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Renin-angiotensin system is involved in embryonic emergence of hematopoietic stem/progenitor cells

Emmanuelle Julien¹, Katia Biasch^{1,2}, Reine El Omar^{1,3}, Jean-Noël Freund², Christian Gachet¹, François Lanza¹, Manuela Tavian^{1,2}

¹University of Strasbourg, INSERM, EFS Grand-Est, BPPS UMR-S1255, Strasbourg, France

²University of Strasbourg, INSERM, IRFAC/ UMR-S1113, ITI InnoVec, FHU ARRIMAGE, FMTS, Strasbourg, France

³IMoPA, UMR7365 CNRS-University of Lorraine, Vandœuvre Les Nancy, France

Correspondence

Manuela Tavian, PhD, University of Strasbourg, INSERM, IRFAC/UMR-S1113, 3, Avenue Molière, 67200 Strasbourg, France. Email: manuela.tavian@inserm.fr

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1 | INTRODUCTION

In higher vertebrates, the embryonic blood system is characterized by two programs that exhibit distinct potential and temporal patterns during development.¹ Primitive hematopoiesis emerges in the extraembryonic yolk sac (YS) and generates essentially short-lived primitive erythrocytes, macrophages, and megakaryocytes.² Next, self-renewing multipotent hematopoietic stem/progenitor cells (HSPCs) are first generated during definitive hematopoiesis in the aorta-gonad-mesonephros (AGM) region and emerge as clusters of cells associated with the ventral endothelium of the aorta and major arteries.³⁻⁵ However, an elusive blood-forming potential is present earlier in the embryo, into the paraaortic splanchnopleura (P-Sp), the presumptive AGM region.^{6,7} The developmental origin of blood cells has been the subject of an intense debate during the last decade. Several studies have well documented that in the AGM, HSPCs are generated through a rare type of endothelial cells, endowed with hematopoietic potential, also known as hemogenic endothelium. The process of generating blood cells from the hemogenic endothelium seems to be evolutionary conserved, as it occurs in many different vertebrates.⁸⁻¹⁴ Nevertheless, the direct precursors of this cell type and the mechanism that governs the early steps of hematopoietic emergence inside the embryo remain poorly understood.

Our recent studies on human embryo document the first evidence for the presence of pre-hematopoietic precursors in the sub-aortical mesenchymal layers, before the endothelium acquires hemogenic capacity. These mesodermal precursors, able to generate in vitro hematopoietic CD45-positive cells, are identified by the expression of CD143/ACE (angiotensin-converting enzyme).¹⁵ ACE is a key element of the renin-angiotensin system (RAS), whose principal role is the regulation of blood pressure and fluid homeostasis. Angiotensinogen (AGT) the only polypeptide precursor of this system is catalyzed by renin (REN) into angiotensin I, which is subsequently cleaved by ACE into the bioactive peptide angiotensin II (Ang II). Ang II is a potent vasoconstrictor and exerts its effects via two receptors, Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors.¹⁶ Inhibitors of RAS, particularly ACE inhibitors and Ang II receptor blockers, are commonly used in the treatment of hypertension, cardiac failure, and renal insufficiency.¹⁷ In addition to this systemic effect, existence of an autocrine or paracrine role of RAS has been well documented.¹⁸ Ang II peptides may act locally as growth factors or inhibitors, and exert effects in cardiac, vascular, renal, and other tissues, in a way similar to cytokines.¹⁹ Regulatory effects of the RAS on hematopoiesis have been reported in a variety of studies in humans and mice (for review²⁰). Knockout mouse for the ACE gene presents severe anemia^{21,22} and defects in myelopoiesis²³ because of the lack of Ang II production.²⁴ In humans, anemia and leukopenia have been reported following the treatment with high doses of ACE inhibitors.^{25,26} Evidence indicates that Ang II can stimulate in vitro the proliferation of hematopoietic progenitors from murine bone marrow (BM) or human umbilical cord blood.²⁷ These studies along with the observation that all RAS components are expressed in the BM-stromal cells and hematopoietic progenitors²⁸ point on a potential role of RAS in regulating aspects of hematopoiesis.²⁹ Other lines of evidence indicate that a local-RAS also contributes to the primitive hematopoietic emergence during ontogenesis.³⁰ In the developing avian embryo ACE, AGT and REN were detected in the vicinity of the blood islands in the YS. Administration of ACE inhibitors resulted in a dramatically lower hematocrit, showing that RAS modulates blood-island differentiation during primitive erythroid cell development.³⁰ Similarly, in vitro studies using human embryonic stem cells reported an impact of the renin-angiotensin pathway directly regulating the genesis of embryonic angio-hematopoiesis.³¹ Hematopoietic colonies from human embryoid body presented robust enzymatic ACE activity and also expressed angiotensinogen and AT1 receptor. Along this line, we have previously demonstrated that ACE/CD143 is a cell surface marker of human HSPC in adult BM as well as in all presumptive and developing blood-forming tissues during human ontogeny, hinting at the role of this enzyme in the physiologic regulation of hematopoiesis.^{15,32} However, the involvement of a local-RAS during the establishment of definitive blood system in mammalian embryos has not yet been described.

Here, using mouse embryo we functionally evaluated the role of RAS at the early step of blood generation. We found that ACE, REN, as well as the AT1 receptor are expressed early in the developing embryo. Interestingly, the addition of exogenous Ang II enhances the emergence of hematopoietic progenitors in organotypic cultures of dissected AGM, whereas a specific AT1 receptor antagonist has the opposite effect. Taken together, this work highlights the existence of a functional local-RAS inside the AGM, whose activation has important consequences for hematopoietic emergence in the developing embryo.

2 - MATERIALS AND METHODS

2.1 Biological samples

Mouse embryos were generated from C57BL/6 mice. The date of vaginal plug discovery was considered E0. Embryos were staged by somite counting and P-Sp/AGM were dissected as previously described.³³

Human embryos were obtained from voluntary abortions performed according to the guidelines and with the approval of the French National Ethics Committee. Written consent for their use in research was obtained from patients. Human embryos were staged using anatomic criteria and the Carnegie classification as previously described.³⁴

2.2 In vitro treatment

P-Sp was dissected from E9.5 (9.5 embryonic days) (ranging from 22 to 24 somite pairs [sp]) mouse embryos and seeded undissociated over permeable Isopore Membrane filters (Merck) for 3 days in a six-well plate containing Glutamax Alpha-MEM medium (Gibco) supplemented with 1% antibiotics and 20% Hyclone defined serum. Explants were cultivated in toto with Ang II peptide 100 nM (every 12 hours) (Sigma-

Aldrich) in the presence of either 30 nM of AT1 receptor antagonist, Losartan (Sigma-Aldrich), or 100 nM of AT2 receptor antagonist PD123.319 (every 24 hours) (Sigma-Aldrich). Control cultures contained neither Ang II, nor Losartan, nor PD123.319. The concentrations of Ang II, Losartan, and PD123.319 were selected basing on previously reported^{30,35} and by our own experiments (Figure S1).

After 3 days of in toto culture (protocol in Figure 4A), P-Sp were dissociated mechanically through a 26-gauge needle, and harvested cells, corresponding to one P-Sp per well, were seeded on a 24-well plate over an OP-9 stromal cell layer and cultivated in Alpha-MEM medium supplemented with 1% antibiotics, 20% Hyclone defined serum and the following mouse recombinant cytokines: 5 ng/mL Flt3-ligand and 2 ng/mL IL-7 (PromoCell). Cells were collected by pipetting and labeled with the following monoclonal anti-mouse antibodies for flow cytometry analysis: CD45-FITC, CD11b-APC, CD19-PE, and c-Kit-APC. One fifth of the harvested cells was seeded back over a fresh OP-9 layer. Analyses were performed at day 15 of culture.

In other experiments (protocol in Figure 5A), after the 3 days of in toto culture, P-Sp were dissociated by collagenase type I and directly stained and processed for cytometry or colony-forming cells assays. For cytometry, cells were incubated with the following monoclonal antibodies: CD45-PE-Cy7, CD31-PE, CD41-PerCP-eFluor 710, CD117-APC, and CD42-Alexa 488. The DAPI viability staining solution was used to exclude dead cells and gates were set using appropriate fluorescence minus one (FMO) controls.

2.3 Colony-forming cell assay

Dissociated P-Sp cells were seeded in 1.1 mL methylcellulose-based medium (Methocult GF M3434, Stem Cell Technologies) at 1 P-Sp/well after 3 days of in toto culture. Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere and colonies were scored after 11 days.

2.4 Cell labeling and sorting

Mouse AGM were dissected at E10.5 and E11.5 and single-cell suspensions were obtained after incubation in medium containing 0.23% w/v collagenase type I (Worthington Biochemical Corporation) for 30 minutes at 37°C and passage through a cell strainer (70 µm, BD Biosciences). Fc receptor was blocked with purified anti-CD16/CD32 antibody (93, eBioscience). Cells were stained for 30 minutes at 4°C with the following anti-mouse monoclonal antibodies: CD45-PE-Cy7 (30-F11), CD31-PE (MEC13.3), biotinylated CD41 (MWR30) (eBioscience), CD117-APC (2B8; BioLegend), and CD42 Alexa 488 (Ram1, home produced³⁶), and then for another 30 minutes at 4°C with streptavidin APC-Cy7 (BioLegend). Cells were then collected in a solution of 7-aminoactinomycin D (2 µg/mL) to exclude dead cells and processed for sorting. Endothelial cells (CD45⁻CD41⁻CD42⁻CD31⁺c-Kit⁻), hematopoietic cells (CD45⁺CD41⁻CD42⁻CD31⁻c-Kit⁻), hematopoietic progenitor cells (CD45⁺CD41⁺CD42⁻CD31⁺c-Kit⁺), and mesenchymal cells (CD45⁻CD41⁻CD42⁻CD31⁻c-Kit⁻) were sorted. Gates were set using appropriate fluorescence FMO controls. Sorting was performed on FACS Aria II equipped with a FACS Diva software (BD Bioscience). Purity for sorted cells was checked before RNA extraction.

2.5 RNA preparation

Total RNA was extracted with RNeasy Plus Mini or Micro kit (Qiagen) according to the manufacturer's instructions. RNA concentrations and purity were assessed by measuring absorbance at 260 and 280 nm on the NanoVue spectrophotometer (GE Healthcare Life Sciences). RNA quality was analyzed using Agilent RNA 6000 Nano LabChip kit with the Agilent 2100 Bioanalyzer (Agilent Technologies).

2.6 Reverse transcription, semi-quantitative, and real-time quantitative polymerase chain reaction (RT-qPCR)

A 80 ng of total RNA was reverse transcribed in a total volume of 20 µL using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) following the manufacturer's instructions. cDNA synthesis was performed in a GeneAmp 9700 PCR System thermocycler (Applied Biosystems) by incubation at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. For negative controls, reverse transcriptase or RNA was omitted.

Semi-quantitative PCR were performed with Qiagen hotStarTaq DNA polymerase Master Mix Kit (Qiagen) in a final volume of 20 µL, using 4 µL of cDNA and 1 µL of each primer. Primers used for PCR (Eurofins Scientific) are shown in Table S1. The reaction conditions were as follows: 95°C for 15 minutes, 35 cycles of amplification (94°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minute) and a further step at 72°C for 10 minutes. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized under UV illumination.

RT-qPCR was carried out in duplicates in 20 µL containing 2 ng of cDNA, 1 µL of primers and 10 µL of SsoAdvanced Universal SYBR Green Supermix, using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Primers used for AGT, REN, AT1, AT2, ACE, Actin, and Gapdh were from PrimePCR SYBR Green Assay (BioRad), following the MIQE guidelines.³⁷ The cycling parameters were: 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. cDNA was omitted for negative controls. Fold changes were calculated by the comparative cycle threshold (C_T) method using $\Delta\Delta C_T$ values with the formula $2^{-\Delta\Delta C_T}$, relative to control values. Gene expression was normalized to the levels of Actin and Gapdh gene expression. The generation of single amplicon products was confirmed by melting curve analysis.

2.7 In situ hybridization

Tissues were fixed in 4% para-formaldehyde (Sigma-Aldrich) at 4°C during 24 hours following the manufacturer's instructions for in situ hybridization using RNAscope assay (Advanced Cell Diagnostics). Tissues were then submerged in 10%, 20%, and 30% sucrose, then frozen in OCT embedding medium and cross sectioned (10 µm sections). Development of signal was performed using the RNAscope 2.5 HD brown detection kit. Probes for mouse and human ACE, AGT, REN, AT1, AT2, Ppib, and Dapb were purchased from Advanced Cell Diagnostics. Ppib

was used as positive control, and Dapb as negative control. Slides were imaged under an Eclipse 50i microscope (Nikon).

2.8 Immunohistochemistry

The immunohistochemistry techniques used have been previously described in detail.^{15,34} Briefly, tissues were fixed in PBS 4% paraformaldehyde solution (Sigma-Aldrich), embedded and stored at -80°C . Five micrometer sections were incubated with primary antibody overnight at 4°C , and then 1 hour at room temperature with biotinylated secondary antibody and finally with peroxidase-labeled streptavidin. Peroxidase activity was revealed with 0.025% 3,3-diaminobenzidine (Sigma-Aldrich) in PBS containing 0.03% hydrogen peroxide. Tyramide signal amplification biotin system (Perkin Elmer) was used to detect low amounts of antigens. Uncoupled primary antibodies were: rat anti-mouse CD31 (MEC13.3; BD Pharmingen), goat anti-mouse ACE (AF1513; R&D System), rat anti-mouse CD41 (MWRReg30; BD Pharmingen), and rat anti-mouse CD117 (2B8; BioLegend). Secondary biotinylated monoclonal antibodies were: goat anti-rat IgG antibody (Southern Biotech), goat anti-mouse (Beckman Coulter), and anti-mouse IgM (Southern Biotech). The slides were examined and imaged using an Eclipse 50i microscope (Nikon).

2.9 Statistical analysis

Differences between experimental groups were analyzed using the Student *t* test. Values of *P* less than .05 were considered statistically significant. Mean and SE were calculated and graphed using GraphPad Prism 7 software.

3 RESULTS

3.1 ACE expression pattern in mouse P-Sp and AGM regions

An immunohistochemistry approach was used to detail the spatiotemporal expression pattern of ACE during mouse embryonic development at stages ranging from E9 (20sp) to E10.5 (36sp) (Figure 1A,C).

At E9 (Figure 1A,A⁴), ACE was expressed in endothelial cells lining the lumen of the dorsal aorta, the omphalomesenteric, and umbilical arteries (Figure 1A⁴), as well as on the surface of epithelial cells forming the hindgut. At this stage, a staining was also detected in the endodermal layer and endothelial cells of the extraembryonic YS (Figure 1A). This ACE expression was maintained at E10.5 in all the endothelial cells bordering the lumen of the aorta (Figure 1C,C³), omphalomesenteric, and umbilical arteries (not shown). Endothelial cells of other vessels, which were CD31-positive, such as cardinal veins, umbilical veins, and vitelline veins (Figure 1B,D), never expressed ACE (Figure 1A,C). At this stage, CD31⁺CD41⁺c-Kit⁺ HSPCs were detected in the AGM region into intra-aortic hematopoietic cell clusters (IAHC) adhering to the endothelial cells bordering the lumen of the DA (Figure 1E). ACE identified these aggregates, confirming that during murine development it is a marker of HSPC, as we had previously discovered in human embryo.¹⁵

3.2 Expression of the RAS components in the mouse embryo

The expression profile of ACE observed in developing blood-forming organs (P-Sp and AGM region) both in mouse and human embryos hints at a role of RAS in controlling hematopoietic emergence. To address this issue, we checked for the simultaneous presence of other RAS elements during mouse development, using embryos at stages between E8 and E11. Because of the unavailability of validated commercial antibodies, a semiquantitative RT-PCR approach was first used to analyze the expression of mRNA encoding all RAS elements in the embryo and the dissected YS. As indicated in Figure 2A, the transcripts of ACE and AT1 receptor, as well as REN at weak level, were detected at all stages analyzed, both in the embryo and in the YS. In contrast, the expression of AT2, not detected in the YS, was turned on in the embryo proper at E11.5. Concerning AGT, the precursor of RAS, its expression was detected in the YS at E10.5 and into the whole embryo at E11.5. Since in the adult, AGT synthesis is almost restricted to the liver, analyses were performed on dissected fetal liver (Figure 2C; Figure S2). This revealed the expression of AGT in this organ as early as at E10.5, indicating that all the elements needed for a functional RAS activity are already present in the developing embryo at this stage.

Next, we analyzed the blood-forming organs separated from the rest of the embryo. P-Sp and the AGM region were dissected from the embryo respectively at E9 (15-20sp) and E10.5, as well as at E11.5, and then processed for RTqPCR analyses (Figure 2B). ACE transcripts were detected in the E9 P-Sp, and their expression increased throughout the development. Already present at E9, the transcripts of AT1—the principal receptor of Ang II—were constantly expressed throughout the development. AGT, present in E9 P-Sp, was also detected in the dissected AGM at E11.5.

3.3 Localization of the transcripts for RAS components inside the AGM region

Having detected transcripts for all RAS elements at various embryonic stages, we aimed at determine the precise cellular localization of these transcripts. To address this, endothelial cells (CD45⁻CD41⁻CD42⁻CD31⁺c-Kit⁻), hematopoietic cells (CD45⁺CD41⁻CD42⁻CD31⁺c-Kit⁻), IAHC

(CD45⁺CD41⁺CD42⁻CD31⁺c-Kit⁺), and mesenchymal cells (CD45⁻CD41⁻CD42⁻CD31⁻c-Kit⁻) were sorted from the AGM region at E10.5 and E11.5 and processed for semiquantitative RT-PCR (Figure 2C) and RTqPCR (Figure 2D) analyses. At E10.5, the transcripts of ACE, AT1, AT2, and REN were observed in the mesenchymal cells which at E11.5 also expressed AGT (Figure 2C). However, the expression patterns were more complex in the other cell populations. Indeed, ACE transcripts detected in both endothelial cells and adhering IAHC at E10.5 were absent in the IAHC at E11.5, according to the protein expression (Figure 1). In addition, although the transcripts for Ang II receptors AT1 and AT2 could not be detected in the endothelial cells at E10.5, they were turned on at E11.5, suggesting the ability of these cells, together with the mesenchymal cells, to activate an Ang signaling.

To strengthen these observations, we performed high-resolution RNAscope in situ hybridization for the REN, ACE, AT1, and AT2 transcripts. The transcripts of ACE observed in both the endothelial cells and adhering IAHC at E10.5 (Figure 3A) were restricted to the endothelial cells at E11.5 (Figure 3B), in line with RT-PCR (Figure 2C). REN was expressed in the mesenchyme underlying the aorta (Figure 3A,B). AT1 transcripts were detected in the mesenchymal cells underneath the IAHC at E10.5 (Figure 3A) and also in the endothelial cells specifically associated with the IAHC at E11.5 (Figure 3B). No expression of AT1 and AT2 receptors was observed in the IAHC whatever the developmental stage. Similar experiments conducted on human embryonic sections confirmed these findings (Figure 3C,D), indicating a conserved pattern in mammals. Indeed, in human AGM region at 28 days of development, the expression of both AT1 and AT2 receptors was observed in endothelial cells (which also express ACE) and in the mesenchyme underneath the IAHC (Figure 3C). At 38 days, no transcripts of AT1 and AT2 receptors were observed on ACE-positive IAHC, but only on the endothelial and surrounding mesenchymal cells (Figure 3D).

Altogether, these results highlight the expression of the components of a local-RAS in the homogenic region of the embryo, precisely in the mesoderm underneath the ventral aspect of the aorta, where hematopoietic emergence takes place.

3.4 Effect of RAS on hematopoietic emergence

The spatiotemporal expression pattern of RAS described at early stages of development, both in mouse and human embryos, suggested that this system might be involved in regulating the process of blood emergence in this region via the activation of Ang signaling. To evaluate the putative functional role of the RAS during early hematopoietic generation, we developed a protocol of in vitro culture of P-Sp explants and incubated them with Ang II or Losartan, a specific AT1 receptor antagonist (protocol in Figure 4A). Firstly, E9.5 P-Sp including the aorta was cultured in toto for 3 days, in the presence of Losartan or Ang II, respectively, the antagonist or the agonist of the AT1 receptor. Tissues were then dissociated and cells were plated on fresh OP-9 stromal cells, which allowed survival, proliferation, and lymphomyeloid differentiation of hematopoietic stem cells.³⁸ The hematopoietic activity developed in the culture was evaluated by means of flow cytometry analysis at day 15 of coculture.

Under these conditions, 43% of the cells cultured from the P-Sp expressed the pan-leukocyte antibody CD45, demonstrating the hemogenic potential of this embryonic region. However, in six independent experiments, blocking the AT1 receptor by addition of Losartan resulted in an almost total absence of hematopoietic cells as indicated by the failure to detect CD45-stained cells (Figure 4B). Conversely, to strengthen the notion that RAS is involved in the emergence of hematopoietic cells, we carried out the inverse approach by adding in the culture exogenous Ang II peptide, the agonist of the AT1 receptor. The presence of Ang II resulted in a strong expansion, up to seven times, of the hematopoietic cell compartment, as evaluated by the number of CD45⁺ cells detected in the culture of nine independent experiments (Figure 4B,C).

Cocultures were then analyzed for the differentiation ability of CD45⁺ cells after 15 days of culture. Despite the considerable increase in the number of hematopoietic CD45-positive cells, the activation of RAS by adding Ang II did not modify the balance between myeloid and lymphoid differentiation, evaluated as the percentage of granulomonocytic cells (CD11b) and B lymphocytes (CD19) which were present in almost the same proportion as in the control (Figure 4D; Figure S3). Interestingly, the number of cells expressing the stem cell-marker c-Kit, still present in the population of CD45⁺ cells after 15 days of culture, was consistently higher in the presence of Ang II, reaching almost 11% on average (range: 6.5%-15%), compared to the control, which never exceeded 1.65% (mean 0.75% of CD45⁺ cells) (Figure 4D,E). These results suggested that Ang signaling could contribute to the definitive hematopoiesis, by increasing the number of hematopoietic progenitors that emerge into the P-Sp. This assumption was evaluated by performing a further set of experiments in which the number of progenitors generated in the culture (identified by the phenotype c-Kit⁺CD41⁺CD31⁺CD45⁺) was directly analyzed after the 3 days of treatment (protocol in Figure 5A). Analysis of six individual experiments showed that after 3 days of treatment with Ang II the number of c-Kit⁺CD41⁺CD31⁺CD45⁺ progenitors in the culture (average 540.3 cells/P-Sp) increased by 61% on average (range: 20%-271%) compared to the control (average 335.7 cells/P-Sp) (Figure 5B; Figure S4). In contrast, the administration of Losartan together with Ang II decreased the number of progenitors (average 298 cells/P-Sp), up to 12% on average, confirming that the activation of AT1 receptor is required to the hematopoietic emergence. Although, most of the effects of Ang II described in vivo are due to the stimulation of the AT1 receptor, the high expression of the AT2 we detected in the AGM region during the hematopoietic emergence (Figures 2 and 3) prompted us to investigate the activity of this receptor in the emergence of hematopoiesis. By this way, experiments were carried out in the presence of PD123.319, a specific antagonist of the AT2 receptor. Addition of PD123.319 in the presence of Ang II increased the number of progenitors at a similar level as Ang II alone (up to 117%, range: 19%-183%), indicating that the

effect of Ang II is not mediated by the AT2 receptor. Altogether, these results suggested that the emergence of hematopoietic progenitors in the AGM requires activation of Ang signaling through the AT1 receptor.

To strengthen the results, methylcellulose assays were carried out 3 days after *in toto* culture with Ang II, in the presence or in the absence of Losartan or PD123.319. The frequency of colonies stemming from P-Sp was significantly increased (on average of 33%) by the addition of Ang II compared to untreated P-Sp (mean 30.74 colonies and 41.05 colonies per P-Sp, respectively) (Figure 5C). Specifically, the number of CFU-GEMM and CFU-GM were increased by 305% and 57.1%, respectively, compared to the control (Figure 5D,E). In addition, the presence of Losartan over Ang II decreased the number of CFU-GEMM, but did not affect other type of colonies. Under these conditions, PD123.319 did not change the number of colonies. Thus, these data confirm the involvement of Ang II through the AT1 but not AT2 receptor in the emergence of hematopoiesis.

3 | DISCUSSION

In the present study, we provide a comprehensive view of the developmental expression pattern of ACE and the other RAS elements in mouse embryo at RNA level by *in situ* hybridization and RT-PCR, and at protein level by immunohistochemistry. In addition, functional assays were performed *in vitro* on dissected P-Sp to investigate the potential role of Ang signaling in the generation of embryonic hematopoiesis. Altogether, these data demonstrate that all RAS elements are early expressed in the developing embryo in the site of hematopoietic emergence and that this system is locally functional and has a direct role in modulating the emergence of definitive hematopoietic lineage.

Our study shows for the first time in the mouse model that ACE expression parallels the formation of the principal vessel in the embryo proper, a pattern we have previously described in developing human embryo.¹⁵ Indeed, inside the AGM region ACE is synthesized within the endothelial cells bordering the lumen of the aorta, umbilical, and omphalomesenteric artery—all vessels known to be endowed with hematopoietic abilities³⁹—as well as in associated hematopoietic progenitor cell aggregates. Moreover, in addition to ACE, RT-qPCR analyses on dissected AGM demonstrate here the presence of all components necessary for bioactive Ang II synthesis (REN and ACE) and signaling (AT1 and AT2 receptors) as early as E10.5. RNAscope *in situ* hybridization confirms the expression of the AT1 receptor precisely in the mesenchyme underneath the IAHC, both in mouse and human embryos, hinting at a novel role of RAS in the hematopoietic emergence. This pattern evolves during development, since at E11.5 AT1 transcripts, still present in mesenchymal cells, are now also expressed in endothelial cells associated with the IAHC, likely hemogenic endothelial cells. Again, this expression is consistent with the existence of a local-RAS playing a functional role during blood emergence, in an autocrine/paracrine manner. A role of RAS in adult hematopoiesis has already been reported and requires the synthesis of Ang II.⁴⁰ Accordingly, the addition of exogenous Ang II peptide affects the proliferation and differentiation of adult murine and human blood progenitors.^{23,29,41,42} The effects of the bioactive Ang II peptide are mediated via specific signaling through its two main receptors, AT1 and AT2. In our present work, we have evaluated the possible role of Ang II and its receptors on blood cell generation in mouse P-Sp cultured explants by addition of Ang II alone or in combination with either Losartan or PD123.319, the specific antagonists of AT1 or AT2 receptor, respectively. These functional assays point out the strong stimulatory effect exerted by Ang signaling on hematopoietic generation mediated by the AT1 receptor.

Therefore, here we demonstrate for the first time the existence in the early embryo of a functional local-RAS involved in the emergence of definitive hematopoiesis. Strikingly, unlike previously shown in adult BM-derived murine and human progenitors cultures,²⁷ Ang II added in embryonic P-Sp culture does not modify the differentiation ability and commitment of hematopoietic progenitors, since no variation in the balance between myeloid and lymphoid lineages is observed. By contrast, it increases the number of immature $c\text{-Kit}^+ \text{CD41}^+ \text{CD31}^+ \text{CD45}^+$ hematopoietic progenitors, capable to generate multipotent CFU-GEMM colonies, suggesting that Ang II-derived mitotic effect does not occur in already committed cells, but earlier at tissue level, before blood cells are released from the P-Sp. This effect is reversed by adding Losartan but not by PD123.319, confirming that Ang signaling via the AT1 instead of AT2 receptor is necessary to the hematopoietic emergence into the P-Sp. The AT2 receptor is abundantly and widely expressed in fetal tissues but it generally mediates growth inhibition, differentiation, and/or apoptosis unlike AT1.^{43,44} Here, we report that AT2 is expressed in the AGM region but its blockade by PD123.319 does not impair the Ang II stimulatory effect, suggesting that this receptor has no major role in the emergence of hematopoiesis. However, as AT2 is known to antagonize AT1 directly, or compete for Ang II binding,⁴⁵ further studies are needed to investigate in detail whether AT2 can refine the activity of AT1 in stimulating hematopoietic emergence into the AGM region in response to Ang II.⁴⁶

No expression of AT1 receptor is observed in IAHC cells emerging in the AGM region, confirming that at early stages the addition of Ang II in the culture cannot directly affect the proliferation of these immature hematopoietic progenitors. By contrast, this receptor is detected at E13.5 in fetal liver-derived SLAM-enriched HSPC (data not shown), supporting the idea that Ang II-mediated stimulation is required later in HSPC, during their maturation and expansion in the fetal liver. Corroborating these results, the expression of the AT1 receptor has previously been described in the adult BM, both in hematopoietic progenitors and in stromal cells, which suggests a contribution, via activation by Ang II, in controlling the autocrine/paracrine interactions between these different compartments.²⁸ Angiotensin peptides may act directly as growth factors on hematopoietic precursors, but could also act indirectly by modifying the commitment or the fate of these precursors by acting on the BM stroma cells.²⁷ Several intracellular signaling pathways well known to be used by many hematopoietic cytokines are also activated by Ang II, such as the JAK-STAT pathway and the mitogen-activated protein kinase pathway.^{47,48}

Furthermore, the most compelling aspect of our data is that AT1 receptor is early present in the mesoderm as well as in the endothelium underneath the IAHC during embryonic development. Thus, its activation could be required to promote the proliferation and/or migration of undifferentiated mesodermal precursors as well as their integration into the hemogenic endothelial cells associated with the IAHC. Although the mechanisms at the base of this process are still unknown, our data show that Ang II signaling via AT1 receptor contributes to increase the number of IAHC emerging in the culture of dissected P-Sp, an effect that is blocked when a specific antagonist of the AT1 receptor is added to the culture.

In conclusion, this study provides the first demonstration of the existence of an active local-tissue RAS into the AGM region, both in mouse and human embryos, sequentially involved in the establishment of definitive hematopoietic system. This system includes the enzymes required for angiotensinogen processing into bioactive Ang II, and the AT1 receptor that predominantly mediates downstream signaling. Rather than acting on the differentiation ability and commitment of hematopoietic progenitors, Ang II signaling directly promotes the emergence of immature hematopoietic progenitor cells. Based on these observations, we propose a multistep scenario for the emergence of HSPC in the AGM region suggesting that RAS promotes directly/indirectly migration/proliferation of pre-hematopoietic precursors localized in the subluminal aortic layers which then integrate the endothelial layer (hemogenic endothelium) (Figure 6). These findings strengthen the idea of a mesodermal precursor at the origin of the blood system, as previously described both in human and mouse embryo^{15,49-53} and are consistent with the arterial source of HSPC in the AGM that has been recently suggested by single-cell transcriptome analysis.^{54,55} Also, worth noting, this study provides crucial clues on the strategies for in vitro generation of HSPC, a major challenge for human therapeutic applications.

4 | CONCLUSION

Here, we have uncovered a functional local-RAS into the AGM region involved in the emergence of the definitive hematopoietic system both in mouse and human embryos. This study provides crucial clues on the strategies for in vitro generation of HSPC, a major challenge for human therapeutic applications.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

E.J., K.B.: collection and assembly of data, data analysis and interpretation, manuscript writing; R.E.: collection and assembly of data; C.G., F.L., J.N.F.: manuscript editing; M.T.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

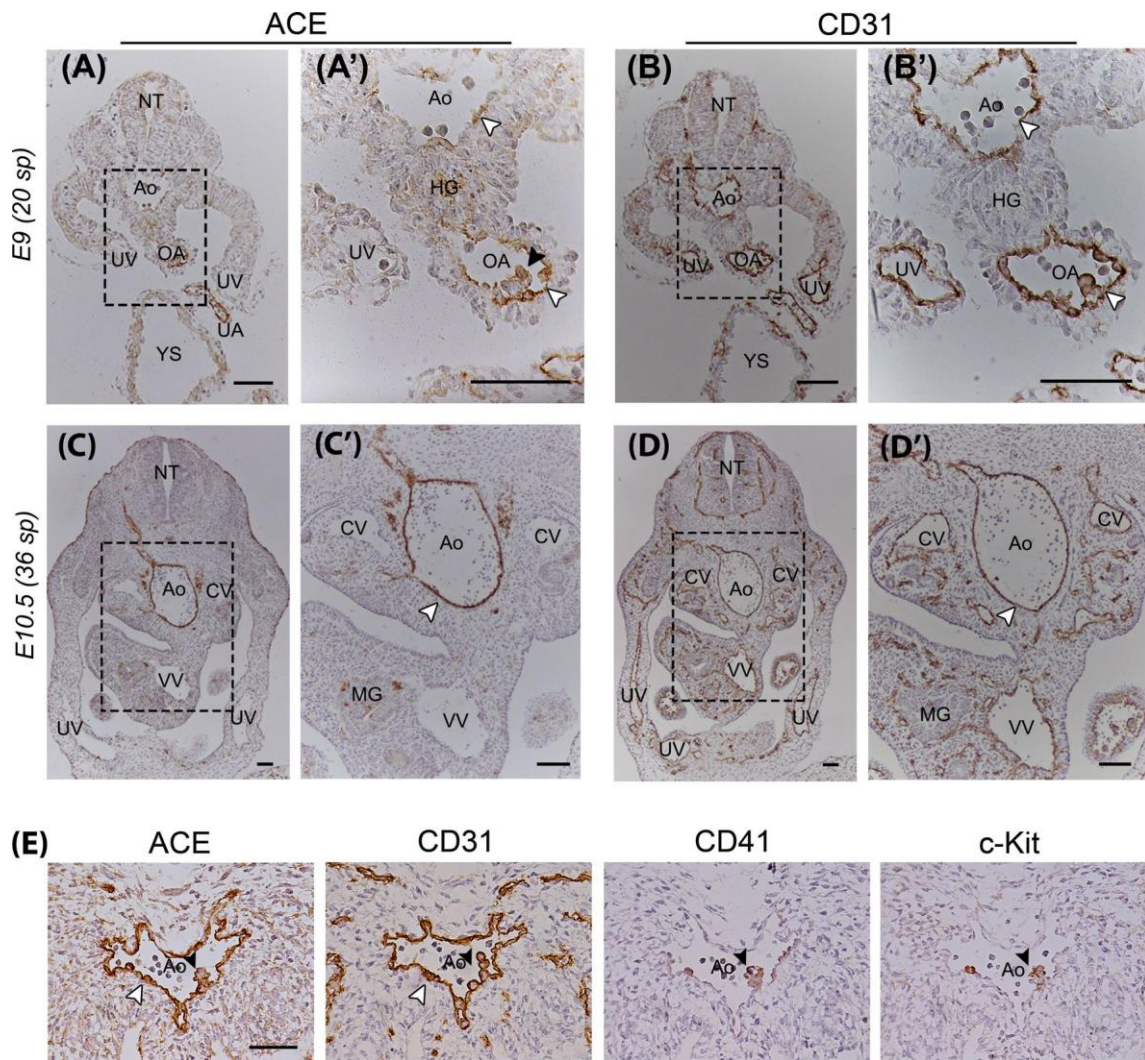


FIGURE 1 Expression pattern of angiotensin-converting enzyme (ACE) in the E9 and E10.5 mouse embryo, and in the hemogenic region. A-D, Transverse sections through E9- and E10.5-mouse embryos immunostained with anti-ACE (A,C) and anti-CD31 antibodies (B,D). A, At E9 (20sp) (n = 5), ACE is weakly expressed at the surface of some endothelial cells bordering the lumen of all the arteries: dorsal aorta, omphalomesenteric, and umbilical arteries (white arrowhead). ACE is also expressed by epithelial cells forming the hindgut, and by the endodermal layer of the extra-embryonic yolk sac. A' and B', and C' and D' represent high magnifications of the splanchnopleura and AGM region dotted in A and B, and in C and D, respectively. C, At E10.5 (36sp) (n = 7), ACE antibody recognizes CD31⁺ endothelial cells bordering all arteries (white arrowhead C' and D'). No ACE expression was detected in endothelium of the veins, which expresses CD31. E, Transverse sections in the AGM region at E10.5. ACE is expressed by CD31⁺CD41⁺c-Kit⁺ hematopoietic progenitor cells (black arrowheads) adhering to the endothelium of the aorta, as well as by endothelial cells (white arrowhead). Ao, aorta; CV, cardinal vein; HG, hindgut; MG, midgut; NT, neural tube; OA, omphalomesenteric artery; sp, somite pairs; UA, umbilical artery; UV, umbilical vein; VV, vitelline vein; YS, yolk sac. Scale bars = 50 μm in A-D, 25 μm in E

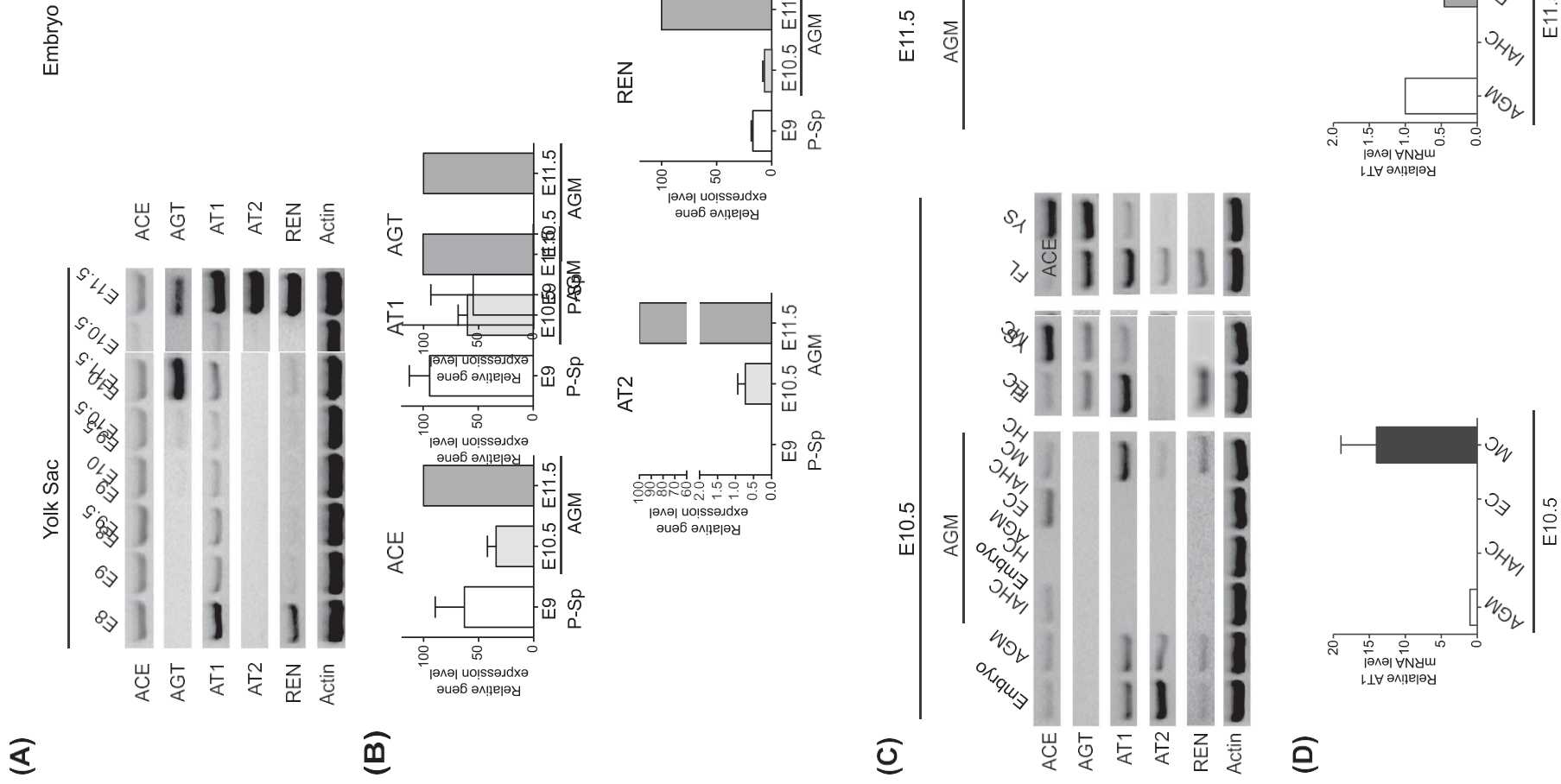


FIGURE 2 Legend on next page.

FIGURE 2 Spatial-temporal expression of renin-angiotensin system (RAS) elements during mouse embryonic development. A, Representative RT-PCR analysis displaying RAS components mRNA in yolk sac and in whole embryo ranging from E8 to E11. Actin is used as endogenous control. E8 (9-10sp); E9 (10-20sp); E9.5 (24-25sp); E10 (28sp); E10.5 (35sp); E11.5 (40-45sp). B, RTqPCR analysis of RAS components in P-Sp (E9) and AGM (E10.5-E11.5). Results are normalized to Gapdh and expressed relative to the E11.5 AGM mRNA level. Values are represented as mean \pm SEM (n = 3). C, Representative RT-PCR analysis displaying RAS components mRNA expression in intra-aortic hematopoietic cell clusters (IAHC), endothelial- (EC), hematopoietic- (HC), and mesenchymal- (MC) cells sorted from E10.5 (30-35sp) and E11.5 (39-45sp) AGM. Yolk sac and fetal liver dissected at the same stages are also analyzed. At E10.5, mesenchyme cells underneath the aorta express all RAS components with the exception of AGT which at this stage is expressed by the liver and the YS. Interestingly, at E11.5 transcripts of the AT1 and AT2 receptors are also expressed in endothelial cells. These data are also confirmed by qPCR. D, RTqPCR analysis of the AT1 receptor in cell populations sorted from E10.5 and E11.5 murine AGM. Results are normalized to Gapdh, and expressed relative to the AGM sample. Values are represented as mean \pm SEM (n = 3). ACE, angiotensin-converting enzyme; AGT, angiotensinogen; AT1, AT1 receptor; AT2, AT2 receptor; AGM, aorta-gonad-mesonephros; P-Sp, paraaortic splanchnopleura; REN, renin; Sp, somite pairs

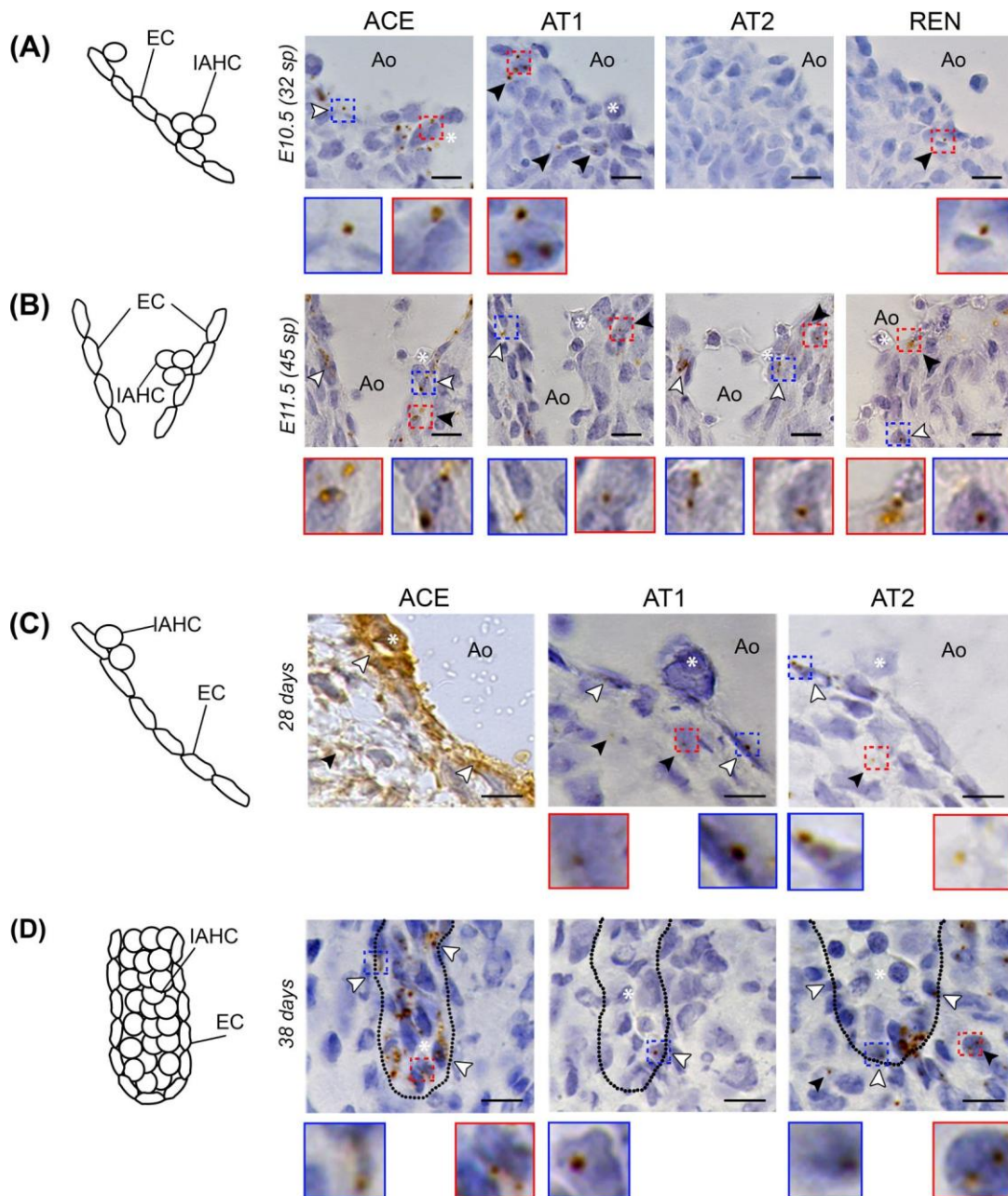
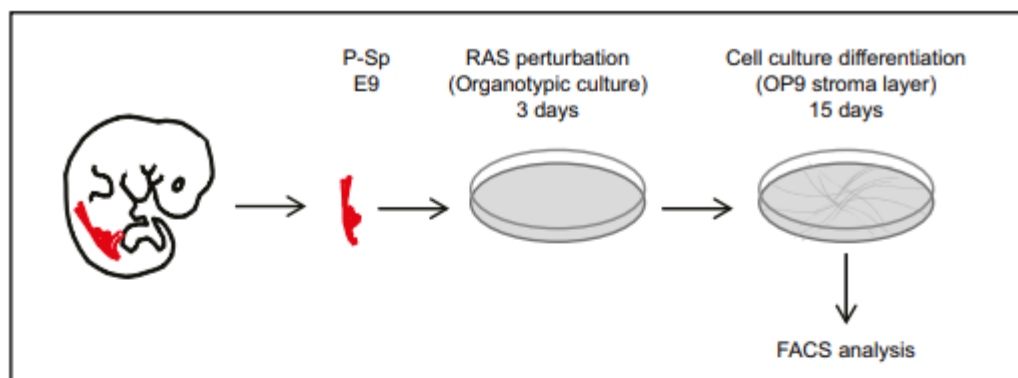
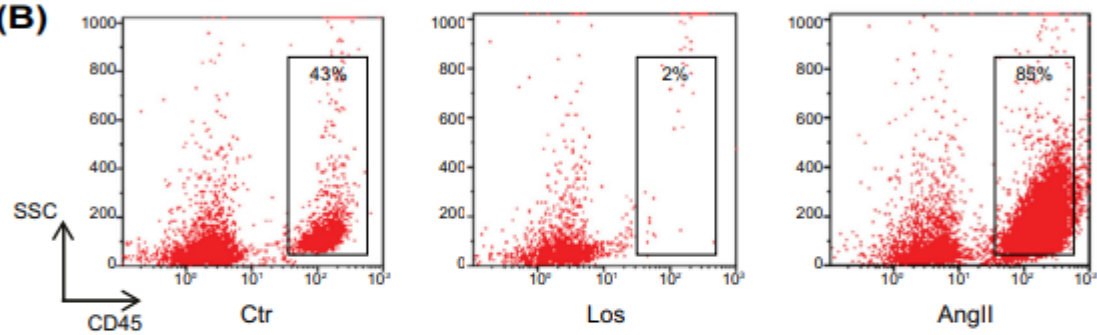


FIGURE 3 Localization of renin-angiotensin system (RAS) components into mouse and human aorta-gonad-mesonephros (AGM) region representative images of RNAscope in situ hybridization in human and murine AGM regions. **A**, Cross sections of a E10.5 mouse embryo ($n = 7$). At this stage, mRNA transcripts of all RAS components are present in the AGM region (as shown by RT-qPCR). Angiotensin-converting enzyme (ACE) is expressed in endothelial cells (white arrowhead) and in IAHC (white star) of the dorsal aorta. AT1 is detected in the mesenchyme (black arrowheads) underneath the IAHC (white star). mRNA transcripts of AT2 are present in a distal part with respect to the lumen of the aorta, not framed in this figure. REN is expressed in the mesenchyme (black arrowhead) underlying the aorta. **B**, Cross sections of an E11.5 mouse embryo ($n = 5$). At this stage, ACE is expressed in endothelial cells (white arrowheads) and in the mesenchyme (black arrowhead), but no longer in the IAHC (white star). AT1 and AT2 mRNA are detected in endothelial cells (white arrowheads) and in the mesenchyme (black arrowheads). Renin (REN) is expressed in cells underlying the endothelium (white arrowhead) as well as in the mesenchyme (black arrowheads). **C**, Cross sections of a 28-day human embryo (CS 12) ($n = 3$). ACE protein detected by immunohistochemistry is localized on endothelial cells (white arrowheads) and on IAHC emerging in the ventral side of the aorta (white star). mRNA transcripts of the AT1 and AT2 receptors are detected by in situ hybridization in some endothelial cells (white arrowheads) and mesenchymal cells (black arrowheads) underneath IAHC (white star). **D**, Transverse sections of a 38-day human embryo (CS 16) ($n = 3$). ACE is expressed on the emerging IAHC (white star), as well as in the surrounding endothelial cells (white arrowheads). Transcripts of AT1 are detected on endothelial cells. AT2 mRNA is detected in endothelial cells (white arrowheads) as well as in mesenchymal cells (black arrowheads). Dotted lines indicate the endothelium. Diagrams on the right are schematic representations of the figures. Scale bar = 10 μm . Ao, aorta; EC, endothelial cells; IAHC, intra-aortic hematopoietic cell clusters; CS, Carnegie Stage. Zoomed images of the dotted regions are shown below each figure

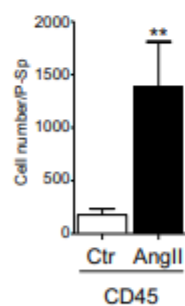
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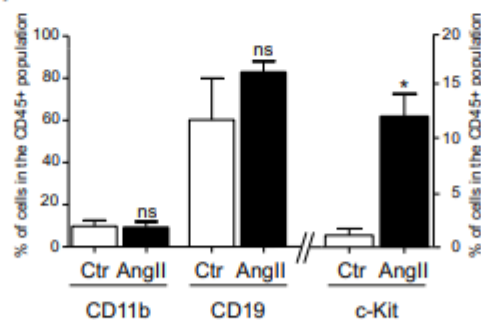
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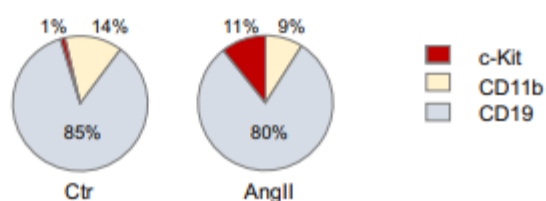
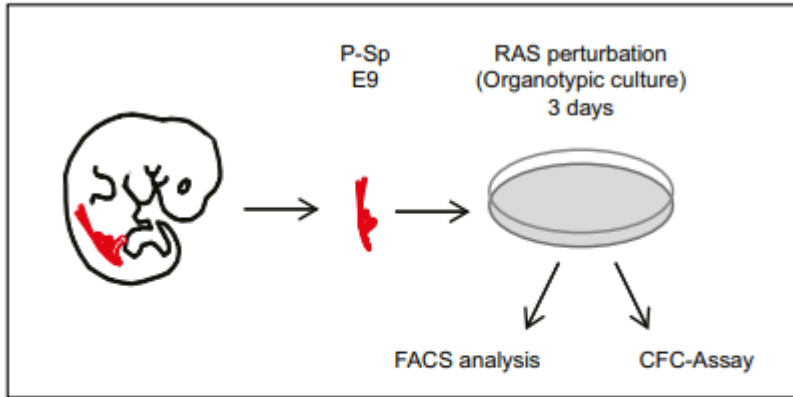
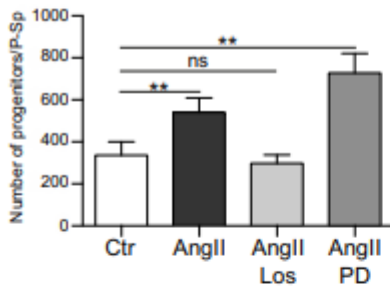


FIGURE 4 Effects of renin-angiotensin system (RAS) perturbation in the paraaortic splanchnopleura (P-Sp) culture. A, Scheme of in vitro assay for studying RAS perturbation during hematopoietic emergence in the early mouse embryo. P-Sp dissected at E9.5 was cultured in toto in presence of either Losartan or Ang II. After 3 days, P-Sp were mechanically dissociated, and cells seeded over an OP-9 stromal cell layer and grown for 15 days then analyzed by FACS. B, Representative example displaying flow cytometric analysis of P-Sp-derived cells after 3 days of treatment and successive 15 days of coculture on OP-9 stromal cells. The administration of Losartan dramatically decreases the percentage of hematopoietic CD45⁺ cells (2%) detected in the culture, compared to the control (43%), which represents P-Sp without any treatment. Conversely administration of Ang II results in a strong expansion of the percentage of hematopoietic CD45⁺ cells (85%). C, Absolute number of hematopoietic CD45⁺ cells obtained in the culture of one single P-Sp treated with Ang II. The bar graph represents the average of six independent experiments. ****P < .01**. D, Percentage of myeloid (CD11b⁺) and lymphoid (CD19⁺) cells in the population of CD45⁺ cells represented in the bar graph C. Ang II does not modify the proportion of CD11b⁺ and CD19⁺ cells, but consistently increases the percentage of more immature CD45⁺c-Kit⁺ cells, by almost 11% of the CD45⁺ cell compartment on average (range: 6.5%-15%), compared to the control, which never exceeded 1.65% (mean 0.75% of CD45⁺ cells). ***P < .05**. E, Pie charts show percentages of hematopoietic population differentiated in the culture. Six independent experiments were performed. Values are represented as mean ± SEM

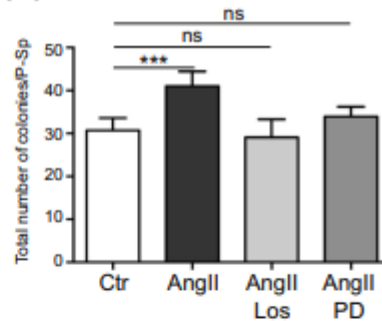
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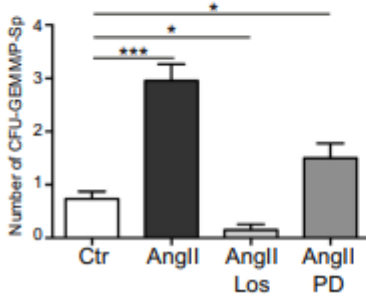
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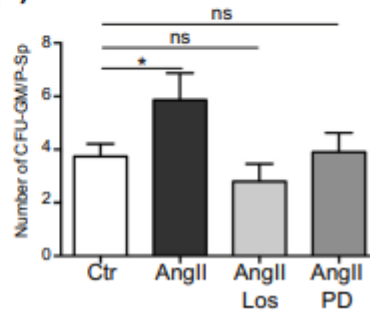


FIGURE 5 Effects of renin- angiotensin system (RAS) perturbation in hematopoietic progenitor cell emergence. A, Scheme of *in vitro* assay for studying RAS perturbation during hematopoietic emergence in the early mouse embryo. Paraortic splanchnopleura (P-Sp) dissected at E9.5 was cultured *in toto* with Ang II administration in presence of either Losartan or PD123.319. After 3 days, P-Sp were mechanically dissociated and cells directly analyzed by flow cytometry or plated in methylcellulose-based medium for colony forming cell assay (CFC). B, Analysis of the number of CD45⁺CD31⁺CD41⁺c-Kit⁺ progenitor cells generated after RAS perturbation during 5 days of P-Sp organotypic culture. Six independent experiments were performed. C-E, Clonogenic assays were established using dissociated P-Sp cells (1P-Sp cell/plate in triplicate). Hematopoietic colonies were scored after 11 days of culture. Six independent experiments were performed. Values are represented as mean \pm SEM. * $P < .05$, ** $P < .01$, *** $P < .001$

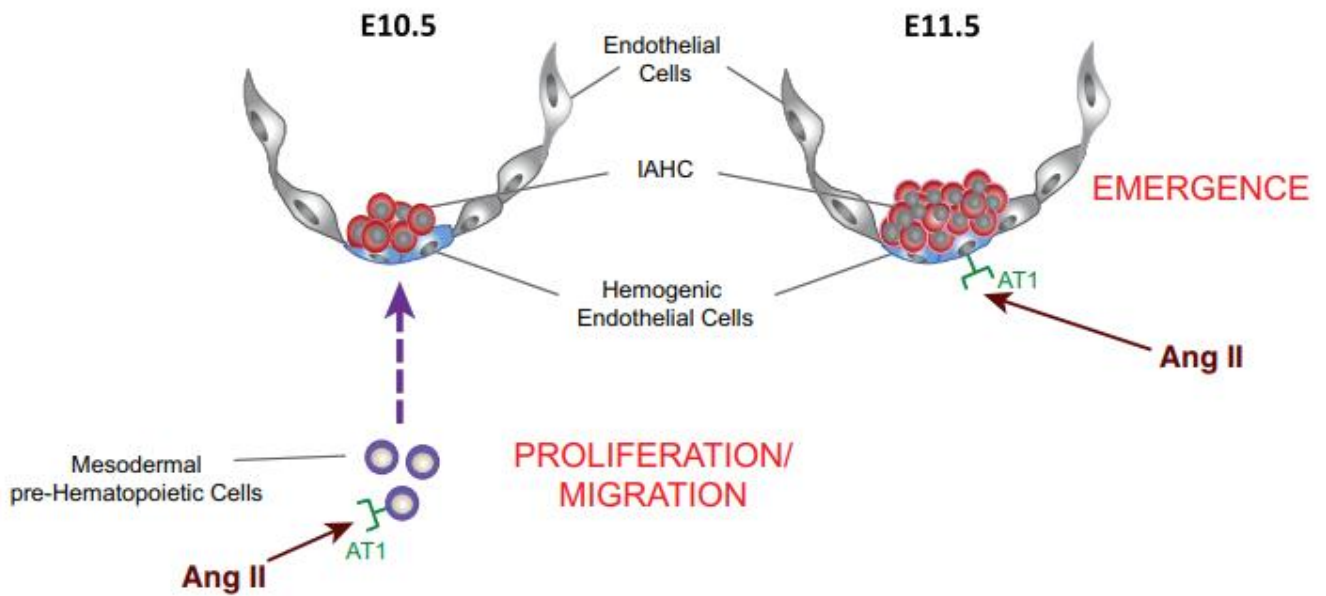


FIGURE 6 Proposed model for renin-angiotensin system (RAS) involvement in hematopoietic stem/progenitor cell emergence in the aorta-gonad-mesonephros (AGM) region. At E10.5, all RAS components required for bioactive Ang II-synthesis and -signaling (AT1 receptor) are synthesized locally in the AGM region. Ang II signaling directly/indirectly promotes the emergence of hematopoietic progenitor cells by stimulating the proliferation/migration of pre-hematopoietic precursor cells (E10.5) localized in the subluminal aortic layers which then integrate the endothelial layer (hemogenic endothelium) (E11.5)

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