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Early Formation of the Coelomo-Cardiovascular Complex in the Chick Blastoderm

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ABSTRACT. Although it is known that small areas in the unincubated avian blastoderm undergo regulation under influence of the surrounding large Rauber's (Koller's) sickle-dependent anlage fields, this seems not to be true for these anlage fields themselves. Indeed, after removal of whole anlage fields or regions in the unincubated avian blastoderms, no restoration of a complete embryo occurs, suggesting predisposition (mosaicism). In the unincubated chicken blastoderm, in the absence of Rauber's (Koller's) sickle horn regions, the isolated median region (with included middle part of Rauber – Koller's sickle) is not able, after culture, to give rise to primary heart tubes. Our present study indicates that the earliest anlage field of the coelomo-cardiovascular system in the unincubated chicken blastoderm (giving rise to both the area vasculosae laterales and the area vasculosa caudalis) is localized in the upper layer between the definitive endoderm anlage field (in the concavity of Rauber – Koller's sickle) and the more rostral and lateral neighbouring sickle-shaped lateral plate anlage.

KEY WORDS: chick embryo, mosaic development, Rauber - Koller's sickle, sickle horns, coelomo-cardiovascular system, coelomates.

INTRODUCTION

Since the experimental studies in ovo of LUTZ (1949) LUTZ et al. (1963) and the in vitro studies of SPRATT & HAAS (1960), it has generally been accepted that the avian blastoderm always presents a highly regulative development, i.e., that any isolated major part of it could develop into a normal symmetrical embryo. However, we have shown that mosaicism also can be provoked, under certain circumstances, in unincubated avian blastoderm parts depending on the spatial distribution of RAUBER - Koller's sickle (1876) material and its relationship with the upper layer (CALLEBAUT et al., 2007). We use here the term mosaic development as originally defined by CONKLIN (1905) in ascidian species: each region of the whole fertilized egg would be able to form more or less independently on its own. The development of the entire embryo was regarded as being the sum of the development of the interacting individual parts. In the present work, we studied the mosaic or regulation phenomena occurring before and during the early formation of the coelomo-cardiovascular system. We define the coelomo-cardiovascular system as the intimate association of blood islands (which will give rise to the cardiovascular system) with the more superficial coelomic vesicles (giving rise to the coelomic cavity) (CALLEBAUT et al., 2004). Both mosaic and regulation phenomena and the development of the coelomo-cardiovascular system are closely related since they are successively influenced by the localization of Rauber - Koller's sickle material (junctional endoblast). Until now, the earliest known localization of the cells of the prospective cardiovascular system in avian embryos has been determined in pregastrular blastoderms (HATADA & STERN, 1994) and in the intermediate primitive streak stages (GARCIA-MARTINEZ & SCHOENWOLF, 1993; LOPEZ-SANCHEZ et al., 2001). The latter authors localized heart and lateral plate precursor cells just lateral to, and parallel with, the cranial part of the primitive streak. In the caudal blastoderm region they found precursors of lateral plate and extraembryonic mesoderm. Explants from the caudal region of pregastrula chicken blastoderms give rise to blood tissue (haemoglobin) (GORDON-THOMSON & FABIAN, 1994). Caudal deep layer cells seem to play a role in cardiac myogenesis in pregastrular upper layer (YATSKIEVYCH et al., 1997). In these studies, however, no precise relationship with Rauber - Koller's sickle material was described, since the fundamental inductive effect on gastrulation (CALLEBAUT & VAN NUETEN, 1994) and of the Rauber - Koller's sickle-derived junctional endoblast on the formation of the coelomo-cardiovascular system was only shown more recently (CALLEBAUT et al., 2002a; 2004). By our present ablation experiments, we were able to localize retrospectively the anlage field of the coelomo-cardiovascular system in the upper layer at the unincubated chicken blastoderm stage.

MATERIALS AND METHODS

We used unincubated chicken (*Gallus domesticus*) eggs. We studied, in culture, the effect on general development or more particularly the development of the coelom and associated cardiovascular system, after ablation experiments in unincubated chicken blastoderm or parts of it. The effect of removal of one or both lateral parts (containing the sickle horns) of the blastoderm was also investigated. Finally the evolution, in culture, of isolated caudal parts (containing the medium part of Rauber - Koller's sickle) was followed. Each experimental procedure is represented in a scheme accompanying the photomicrographs. The blastoderm parts were cultured according to the technique of SPRATT (1947). The semi-

solid culture media allow microsurgery and further culture on the same substrate. Stereomicroscopic photographs were taken in the same direction at the beginning, during and at the end of the culture period. After fixation, the blastoderms were stained with Unna in toto to visualize the localization of blood-containing structures in surface views (VAKAET, 1962). Embedding in paraffin was performed as mentioned in earlier studies (CALLEBAUT et al., 2004). The blastoderms were sectioned perpendicularly to the visible or presumed axis. The deparaffinized sections were stained with Harris's or Heidenhain's haematoxylin and eosin.

RESULTS

1/ Unilateral excision of a sickle horn region and accompanying lateral tissue, according to the procedure schematically represented on the map of the anlage fields (CALLEBAUT et al., 2000) in Fig. 1A (n=6)

After culture, fixation and in toto staining with Unna, an apparently normal embryo is seen (Fig. 1B). However, the coelomo-cardiovascular system is only normally developed in the unoperated side (containing the remaining sickle horn region) and in the caudal region (median part of Rauber - Koller's sickle), respectively visible as an area vasculosa lateralis and an area vasculosa caudalis. In the operated side the area vasculosa lateralis is absent. Sections confirmed these observations and also revealed that in the operated side no coelomo-cardiovascular anlage was seen and the intraembryonic cavity was closed laterally (Fig. 1C).

2/ Bilateral removal of the sickle horns and neighbouring lateral parts as represented schematically on the map of the anlage fields of the unincubated chicken blastoderm (CALLEBAUT et al., 2000) (Fig. 2A) (n=7)

In Fig. 2B, such an operated chicken blastoderm is seen at the start of the culture period. After thirty hours of culture (Fig. 2C) no area vasculosae laterales were visible. Only in the caudal region was a reduced area vasculosa caudalis discernable. In sections, it is seen that a neural plate has developed in the cranial half of the blastoderm. In and below the middle part of this neural plate, the so-called midline structures (floor plate, prechordal plate, notochord, pharyngeal endoderm) are seen (Fig. 2D). They are formed from Hensen's node material (LE DOUARIN et al., 2000) and are originally derived from median upper layer cells localized in the concavity of Rauber - Koller's sickle and induced by the median part of the latter (CALLEBAUT et al., 1996; CALLEBAUT et al., 2006). In the caudal half of the blastoderm a short primitive streak and two paraxial mesoblast mantles are obvious (Fig. 2E). These are derived from the presomite material that is amply present in the truncated fate map of the unincubated chicken blastoderm (Fig. 2A). Laterally, these mesoblast mantles end abruptly near the cut edge of the blastoderm (Fig. 2E). Since neither junctional endoblast nor lateral plate anlage is present and no regulation occurs, no blood islands or coelomic vesicles develop more laterally, which explains the absence of area vasculosae laterales (Fig. 2C). By contrast, more caudally, blood islands and associated coelomic vesicles are present, forming the caudal area vasculosa. In some bilaterally-truncated blastoderms, after prolonged culture, the cranial part (with neurulation phenomena) and the caudal part (with reduced gastrulation and coelomo-cardiovascular phenomena) are only joined by a narrow tissue bridge along which individual somites can be seen bilaterally (Fig. 2F). The morphogenetic movements that occur in bilaterally truncated blastoderms after culture are represented in Fig. 2G. The peripheral anlage fields (definitive endoderm, cardiovascular tissue, lateral plate and finally somites) in the early incubated chicken blastoderm are successively induced point by point by the Rauber - Koller's sickle material in their immediate neighbourhood by sliding along its concavity (CALLEBAUT et al., 1996; CALLEBAUT et al., 2003a) and finally they ingress in the primitive streak. This results in the so-called "polonaise" movement of the upper layer cells in the area centralis (GRÄPER, 1929; WETZEL, 1929; PASTEELS, 1937). Since the bulk of the lateral plate anlagen and cardiovascular system are localized in the immediate neighbourhood of the inducing sickle horns (Figs 1A; 3), it is quite obvious that in the absence of sickle horns and associated lateral plate upper layer cells, no coelomo-cardiovascular system (area vasculosae lateralis) can develop. Thus our experiments indicate that the median part of Rauber - Koller's sickle alone can not regulate the formation of a whole embryo. Thus no reorganization takes place in the remaining upper layer after unilateral or bilateral removal of the sickle horn regions.

DISCUSSION

The sickle horn regions have thus an indispensable role in the development of the area vasculosae laterales, which give rise to the latero-cranial part of the coelomo-cardiovascular system including the heart anlage. Indeed, bilateral removal of the sickle horn regions and neighbouring lateral blastoderm parts (experiment 2) results in the absence of a coelomo-cardiovascular system in the embryo. Thus, no heart develops when no sickle horn regions are present. Moreover, our experiments suggest that a local, direct influence (by positional information; CALLEBAUT et al., 2003a) of the sickle horns on the close-by upper layer cells (anlage of the lateral plates) already exists at the unincubated stage. The different parts of Rauber - Koller's sickle behave as an early preformed skeleton, and by positional cues, build up the whole blastoderm (CALLEBAUT et al., 2003b). That the median massive part of Rauber - Koller's sickle induces blood and large blood vessels but no heart (comparable with the area vasculosa caudalis) has been observed after transplantation of the median part of Rauber - Koller's sickle (responsible for the formation of the area vasculosa caudalis) on the isolated uncommitted upper layer of the anti-sickle region (CALLEBAUT et al., 2002b). Also, LOPEZ-SANCHEZ et al. (2001) found lateral plate and extraembryonic mesoderm but no heart precursor cells in the most caudal median region of the intermediate primitive streak chicken blastoderm. Indication of the performed surgical

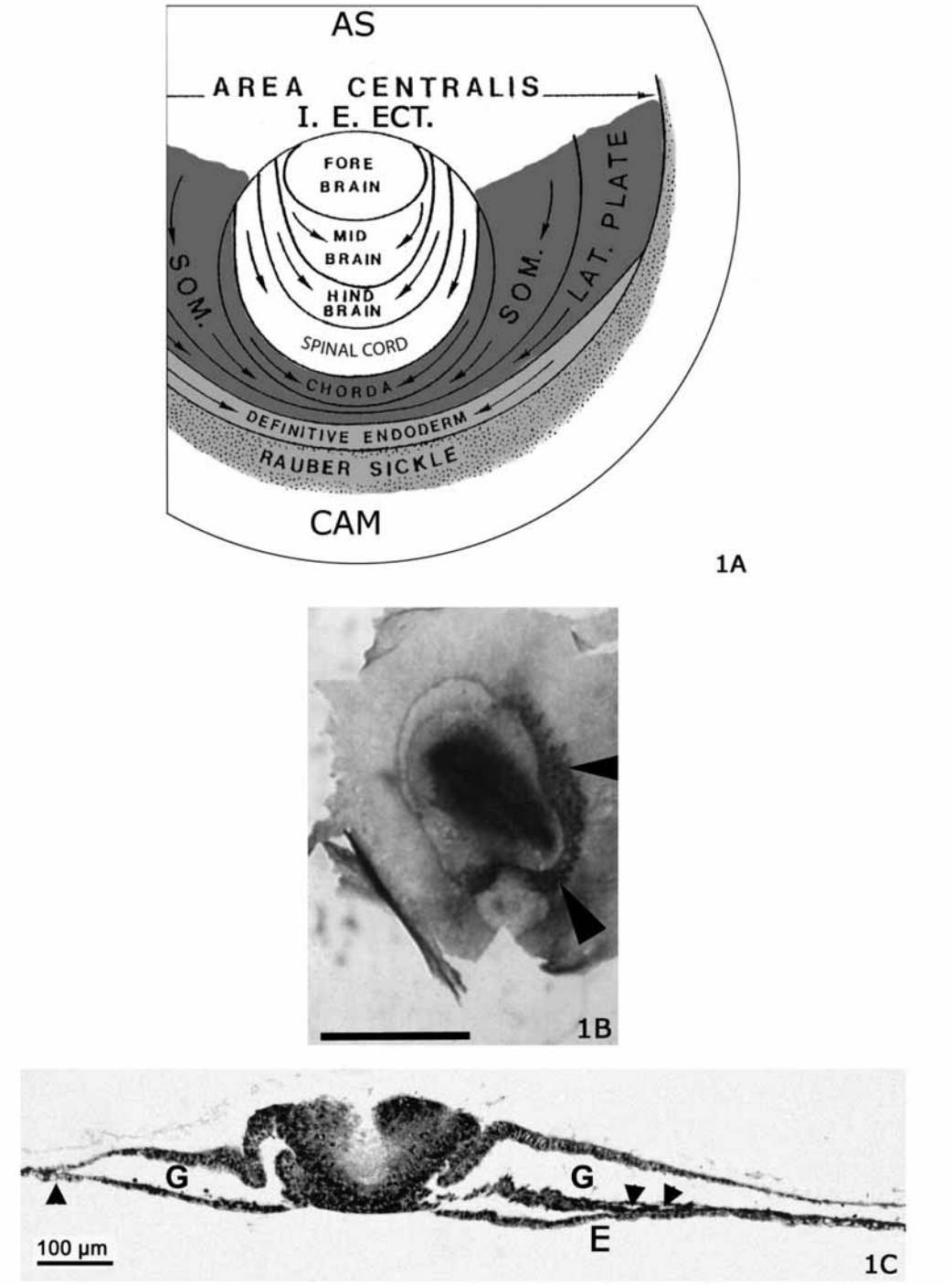

Fig. 1A. – Procedure of the unilateral excision of a sickle horn region and neighbouring lateral tissue, schematically represented on the map of the anlage fields in the upper layer of an unincubated chicken blastoderm (ventral view). The endophyll layer is not represented. AS, anti-sickle region; I.E. ECT, intraembryonic ectoderm; SOM, somites; CAM, caudal marginal zone.

Fig. 1B. – Blastoderm treated as represented in Fig. 1A, after 28h of culture, fixation and in toto staining with Unna. A normal area vasculosa caudalis (caudal arrowhead) and area vasculosa lateralis (lateral arrowhead) are seen in the unoperated side. At the operated side no area vasculosa lateralis is seen as the consequence of the removal of the Rauber's sickle horn in this region and thus no heart can be formed in this side. Bar=2mm.

Fig. 1C. – Section through a similar embryo after 28h of culture. G, intraembryonic cavity closed at the operated side (indicated by the arrowhead directed upwards) contains no mesoblast. In the unoperated side the intraembryonic cavity (G) extends far peripherally. It contains a mesoblast mantle, which, laterally, forms blood islands close to the endoderm (E) that are domed by coelomic vesicles (indicated by 2 arrowheads directed downwards). Bar=100µm.

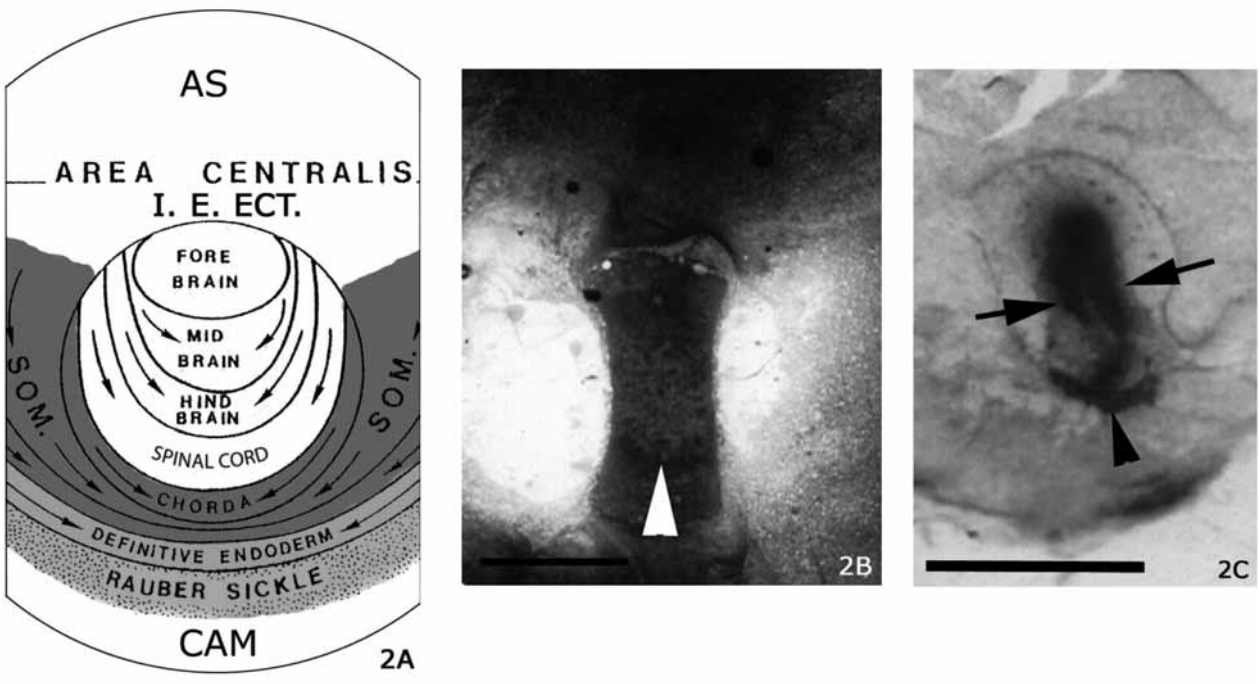

Fig. 2A. – Procedure of the bilateral removal of the sickle horn regions and neighbouring lateral tissue, schematically represented on the map of the anlage fields in the upper layer of the unincubated blastoderm (ventral view) (identical indications as seen in Fig. 1A).
Fig. 2B. – Unincubated blastoderm treated as represented in Fig. 2A., at the start of the culture. White arrowhead indicates median part of Rauber's sickle. Bar=2mm.
Fig. 2C. – The same blastoderm after 30h of culture after fixation and in toto staining with Unna. Bilaterally no area vasculosae laterales and thus no heart tubes have formed because both sickle horn regions have been removed and no junctional endoblast has developed. Only the caudal area vasculosa (arrowhead) has developed because the median part of Rauber's sickle remained intact. In the middle region of the embryo proper, two paraxial denser zones of presomitic material (bilaterally indicated by two arrows) are seen; these correspond to the two paraxial tissue condensations seen in sections (Fig. 2E). Bar=2mm.

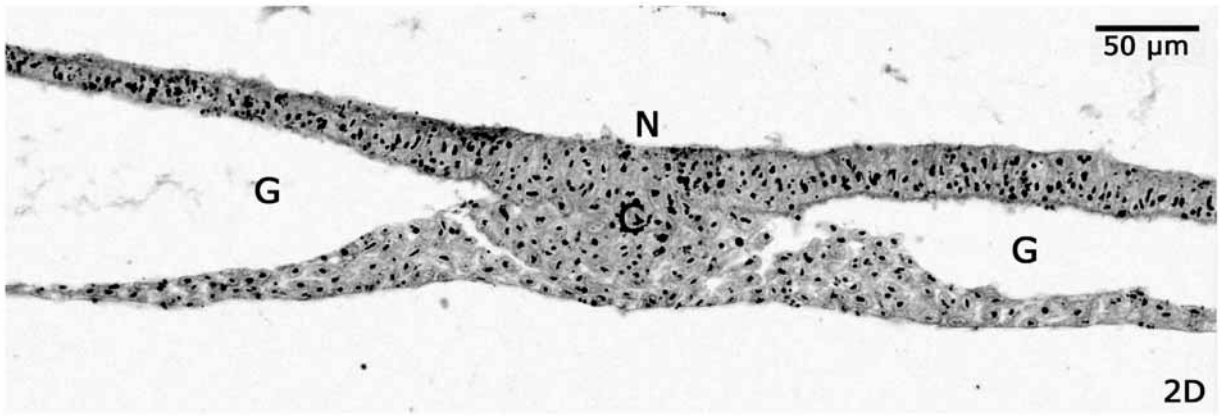

Fig. 2D. – Section through the cranial part of the embryo seen in Fig. 2C; N, neural plate; C, prechordal plate and notochord; G, intraembryonic cavity. Heidenhain hematoxylin. Bar=50µm.

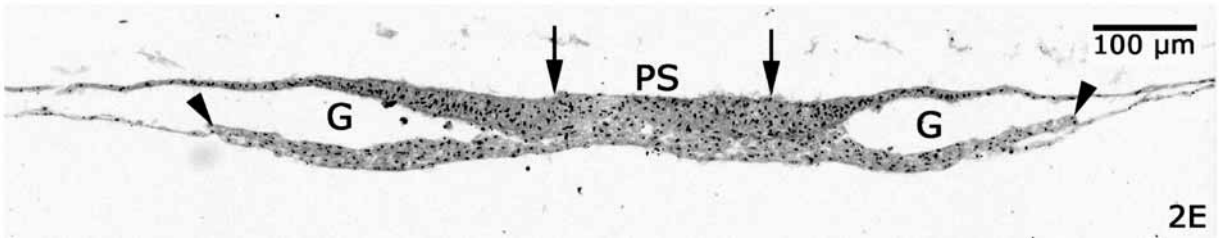

Fig. 2E. – Section through the middle region of the embryo seen in Fig. 2C. The lateral arrows indicate paraxial condensations of tissue (probably presomite material) laterally from an atypical primitive streak (PS). Arrowheads indicate the lateral cut ends of the mesoblast mantle in the intraembryonic cavity (G). More laterally neither blood islands nor coelomic vesicles are present, because the junctional endoblast (normally derived from the Rauber's sickle horns) is absent. Heidenhain hematoxylin. Bar=100µm.

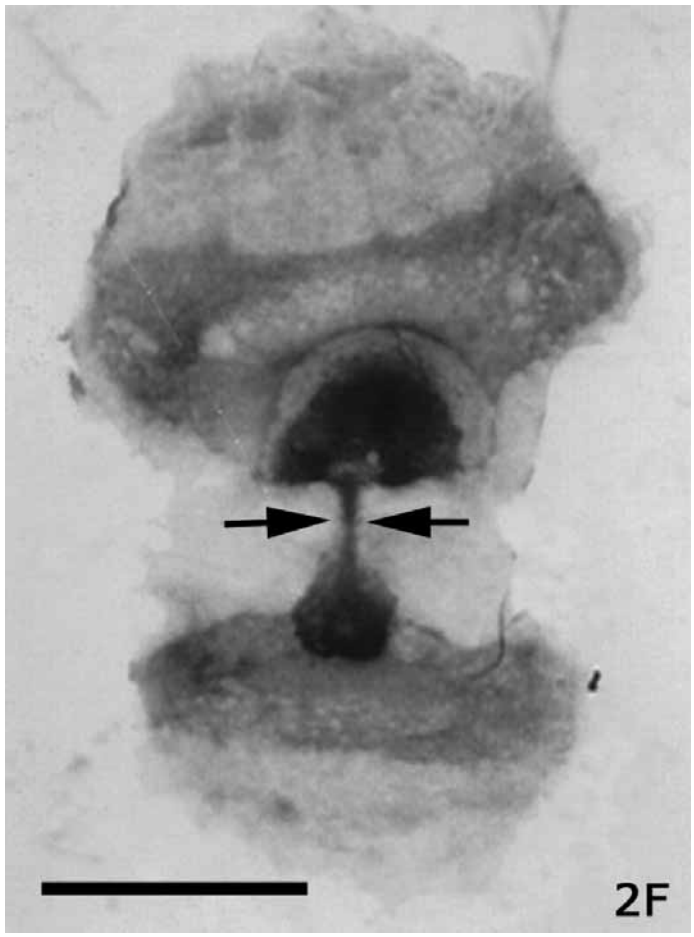

Fig. 2F. – Chicken embryo operated as represented in Fig. 2A after 28h of culture (fixation and Unna staining). A narrow bridge (indicated by arrows) has formed between the cranial and caudal part of the embryo. Bar=2mm.

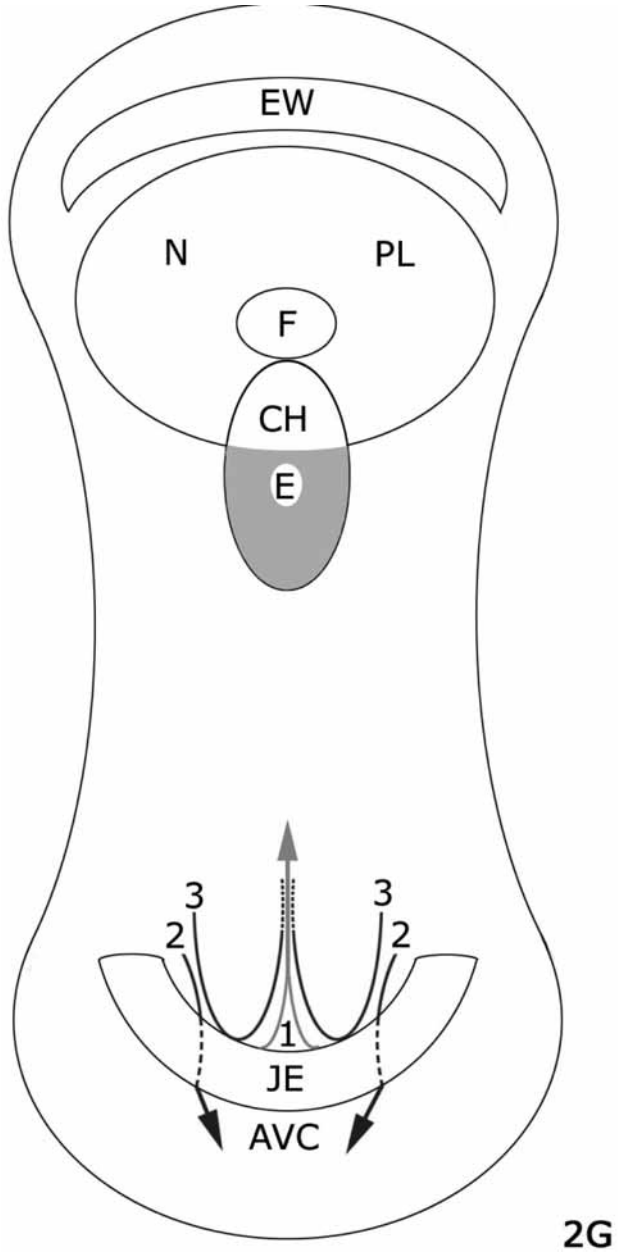

Fig. 2G. – Schematic representation (ventral view) of the successive migration pathways (1-3) of the different cell groups in bilaterally truncated embryos after the excisions represented in Fig. 2A. 1: Bilateral "polonaise" movement (GRÄPER, 1929): the cells of the anlage field of the definitive endoderm ingress into the primitive streak, migrate cranially (median arrow) and become finally localized in E (endoderm). The anlage fields of the so-called midline structures; floorplate (F), notochord (CH), are also displaced cranially. (N PL) neural plate; (EW) endophyll wall. 2: Remainder of the lateral plate mesoderm sliding medially between remainder of the junctional endoblast (JE) and the epiblast, forming the area vasculosa caudalis (AVC). 3: Finally the "polonaise" movement (GRÄPER, 1929) of the anlage field of the paraxial mesoderm, forming somites (interrupted lines).

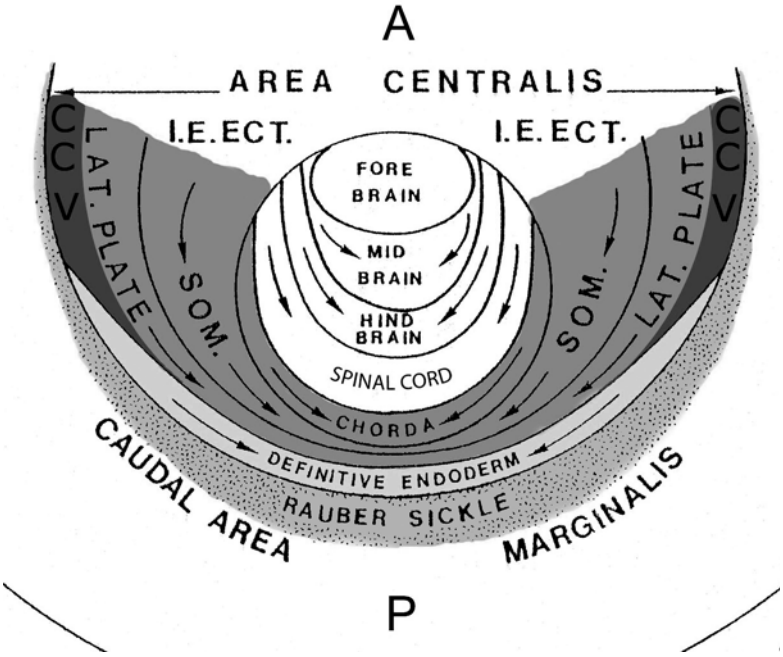

Fig. 3. Adapted schematic representation of the main localization of the predisposed anlage fields in the upper layer of a chicken unincubated blastoderm (ventral view, endophyll not represented). CV, anlage of the coelomo-cardiovascular system localized between definitive endoderm and lateral plate anlagen.

ablations on the map of the anlage fields of the unincubated chicken blastoderm (according to CALLEBAUT et al., 1996; CALLEBAUT et al., 2000), allows the evolution of the remaining blastoderm parts after culture to be better be followed, understood and sometimes predicted. In this anlage field map, we could now add the localization of the earliest anlage field of the cardiovascular system (Fig. 3).

In his fate map of birds (unincubated blastoderm stages; -1,0,1), VAKAET (1985) localized the area vasculosa (extraembryonic mesoblast, according to him), outside the area centralis, thus already early in utero, exterior to the Rauber - Koller's sickle. According to our experiments, however (CALLEBAUT et al., 2002a; b; 2004), the area vasculosa (coelomo-cardiovascular system in our terms) is only formed much later, after primitive streak formation and by peripheral migration of cells of the mesoblast mantle below the epiblast and over the junctional endoblast, under inductive influence of the latter. That the coelomo-cardiovascular system (typically found in coelomates; DOLLANDER & FENART, 1973), forms one embryological entity has been demonstrated in avian blastoderms (CALLEBAUT et al., 2002a; 2004). Thus, the coelomo-cardiovascular system develops both phylogenetically and ontogenetically later than the general body structures. It is part of three associated elements; endoderm, blood islands and coelomic epithelium. Later, even in adult structures, this epithelial covering of coelomic organs can contribute to the vasculature of the heart (MIKAWA & GOURDIE, 1996; MÄNNER, 1999) or, in the intestinal tract, by developmental processes of epithelial-mesenchymal transition, migration and differentiation into vascular endothelial cells, vascular smooth muscle cells and pericytes (KAWAGUCHI et al., 2007). We observed the effects of the most cranially- and laterally-extending parts of the Rauber - Koller's sickle (later junctional endoblast) on the formation of the hemi-pericardial cavities and endocardium on both sides. The far-extending lateral localization of the sickle horns is probably the reason for the original double anlage of the heart. At stage 7 (HAMBURGER & HAMILTON, 1951) (23-26h incubation) the first reported markers of terminal myocardial differentiation were detected in the primary heart fields (BISAHA & BADER, 1991; HAN et al., 1992). MARTINSEN (2005) localizes gastrulated precardiac cells at stage 7 (HAMBURGER & HAMILTON, 1951) in an elongated, sickle-shaped region that corresponds to the also-sickle-shaped region at the lateral border of the embryo proper, i.e., at the inner limit of the area vasculosa (CALLEBAUT et al., 2002a; 2004).

Heart and pericard development

LUTZ (1949) described experiments in which the median part of unincubated chicken blastoderms had been transplanted on a chorioallantoic membrane and cultured in ovo for a prolonged growth period. After histological examination of the graft, he found axial structures; nervous system, notochord, pharynx and hypohysis - but no heart. By contrast, grafts of lateral parts of unincubated blastoderms developed rudimentary hearts. SPRATT & HAAS (1960) excised triangular caudal segments (less than one fourth of the whole surface of unincubated blast-

oderms). The segments were cultured. Histological examination revealed that these explants formed embryonic axial systems, many of which were bilaterally-symmetrical bodies containing brain, spinal cord, notochord, pairs of somites but obviously no heart. We can explain the absence of heart formation in both mentioned cases in view of our present results. Indeed, we demonstrated here that in the absence of sickle horns no area vasculosae laterales developed and thus no coelomo-cardiovascular system appeared, at that level leading to the absence of heart and pericard formation. We made a distinction in localization and function between the area vasculosae laterales (extending cranially, giving rise to the primary heart tubes) and the area vasculosa caudalis. 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. (2002). The first population gives rise to embryonic blood and vitelline veins and to the endocardium of the heart in the anterior ventral blood island. The second population resides in the dorsal lateral plate mesoderm and contains precursor adult blood stem cells and the major vessels.

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