Trends in Developmental Biology
Vol. 7, 2013

Original Communication

Earliest Anlagen of the area vasculosa and heart: Regional influences of "extraembryonic" (yolk rich) tissues

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ABSTRACT

In the avian blastoderm four (hemi) concentrically disposed, deep yolk-rich "extraembryonic" structures (Rauber's sickle and sickle horns, caudo-lateral marginal zone, area opaca, perigerminal zone) are successively involved in the early formation of the coelomo-cardiovascular system. Our experiments indicate that two hemangioblastic foci exist in the young avian blastoderm. The caudal (median) part of Rauber's sickle induces the formation of the area vasculosa caudalis, giving rise to the aorta dorsalis and the arteriae vitellinae. The sickle horn material by direct induction gives rise bilaterally to the formation of the primary heart tubes, pericard and associated venae vitellinae. After partial or total removal of the area opaca no regeneration occurs, indicating mosaicism. The spatial relationship between the inducing Rauber's sickle material (y−ooplasm surrounding the area centralis) and the blood islands in the caudal marginal zone giving rise to the early area vasculosa can be clearly visualized by histochemical staining (Unna) *in toto*. The further horse-shoe shaped expansion of the area vasculosa occurs in the lateral marginal zone and not in the area opaca (as was formerly assumed). However for the further expansion an intact anchoring on the area opaca must be present. In culture *in vitro* the area opaca (expanding spherically around the caudal, lateral and rostral marginal zones) is characterized by enlarging vacuolized zones and finally forms the area vitellina interna. The perigerminal zone plays a role in the development of the area vitellina externa and further expansion of the blastoderm.

KEYWORDS: chick embryo, area vasculosa, area opaca, caudo-lateral marginal zone, Rauber's sickle, heart Anlage

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INTRODUCTION

Pander [1] first distinguished blood islands as distinct masses (first yellow, finally red stained) derived by fragmentation of the peripheral middle or "vascular" layer (mesoblast). They were first visible in the chicken blastoderm after approximately 20h of incubation. At that time the general cell theory (Schwann, 1839) [2] was not yet established and blood islands were not considered as distinct cell groups but belonging to one of the three embryonic membranes (vascular layer) forming the avian blastoderm. The sickle-shaped localization of the erythrocyte-forming areas in the early chicken blastoderm, cultivated *in vitro*, was described by Settle [3]. Since the first blood islands already will appear early in the immediate neighborhood of the deep extraembryonic tissues of the unincubated blastoderm, we focused here first on recently obtained data of these structures. After separation of the unincubated blastoderm from the egg yolk ball, the caudal part of this blastoderm is usually recognizable as a sickle-shaped broader less transparent region (Fig. 1),

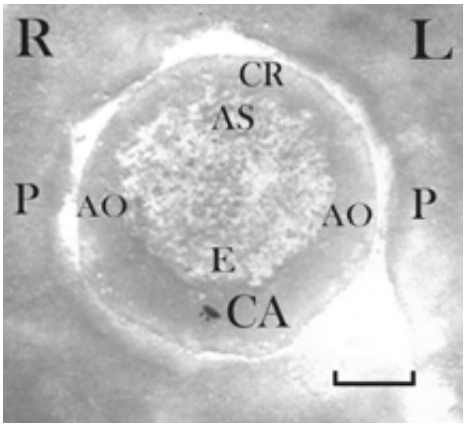

Fig. 1. Vegetal (ventral) view of unincubated chicken blastoderm removed from its egg yolk ball. The Rauber sickle and caudal marginal zone are not visible, since they are covered by adhering caudal subgerminal ooplasm, forming the so-called "caudal germ wall" (CA), in contrast with the narrower cranial germ wall (CR) in front of the anti-sickle (AS): AO: area opaca; E: endophyll; P: perigerminal zone. Bar = 1 mm. R: right side and L: left side.

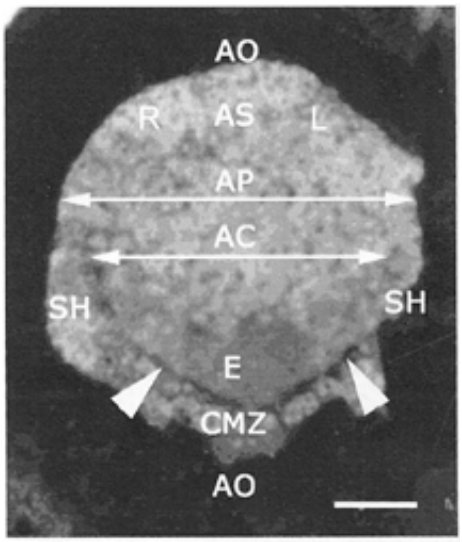

Fig. 2. After careful removal of the caudal adhering yolky ooplasm (seen in Fig. 1), the structural details of the area pellucida become visible. Transparent area pellucida (AP) from a living chicken blastoderm, seen from below, showing deep layer components, adherent to the deep side of the transparent upper layer. E: endophyll in the caudal concavity of the denser Rauber's sickle (indicated by white arrowheads); SH: sickle horns; AC: area centralis in the concavity of Rauber's sickle; AS: anti-sickle; AO: area opaca. The caudal marginal zone (CMZ) (localized just peripheral to Rauber's sickle), in which the first blood islands will develop after incubation, under influence of caudal Rauber's sickle material. Bar = 1 mm. R: right side and L: left side.

which gives the impression that the so-called "caudal germ wall" is larger than the cranial one [4, 5]. This seems however to be due to the presence of more adhering subgerminal ooplasm in this region provoked by the caudal shifting and pressure of the Nucleus of Pander against the overlaying upper layer before laying [6]. Indeed after careful removal of the adhering underlying ooplasm, three structures become more clearly visible: the endophyll (δ−ooplasm) in the caudal part of the area centralis, the Rauber-Koller's sickle (γ−ooplasm) which surrounds the area centralis and the real caudal marginal zone (with transparent upper layer) behind it (Fig. 2). In earlier studies we observed experimentally that in the absence of Rauber's sickle material, no blood islands were formed in the avian blastoderm [7]. Also we observed [8, 9] that the induction of blood islands and coelomic vesicles occurred when mesoblast cells migrate peripherally over and in close contact with Rauber's sickle (junctional endoblast) material. Blood islands and coelomic vesicles can be induced in any neighboring part of the upper layer of the area centralis (stem cells containing β−ooplasm) by aposal of a fragment of Rauber's sickle material [10, 11, 7]. It was recently shown [12] that Rauber's sickle material contains Vg1. In recent ablation experiments of part of the area centralis [13, 14], we recognized the existence of two kinds of areae vasculosae: the area vasculosa caudalis and two (left and right) areae vasculosae laterales, from which the primary heart tubes are derived. In the present study, also by labelling and ablation experiments we investigated the influence of concentrically disposed more peripheral yolk - rich structures (caudal marginal zone, area opaca, perigerminal zone) on the extension of the vitelline blood circulation and coelom. In earlier studies [13, 15] we found indirect evidence (by ablation experiments) for the inducing effect of Rauber's sickle horns on the early development of the primary heart tubes. In the present study we demonstrated unequivocally that the earliest Anlagen of the primary heart tubes, pericardial cavities and venae omphalomesentericae are formed by the direct

inducing effect of material from the far cranially extending flattened Rauber' sickle horns.

MATERIAL AND METHODS

We started our experiments with unincubated chicken blastoderms. These unincubated chicken blastoderms (operated or not) were incubated *in vitro* during 1 or 2 days, according to the technique of Spratt [16]. The used semi-solid culture medium allows microsurgery and further culture on the same substrate. Stereomicroscopic views were taken with a Sony KX-14CPI color video printer (Mavigraph) in the same direction at the beginning, during and at the end of the culture period. Fixation of the embryos was performed in a modified Heidenhain's fixative containing 0.5 g NaCl, 80 ml Aqd, 2 g trichloracetic acid, 4 ml acetic acid and 20 ml formalin. After 1 day of fixation, the blastoderms were stained *in toto* with Unna solution to visualize the localization of blood-containing structures in surface views. After dehydration in an alcohol series and during their stage in alcohol 100% bath, surface photographs were taken and compared with the aspect after clearing in xylene or in the living state. After embedding in paraffin wax, the blastoderms were sectioned perpendicularly to the already formed or presumed axis. The deparaffinized 8 µ thick sections were stained by Harris' or Heidenhain's Hematoxylin and Eosin. Different kinds of labelling or ablation of parts of the caudo-lateral marginal zone, area opaca or perigerminal zone were performed *in vitro* to investigate after incubation the evolution and role of these structures. For the exact orientation with reference to the middle of the RS and later to the PS, a graphite particle was placed usually at the start of the culture in the median outer region of the area opaca. For the study of the evolution of the area opaca *in vitro* culture, it is better to start the explantation of the blastoderm at the unincubated stage and not at more advanced stages. So the transformation of the dense area opaca into a thinny vacuolized membrane (giving rise to the area vitellina interna) can better been followed *in vitro* since then it is not obscured by adhering yolk.

RESULTS

Normal development of the area vasculosa (n = 7)

In Fig. 3A we see an unincubated chicken blastoderm *in vitro*, at the start of the culture.

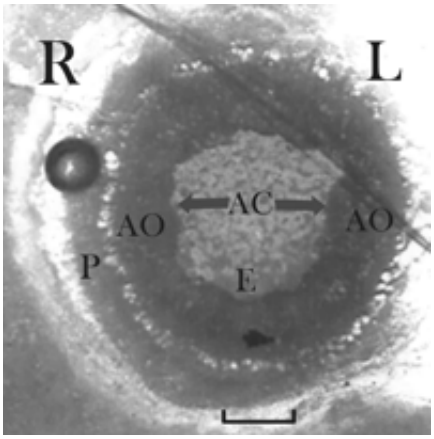

Fig. 3A. Unincubated chicken blastoderm at the start of *in vitro* culture; E: endophyll; P: perigerminal zone with yolk; the area opaca (AO), labelled caudally with graphite particle, forms an opaque ring round the area centralis (AC); the Rauber sickle and caudal marginal zone are covered by caudal subgerminal ooplasm. Bar = 1 mm. R: right side and L: left side.

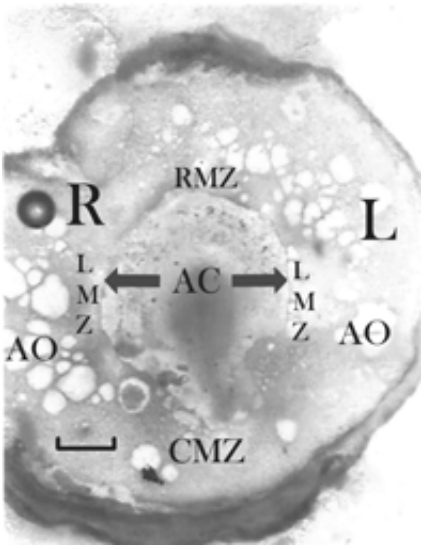

Fig. 3B. The same blastoderm as in Fig. 3A, fixed after 20 h of culture : note the tennis racquet-like aspect of the area centralis (AC), surrounded from rostrally to caudally by the rostral marginal zone (RMZ), the lateral marginal zone (LMZ) and the caudal marginal zone (CMZ); more peripherally the more transparent (vacuolized), spherically extending area opaca (AO) progressively transforming in the area vitellina interna; blood islands and area vasculosa are not distinctly visible before Unna staining *in toto.* Bar = 1 mm. R: right side and L: left side.

In Fig. 3B we see the same blastoderm after 20 h of culture (head process stage 7 V). The area opaca with large empty spaces has much enlarged and has become transparent forming the area vitellina interna. Blood islands are not yet visible after simple fixation. In Figs. 3C, 3D, the same blastoderm after histochemical Unna staining *in toto*, is seen in an alcohol 100% bath: the exact localization of the blood islands with reference to the Rauber's sickle material is now clearly visible. The largest blood islands are localized at the border line between area opaca (area vitellina interna) and the caudal marginal zone. The whole area vasculosa extends "en bloc" in the caudal marginal zone and not in the area vitellina interna. We see that part of the V-shaped Rauber's sickle material (junctional endoblast) is eroded and is mixed with the forming blood islands in the area vasculosa. The area centralis containing the primitive streak takes on a form, resembling a tennis racquet. In view of our recent investigations this can be explained by the out-growth (flux) of mesoblast cells (precursors of the blood islands and coelomic vesicles) over the V-shaped Rauber's sickle material and their accumulation outside the Rauber sickle in the caudal marginal zone (forming the early area vasculosa). At the same time some parts of the Rauber sickle are "eroded" and also displaced and mixed with the blood islands by the outside cell flux. The graphite particle originally placed in the area opaca (in Fig. 3A) indicates that the blood islands do not extend into the area opaca. After clearing in xylene (Fig. 3E), the blood islands and Rauber's sickle material are not or less

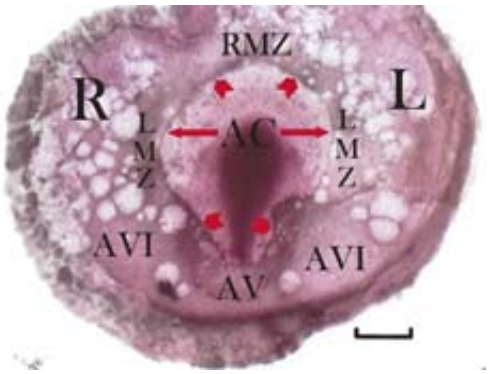

Fig. 3C. The same blastoderm after staining *in toto* with Unna and dehydration in alc.100; the distribution of the blood islands with reference to the inducing V-shaped Rauber's sickle (caudal arrowheads) is clearly seen in the early area vasculosa (AV) derived from the caudal marginal zone; the rostral junctional endoblast (cranial arrowheads) is continuous with/and has the same aspect as the caudal Rauber's sickle material; both Rauber's sickle and rostral junctional endoblast surround the racquet-shaped area centralis (AC). Both Rauber's sickle material and rostral junctional endoblast seem to belong to the original circular y−ooplasm; area vitellina interna (AVI: containing a graphite particle) derived from the area opaca; RMZ: rostral marginal zone; LMZ: lateral marginal zone. Bar = 1 mm. R: right side and L: left side.

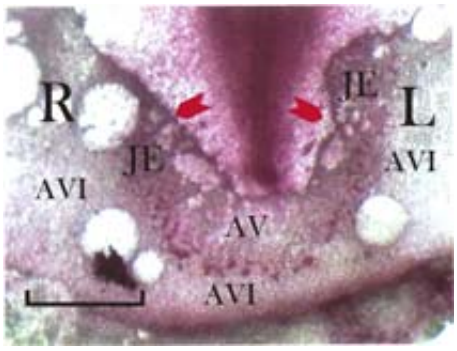

Fig. 3D. Higher magnification of the caudal region of the blastoderm seen in Fig. 3C; (JE) junctional endoblast (Rauber's sickle material indicated by 2 arrowheads) mixed with blood islands of the early area vasculosa (AV), presenting large blood islands at the borderline with the area opaca (area vitellina interna: AVI) labelled with graphite particle. Bar = 1 mm. R: right side and L: left side

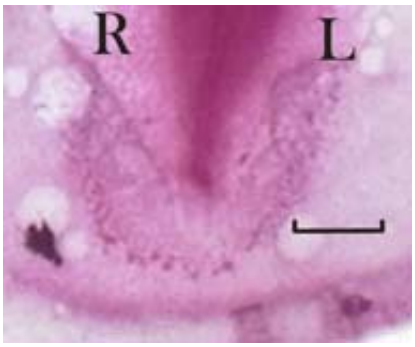

Fig. 3E. The same blastoderm now seen after a xylene bath, shows no longer the same details as seen in Figs. 3C, 3D. Bar = 1 mm. R: right side and L: left side.

distinctly visible. In sections of older blastoderms the intimate relationship between the blood islands and the inducing Rauber's sickle material is visible (Fig. 4). Medially the blood islands and the coelomic vesicles slide freely over the Rauber's sickle material and under the epiblast. More peripherally the larger blood islands get stuck into the deep yolky layer (so-called yolk endoblast). The latero cranial extension of the blood islands is possible by the ingression in the sickle-shaped adhering yolky ooplasm (so called broader, caudal germwall: Fig. 1). So the large blood islands become in close contact via narrow channels with the nutritive substances from the subgerminal space. In Fig. 3C we see that there exists continuity in structure and histochemical aspect of the Rauber's sickle material (caudally) and the rostral junctional endoblast. In sections through the rostral junctional endoblast, one sees similar large, round yolk spheres, in contrast with the median deep layer cranial from the anti-sickle region (Fig. 5). This continuity seems also to exist in the living cultured embryo (stage 4 to 8) [5]. Both structures are probably derived from the same original y−ooplasm encircling the central circular part of the blastoderm (area centralis) [9]. During the culture *in vitro* the rostral (cranial) region of the area opaca differentiates progressively into the rostral part of the area vitellina interna and in the rostral marginal zone separated from the rostral part of the area centralis by rostral junctional endoblast. The further evolution and general aspect of the junctional endoblast, area centralis and area vasculosa in a normal chicken blastoderm, cultured *in vitro* for 44 h, is seen in Fig. 6.

Experimental results

Transformation of the caudal marginal zone from the unincubated chicken blastoderm into the area vasculosa (n = 5)

This can be demonstrated by aposing graphite particles on the caudal marginal zone. After further culture *in vitro*, graphite particles are found over the developing area vasculosa caudal to the Rauber sickle and not in the area vitellina interna, derived from the area opaca (Figs. 7A, 7B).

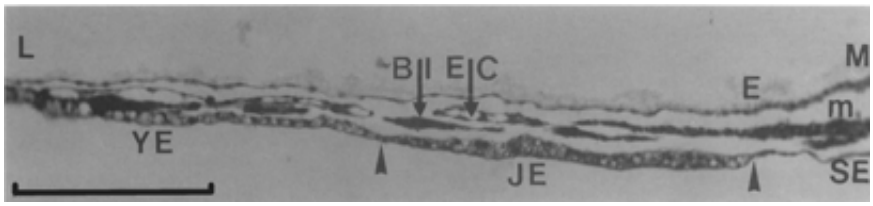

Fig. 4. Low-power view of a section through a chicken blastoderm (after 2 days incubation) to show the relationship between blood islands (BI) and extraembryonic coelomic vesicles (EC) and their progressive development from medially (M) to laterally (L). The blood islands first appear in the region of the junctional endoblast (JE). Its inner and outer border are indicated by arrowheads. There is a narrow zone (lateral arrowhead) between the junctional endoblast and the yolk endoblast (YE). SE: sickle endoblast; more laterally the blood islands become trapped between the yolk endoblast and the dilated extraembryonic coelomic vesicles; E: epiblast; m: mesoblast. Harris Hematoxylin and Eosin. Bar = 200 µm.

Fig. 5. Section through the most rostral part of the area centralis; the arrow indicates the rostral junctional endoblast, the arrowheads indicate the median deep layer in front of the anti-sickle; EP: epiblast. Bar = 100 µm.

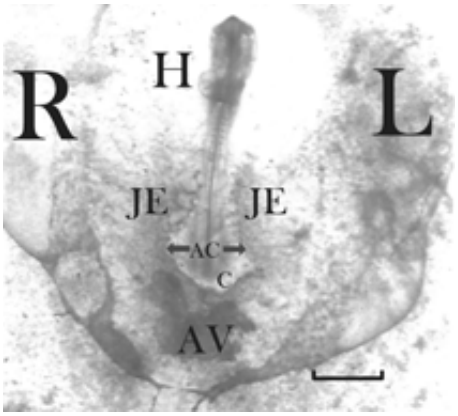

Fig. 6. Ventral view of a normal chicken blastoderm after 44 h of culture *in vitro* starting from the unincubated stage to stage 12-13; Hamburger and Hamilton 1951) H: heart presenting a normal ventricular looping to the right. AC: area centralis surrounded by junctional endoblast (JE) derived from the Rauber sickle, indicated on both sides by an arrowhead. AV: area vasculosa; C: sickle canal visible as an U-shaped clear zone in the concavity of Rauber sickle. Bar = 1 mm. R: right side and L: left side.

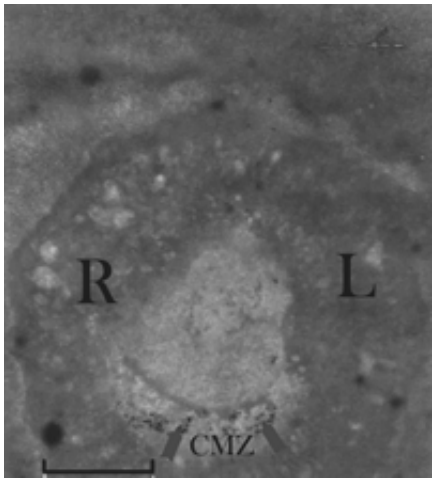

Fig. 7A. Living unincubated chicken blastoderm (ventral view) *in vitro* after placing some graphite particles (arrowhead) on the deep side of the caudal marginal zone (CMZ) behind the Rauber sickle. Bar = 1 mm. R: right side and L: left side.

Thus the area vasculosa is formed in the caudal marginal zone by ingrowing blood islands and erodes Rauber's sickle material (junctional endoblast).

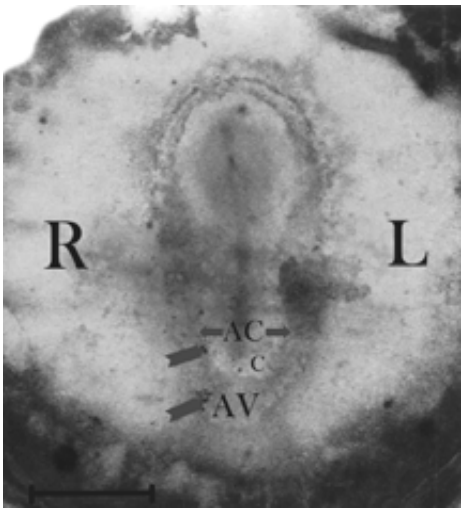

Fig. 7B. The same blastoderm a seen in Fig. 7A, after 28 h of culture (head process stage): graphite particles are now visible in the early area vasculosa (arrowheads), indicating that the area vasculosa (AV) is derived from the caudal marginal zone; C: sickle canal visible as an U-shaped clear transparent zone in the concavity of Rauber sickle; AC: area centralis. Bar = 1 mm. R: right side and L: left side.

Lateral partial excision of the area opaca (n = 7)

The lateral excision of a part of the area opaca usually also provokes loss of the neighboring perigerminal zone on which it is loosely fixed (Fig. 8A). Indeed both structures form together the so-called germ wall of the blastoderm and both are covered by the vitelline membrane. After one day of culture the difference between the operated and the not-operated side of the blastoderm is obvious: a structural empty space is seen at the operated side (Fig. 8B). No area vitellina interna and no perigerminal zone are seen. The unoperated area is composed of area vitellina interna and is surrounded by a large dense perigerminal zone. After 2 days of culture an apparently normal embryo (with eventual beating heart) is seen (Fig. 8C). In the unoperated region the area vasculosa has fully developed in the lateral marginal zone. In the operated side no or very few blood vessels are present.

Cranial excision of the area opaca in front from the anti-sickle region (n = 4)

Here also by the local excision of the area opaca usually the neighboring perigerminal zone

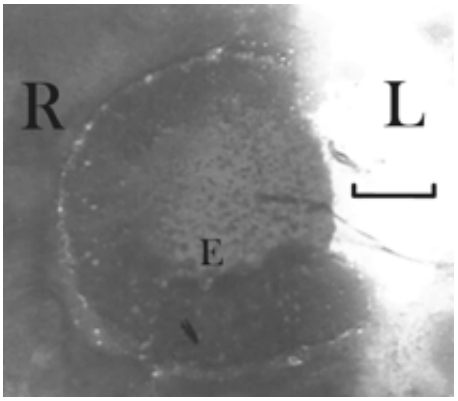

Fig. 8A. Ventral view (in culture) of an unincubated chicken blastoderm from which a lateral part (left) of the area opaca is excised; Rauber sickle and caudal marginal zone are partially hidden by adhering deep ooplasm (so-called "caudal germwall"); note the graphite particle in the area opaca. E: endophyll. Bar = 1 mm. R: right side and L: left side.

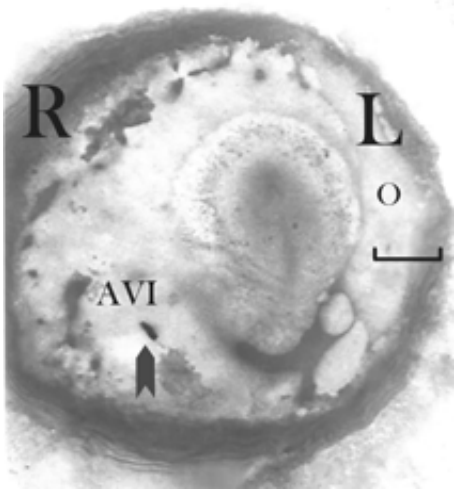

Fig. 8B. The same blastoderm as seen in Fig. 8A, after 20h of culture: a structureless empty, narrow zone is seen in the operated side (O): note the localization of the graphite particle (arrowhead) in the area vitellina interna (AVI). Bar = 1 mm. R: right side and L: left side.

is lost (Fig. 9A). After approximately 2 days of culture a normal embryo (with beating heart) and a normal bilateral development of the vitelline bloodvessels are observed (Fig. 9B). This seems to be due to the expansion of the area vasculosa into the lateral marginal zones. The vitelline blood vessels do not extend in the area vitellina interna

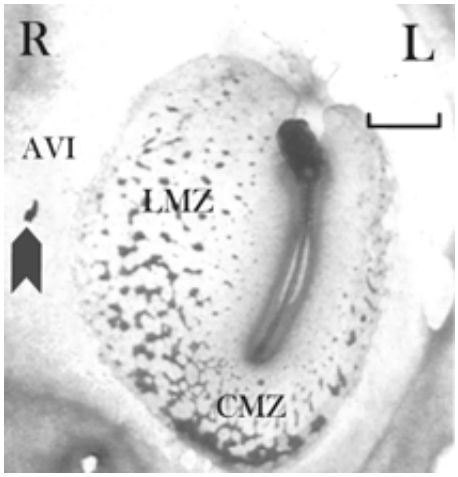

Fig. 8C. The same blastoderm as seen in Fig. 8B, after 44 h of culture: there is an obvious asymmetry of the vitelline blood circulation, at the not operated side the normal cranial extension of the area vasculosa in the lateral marginal zone (LMZ) is visible; Unna staining *in toto*; the graphite particle (arrowhead) is localized in the area vitellina interna(AVI), which demonstrates that the area vasculosa has extended in the lateral marginal zone and not in the area opaca. Bar = 1 mm. R: right side and L: left side.

(labelled by a graphite particle) and remain localized in the marginal zones.

Partial lateral removal by scraping away the deep (yolky) syncytial layer of the area opaca (n = 5)

This procedure is performed to avoid coming off or total loss of the neighboring perigerminal zone (Fig. 10A). This results in an underdevelopment of the operated side (Fig. 10B), comparable with the results obtained after complete lateral removal of the area opaca. After culture the surrounding perigerminal dense zone remains visible.

Removal of a lateral part of the area opaca and neighbouring sickle horn region (Fig. 11A) (n = 5)

After culture during approximately 1 day, fixation and *in toto* staining with Unna, it was seen that in the unoperated side the area opaca has transformed into the area vitellina interna, characterized by round empty areas (Fig. 11B). In the operated side, no such structures are visible and no area vitellina interna has formed. At the not-operated side (with intact sickle horn) we found in sections the presence of the earliest Anlage of the heart region in the splanchnopleura: blood islands and coelomic

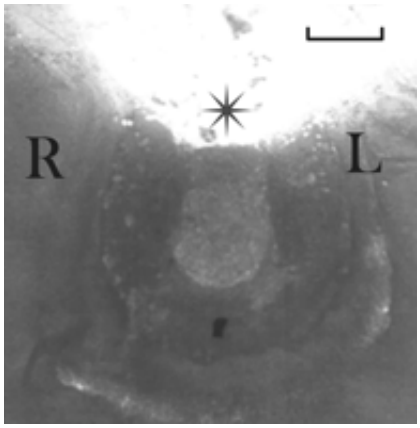

Fig. 9A. Ventral view of an unincubated chicken blastoderm after cranial excision of the area opaca and adherent perigerminal zone (*) at the start of the culture: a graphite particle has been placed in the inner part of the area opaca. Bar = 1 mm. R: right side and L: left side.

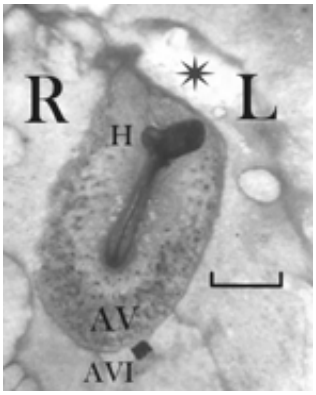

Fig. 9B. Ventral view of the blastoderm seen in Fig. 9A after 43 h of culture: the area vasculosa (AV) on the left and right side have symmetrically extended in the lateral marginal zones; the graphite particle is still localized in the area opaca now transformed in area vitellina interna (AVI) and not in the area vasculosa, the empty space (*) in front of the embryo is still visible. H: heart. Bar = 1 mm. R: right side and L: left side.

vesicles in intimate contact with the inducing Rauber's sickle horn material (Figs. 11C, 11D). By contrast in the contra-lateral (operated) side no Rauber sickle (or junctional endoblast) was present. Here the mesoblast layer ends laterally abruptly in two short lateral plates, encircling a rudimentary coelomic cavity. Neither blood islands nor coelomic vesicles are seen (Fig. 11E).

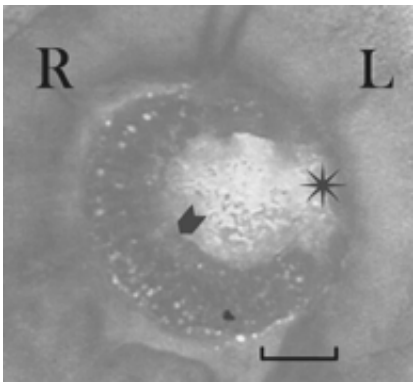

Fig. 10A. Ventral view of unincubated chicken blastoderm at the start of the culture: in one part (left) of the area opaca the deep syncytial layer has been scraped away (*), but the upper layer and neighboring perigerminal zone remained intact; the Rauber sickle (arrowhead) is just visible in front of the so-called "caudal germ wall" but the caudal marginal zone is hidden by adhering ooplasm. Bar = 1 mm. R: right side and L: left side.

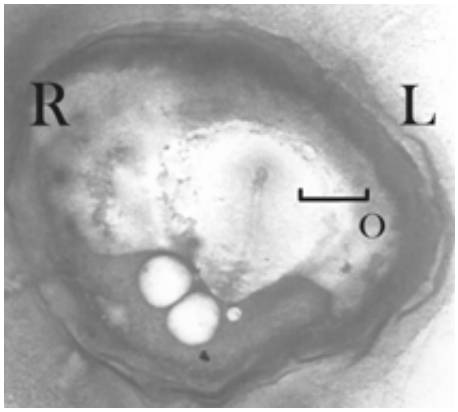

Fig. 10B. The same living blastoderm as seen in Fig. 10A after 20 h of culture: the operated side (O) is clearly less developed then the unoperated side. Bar = 1 mm. R: right side and L: left side.

(Sub) total removal of the whole area opaca and perigerminal zone (n = 4)

The remaining blastodermal structures placed *in vitro* culture (Fig. 12A) are the area centralis, encircled by Rauber's sickle and horns, the caudal marginal zone and eventually a small rim of the area opaca. After culture during approximately 1 day a primitive streak developed in the area centralis, starting from the middle region of the

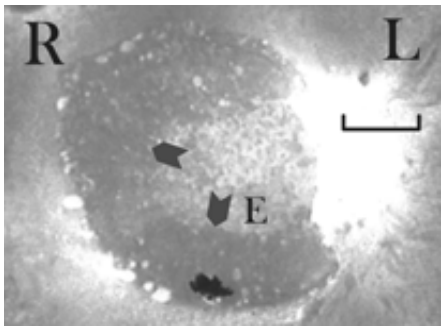

Fig. 11A. Unincubated chicken blastoderm from which unilaterally the area opaca is excised together with the neighboring left sickle horn region; the median part of Rauber sickle (median arrowhead) and right sickle horn (other arrowhead) are visible; E: endophyll. Bar = 1 mm. R: right side and L: left side.

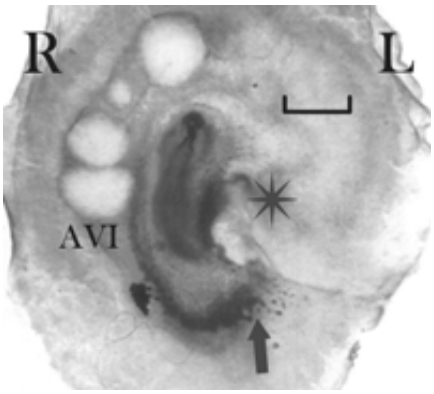

Fig. 11B. The same blastoderm (2 somite embryo) as seen in Fig. 11A after culture of approximately 27 h; Unna staining *in toto* in xylene bath: at the operated (left side) an empty space (*) and no area vitellina interna is seen; the area vitellina interna (AVI) is derived from the right area opaca (containing a graphite particle); the arrow indicates a group of caudal blood islands. Bar = 1 mm. R: right side and L: left side.

Rauber sickle (Fig. 12B). On sections, blood islands and coelomic vesicles are found behind the Rauber sickle region. However after further culture, the blastoderm stops growing and no normal vitelline blood circulation appears.

Total removal of the perigerminal zone (Fig. 13A) (n = 4)

After 44 h of culture usually an apparently normal smaller embryo develops (usually with beating

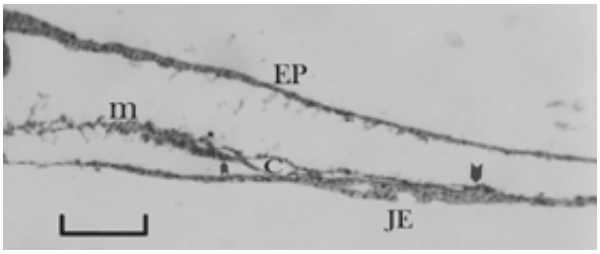

Fig. 11C. Section through the earliest heart region (right side) of the embryo seen in Fig. 11B: under the direct inductory influence of the junctional endoblast (JE), derived from Rauber's sickle, coelomic vesicles (C) and blood islands (arrowheads) are formed in the laterally sliding mesoblast (m): these are the first visible Anlagen of the right primary heart tube and pericard; EP: epiblast; Hematoxylin and Eosin staining. Bar = 100 µm.

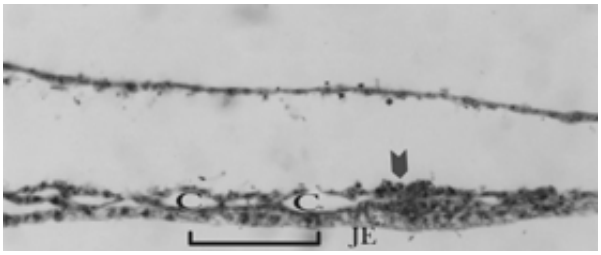

Fig. 11D. Magnification of the right heart region of the embryo of Fig. 11B: coelomic vesicles (C) and blood islands (arrowhead) are induced by the neighboring junctional endoblast (JE) derived from Rauber's sickle material. Bar = 100 µm.

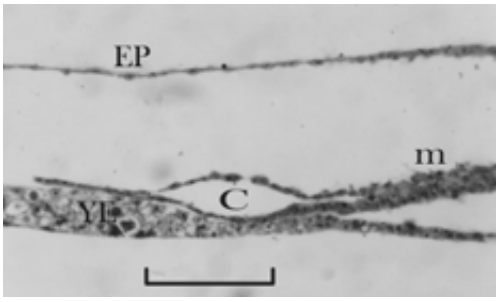

Fig. 11E. Section through the operated contralateral (left) region; the thickened mesoblast layer (m) divides laterally in two short, sectioned lateral plates encircling a rudimentary coelomic cavity (C); no junctional endoblast and no coelomic vesicles or blood islands are present, only yolk endoblast (YE); EP: epiblast. Bar = 100 µm.

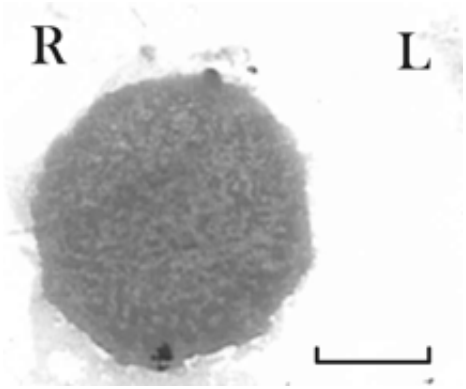

Fig. 12A. Unincubated chicken blastoderm from which area opaca and perigerminal zone are (sub)totally removed. Bar = 1 mm. R: right side and L: left side.

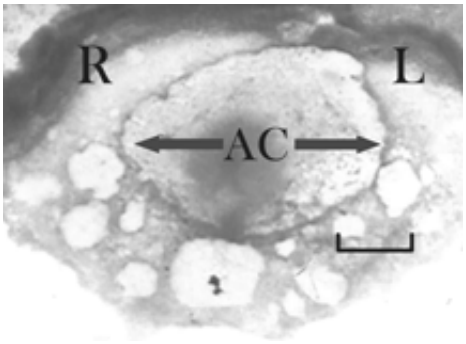

Fig. 12B. The same blastoderm as seen in Fig. 12A after approximately 1 day of culture; a primitive streak with neurogastrulation phenomena have developed in the area centralis (AC). Further development takes not place. Bar = 1 mm. R: right side and L: left side.

heart) and a symmetrical vitelline blood circulation is seen (Fig. 13B) surrounded by a broad area vitellina interna (derived from the intact area opaca). The embryo and area vasculosa are smaller than after culture with still associated perigerminal zone (compare with Fig. 6).

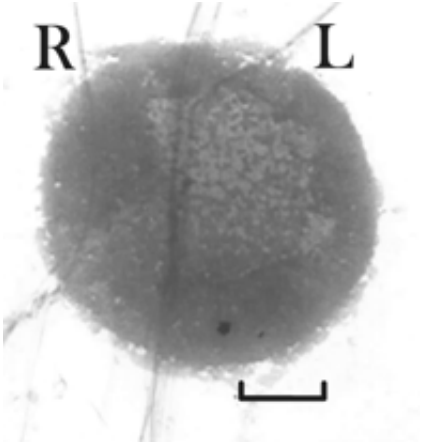

Fig. 13A. Unincubated chicken blastoderm (ventral view) from which the perigerminal area was removed. Bar = 1 mm. R: right side and L: left side.

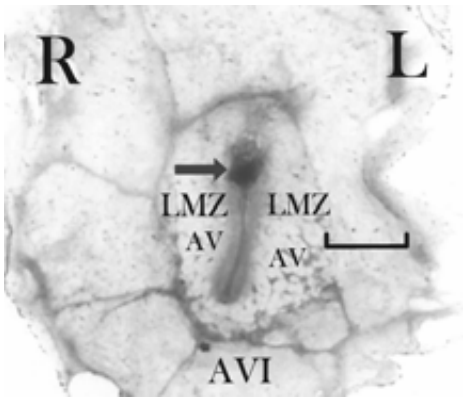

Fig. 13B. The same chicken blastoderm as seen in Fig. 13A after 44 h of culture; Unna staining; the embryo with beating heart (arrow) and symmetrical area vasculosa (AV) are smaller than when cultured with perigerminal zone (compare with Fig. 6). Note the graphite particle at the inner rim of the area opaca (area vitellina interna; AVI) and not in the area vasculosa, demonstrating that the area vasculosa extends in the lateral marginal zones (LMZ) and not in the area opaca. Bar = 1 mm. R: right side and L: left side.

DISCUSSION

From our present observations and experiments it is clear that the vegetal deep yolky tissues play a preponderant role during the development of the heart and area vasculosa in birds. Successively from centrally to more peripherally first the junctional endoblast (derived from Rauber's sickle material) induces the development of the blood islands and coelomic vesicles. They become more or less mixed with Rauber's sickle material by "erosion" (Figs. 3C, 3D, 14A, 14B). So a unidirectional outflow of precursors of mobile isolated blood islands sliding over the junctional endoblast occurs at the exterior side of the Rauber sickle into the caudal marginal zone. The real role

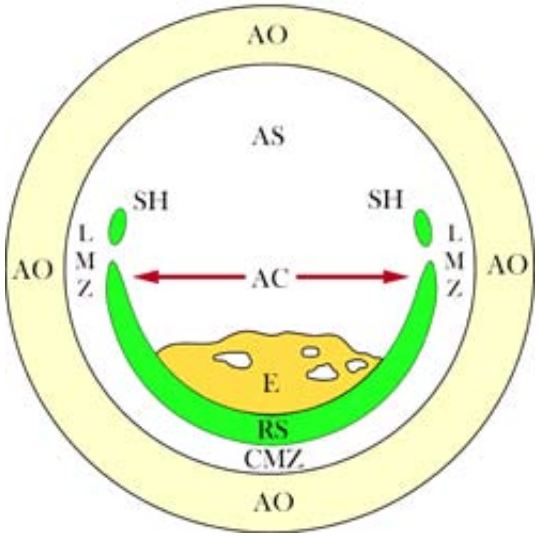

Fig. 14A. Schematic representation of the components of the unincubated avian blastoderm (ventral view): the three elementary tissues, Rauber's sickle (RS) and sickle horns (SH); endophyll (E) and the upper layer in the area centralis (AC) are seen; CMZ: caudal marginal zone; LMZ: lateral marginal zones; AS: anti-sickle; AO: area opaca.

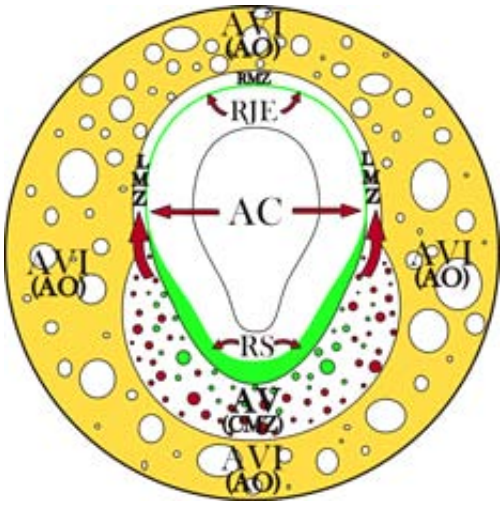

Fig. 14B. Schematic representation of the structures visible on the deep side of an avian blastoderm after 20 h incubation; Rauber's sickle (RS) in continuity with the rostral junctional endoblast (RJE), containing y−ooplasm, encircles the area centralis (AC); RMZ: rostral marginal zone in continuity with the lateral marginal zones (LMZ) and caudal marginal zone (CMZ) in which the area vasculosa (AV) develops; after further incubation the area vasculosa extends cranially in the lateral marginal zone; at the same time the area opaca (AO) extends spherically and differentiates in area vitellina interna (AVI) which typically contains rounded empty spaces.

of the caudal marginal zone (as defined here) is not inducing a primitive streak but it forms a transition and harbour zone for blood islands induced by the neighboring Rauber's sickle material so giving rise to the area vasculosa. Our *in toto* histochemical staining procedure (Unna) seems to visualize the real existence of an original circular y−ooplasm [9] later becoming a racquet-like-shaped ooplasm around the area centralis (Figs. 3C, 3D, 14B). After the arrival of the blood islands in the deep yolky part of the caudal marginal zone, the enlarging blood islands and coelomic vesicles get stuck in this layer. So these fixed blood islands, become doomed by larger coelomic vesicles, lodged directly below the epiblast. This plays a role for the exchange of respiratory gases through the surface epiblast. In the depth the blood islands are in contact via narrow channels, with the nutritive substances present in the subgerminal space [15]. Also transport across endodermal cells of the chick yolk sac has been described [17]. So the yolk elements of the caudal and lateral marginal zones and the sickle-shaped adhering yolky ooplasm of the caudal germ wall form the second factor for the establishment of the area vasculosa. From the caudal marginal zone, blood vessels extend and bifurcate in the lateral marginal zones and encircle the area centralis (Fig. 14B). From graphite labelling of the area opaca we can conclude that the vitelline blood circulation does not extends directly into the area opaca, but remains surrounded "en bloc" by it. Graphite labelling indicates that the area opaca progressively transforms into the area vitellina interna (characterized by the presence of large rounded vacuoles), distinctly separated from the area vasculosa. Indeed we observed that the largest blood islands or blood vessels are localized at the peripheral border line between area vasculosa and area opaca (Figs. 3C, 3D). This borderline seems to permit surface expansion of the area vasculosa at the expense of the area opaca. This no longer occurs when the anchoring between both zones no longer exists by the removing of the area opaca or more particularly its deep yolky part (syncytial layer) containing

giant chromosome groups [18]. Indeed if the deep yolky layer is scraped away, blood vessels no longer develop. This deep syncytial layer of the area opaca is thus the thirth yolky-rich factor for the formation of the vitelline blood circulation. After unilateral removal of a Rauber's (Koller's) sickle half and culture, the corresponding primary heart tube is often absent [15]. Our present experimental results confirm with more precision our earlier observations, [13, 14] that in the unincubated chicken blastoderm, in the absence of Rauber's (Koller's) sickle horns regions, the more caudal middle part of Rauber sickle is not able, after culture, to give rise to primary heart tubes. So we must made a distinction between two hemangioblastic foci: the areae vasculosae laterales (induced directly by Rauber sickle horns material extending cranially) giving rise to the primary heart tubes and associated venae vitellinae derived from mesoblast cells ingressing through the cranial part of the primitive streak [19]. In contrast the area vasculosa caudalis gives rise mainly to the arteriae vitellinae. Earlier studies [13] and our present study suggest that area vasculosa (caudalis) is formed without the mediation of the primitive streak *strictu sensu* by direct sliding between the Rauber's sickle material and the epiblast into the caudal marginal zone, outside the area centralis. Morphologically and physiologically there seems to exist a rostrocaudal polarity in the early vitelline blood circulation. The primary heart tubes and associated venes (venae vitellinae craniales and venae vitellinae omphalomesentericae) arise cranially. The arterial trunci arise in the caudal vascular network forming finally the arterial vitelline branches of the dorsal aorta [20, 21]. The sinus marginalis forms an extraembryonic shunt between both systems, permitting a closed vitelline circulation. Recent papers present evidence that the fetal and adult blood stem cells were actually part of the aorta dorsalis (cell clusters in its floor) which undergo a transdifferentiation from intra-aortic cells into a blood stem cell. In birds it was shown by Dieterlen-Lièvre *et al.* [22] that intra-aortic clusters (a characteristic vertebrate feature) are derived from the splanchnopleural (ventral) lateral plate mesoderm which has the potential to give rise to both angioblasts and hemopoietic cells. This suggests that the floor region of the aorta must be considered as the most medial continuation of the primitive splanchnopleura giving rise to the vitelline circulation. In contrast the somitic mesoderm was shown to give rise to angioblasts only (into the roof and sides of the aorta). This suggests some similarity with the two spatially-distinct populations of progenitors for blood and endothelial cells described in developing *Xenopus* embryos by Walmsley *et al.* [23]. The first population gives rise to embryonic blood, vitelline blood, vitelline veins and the endocardium of the heart in the anterior ventral blood islands. The second population resides in the dorsal lateral plate mesoderm and contains precursor adult stem cells and the major vessels. Junctional endoblast cells have been found in the splanchnopleuric part of the pericardium [16] after 2 days of culture. This suggests that the junctional endoblast cells remain present in the pericard. Heart and lateral plate precursor cells have been localized just lateral and parallel with the cranial part of the primitive streak [19]. In the caudal blastoderm region the same authors found precursors of lateral plate and extraembryonic mesoderm. In their study, however, no precise relationship with Rauber-Koller's material was described, since the fundamental inductive effect for gastrulation [24] and of Rauber-Koller's sickle derived junctional endoblast on the formation of the coelomo-cardiovascular system was only shown more recently [7, 9]. Moreover our present study indicates that the laterally sliding mesoblast cells, derived from the cranial part of the primitive streak, come in direct intimate contact with Rauber's sickle material of the sickle horns and so induce the earliest Anlage of the heart tubes and pericard. Our present study indicates that at different levels and moments the successive interaction with different yolk rich structures has an influence on the directly concerned blood circulation and coelom. Since the original descriptions by Malpighi [25], it was generally assumed that the earliest vitelline blood circulation develops in the proximal part of the area opaca. This conception is still propounded in modern embryology books [26]. Here we demonstrate unequivocally that this occurs in the caudal marginal zone and not in the area opaca.

ACKNOWLEDGMENTS

The authors thank Mr. J. Callebaut for photographic processing and Mr. D. Malan for artwork. We thank also Prof. Dr. K. D'Herde of the Laboratory of Human Anatomy and Embryology, University Ghent for valuable suggestions.

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