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Matrix Gla Protein expression pattern in the early avian embryo

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ABSTRACT MGP (Matrix Gla Protein) is an extracellular matrix vitamin K dependent protein previously identified as a physiological inhibitor of calcification and shown to be well conserved among vertebrates during evolution. MGP is involved in other mechanisms such as TGF-β and BMP activity, and a proposed modulator of cell–matrix interactions. MGP is expressed early in vertebrate development although its role has not been clarified. Previous work in the chicken embryo found MGP localization predominantly in the aorta and aortic valve base, but no data is available earlier in development. Here we examined *MGP* expression pattern using whole-mount *in situ* hybridization and histological sectioning during the initial stages of chick development. *MGP* was first detected at HH10 in the head and in the forming dorsal aorta. At the moment of the onset of blood circulation, *MGP* was expressed additionally in the venous plexus which will remodel into the vitelline arteries. By E2.25, it is clear that the vitelline arteries are *MGP* positive. *MGP* expression progresses centrifugally throughout the area vasculosa of the yolk sac. Between stages HH17 and HH19 *MGP* is seen in the dorsal aorta, heart, notochord, nephric duct, roof plate, vitelline arteries and in the yolk sac, beneath main arterial branches and in the vicinity of several vessels and venules. *MGP* expression persists in these areas at least until E4.5. These data suggest that *MGP* expression could be associated with cell migration and differentiation and to the onset of angiogenesis in the developing chick embryo. This data has biomedical relevance by pointing to the potential use of chick embryo explants to study molecules involved in artery calcification.

KEY WORDS: *MGP, angiogenesis, heart development, brain development, yolk sac*

Matrix Gla Protein (MGP) is a small secreted protein present in the extracellular matrix (ECM) that is known to be a physiological inhibitor of calcification. The correlation between the absence of functional MGP and aberrant calcification of both arteries and cartilage was clearly established following the development of the MGP knock-out mice (Luo *et al.,* 1997). In humans, MGP mutations are the genetic basis of the Keutel syndrome (KS), a rare autosomal recessive disease characterized by abnormal cartilage calcification, brachytelephalangism, peripheral pulmonary artery stenosis, hearing loss, and facial abnormalities (Keutel *et al.,* 1971; Hur *et al.,* 2005).

Multiple functions have been associated to MGP. These functions include the regulation of calcium phosphate transport (Price *et al.,* 2002), the activity of TGF-β1 (Bostrom *et al.,* 2004), the inhibition of BMP2 (Wallin *et al.,* 2000), BMP4, and BMP7 by direct binding (Yao *et al.,* 2011), the maintenance of BMP and Notch signaling balance and the maintenance of normal endothelial cell function (Sharma and Albig, 2013; Yao *et al.,* 2013).

Nonetheless, *MGP* appears early in development, being expressed in a specific pattern in embryonic tissues, suggesting a developmentally regulated expression which could be associated to cell differentiation and proliferation (Cancela *et al.,* 2014). In the avian embryo its expression was reported at E 14 in the aorta and at the base of the avian aortic valve predominantly in proteoglycan-rich spongiosa region and on the fibrosa surface of the valve (Alfieri *et al.,* 2010).

Despite a lower homology of chick (Gene ID: 395912) and

Abbreviations used in this paper: ECM, extracellular matrix; MGP, matrix gla protein.

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mammalian MGP when compared to homology among mammalian MGPs (Wiedemann 1998), they possess identical protein domains (signal peptide; phosphorylation domain (SxxSxxS); γ-glutamyl carboxylase recognition site; Gla domain) and there is a conservation of all residues thought to be critical for function such as the Gla residues, the ANxF proteolytic cleavage site, the cysteines required for disulphyde bridge and the C terminal arginine (RR) cleavage site (Cancela *et al.,* 2014). In addition, MGP has been shown to be involved in chondrocyte cell differentiation during limb development of chick embryo (Yagami *et al.,* 1999). The purpose of this study was to investigate the sites of MGP mRNA expression in the initial stages of chick development for which no information is currently available, in order to access the potential use of chick embryo explant cultures to test the activity of novel drugs/molecules with anti-artery calcification potential.

Results

MGP mRNA transcripts were first seen as two longitudinal parallel stripes, equally distant from the notochord, near the somites at HH10+ (Hamburger-Hamilton staging; Fig. 1A, black arrows). At HH13 it remains on the two stripes beside the first seven pairs of somites (Fig. 1B-black arrows). By HH15 (Fig. 1C) no staining is visible in the dorsal aorta, however it is present in some portions of the notochord (Fig. 1ii-black arrow and pink arrow, respectively). At HH18 *MGP* transcripts are seen in the dorsal aorta and notochord, but also in the vicinity of the internal carotids, which are the anterior extension of the dorsal aorta. This expression is present in the surrounding areas of the internal carotids facing the core of the embryo (Fig. 3Da-De). Further in development (HH21-23; Fig. 3Ca-black arrow), the dorsal aorta remains positive for *MGP* transcripts. The notochord also maintains *MGP* expression, however now the expression seems to transcend its limits (Fig. 3Ca-pink arrows). By HH25 *MGP* expression in the dorsal aorta and notochord was no longer observed (data not shown).

MGP is expressed on a plexus of small caliber sized vessels (18ss; Fig. 1B, red arrows) that constitute the future vitelline arteries (le Noble *et al.,* 2004). In Fig. 1C (red arrows; HH15), the newly formed vitelline arteries can be seen expressing *MGP* transcripts. This intense expression occurs evenly in the wall of the lateral vitelline arteries (red arrows in Fig. 1ii and Fig. 1iii), in the area pellucida. As soon as (or shortly after) the vitelline artery branches enter the area vasculosa of the area opaca, a change on the expression of *MGP* can be observed, becoming then restricted to the dorsal part of these vessels (Fig. 1 i-iii, red arrowheads and Fig. 2B, red arrows and red arrowheads).

As embryonic day 2 advances, *MGP* expression spreads through the area vasculosa. By HH18 *MGP* expression can be seen covering the full-extension of the area opaca, reaching up to the sinus terminalis. *MGP* transcripts were not detected neither in the sinus terminalis nor in the vitelline veins (Fig. 2B, red hollow arrows) at any developmental stage analyzed. *MGP* expression in the area vasculosa not only appears in the dorsal part of vitelline artery branches but also in the vicinity of several vessels and venules, as well as in what seems to be small vessels beneath main arterial branches (Fig. 3 Aa-Bc). At this developmental

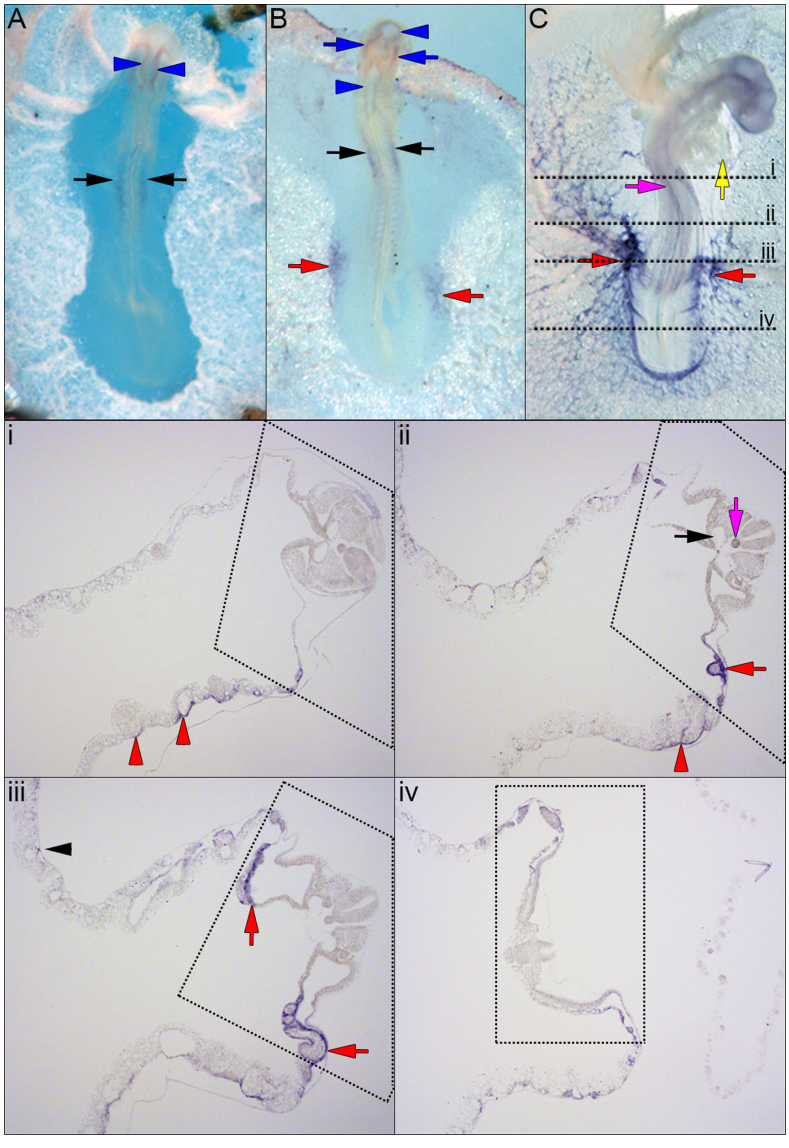

Fig. 1. *MGP* expression during pre-circulatory and early circulatory stages. *WISH using DIG labelled* MGP **(A-C)** *was performed on embryos from HH10+ to 15.* **(A)** *At HH10+,* MGP *is expressed as two stripes alongside the first 7 pairs of somites (black arrows) and in the mesencephalon (blue arrowheads).* **(B)** MGP *expression, at HH13, is present in the prosencephalon and rhombencephalon (blue arrowheads), in two stripes bilateral to the mesencephalon (blue arrows), on a plexus of small caliber sized vessels (red arrows) that constitute the future vitelline arteries, and it also remains on the two stripes beside the first 7 pairs of somites (black arrows).* **(C)** *By HH15,* MGP *expression is seen in some portions of the notochord (pink arrow; ii), with great intensity in the early vitelline arteries (red arrows). This expression is observed uniformly throughout the lateral wall of the vitelline arteries (ii-iii; red arrows), at least in the area pellucida. The expression in the area opaca seems to be restricted to the dorsal part of these vessels (i-ii; red arrowheads). No expression was detected in the heart (C; yellow arrow), anterior vitelline vein (C; hollow red arrow) or dorsal aorta (ii; black arrow). Embryo images (A-C) from a dorsal view. (i-iv) transverse sections of embryo in (C), with dorsal side displayed to the right. Dashed lines delimitate the area pellucida.*

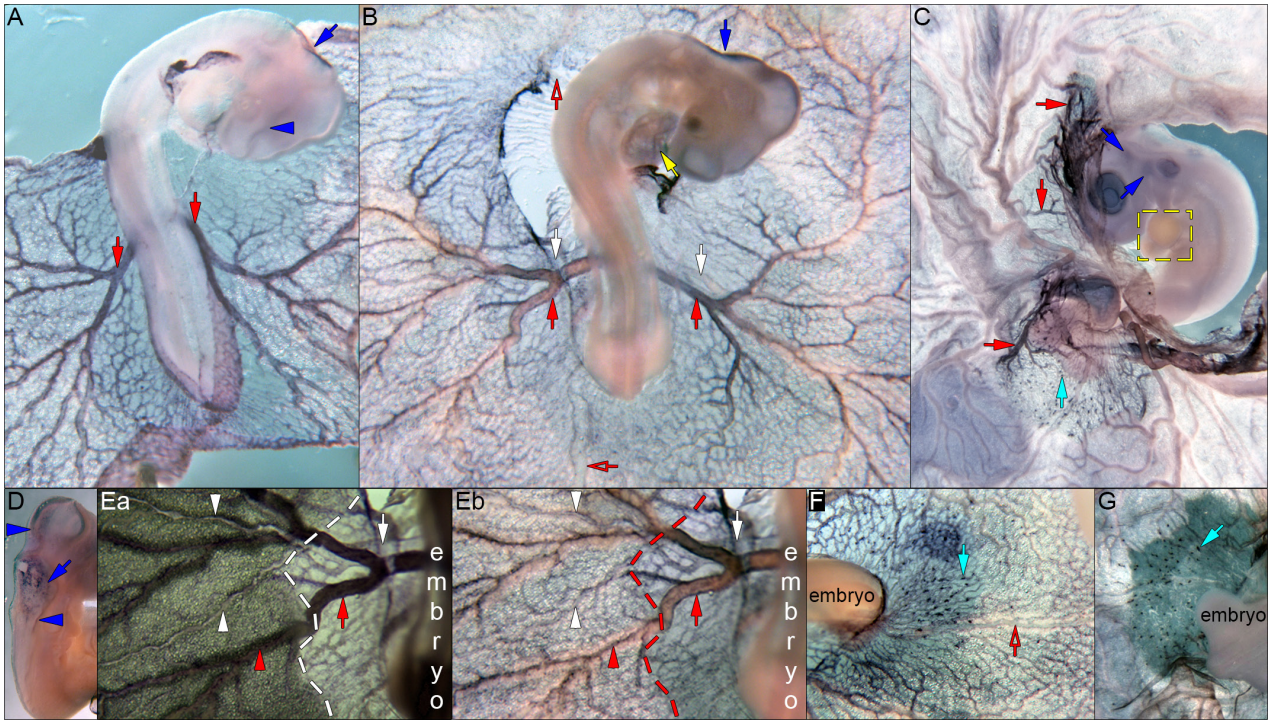

Fig. 2. *MGP* expression during yolk sac vasculature remodeling. *WISH using DIG labelled* MGP **(A-G)** *was performed on embryos from HH17 to HH21.* **(A)** *At HH17,* MGP *is expressed in the vitelline arteries (red arrows) and throughout the yolk sac vasculature, in the roof plate (blue arrow) and in the forming diencephalon (blue arrowheads).* **(B)** MGP *expression, at HH18, remains in the roof plate (blue arrow), in the vitelline arteries (red arrows) and throughout the yolk sac vasculature. Additionally it appears in the heart (yellow arrow).* MGP *expression is absent from the anterior vitelline vein (red hollow arrow) and from the newly formed posterior vitelline vein (red hollow arrow) and lateral vitelline veins (white arrows).* **(C)** *By HH21,* MGP *is observed predominantly in the vasculature of the area pellucida (red arrows), excluding the embryo. Still, in the area pellucida, blood islands can be observed (cyan arrow). Although, staining is not visible in the roof plate at this developmental stage, same regions appear positive for* MGP *in the head (blue arrows). The expression in the heart is maintained (yellow dashed boxed detail).* **(D)** *Detail of an HH18 roof plate.* **(E a,b)** *Detail of the left side of the embryo in (B), respectively in dark field view and bright field view. Dashed lines delimitate the area pellucida. White arrows, lateral vitelline veins in the area pellucida; white arrowheads, lateral vitelline vein branches in the area opaca; red arrows, vitelline arteries in the area pellucida; red arrowheads, vitelline artery branches in the area opaca.* **(F,G)** *Detail of blood islands (cyan arrows) and posterior vitelline vein (red hollow arrow) of embryos at HH19 and HH21, respectively. All images show embryos in a dorsal view. A, anterior; P, posterior.*

stage, there is a partial overlap of *MGP* and *Ephrin-B2* expression (Fig. 3Ha-Id). Both genes seem to be expressed in the dorsal aorta, in the vitelline arteries and in the yolk sac. In Fig. 3Ha, an even expression of *Ephrin-B2* can be seen (purple staining, blue arrow) in the endothelium of the artery. On the other hand, *MGP* expression is seen only in the dorsal part of this vessel (red staining, red arrows, Fig. 3Ha and Hb). *MGP* expression level in the embryo is very subtle but very intense in the vasculature of the yolk sac. Interestingly, at this same developmental stage, *Ephrin-B2* expression levels are the complete opposite, being more intense in the embryo. *MGP* was also observed at different developmental stages (in-between E2.5 - 3 and E3 - 3.5) in the posterior part of the embryo, being expressed in what looks like blood islands (Fig. 2C, F and G).

At HH10 a very weak staining is detected in the mesencephalon (Fig. 1A-blue arrowheads). By the end of HH12 *MGP* appears to be absent from the mesencephalon but present very weakly in the prosencephalon and rhombencephalon (Fig. 1B-blue arrowheads). In addition to this expression, two stripes bilateral to the mesencephalon can be observed, possibly of mesenchymal nature (Fig. 1B-blue arrows). By the end of E2, from HH17 to HH19, the roof plate of the hindbrain becomes positive for *MGP* transcripts (Fig. 2A, B and D).

By HH18, *MGP* expression becomes discernible in the heart (Fig. 2B). Particularly, *MGP* is noticeable in the endocardium (Fig. 3Ea-Ed, yellow arrow), atrio-ventricular canal (AVC; Fig. 3Ea and Eb, yellow asterisk), and in some regions of the atrium (Fig. 3Ec, yellow hashtag), sinus venosus (Fig. 3Ed, yellow square) and trabeculae (Fig. 3Ea and Eb, yellow arrowhead). At HH25 (or E4.5 - E5) our results show *MGP* being expressed partially in the atria, sinus venosus and trabeculae (Fig. 3Ga-Gc", respectively yellow hashtag, yellow square and yellow arrowheads), but no longer in the AVC (Fig. 3Gc, yellow asterisk).

We were able to identify *MGP* expression in the nephric duct (Fig. 3Ba-Ca and Ia-Id, green arrows) between HH18 and HH23.

Discussion

This is the first report of the expression pattern by whole-mount *in situ* hybridization (WISH) of *MGP* during the early stages of development of the avian embryo. Based on its expression pattern, we can speculate on its putative functions during organogenesis. *MGP* expression can be observed at the time and place where the preliminary dorsal aorta develops a vascular appearance.

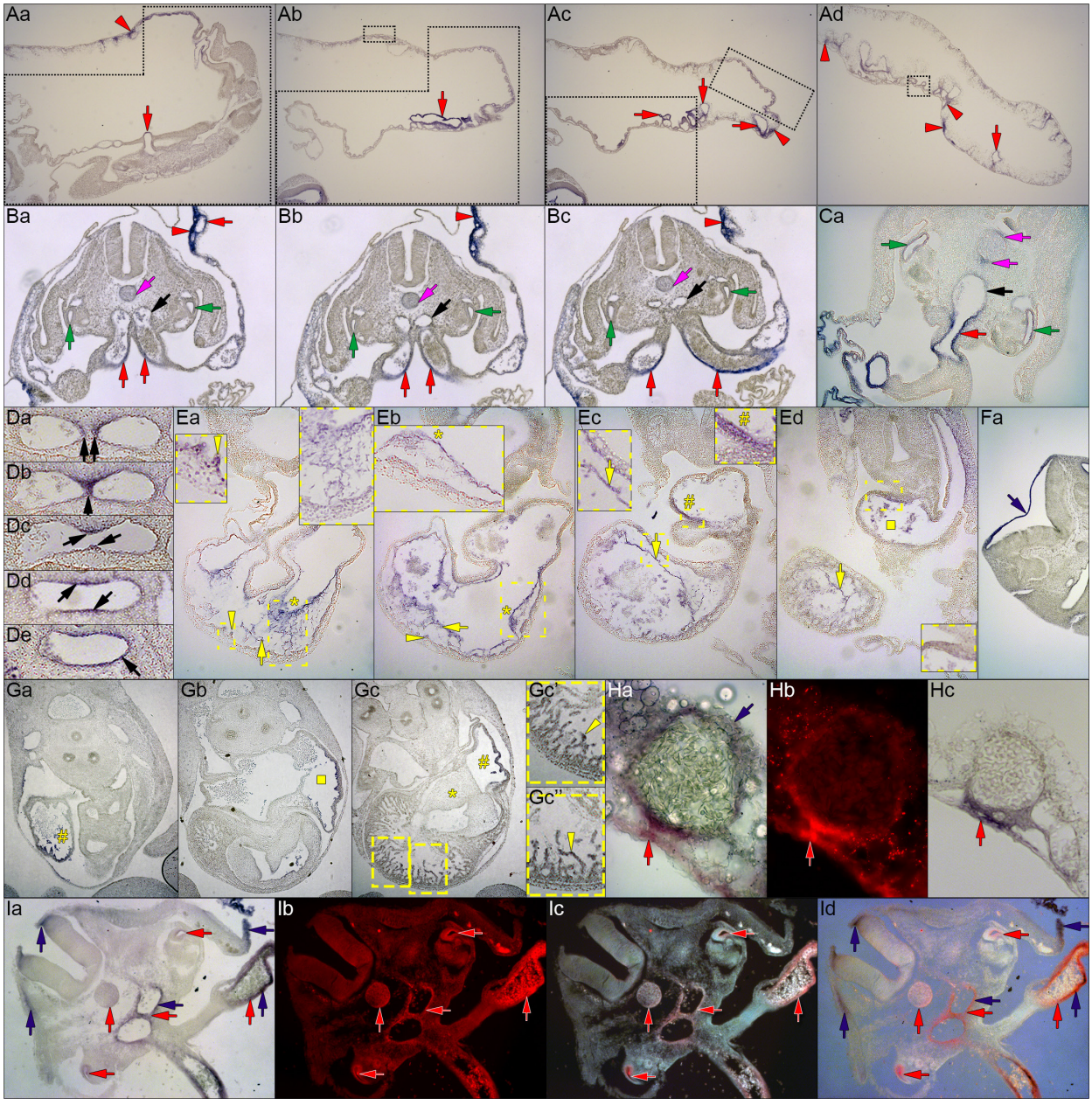

Fig. 3. *MGP* expression during yolk sac vasculature remodeling *(histological sections). WISH using DIG labelled* MGP **(Aa-Gc")**, *and double WISH using DIG labelled* MGP *and fluorescein labelled* Ephrin-B2 **(Ha-Id)**. **(A a-d)** *Longitudinal sections of an HH18 embryo (Figure 2B). Dashed lines delimitate the area pellucida. Red arrows, uniformly stained vessel; red arrowheads, dorsal part of vitelline artery branches and vicinity of several vessels and venules.* **(B a-c)** *Tranverse sections of an HH18 embryo.* MGP *expression is seen in the notochord (pink arrows), dorsal aorta (black arrows), nephric duct (green arrows), vitelline arteries and vessels, venueles (red arrows) and its vicinity in the area opaca (red arrowheads).* **(Ca)** *Tranverse sections of an HH23 embryo.* MGP *expression is seen in the vicinity of the notochord (pink arrows), dorsal aorta (black arrow), nephric duct (green arrows), and vitelline arteries (red arrow).* **(D a-e)** *Magnification of the carotids and dorsal aorta from an HH18 embryo (Figure 2 panel B). Black arrows show how* MGP *expression changes across the embryo, from the head (carotids) until beneath the heart (dorsal aorta).* **(E a-d)** *Transverse sections of an HH18 embryo (Figure 2B) at the heart level.* MGP *expression is observed in the endocardium (yellow arrow), atrio-ventricular canal (yellow asterisk), trabeculae (yellow arrowhead), in some regions of the atrium (yellow hastag) and in the sinus venosus (yellow square). Insets show higher magnifications of the areas within the yellow dashed boxes.* **(Fa)** *Transverse section of a roof plate (blue arrow) from an embryo at HH23.* **(G a-c)** *Transverse sections of an HH25 embryo at the heart level.* MGP *expression is observed partially in both atria (yellow hastag), sinus venosus (yellow square) and trabeculae (yellow dashed line box), but no longer in the AVC (yellow asterisk).* **(G c'-c")** *Magnification of yellow dashed line boxes in panel Gc showing* MGP *expression in the trabeculae (yellow arrowhead).* **(Ha)** *Double WISH section of a vessel in the area opaca, in bright field. HH18 embryo. Red arrow,* MGP *expression; blue arrow,* Ephrin-B2 *expresion.* **(Hb)** *Image in panel Ha using fluorescence. Red arrow,* MGP *expression.* **(Hc)** *Detail of a vessel in the area opaca positive for* MGP *(red arrow). WISH section from another embryo at HH18, in bright field.* **(I a-d)** *Double WISH transverse section of an embryo at HH18. In bright field* **(Ia)**, *fluorescence (Ib, only red channel; Ic, red channel and empty channel), combined view of fluorescence and bright field view* **(Id)**. *Red arrows,* MGP *expression; blue arrows,* Ephrin –B2 *expression.*

This process is known to take a few hours, and occurs at E1.5 or by HH10 (Sato 2013). The anterior ends of the dorsal aortae are connected with outflow tracts, and the posterior ends gradually elongate toward the tail, giving rise to the primary dorsal aortae that posteriorly connect to the vitelline arteries (also known as omphalo-mesenteric arteries; Sato 2013).

At E2, at the moment of the onset of circulation, *MGP* expression can still be seen on what appears to be the anterior part of the dorsal aorta. These *MGP* positive areas are situated along the first 7 somites (Fig. 1A and B, black arrows). Although it seems likely that the presence of *MGP* in these areas is related to dorsal aortae rearrangement, other hypotheses emerge. (1) *MGP* could be involved in the epithelial-mesenchymal transformations that occur by this developmental stage in the trunk of the chick embryo: the formation of neural crest from neural tube and formation of sclerotome mesenchyme from ventromedial somite wall. Other matrix proteins, such as type I collagen, have been proposed to play a role in directing neural crest migration. Interestingly, type I collagen and *MGP* are both skeletogenesis-related proteins. (2) *MGP* could be expressed in the splanchnic and intermediate mesoderm, in a similar fashion as the transcription factor capsulin, playing a role in the urinary system development.

Previous studies show that the right and left dorsal aortae begin to fuse together, at HH15 (or E2,25; Fig. 1C). This process seems to be allowed by an anteroposterior wave of Chordin mRNA attenuation in the notochord, prior to dorsal aorta fusion (Garriock *et al.,* 2010). Interestingly, at this developmental point, *MGP* expression is seen in the notochord and not in the dorsal aorta (Fig. 1C and Fig. 1ii, respectively pink and black arrows).

MGP may have a significant role in the formation of the yolk sac angiogenesis since it is expressed from the beginning on a plexus of small caliber sized vessels that will give rise to the vitelline arteries. *MGP* expression in the area vasculosa not only appears in the dorsal part of vitelline artery branches but also in the vicinity of several vessels and venules, as well as in what seems to be small vessels beneath main arterial branches (Fig. 3Hc, Aa and Bb). At 30ss, le Noble and colleagues (2004) described the presence of "blood-filled spots" identified as being small arteriolar side-branches of the vitelline artery that were selectively disconnected and broken off the arterial tree. These small arteriolar side-branches make sprouts perpendicular and dorsal to arteries, and reconnect to the primary venous system, being reperfused. These "blood-filled spots" represent the origin of the first embryonic veins to run parallel and dorsal to an artery, i.e. the lateral vitelline veins, as well as of all the other veins of the secondary circulation. These findings may suggest that *MGP* could be somehow involved in this remodeling of arterial and venous trees, an hypothesis that is supported by the fact that spots of *MGP* expression are seen in several vessels in the yolk sac (Fig. 2B). Another hypothesis that seems reasonable to explain *MGP* expression in the vicinity of yolk sac vessels is the possibility that *MGP* plays a role in early definitive erythropoiesis. Hematopoietic and erythropoietic clusters in the yolk sac, residing outside the vasculature after the initiation of circulation at E2, have been suggested as a possible source for definitive erythrocytes (Sheng 2010). *MGP* was also observed on what appears to be another hematopoietic/erythropoietic cell population, the blood islands. Furthermore, it has been described that new blood islands are continuously being formed in the posterior part of the developing embryo, even at E3; and that these blood islands are molecularly distinguishable from those generated earlier (Sheng 2010).

MGP transcripts were also seen in the chick embryo's head at different moments in development. This fact is strengthened by a recent study, which reports that *MGP* gene deletion, a known BMP inhibitor, causes cerebral arteriovenous malformations (AVMs) in mice by activating activin receptor-like kinase 1. The increase of this BMP type I receptor enhances the expression of Notch ligands Jagged 1 and 2, which increases Notch activity and alters the expression of Ephrin-B2 and Ephrin receptor B4, arterial and venous endothelial markers, respectively. MGP is responsible for maintaining the balance between BMP and Notch signaling and promotes a normal brain vasculature (Yao *et al.,* 2013). Furthermore, earlier in 2013 another study highlighted MGP importance during angiogenesis, namely in promoting angiogenic resolution and vascular stabilization (Sharma and Albig 2013).

By the end of embryonic day 2, at HH18, *MGP* expression in the heart becomes discernible (Fig. 2B). Particularly, *MGP* is noticeable in the endocardium (Fig. 3Ea-Ed, yellow arrow), atrioventricular canal (AVC; Fig. 3Ea and Eb, yellow asterisk), and in some regions of the atrium (Fig. 3Ec, yellow hashtag), sinus venosus (Fig. 3Ed, yellow square) and trabeculae (Fig. 3Ea and Eb, yellow arrowhead. At HH25 (or E4.5 - E5) our results show *MGP* being expressed partially in the atria, sinus venosus and trabeculae (Fig. 3Ga-Gc", respectively yellow hashtag, yellow square and yellow arrowheads), but no longer in the AVC (Fig. 3Gc, yellow asterisk). During heart development, the AVC and the outflow tract region undergo an epithelial-mesenchymal transformation (EMT) to form the valves of the heart. These cushion areas are initially formed from cardiac jelly (ECM) which then becomes invaded by cells from the endocardium. The invasion is a result of molecular cues from the myocardium that signal the endothelium to activate. During activation, endothelial cells lose cell-cell contacts, undergo hypertrophy and polarization, and increase the expression of ECM molecules. In the chick embryo this invasion begins at the end of HH16 or beginning of HH17, after which activated endothelial cells become mesenchymal cells. Towards the end of E5, the AVC cushions meet and fuse in the midline, leaving a canal. Overall, this would indicate that *MGP* is being expressed by activated endothelial cells, being seen both in the endocardium and in the AVC. We can observe *MGP* also in the trabeculae, which are formed in an event where endocardial cells participate (DeLaughter *et al.,* 2011). Interestingly, in addition to the fact that it is expressed in a layer of endocardium that covers the interior of the heart, it is known that Notch intracellular domain (NICD) is also detected in other endocardial populations such as the atrium and ventricles (DeLaughter *et al.,* 2011). This expression pattern seems to correlate with MGP's, which could be modulating Notch pathway in those tissues.

During embryonic development, the intermediate mesoderm differentiates into tubular epithelial tissues of the kidney and genital system, and is first seen at HH8+ between the segmental plate and lateral plate (Hiruma and Nakamura 2003). By HH9 to HH10 it is a cell cord that goes from the level of the 6th to the presumptive 13th somite. This cell cord then separates into dorsal and ventral parts, becoming respectively the nephric duct and the tubules by HH14 (Hiruma and Nakamura 2003). Interestingly, the nephric duct is the outcome of one of the first mesenchymal-to-epithelial conversions (MET) to occur during development. The nephric

duct induces surrounding intermediate mesoderm to differentiate into nephrons of the mesonephric kidney, the excretory organ of a majority of vertebrate species and a developmental intermediary excretory organ of birds and mammals. *MGP* expression in the nephric duct (Fig. 3 green arrows) is very interesting because: (1) it occurs in another epithelial tissue that endures a change in cellular nature; and (2) it is present in a structure that induces differentiation in its neighbor tissues.

Interestingly, MGP has been previously suggested to be associated in other biological systems, to endothelial-to-mesenchymal conversions (Yao *et al.,* 2013), an hypothesis that is strengthened by the present results.

Materials and Methods

The studies involving animal experiments are in accordance to the ethical regulation for clinical research and European Union (EU) guidelines for animal research. All animal work performed in this study was conducted in compliance with the Portuguese law and approved by the Consultive Commission of the Veterinary Agency from Portuguese Ministry of Agriculture (Directive 2010/63/EU of the European Parliament), the Agency responsible for issuing approval for experimental animal studies, following the EU guidelines for animal research and welfare.

Chick embryo collection

Fertilized chicken eggs (Sociedade Agrícola Quinta da Freiria, SA, Torres Vedras, Portugal) were incubated for 1–4.5 days at 38ºC in a humidified incubator. Embryos were staged according to Hamburger and Hamilton.

Whole-mount in situ *hybridization*

MGP and EprinB2 antisense RNA (asRNA) probes were generated by incubating a DIG or FLUO-labeled ribonucleotide mix (Roche) with T3 or T7 RNA Polymerase (Roche) and the linearized DNA template, according to the manufacturer instructions. Template DNA used in the transcription reactions for asRNA production was generated by PCR using specific primers designed to obtain the complete coding sequence (CDS) for MGP, and a partial one for Ephrin-B2. Primer sequences are available upon request.

Embryos were processed for whole-mount *in situ* hybridization using a standard protocol as previously described (Furtado *et al.,* 2014). MGP labeling was detected using BM purple alkaline phosfatase AP substrate. After *in situ* hibridization embryos were washed in PBS, dehydrated in a methanol series and paraffin embedded. Serial 8 - 12 μm sections were taken (microtome Leica-RM 2135), dewaxed and rehydrated, mounted with DPX Mountant (Sigma-Aldrich), and analysed with Zeiss Axioimager Z2 microscope (Carl Zeiss Group). Double whole-mount *in situ* hybridization was performed for MGP and Ephrin-B2 and embryos processed for cryosectioning by embedding in tissue-tek®O.C.T.™ compound (Sakura). Serial 16 μm sections were mounted with Mowiol Mountant Medium. MGP probe detection was performed by using the alkaline phosfatase (AP) substrate Texas Red (Roche) and visualized under fluorescent light; Ephrin-B2 was detected with BM Purple AP substrate and visualized in bright field. To identify unspecific auto-fluorescent signal green-channel images were obtained and juxtaposed. The imaging of the double-labeled criosections was performed with a Zeiss Axioimager Z2 microscope (Carl Zeiss Group) with a coupled Axiocam icc3 Digital Camera.

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