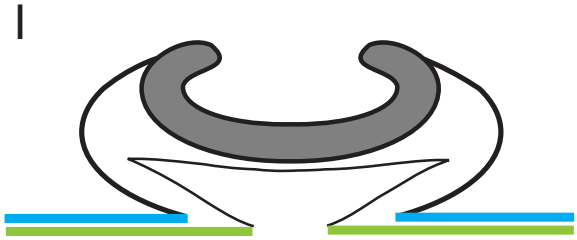
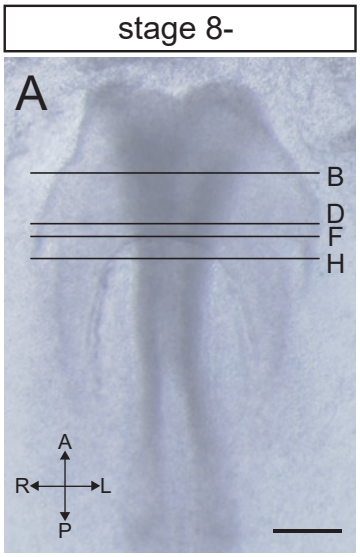
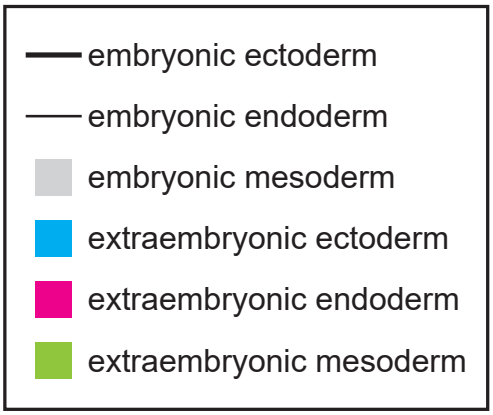
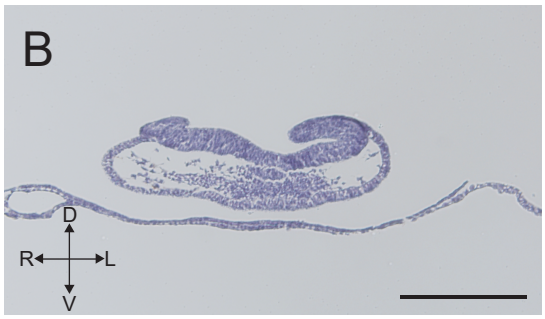

I

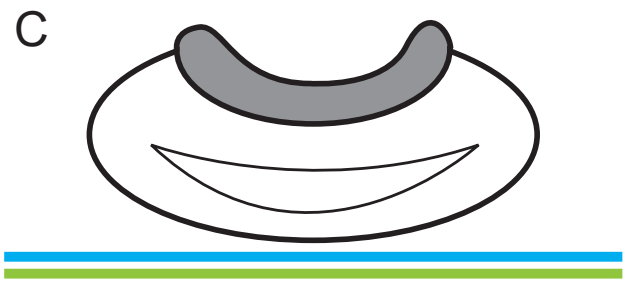
Figure 3. The anterior end of the exe/em border exists in the right side anterior of AIP in stage 8-.

(A) Bright field image of an anterior part of chicken embryo at stage 8- from ventral. (B, D, F and H) Transverse sections stained by hematoxylin through the embryo in panel A. Each section is 175μm (B),329μm (D), 392μm (F) and 427μm (H) posterior from the anterior end of embryo. (C, E, G and I) Schematic figure of B, D, F and H, respectively. (D and E) The anterior end of exe/em border exists in the right side (arrowheads). em- and exe-mesoderm were connected in this site (asterisk). (F and G) The anterior end of AIP exists in the ventral midline (arrows). A, anterior; P, posterior, R, right; L, left; D, dorsal; V, ventral. Scale bars = 200μm.


stage 8-
A
B
D
F
H
A
R
L
P


embryonic ectoderm
embryonic endoderm
embryonic mesoderm
extraembryonic ectoderm
extraembryonic endoderm
extraembryonic mesoderm


B
D
R
L
V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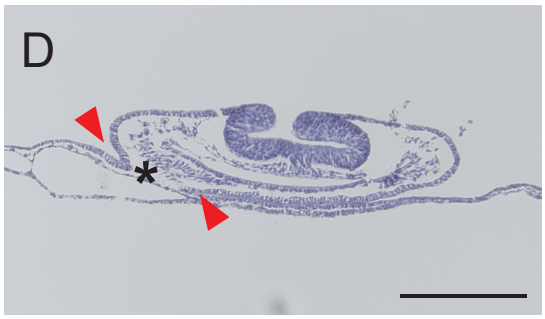
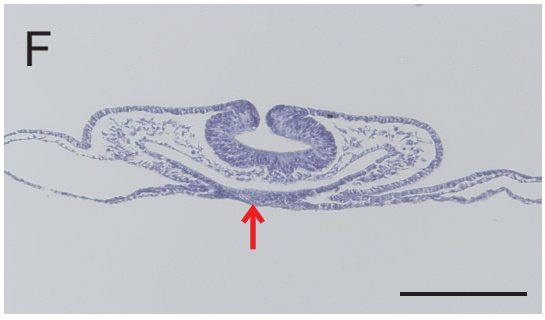

D


E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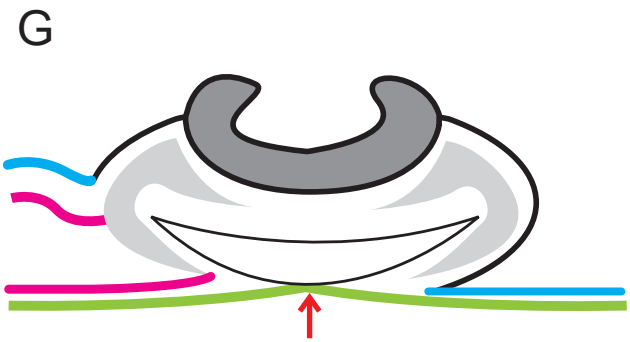
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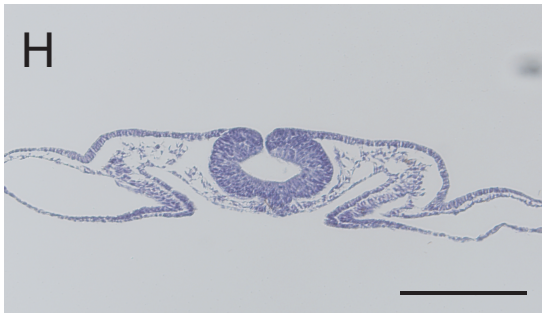
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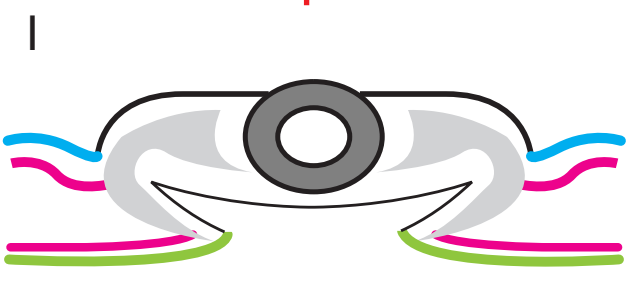
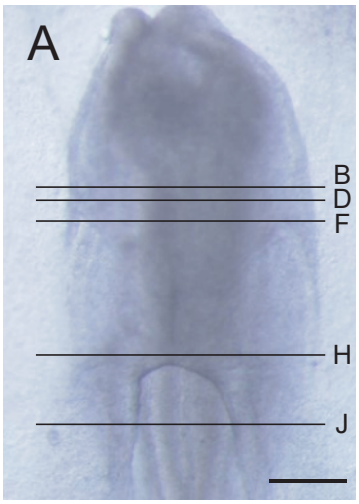
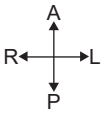

I

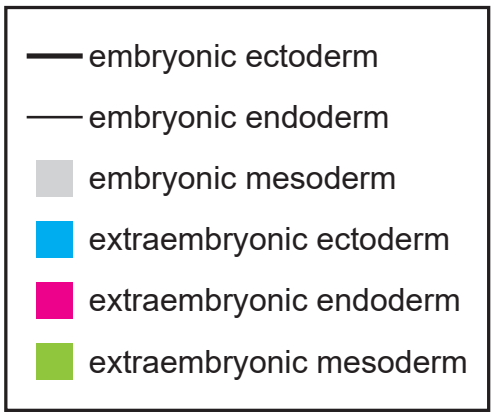
Figure 4. The anterior end of the exe/em border exists in both lateral side anterior of AIP in stage 9.

(A) Bright field image of an anterior part of chicken embryo at stage 9 from ventral. (B, D, F, H and J) Transverse sections stained by hematoxylin through the embryo in panel A. Each section is 370μm (B), 400μm (D), 460μm (F), 690μm (H) and 730μm (J) posterior from the anterior end of embryo. (C, E, G, I and K) Schematic figure of B, D, F, H and J respectively. (D and E) The anterior end of exe/em border exists in the right side (arrowheads). Em- and exe-mesoderm were connected in this site (asterisk). (F and G) The anterior end of AIP exe/em border exists in the left side (arrows). (H and I) The anterior end of AIP exists in the ventral midline (arrows). Em- and exe-mesoderm were connected in this site (asterisk). A, anterior; P, posterior, R, right; L, left; D, dorsal; V, ventral. Scale bars = 200μm.

stage 9


A
B
D
F
H
J


A
R
L
P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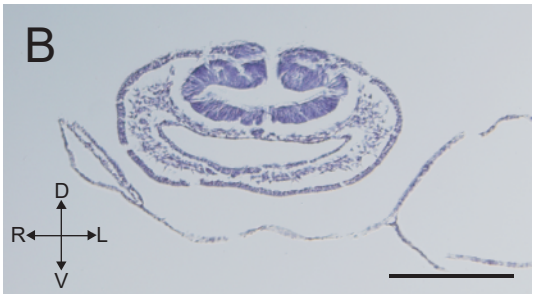
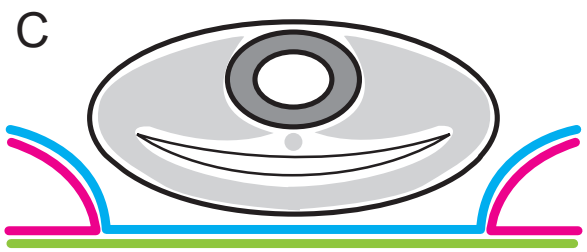
embryonic endoderm
embryonic mesoderm
extraembryonic ectoderm
extraembryonic endoderm
extraembryonic mesoderm


B
D
R
L
V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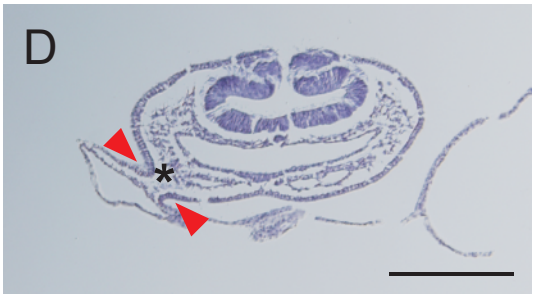
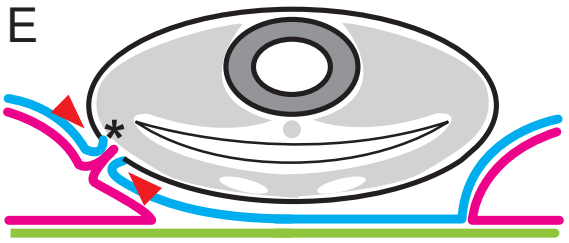

D
*


E
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F
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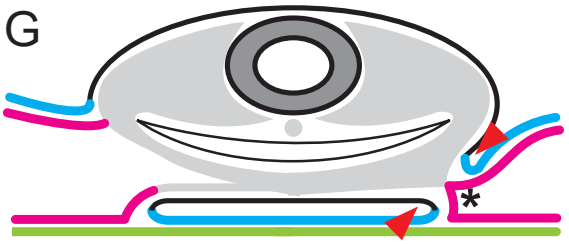
G
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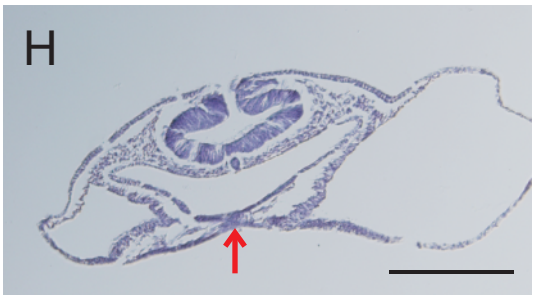
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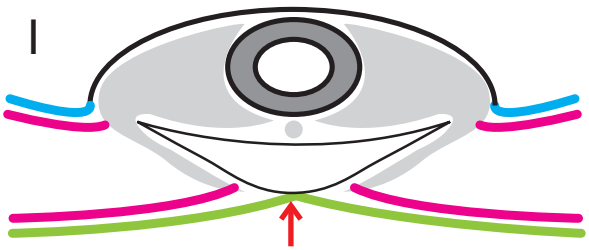
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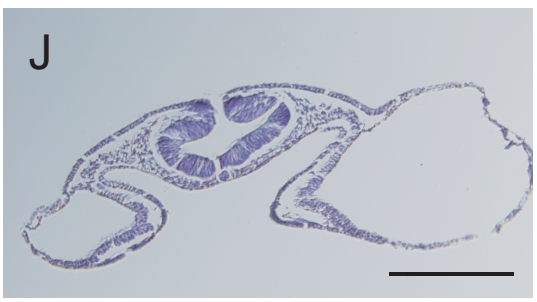
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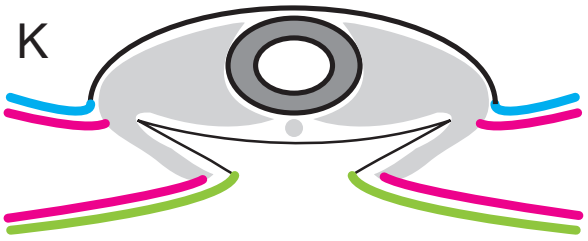
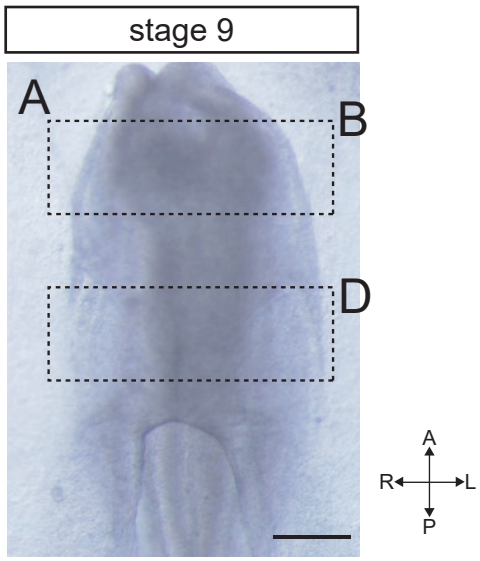
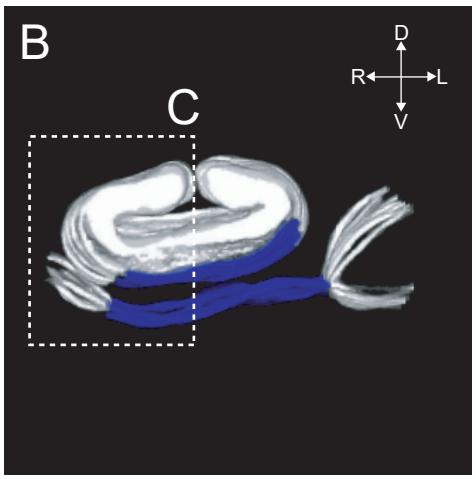

K

Figure 5. Em- and exe-ectoderm and mesoderm are connected respectively at the amniotic collar.

(A) Bright field image of an anterior part of the embryo using in Figure 4. This is the same photo as Figure 4 (A). (B-F) 3D reconstruction of serial transversal sections of the embryo in A. Blue part showed em-ectoderm (dorsal) and exe-ectoderm and endoderm (ventral). (B and C) B is the frontal view of the anterior head (dashed box in A). C is the anterolateral view of the right half of B (dashed box in B). There was no connection between em- and exe-tissues. (D and E) D is the frontal view of the pharynx region (dashed box in A). E is the anterolateral view of the right half of D (dashed box in D). Em- and exe-ectoderm were connected in the ventral (red arrows) and the lateral (red arrowheads) on both side of the embryo. The subcephalic pocket was located in the ventral side of the embryo (white arrowhead). (F) Ventral view of D. The border was V-shape. A, anterior; P, posterior, R, right; L, left; D, dorsal; V, ventral. Scale bar = 200μm.


stage 9
A
B
D
A
R
L
P


B
C
D
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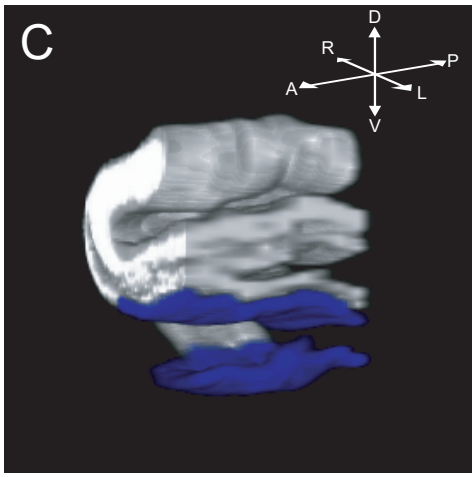
V


C
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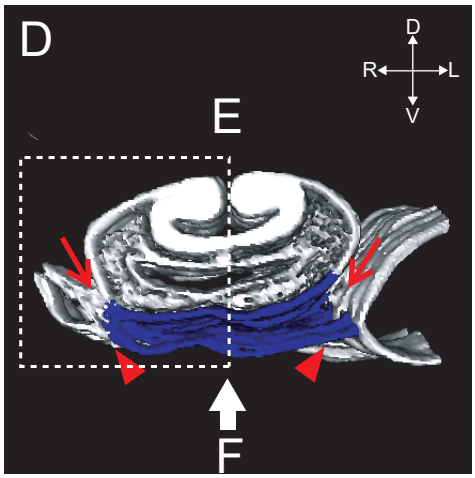
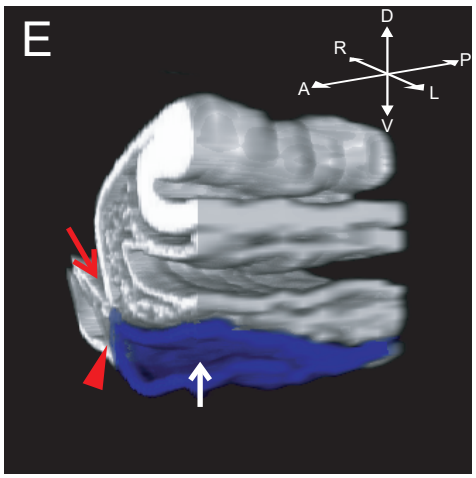
R
P
A
L
V


D
E
D
R
L
V
F


E
D
R
P
A
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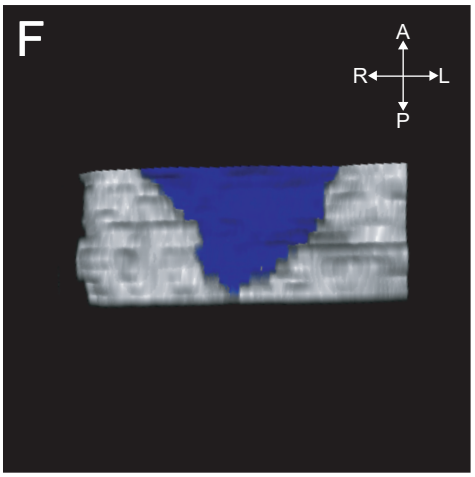
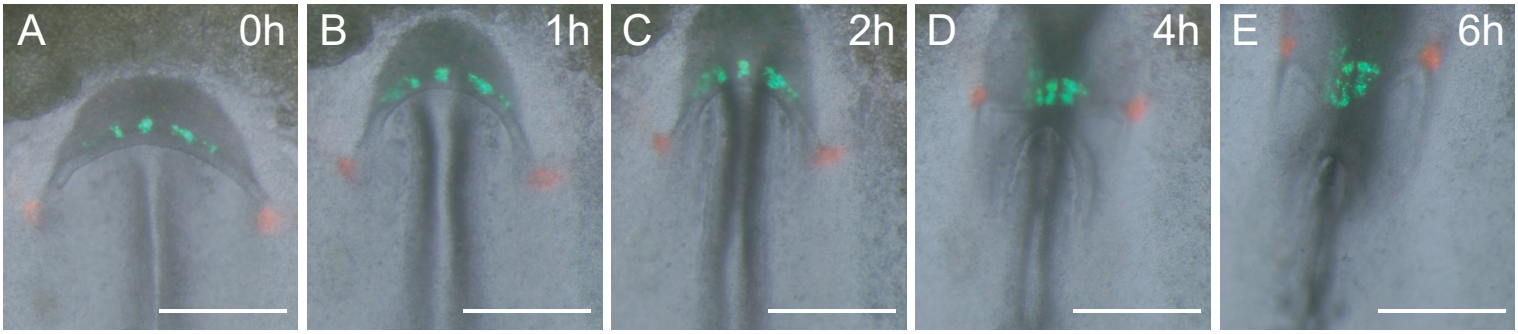

F
A
R
L
P

Figure 6. Cells at the border at stage 6 become the amniotic collar.

(A-E) Sequential images of the dye-labeled cells in exe/em border (ventral view). Both lateral sides were labeled with DiI (red), and near the midline were labeled with DiO (green) at stage 6 (A). After 6 hours, the embryo became stage 9 (E) and the amniotic collar was formed. (F-J) Schematic figure of A-E respectively. A, anterior; P, posterior; R, right; L, left. Scale bars = 500μm.


A
0h
B
1h
C
2h
D
4h
E
6h

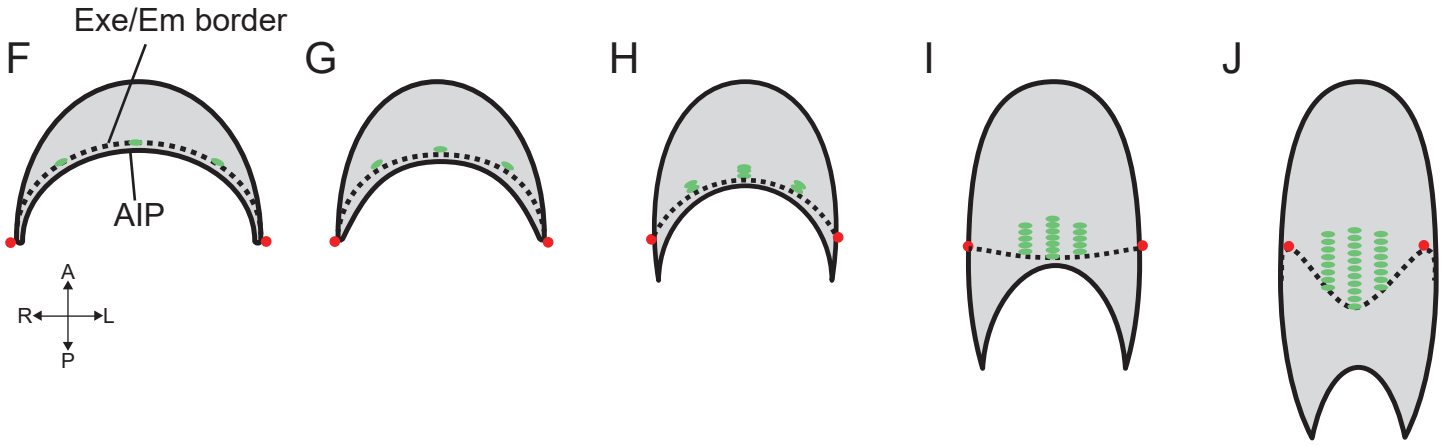
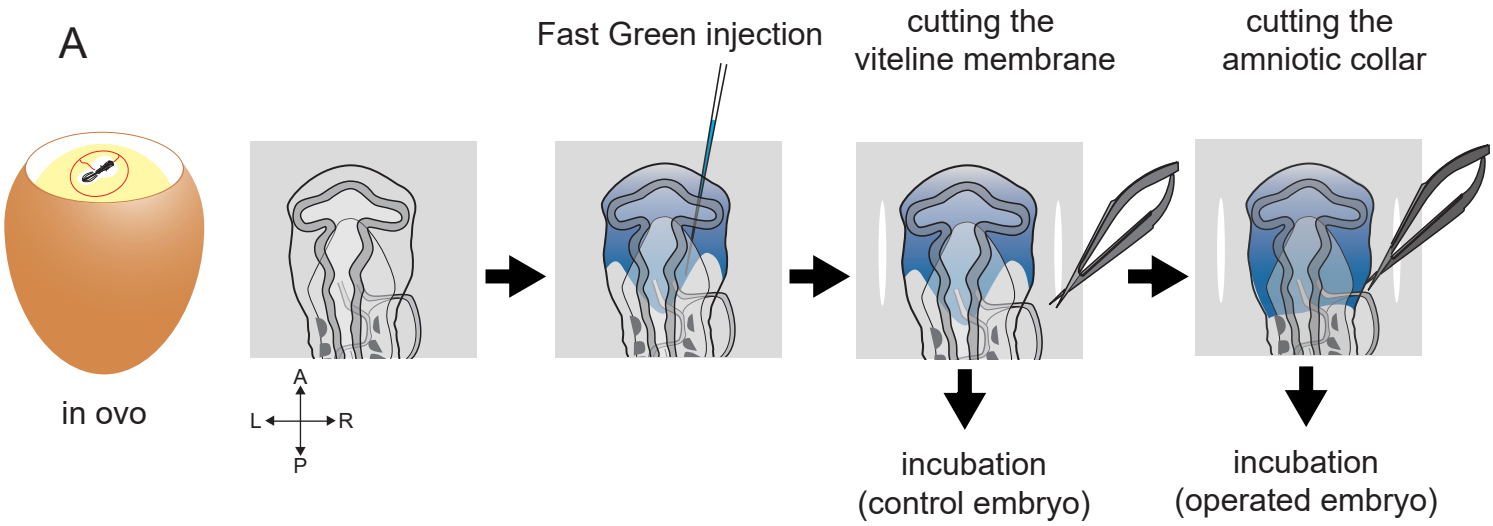
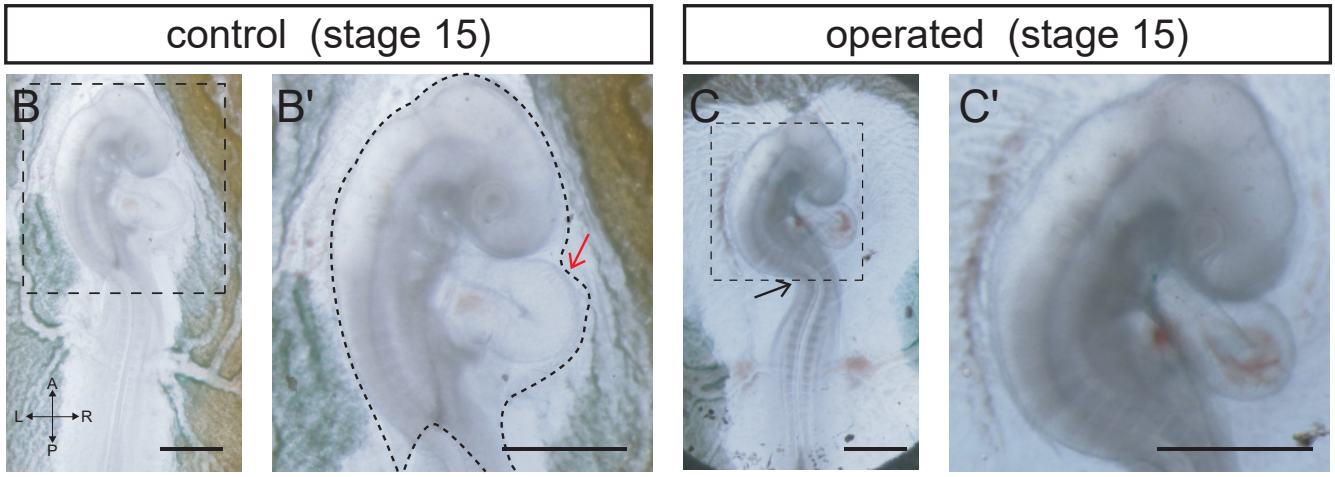
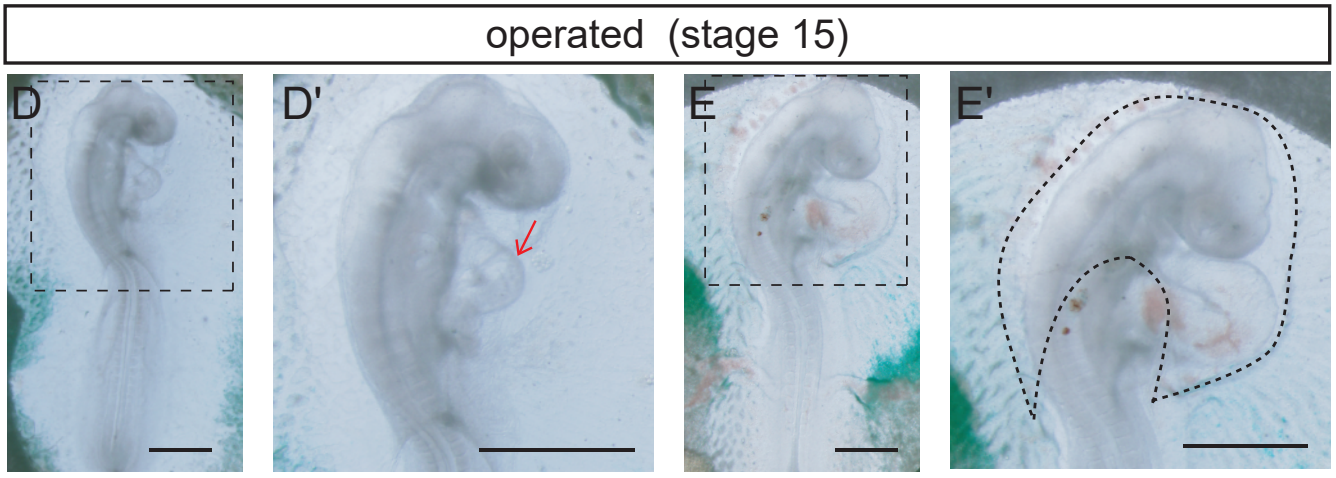

Exe/Em border
F
AIP
A
R
L
P
G
H
I
J

Figure 7. Ablation of the amniotic collar affects the embryogenesis.

(A) A schematic figure of cutting the amniotic collar. (B-E) Bright field images of control embryo (B) and operated embryos (C-E) at stage 15 incubated after 24 hours (ventral view). (B'-E') High magnification of B-E respectively. (B and B') The amnion (the region surrounded by the dashed line) and the heart (arrow) were formed normally. (C and C') Abnormal bent was observed near AIP. (D and D') Abnormal heart (arrow) was formed. (E and E') The area covered with amnion (the region surrounded by the dashed line) was narrow. (F) A number of embryos with no phenotype (light gray bars), phenotype (dark gray bars) and nonspecific defects (black bars) in control and operated embryos. (G) Numbers of embryos with an abnormal bent in control and operated embryos. Light gray bars show the number of embryos with normal bent, and dark gray bars show the number of embryos with abnormal bent. (H) Numbers of embryos with abnormal heart in control and operated. Light gray bars show the number of embryos with a normal heart, and dark gray bars show the number of embryos with an abnormal heart. (I) Numbers of embryos with abnormal amnion in control and operated. Light gray bars show the number of embryos with normal amnion, and dark gray bars show the number of embryos with abnormal amnion. A, anterior; P, posterior; R, right; L, left. Scale bars = 1mm.


A
Fast Green injection
cutting the viteline membrane
cutting the amniotic collar
in ovo
A
L
R
P
incubation (control embryo)
incubation (operated embryo)


control (stage 15)
operated (stage 15)
B
B'
C
C'
A
L
R
P


operated (stage 15)
D
D'
E
E'

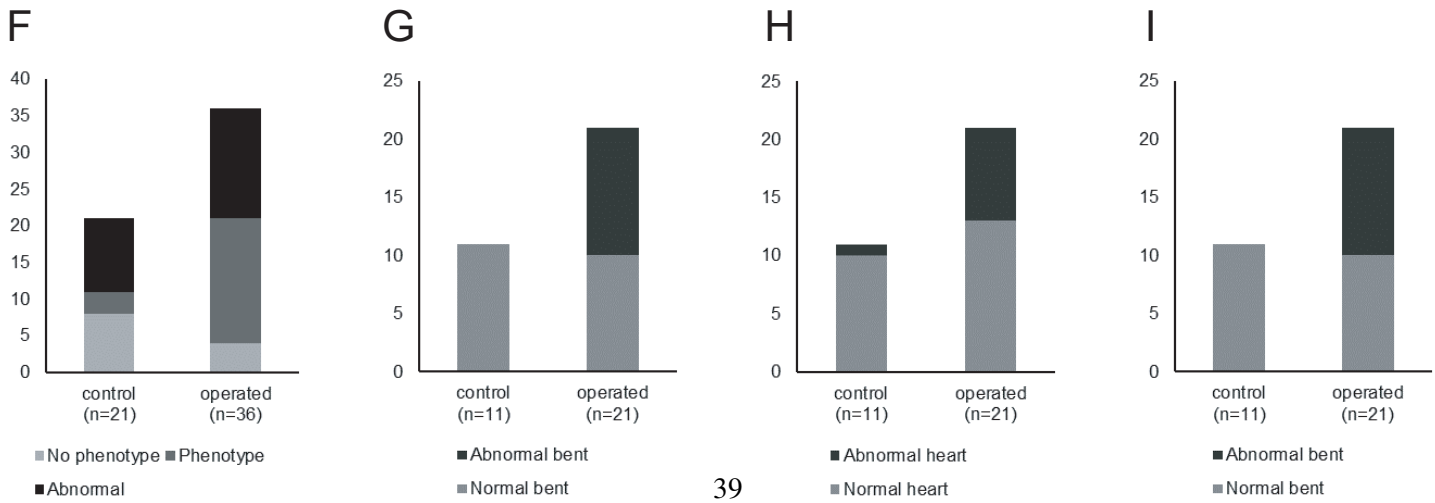
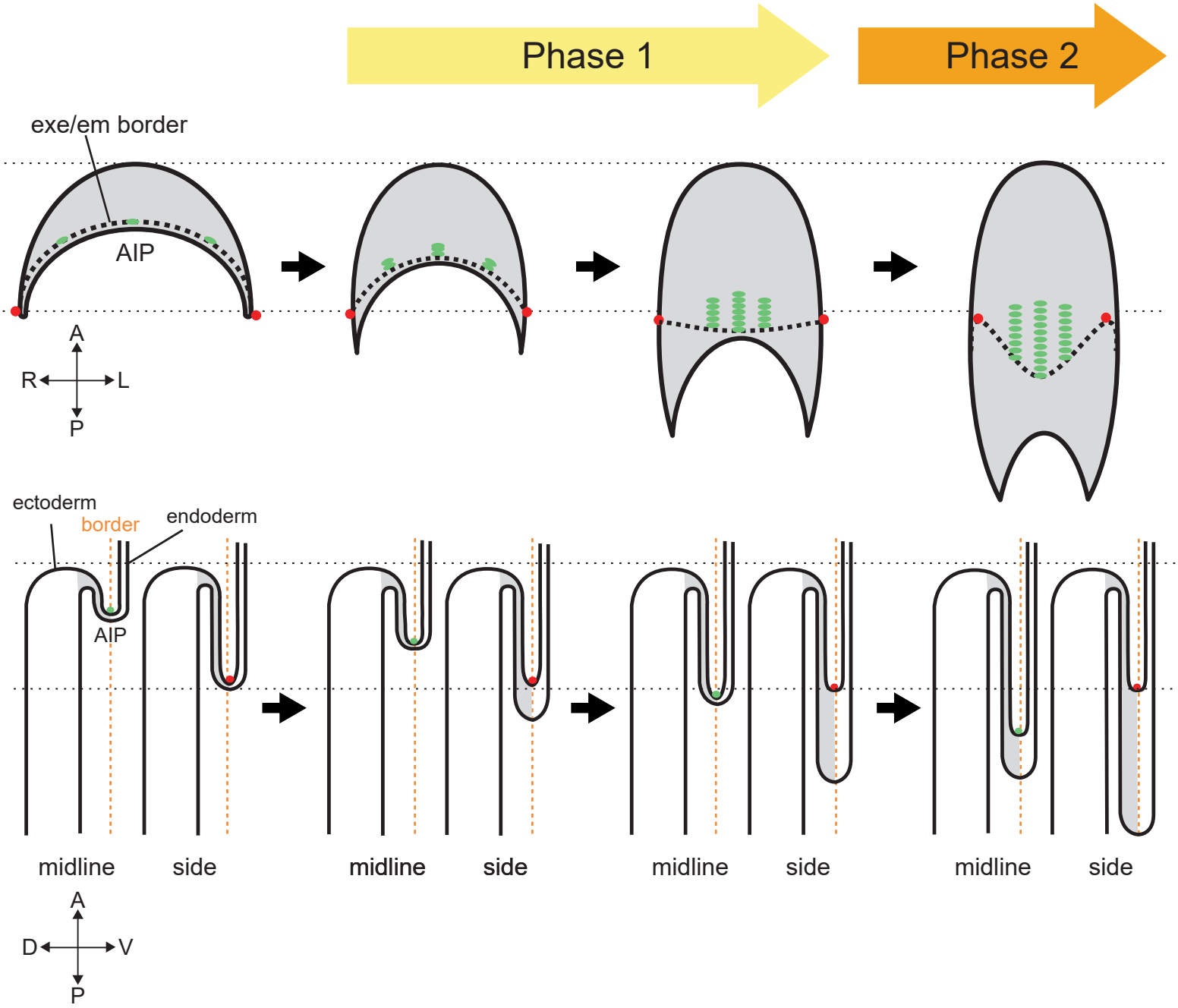

F
40
35
30
25
20
15
10
5
0
control (n=21)
operated (n=36)
No phenotype
Phenotype
Abnormal
G
25
20
15
10
5
0
control (n=11)
operated (n=21)
Abnormal bent
Normal bent
H
25
20
15
10
5
0
control (n=11)
operated (n=21)
Abnormal heart
Normal heart
I
25
20
15
10
5
0
control (n=11)
operated (n=21)
Abnormal bent
Normal bent

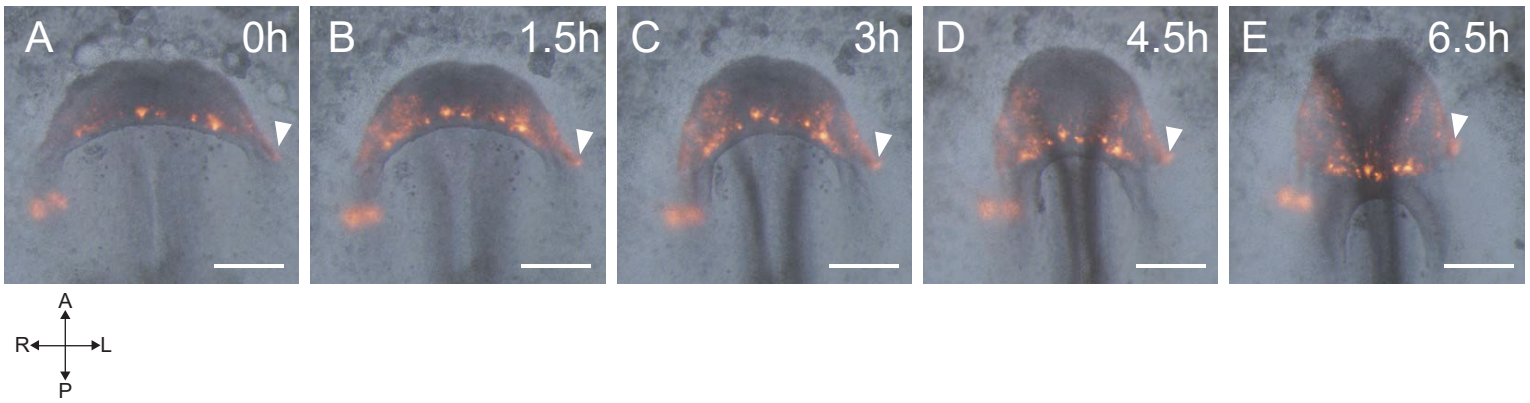
Figure 8. Schematic model showing the formation of the amniotic collar.

The diagrams above show the ventral view of the head from stage 6 to 9. The diagrams below show the sagittal sections in the midline and lateral side at each time points. The gray part of the diagrams above corresponds to the gray part of the diagrams below. Red points show DiI labeled cells in the lateral border, and green points show DiO labeled cells in the middle border. Before the formation of the amniotic collar, exe/em border and AIP extend posteriorly at the same speed both in the midline and lateral side. At phase 1, the border and AIP extend posteriorly at the same speed in the midline, but the lateral side of the border stop extension. At phase 2, AIP continues to extend in both lateral and midline, but the extension speed of the border in the midline becomes slower than that of AIP. Due to the difference in these extension speeds, the amniotic collar is formed. A, anterior; P, posterior; R, right; L, left; D, dorsal; V, ventral.


Phase 1
Phase 2
exe/em border
AIP
A
R
L
P
ectoderm
border
endoderm
AIP
midline
side
midline
side
midline
side
midline
side
A
D
V
P

Supplemental figure 1. The position of labeled cells in the lateral side of the border did not change.

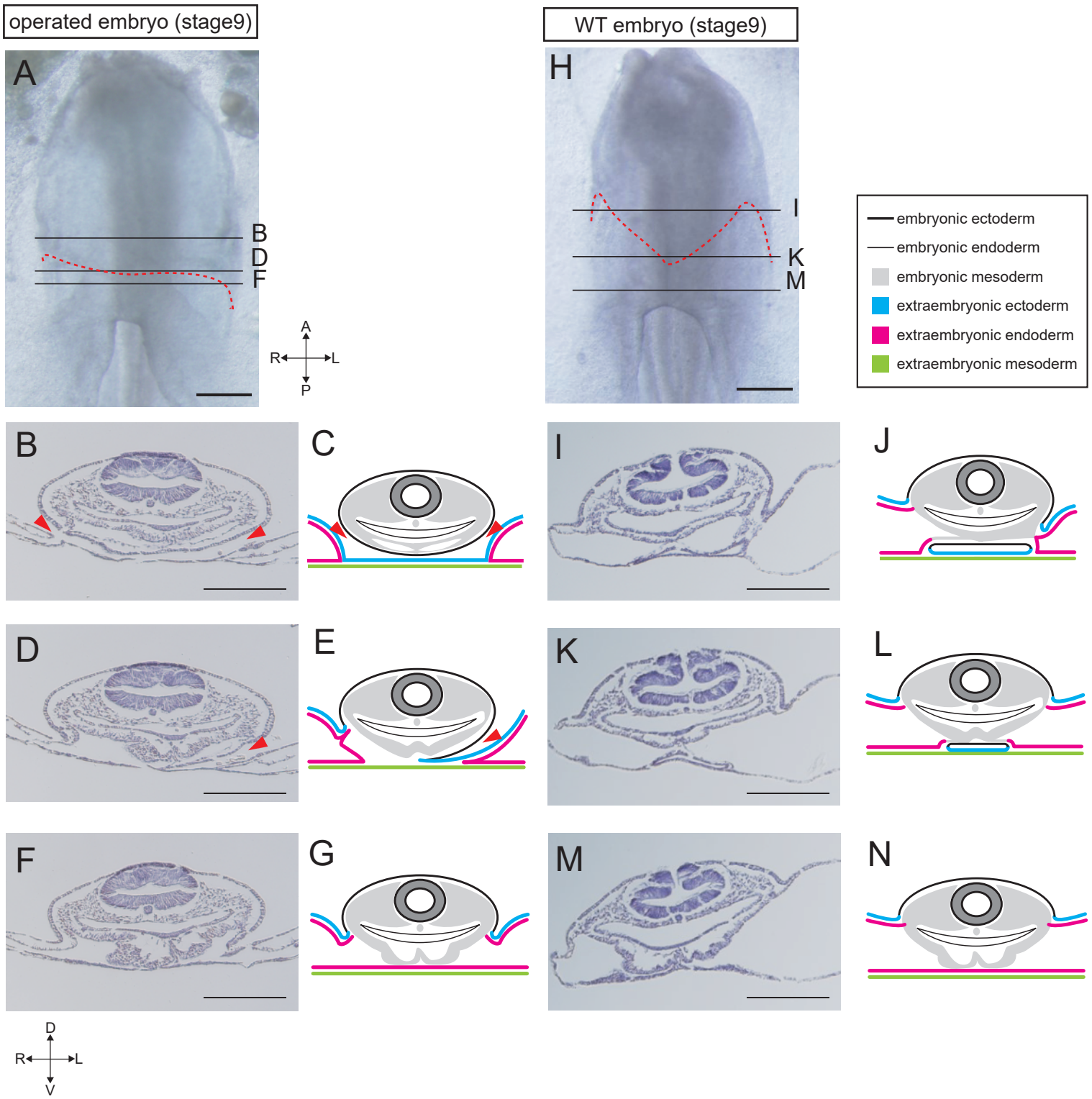
(A-E) Selected images from time-lapse recordings (ventral view). Some points of the border were labeled with DiI (red) at stage 6 (A). After 6.5 hours, the embryo became stage 8+ (E) and the amniotic collar was formed. Labeled cells in the left side of the border hardly moved (arrowheads). A, anterior; P, posterior; R, right; L, left. Scale bars = 500μm.


A
0h
B
1.5h
C
3h
D
4.5h
E
6.5h

A
R
L
P

Supplemental figure 2. The amniotic collar was ablated in the operated embryo.

(A) Bright field image of the operated embryo at stage 9 just after the ablation of the amniotic collar (ventral view). Red dashed line shows exe/em border. (B, D and F) Transverse sections stained by hematoxylin through the embryo in panel A. Each section is 490μm (B), 567μm (D) and 609μm (F) posterior from top of the head. The amniotic collar is ablated (red arrowheads). (C, E and G) Schematic figure of B, D and F respectively. (H) Bright field image of WT embryo at stage 9 using in Figure 4 (ventral view). Red dashed line shows exe/em border. (I, K and M) Transverse sections stained by hematoxylin through the embryo in panel H. Each section is 460μm (I), 510μm (K) and 660μm (M) posterior from top of the head. (J, L and N) Schematic figure of I, K and M. A, anterior; P, posterior; R, right; L, left; D, dorsal; V, ventral. Scale bars = 200μm.


operated embryo (stage9)
WT embryo (stage9)
A
B
D
F
A
R
L
P
H
I
K
M
embryonic ectoderm
embryonic endoderm
embryonic mesoderm
extraembryonic ectoderm
extraembryonic endoderm
extraembryonic mesoderm
B
C
I
J
D
E
K
L
F
G
M
N
D
R
L
V

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