

Tissue Growth Pressure Drives Early Blood Flow in the Chicken Yolk Sac

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Background: Understanding how molecular and physical cues orchestrate vascular morphogenesis is a challenge for developmental biology. Only little attention has been paid to the impact of mechanical stress caused by tissue growth on early blood distribution. Here we study the peripheral accumulation of blood in the chicken embryonic yolk sac, which precedes sinus vein formation. **Results:** We report that blood accumulation starts before heart-induced blood circulation. We hypothesized that the driving force for the primitive blood flow is a growth-induced gradient of tissue pressure in the yolk sac mesoderm. Therefore, we studied embryos in which heart development was arrested after 2 days of incubation, and found that yolk sac growth and blood peripheral accumulation still occurred. This suggests that tissue growth is sufficient to initiate the flow and the formation of the sinus vein, whereas heart contractions are not required. We designed a simple mathematical model which makes explicit the growth-induced pressure gradient and the subsequent blood accumulation, and show that growth can indeed account for the observed blood accumulation. **Conclusions:** This study shows that tissue growth pressure can drive early blood flow, and suggests that the mechanical environment, beyond hemodynamics, can contribute to vascular morphogenesis. *Developmental Dynamics* 000:000–000, 2017. © 2017 Wiley Periodicals, Inc.

Key words: vascular morphogenesis; hemodynamics; vasculogenesis; tissue mechanics; chicken embryo; sinus vein

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Introduction

Understanding how vascular networks are established during embryogenesis is a great challenge of developmental biology and a major field of investigation. During a process called vasculogenesis (Eichmann et al., 2005; Swift and Weinstein, 2009), mesodermal cells differentiate into blood and endothelial progenitor cells or “angioblasts.” They cluster and form blood islands that undergo further morphogenesis to form endothelial tubular networks, the so-called capillary plexus. Sprouting angiogenesis and intussusceptive angiogenesis (Djonov et al., 2000; Potente et al., 2011; Ribatti and Crivellato, 2012) allow the capillary plexus to expand. This lattice of small vessels prefigures the future hierarchic vascular network. By remodeling of the vessel wall (le Noble et al., 2004; Swift and Weinstein, 2009) and by disconnecting side branches from the remodeling vessels (le Noble et al., 2005; Nguyen et al., 2006), this primitive network matures into a hierarchical, functional network with arteries, capillaries and veins. In the past two decades, many studies have shown that these processes are established by molecular signaling

pathways that involve genetically determined mechanisms (for reviews, see Park et al., 2013; Ribatti, 2006) and hemodynamics (le Noble et al., 2004; Lucitti et al., 2007; Nguyen et al., 2006; for a review see Jones et al., 2006).

That the local mechanical environment, and in particular shear stress exerted by the flowing blood, is involved in the remodeling of vascular networks has long been recognized (Thoma, 1893). Biophysical models of both plexus formation (Ambrosi et al., 2005) and vascular wall remodeling (Rachev et al., 1996; Taber, 1998) have been proposed, but very little attention has been given to the contribution of mechanical stress exerted by the surrounding growing tissue on vascular morphogenesis. A mechanical model for the formation of the capillary plexus was developed by Manoussaki and colleagues (1996). In their model, they were able to reproduce in vitro cellular patterns emerging from the interaction between traction forces of endothelial cells and the elasticity of the extracellular matrix. In a model study using a diffusion limited aggregation approach, Nguyen et al. (2006) suggested that, besides blood flow, stress generated by tissue growth might deform the blood vessels. They further proposed that such a deformation of blood vessels might be essential to remodel the capillary plexus in a realistic arterial pattern. The same group suggested that remodeling in the vicinity of newly formed

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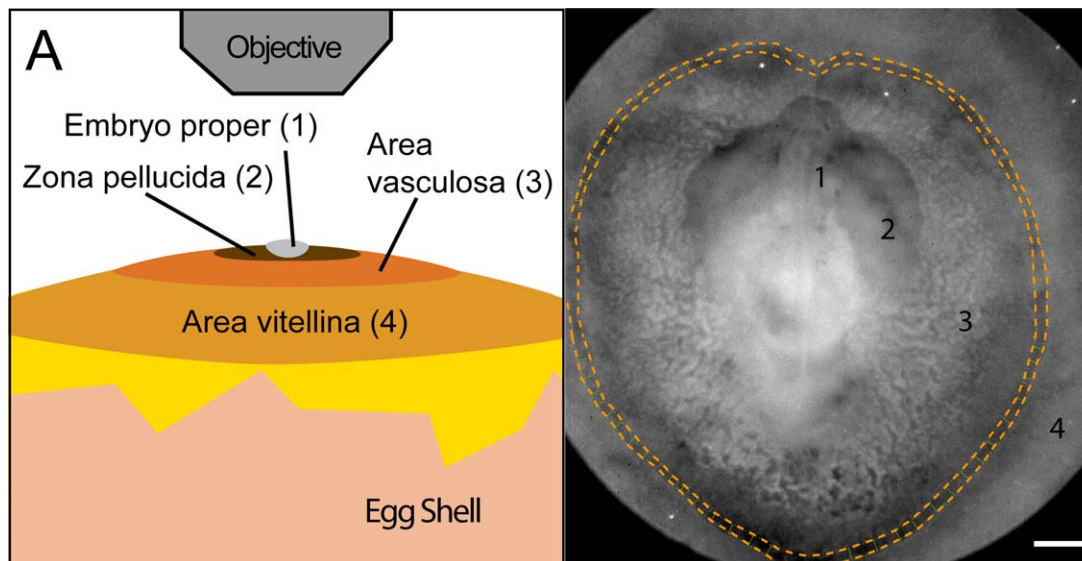


Fig. 1. **A:** Schematics of the set up and side view of the chicken embryo and the yolk sac tissues. The embryo proper (1) is surrounded by the zona pellucida (2), which in turn is surrounded by the area vasculosa of the yolk sac (3) in which the mesoderm is present and the blood vessels develop. The area vasculosa is surrounded by the area vitellina (4), in which no mesoderm is present yet. The yolk sac tissues 2–4 form a flat, round layer on the egg yolk. **B:** Top view of the embryo and yolk sac before the sinus vein is formed, and before heart perfusion (HH13). The sinus vein is in between the two orange dotted lines. Scale bar = 1 mm.

arteries might mechanically prepattern venous development in the capillary plexus (Al-Kilani et al., 2008). More recently, it was observed that stretch in the chicken chorioallantoic membrane induces axial growth and realignment of conducting vessels as well as intussusceptive and sprouting angiogenesis in the capillary bed (Belle et al., 2014).

In this study, we investigate how yolk sac growth pressure drives blood to the area where the sinus vein will form during early chicken embryogenesis before the heart beats strong enough to pump blood around.

In the following, we refer to the different developmental stages of the chicken as proposed by Hamburger and Hamilton (HH) (1951) or, occasionally, we refer to the days of development (E) after the egg is laid. The yolk sac is an embryonic tissue surrounding the developing embryo that provides it with nutrients (Fig. 1). The yolk sac is formed (Romanoff, 1960; Eichmann et al., 2005; Sheng, 2010; Bauer et al., 2013) by rapidly dividing ectodermal cells that start to spread from the embryo over the surface of the yolk. Closely behind the migrating front, between the yolk surface and the ectoderm, the proliferating endodermal epithelial cells of the yolk sac follow the ectodermal cells and form a tight epithelial layer in close contact with the yolk.

Mesodermal cells from the splanchnic mesoderm migrate into the space between the ectodermal and endodermal layers from stage HH2 to at least HH9 (Sheng, 2010). During and after migration the precursor cells of endothelial and hematopoietic cells cluster into so called blood islands. Earliest blood islands are observed at stage HH6. The cells located at the blood island's edges differentiate into endothelial cells and the cells inside differentiate into haematopoietic cells (Sabin, 1920). Soon after differentiation, starting at HH9–HH10 (Sheng, 2010), the endothelial cells anastomose to form the capillary plexus. When the capillary plexus forms, the blood cells are still adhering to each other and to the endothelial cells (Sabin, 1920).

From stage HH10–HH11, they start to segregate into individual blood cells (Sheng, 2010). At HH12, the heart starts to contract sufficiently to allow individual blood cells to circulate and subsequently the plexus remodels progressively into a hierarchical network by the processes described above. Blood circulating in the yolk sac flows toward the periphery of the area vasculosa (Fig. 1) of the yolk sac, where the sinus vein has formed (Fig. 2A3–5). From there, it eventually flows back to the heart first through the cranial vein, later also through the caudal vein. The area vitellina (Fig. 1) is the area of the yolk sac where the mesodermal tissue with blood vessels has not yet entered between the endodermal and ectodermal layers. While at stage HH26/27 (E5) the area vitellina covers most of the yolk surface, the area vasculosa reaches only the equator of the egg yolk. By E15, the area vasculosa fully covers the yolk and the area vitellina has disappeared.

We report here that before circulation and subsequent remodeling of the capillary plexus into arteries and veins, around stage HH12, a preliminary accumulation of blood in the distal area vasculosa of the yolk sac precedes the formation of the so-called sinus vein. We propose that this early radial blood flow is caused by a radial tissue pressure gradient associated to growth in the yolk sac tissue. Considering the yolk sac mesoderm growth as a 2D viscous flow, we infer the pressure gradient in the tissue directly from quantitative growth rate measurements performed by particle image velocimetry (PIV). Then, modeling blood flow in the capillary plexus as a porous flow, we show that the tissue pressure gradient can indeed cause a radial flow, eventually leading to peripheral blood accumulation.

To confirm that tissue growth alone is sufficient to initiate blood accumulation, we sought experimental conditions in which the role of tissue growth could be isolated. As it has been long known, growth of the yolk sac does not require the embryo to be alive (Romanoff, 1960). In eggs in which the heart development was arrested by incision on the second day after incubation at HH11, we found indeed that the yolk sac tissue still grows for

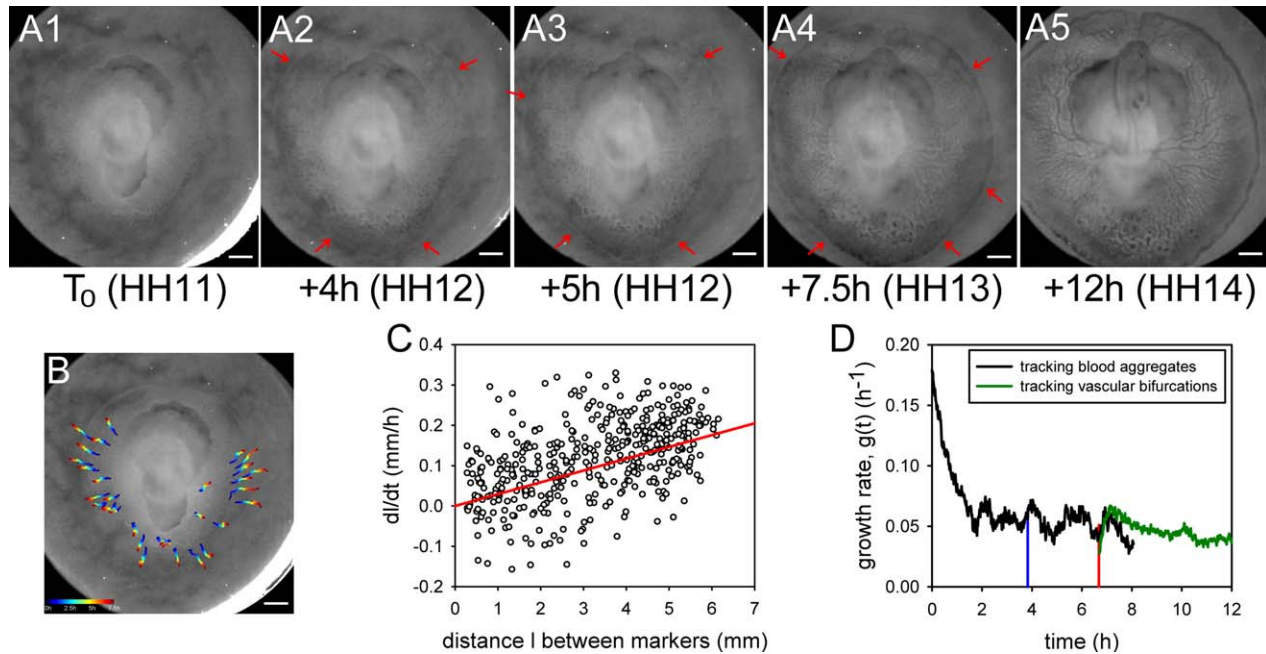


Fig. 2. **A:** Formation of the sinus vein. Blood progressively accumulates at the periphery of area vasculosa of the yolk sac (A2–A3, red arrows). When the heart contracts sufficiently efficient, circulation starts and the sinus vein and the other vessels remodel to form a hierarchical vascular network (A4–A5). Extraction of 5 frames of a time lapse movie of 20 hr with frame rate 60 images/hr (Supplementary Movie S1). Scale bar = 1 mm. **B:** Trajectories of the tracked points between 0 and 7.5 hr, superimposed on a frame of the timelapse video at T_0 . Growth causes radial displacements. **C:** $\Delta l/\Delta t$ as a function of $l(t)$ at $t=2h22'$. $l(t)$ is the distance between each pair of tracked points. The associated growth rate equals $0.029h^{-1}$ and the coefficient of determination r^2 equals 0.24. **D:** Growth rate $g(t)$ extracted from linear fits of $\Delta l/\Delta t$ vs. $l(t)$ performed over the entire experiment. The vertical blue line indicates the time of the first noticeable heart contractions and the onset of visible blood accumulation, which occur approximately at the same time ($t=3h50'$). The vertical red line shows the onset of blood circulation ($t=6h50'$) (see also Supplementary Movie S1).

approximately 9 hr. We observed that peripheral blood accumulation still occurs in these eggs. The model simulations signify that the tissue pressure gradient built up by the growth of the tissue is sufficient to allow blood to accumulate at the periphery of the area vasculosa.

Although tissue growth pressure is proposed to play a role in vascular morphogenesis (Nguyen et al., 2006; Al-Kilani et al., 2008) it was to our knowledge not demonstrated that tissue growth pressure can be sufficient to drive blood flow. We propose that this simple physical mechanism induces peripheral blood accumulation that precedes sinus vein formation. More generally, this work illustrates that the impact of the physical environment on the formation of the primitive vasculature might extend to more than hemodynamics, for instance here to tissue pressure gradients caused by tissue growth.

Results

Sinus Vein Formation

The formation of the sinus vein is visualised in a timelapse (Figure 2A and Supplementary Movie S1). Images were taken with a 1-min interval for almost 20 hr, approximately depicting the course of stages HH11 to HH14. The formation of the sinus vein starts with accumulation of blood in the periphery of the area vasculosa of the yolk sac. This blood accumulation starts to become visible around the same time as the contraction of the heart becomes visible, after 4 hr of growth (Fig. 2A2; the blue line in Fig. 2D; Supplementary Movie S1). Remodeling of the capillary plexus into larger vessels starts approximately after 6.5 hr of

growth (Fig. 2A3–4; Supplementary Movie S1). The increasing volume of blood accumulated at the yolk sac periphery (Fig. 2A2–A3; Supplementary Movie S1) suggests that an outward radial flow of blood exists in the primitive lattice before the heart contracts with enough strength to make the blood circulate. The accumulation of blood leads eventually to the formation of the sinus vein. Upon circulation, the primitive lattice is remodeled as flow increases in the vitelline arteries, and the venous return is established through the cranial vein (A4–A5). By then, blood clusters have completely disappeared and individual blood cells circulate in the vasculature.

Tissue Growth Causes a Radial Stress Gradient

We hypothesize that the accumulation of blood at the yolk sac periphery is driven by a gradient of mechanical stress caused by tissue growth. In this section, we will develop the equations relating growth to tissue pressure and provide measurements of growth.

The yolk sac mesoderm at stages HH11–HH15 is an approximately $35\mu\text{m}$ -thick layer (Mobbs and McMillan, 1979). Mesenchymal tissues are viscoelastic materials with an elastic response at short time scales (seconds) and a viscous response at long time scale (Forgacs et al., 1998; Gonzalez-Rodriguez et al., 2012). As tissue growth is slow compared to the elastic response, we will model the growth of the yolk sac mesodermal tissue as a viscous hydrodynamic flow between the endodermal and ectodermal layers. Hydrodynamics has been shown to be a relevant framework to describe tissue morphogenesis at developmental time

scales (He et al., 2014; Popović et al., 2016). We thus assume that the mesoderm flows between the ectodermal and endodermal layers, which we assume static because they move slowly compared with the mesoderm. In the absence of growth, the local mass balance suggests that the divergence of the velocity field $\vec{u}_t$ of the tissue is zero: what comes out of an arbitrary volume, is equal to what comes in. In presence of growth, new tissue is created inside the volume and the balance should include a growth rate. In that case, a nonzero divergence equation can accurately account for tissue growth (Clément and Mauroy, 2014), and the divergence is instead equal to the growth rate $g(r, t)$. Assuming the system is axisymmetric and neglecting the thickening of the tissue, the so-called continuity equation in the yolk sac mesoderm thus reads:

$$\text{div } \vec{u}_t(r, t) = \frac{1}{r} \frac{\partial}{\partial r} (r u_t(r, t)) = g(r, t). \quad (1)$$

We hypothesize that, from stage HH11, yolk sac mesoderm grows by proliferation of cells and production of extracellular matrix, because at this stage mesodermal cells entering the yolk sac by migration are negligible. In the absence of specific spatial cues controlling growth in the yolk sac, the growth rate g should be, to a first approximation, spatially homogeneous. To test this, we predict the evolution of the distance between any two points of the yolk sac in case of homogeneous growth, and compare it with experimental data. If g is spatially constant, integrating Equation [1] yields:

$$\vec{u}_t(r, t) = \frac{1}{2} g(t) r \vec{e}_r, \quad (2)$$

and the distance l between any two points in the tissue should increase as:

$$\frac{dl}{dt} = \frac{1}{2} g(t) l. \quad (3)$$

To measure the velocity of the yolk sac mesoderm we used digital PIV (dPIV) techniques (Cardoso, 2013). Up to stage HH12, we tracked blood aggregates. The aggregates reside either in the blood islands or in the developing capillary plexus. When the circulation starts and blood vessels can be distinguished, we used vascular bifurcations as markers for mesodermal tissue growth. The markers are indicated in the Supplementary Movie S1 with orange circles. The individual tracks of the markers up to 7.5 hr are shown in Figure 2B. For each pair of markers, we measured the distance l as a function of time, as well as the distance increase rate $\Delta l / \Delta t \simeq dl / dt$ (see the Experimental Procedures section). The results do follow a linear trend (Fig. 2C), and the slope of the linear fit provides a measurement of g at time t (Eq. [3]).

Repeating this procedure over the entire experiment, we were able to measure how g varies over time (Fig. 2D). Figure 2D shows that the growth curve measured by tracking the blood aggregates (black line) is consistent with the growth curve measured by tracking vascular bifurcations (green line). This suggests that the blood aggregates indeed do not move relatively to the endothelial cells of the capillary plexus, as described by Sabin (Sabin, 1920) who observed that the blood aggregates in the plexus adhere to the endothelial cells. Therefore, the movements of both markers seem to reflect well the growth of the mesoderm. The coefficient of determination for the linear fit (Fig. 2C) is low because of the noise in determining the displacement of the markers using the

dPIV algorithm, resulting in a large spread of distance increase rates as a function of distance between the markers.

Therefore, as an additional control, we used the measurement of the growth rate $g(t)$ to predict how the yolk sac should expand. To do so, we applied Equation [3] to a series of points at the moving front of the mesoderm, paired with their center of mass which is not moving with time. We then superimposed their predicted trajectories (Supplementary Movie S1, yellow squares) to the movie. The good agreement between predicted and actual expansion confirms that the time dependent, but spatially homogeneous growth rate $g(t)$ is a good approximation.

Because the mesoderm consists in a thin layer growing between the static ectodermal and endodermal layer, we can use the hydrodynamic analogy of plane flow between two parallel plates to approximate the pressure field in the mesoderm. The local gradient of tissue pressure p exerted by growth reads:

$$\vec{\text{grad}} p_t(r, t) = -\frac{8\eta_t}{Ah^2} \vec{u}_t = -\frac{4\eta_t g(t)}{Ah^2} r \vec{e}_r \quad (4)$$

with A and h depending on the velocity profile in the mesoderm. A is the ratio between the mean velocity, u_r , and the maximum velocity. $A = 2/3$ for a parabolic flow profile (Poiseuille flow) and $A = 1$ for a perfect plug flow. h is the total height on which there is a shear. With H being the thickness of the yolk sac mesoderm, then $h = H$ for a parabolic flow and $h = 0$ for a perfect plug flow ($A = \frac{3H-h}{3H}$, see the Supplementary Materials for details). η_t is the tissue viscosity in the sheared layers h at long time scales. For a parabolic flow η_t represents the global tissue viscosity. The actual shape of the tissue velocity profile is unknown and depends on the friction the yolk sac tissues encounter. There are two sources of friction: the friction inside the tissue between the mesodermal cells and the friction between the mesoderm and the ecto- and endodermal layers. When they are approximately the same, or when the friction between the mesodermal cells is lower, it approaches a parabolic velocity profile. When there is more friction between the mesodermal cells than at the interfaces, the velocity profile flattens up. At the extreme, it can reach a perfect plug flow. The pressure gradient derived from a hydrodynamics parabolic flow is then the lower possible limit, and when the tissue becomes more rigid the pressure gradient can become much larger.

Assuming that the pressure at the yolk sac circumference ($r = R_{AV}$) is constant and equal to p_0 , the pressure field thus reads:

$$p_t(r, t) = p_0 + \frac{2\eta_t g(t)}{Ah^2} (R_{AV}(t)^2 - r^2) \quad (5)$$

where $R_{AV}(t)$ is the radius of the area vasculosa of the yolk sac at time t . Hence the pressure caused by tissue growth in the yolk sac mesoderm is higher in the proximal yolk sac than near the outer border, and increases as the yolk sac grows. This is rather intuitive because confinement is higher close to the center. The velocity and pressure fields caused by tissue growth alone are schematized Figure 3.

Tissue Growth is Sufficient for Blood Accumulation

Let us now investigate how tissue growth pressure impacts the blood flow. As we showed above, the aggregates do not move relatively to the plexus and we assume that only free flowing blood passes through the capillary plexus in between the aggregates.

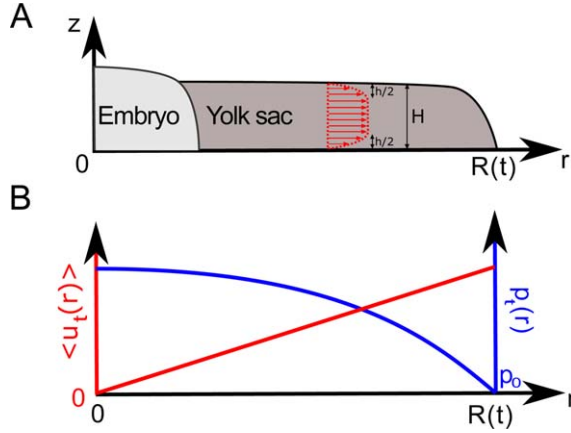


Fig. 3. **A:** Schematics of the growth-induced flow. As the yolk sac tissue grows, the tissue flows radially. The mesoderm is a thin layer flowing between the ectoderm and the endoderm, the z -profile is unknown and could vary from a parabolic profile to a plug profile. The velocity profile depends on A , the ratio between max and mean velocity, and on h , the total height of the mesoderm on which there is shear. H is the mesodermal thickness. In the model the velocity at radius r is averaged over z . **B:** Mean tissue velocity $\langle u_t(r) \rangle$ increases linearly with the distance r to the center of the embryo, while the pressure p_t is maximum at the center and decreases with the radius r .

Blood is generated by the production of plasma by the endothelial cells followed by segregation of individual blood cells from the aggregates (Sabin, 1920; Ferkowicz and Yoder, 2005). To model their flow generated by the growth-driven tissue pressure, we assume that blood flows in the capillary plexus in between the aggregates as in a porous medium. This is justified because yolk sac geometry changes due to growth are slow compared with the motion of blood. This also assumes that space for blood transport in the capillary plexus is not deformed by the blood flow.

The effective permeability, considering that the transport space is randomly oriented, is given by $\kappa = \psi b / 96$ (Guyon et al., 2001). ψ is the volume fraction of the space available for blood transport in the mesodermal tissue, and b the typical pore size. Flow in a medium with heterogeneous pore size is limited by the smallest pores; hence, we assume b to be the typical diameter of capillaries. Darcy's law relates the velocity $\vec{u}_b$ of the porous flow of blood with the gradient of growth pressure:

$$\vec{u}_b = -\frac{\kappa}{\eta_b} \text{grad} p_t \quad (6)$$

with η_b being the viscosity of blood. Using Equation [4] and substituting κ in Equation [6] yields:

$$\vec{u}_b = \frac{\psi b^2 \eta_t}{24 A h^2 \eta_b} g(t) \vec{r} e_r = \frac{1}{2} \psi C g(t) \vec{r} e_r \quad (7)$$

with C being the dimensionless tissue-blood resistance ratio. Note that $\vec{u}_b$ is the velocity of the blood relative to the tissue. In an external frame of reference, the tissue is also in motion, with velocity $\vec{u}_t$, and total blood velocity equals $\vec{u}_b + \vec{u}_t$. This suggests that because of growth pressure, blood in the capillaries will flow faster than the tissue itself, and, therefore, might accumulate at the periphery. To test that blood can indeed accumulate at the periphery because of tissue growth alone, we designed experimental conditions in which the effect of growth could be isolated, in particular from the pressure generated by heart contractions. It

has been long known that eggs in which the embryo dies can still display yolk sac growth (see Romanoff, 1960 and references therein). Therefore, we studied embryos ($n=3$) in which heart development was arrested by incision on the second day after incubation at HH11, before heart contraction was large enough to allow blood to remodel the capillary plexus.

As expected, in all cases, the yolk sac not only survives but also continues to grow for some time (Fig. 4A; Supplementary Movie S2). In the absence of heart-driven blood flow through the vitelline arteries, no remodeling of the primitive blood lattice is observed, and the majority of blood cells remain organized in aggregates. This confirms that shear stress caused by the blood circulation is crucial for vascular remodeling, as abundantly reported in literature (Lucitti et al., 2007; Garcia and Larina, 2014). In addition, we observe that yolk sac peripheral blood accumulation also occurs, which shows that heart contractions or subsequent blood flows are not required for peripheral blood accumulation.

We also quantified the growth rate using dPIV (Fig. 4B,C; Supplementary Movie S2). Note that the same controls, involving predictions of yolk sac expansion, were performed (Supplementary Movie S2). To compare the yolk sac growth of the control embryo with the incised embryo, we shifted the growth curve of the control to have the same initial growth rate for both cases. Note that at this time, the yolk sac area is also similar in both the control and the incised embryos. Our quantifications show that yolk sac growth was slower than in the control embryo: $g(t)$ slows down slower, but drops to zero and eventually becomes negative as growth stops and the yolk sac shrinks, while the control yolk sac continues to grow at a rate of 0.05 h^{-1} (Fig. 4D).

In addition, we measured the average width of the stripe of accumulated blood at the periphery in both control and incised eggs after 7.5 hr. We found that it was approximately 0.18 mm in the former while it was slightly smaller, approximately 0.16 mm, in the latter (Fig. 5A). Width of the blood accumulation was measured as described in the Experimental Procedures section.

Simulations

To test whether our theoretical model could qualitatively account for our experimental observations, we designed simulations of blood transport in the yolk sac tissue. For the sake of simplicity, we used an axisymmetric description and modeled the presence of free flowing blood in the yolk sac using the blood volume fraction in the tissue as a single local parameter $\alpha(r, t)$. In between the blood aggregates in the capillary plexus, blood cells and plasma can flow, which we refer to as free flowing blood. $\alpha(r, t)$ is defined as the average ratio between the volume of this free flowing blood and the total mesodermal tissue volume at distance r from the center of the embryo. In other words, if one draws a ring of infinitesimal width dr at radius r in the yolk sac, a fraction $\alpha(r, t)$ of its volume is occupied by blood. Because we defined ψ as the volume fraction available for blood transport between the blood aggregates in the capillary plexus, we will assume in the following that this space is filled with blood cells and plasma, and thus that $\psi = \alpha$.

We seek to describe the time evolution of the fraction of blood in the tissue $\alpha(r, t)$ in the growing area vasculosa of the yolk sac. Locally, α variation results from the transport of blood at velocity $\vec{u}_b + \vec{u}_t$, and from the creation rate of free flowing blood g_x . The source for blood cells in the free flowing blood are the blood

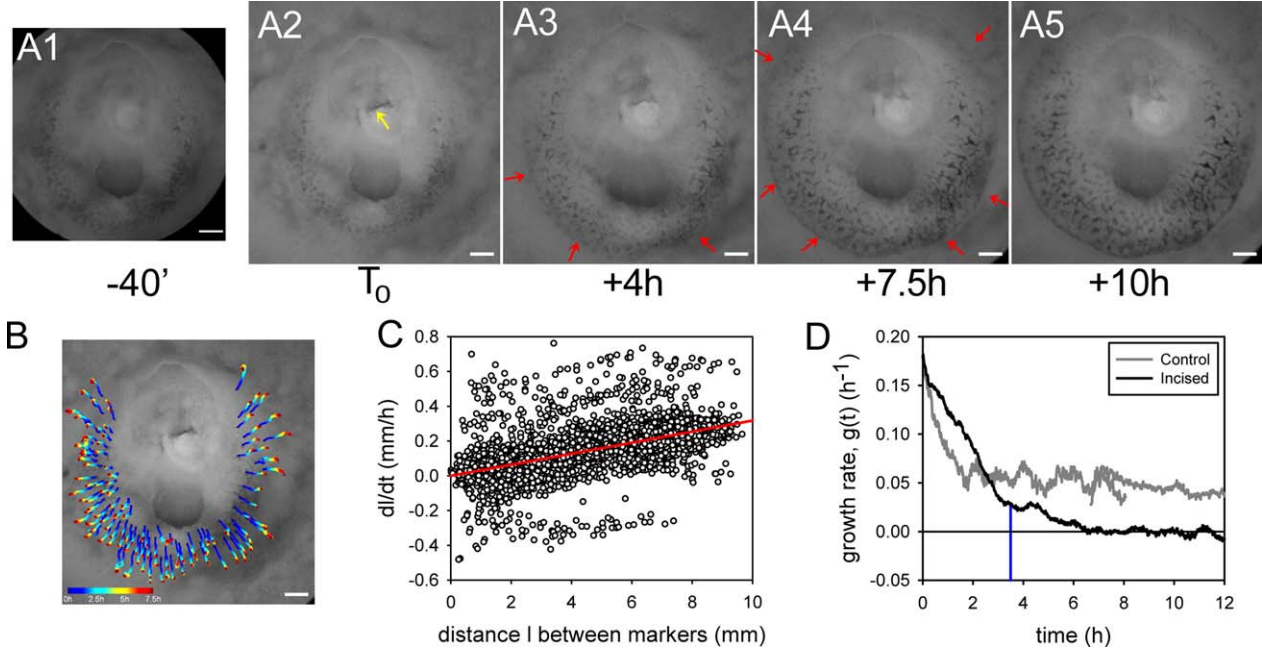


Fig. 4. **A:** Yolk sac development for the incised embryo. A1 shows the yolk sac 40 min before incision. A2–A5 show the yolk sac development after incision. The yellow arrow in A2 indicates the incision. While the yolk sac still grows, growth is slower than in the control, and eventually stops (A4–A5). Blood accumulation can yet be observed, after approximately 3.5 hr (A3–A4, red arrows). Extraction of 5 frames of a time lapse movie of 15.5 hr with frame rate 60 images/h (Supplementary Movie S2). Scale bar = 1 mm. **B:** Trajectories of the tracked points between 0 and 7.5 hr, superimposed on a frame of the timelapse video at T_0 . Growth causes radial displacements. **C:** $\Delta l/\Delta t$ as a function of $l(t)$ at $t = 2h22'$. $l(t)$ is the distance between each pair of tracked points. The associated growth rate equals $0.063h$ and the coefficient of determination r equals 0.35. **D:** Black line: Growth rate $g(t)$ extracted from linear fits of $\Delta l/\Delta t$ vs. $l(t)$ performed over the entire experiment. The growth rate becomes negative beyond 11.5 hr, as the embryo and yolk sac start to shrink. The vertical blue line indicates the onset of noticeable blood accumulation in the periphery of the area vasculosa. Gray line: Growth rate of the control embryo. The start of the control experiment is shifted such that the initial growth rates for control and incised embryos are the same.

aggregates. Blood plasma is produced by the endothelial cells of the anastomosed blood islands (Sabin, 1920; Ferkowicz and Yoder, 2005). The continuity equation for $\alpha(r, t)$ thus reads:

$$\frac{\partial \alpha}{\partial t} = \underbrace{g_\alpha}_{\text{new blood}} - \underbrace{\text{div}(\alpha(\vec{u}_b + \vec{u}_t))}_{\text{blood transport}}. \quad (8)$$

For the sake of simplicity, we chose the blood production, g_α to be independent of position (r) and time (t): $g_\alpha = cst > 0$, and the initial amount of free flowing blood to be zero: $\alpha(r, 0) = 0$. This is obviously a simplification, which we will address in the Discussion section. Blood transport is limited by the outer border of the area vasculosa of the yolk sac, located at radius $R_{AV}(t)$. Near the periphery, blood velocity $u_b(R_{AV}(t))$ is higher than tissue velocity; hence, blood is accumulating. During a short time dt , blood near the periphery travels a small distance $dr = u_b(R_{AV}(t))dt$. Therefore, during time dt , the blood located between the radii $R_{AV}(t) - dr$ and $R_{AV}(t)$ reaches the periphery and contributes to the accumulation. Then, accounting for a circular yolk sac with thickness H , and for an inner blood fraction in the tissue of $\alpha(R_{AV}(t), t)$, the volume dV of blood accumulating at the periphery during dt is:

$$dV = 2\pi R_{AV}(t) \times H \times u_b(R_{AV}(t))dt \times \alpha(R_{AV}(t), t). \quad (9)$$

The total volume of blood accumulated at the periphery at time t is then $V(t) = \int_0^t dV$.

In the simulations, the displacement of the tissue boundaries, the boundary between the area pellucida ($R_{ZP}(t)$) and the area

vasculosa and the boundary between the area vasculosa and the area vitellina ($R_{AV}(t)$) is set by the growth rate $g(t)$ measured earlier, using Equation [2]. The spatiotemporal evolution of $\alpha(r, t)$ is calculated within these moving boundaries using Equation [8] and Equation [7]. From there, the volume increase dV of blood accumulating at the periphery is obtained at each time step. Importantly, the value of the growth rate $g(t)$ is plugged directly from our experimental measurements.

The parameters used for the numerical resolution of the sinus vein width comprise the tissue–blood resistance ratio, C , the free blood creation rate, g_α , the tissue growth rate, $g(t)$, and the initial conditions for the radius at the yolk sac zona pelucida, $R_{ZP}(0)$, the radius of the yolk sac at the area vasculosa, $R_{AV}(0)$, and the free blood volume fraction, $\alpha(r, 0)$. The value for parameter $C = \frac{b^2 \eta_t}{12Ah^2 \eta_b}$ is estimated from the literature. There are no quantitative data available for the tissue velocity profile (the parameters h and A) nor for the viscosity in the shear layers of the mesodermal tissue (η_t). But blood pressure measured in the vitelline artery just when the primitive heart starts to contract at HH12 (Hu and Clark, 1989) provides us the order of magnitude of tissue pressure close to the embryo. Therefore, in Equation [5], we introduce the resistance to tissue flow: $\Omega_t = \frac{8\eta_t}{Ah^2}$.

Using the measured growth rate $g(t)$ in the control situation, the tissue resistance is tuned such that the temporal average of tissue pressure at $r = R_{ZP}$ is equal to 0.03 kPa, the diastolic pressure in the vitelline artery at HH12 (Hu and Clark 1989). The parameters related to the resistance to blood flow, blood viscosity (η_b) and the effective pore size (b), are also unknown at these

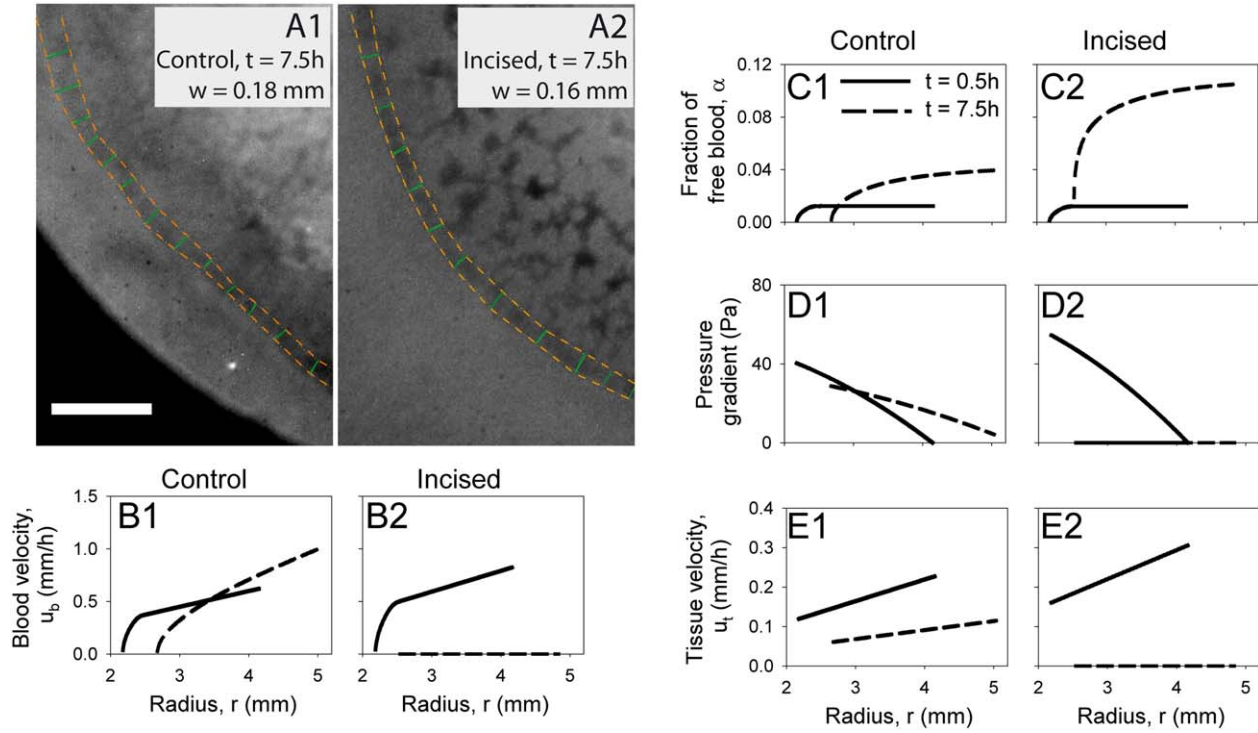


Fig. 5. **A:** Measurements of the width of the sinus vein at $t = 7.5$ h for the control (A1) and incised (A2) embryo. Scale bar = 1 mm. **B–E:** Model variables (blood velocity, u_b , B; tissue fraction of free flowing blood, α , C; tissue pressure, p_t , D; and tissue velocity, u_t , E) as a function of position in the yolk sac, r at $t = 0.5$ hr (solid line) and at $t = 7.5$ hr (dashed line). B1–E1: The variables using the experimental control growth curve. B2–E2 show the variables using the experimental growth curve for the incised embryo.

early embryonic stages, but their values are extrapolated from the available literature at later embryonic stages as argued in the supplemental materials.

The tissue growth rate, $g(t)$, the radii $R_{AV}(0)$ and $R_{ZP}(0)$ are measured (Figs. 2C, 4C). The parameters related to blood production, the initial free blood volume fraction, $\alpha(r, 0)$, is assumed to be zero and the blood production rate, g_x is tuned such that it simulates the measured width of the sinus vein as explained in the following.

Numerical simulations confirm that blood is indeed transported toward the periphery and that the total volume $V(t)$ accumulated at the periphery increases over time. We can estimate the typical width $w(t)$ of the peripheral ring of blood:

$$w(t) = \frac{V(t)}{2\pi HR_{AV}(t)}. \quad (10)$$

In the incised embryo, we measured that at 7.5 hr, the typical width of the peripheral blood accumulation was 0.16 mm (Fig. 5A2). In the model we calibrate g_x such that $w = 0.16$ mm after 7.5 hr growth of the incised embryo. Using the same parameters except for the experimentally measured growth rate, the model predicts that $w = 0.24$ mm in the control embryo as compared to a measured width of 0.18 mm (Fig. 5A1). The calibration of g_x yields a value of 0.027 h^{-1} . A table including all physical parameters and variables is provided in the Experimental Procedures section, together with details on the simulation model.

Figures 5B–E show the distribution of the blood and tissue velocity, the pressure gradient and the tissue fraction of blood for the simulations after 0.5 hr and 7.5 hr of growth for the control and incised embryo. The pressure that is built up (Fig. 5D) depends on the tissue growth rate and is around 0.04 kPa

at the start of the experiment and reduces to approximately 0.03 kPa at 7.5 hr for the control embryo and to 0 for the incised one.

The small pressure gradient is enough to generate a radial blood velocity in the order of mm/hr (Fig. 5B). There is only a small fraction of free flowing blood that flows at a very low speed, which explains why it is hard to observe the moving red blood cells experimentally.

To assess whether our estimate of blood production rate, g_x , is within a reasonable order of magnitude, we can estimate the fraction of red blood cells that accumulate in the sinus vein with respect to the estimated volume of the red blood cells in the blood aggregates, the source for the blood cells. Roughly, the blood aggregates at $t = 0$ of the incised embryo occupies $1/5$ of the total surface (Fig. 4A2). The estimated volume of blood cells in the aggregates equals thus: $1/5 \times \pi \times ((R_{AV}(0)^2) - (R_{ZP}(0)^2)) \times H = 0.25 \text{ mm}^3$. The simulated blood volume that accumulates in the sinus vein after 7.5 hr equals 0.28 mm^3 . Assuming a hematocrit between 5 and 15%, the accumulated volume of red blood cells in the sinus vein is in between 0.013 and 0.041 mm^3 , which represents only 5 to 16% of the blood aggregates. This is consistent with the fact that the blood aggregates do not change much their shape, nor their grey level (which relates to the amount of blood cells in the tissue) in the course of growth of the incised embryos (Fig. 4A; Supplementary Movie S2).

In addition, the blood production rate $g_x = 0.027 \text{ h}^{-1}$ is in the same range as our measurements for the tissue growth rate, $g(t)$ (Fig. 4D). Overall, the simulations show that growth-induced tissue pressure can indeed drive an accumulation of blood in the periphery of the yolk sac area vasculosa commensurate with that observed in vivo.

Discussion

The results presented in this study show that heart-induced flow in the yolk sac is not required to accumulate blood in periphery of the yolk sac area vasculosa, which begins before circulation. We proposed that a mechanical pressure gradient caused by homogeneous tissue growth might drive the radial flow of blood that eventually leads to peripheral blood accumulation. This hypothesis is supported by experiments in which the heart is arrested by incision before circulation starts: the yolk sac still grows and the accumulation of blood is still observed. Based on quantitative growth measurements, a simple theoretical model was built that predicts the peripheral accumulation of blood due to tissue growth pressure.

The theoretical model reveals the basic, qualitative mechanisms of peripheral blood accumulation. In the following, we discuss our theoretical assumptions and results and point out limitations.

Spatially Homogeneous Growth

Our analyses show that the growth of the yolk sac mesoderm is well described by a spatially homogeneous growth (Figs. 2C, 4C; Supplementary Movies S1 and S2). This suggests that the cells are proliferating at the same rate, independent of their location. Phosphohistone H3 staining, marking all mitotic cells, indicates that at E3 and at E5 yolk sac mitotic cells are distributed homogeneously in the yolk sac (Fig. 6A,B in Nagai and Sheng, 2008). This shows that the yolk sac is growing spatially homogeneous at these stages. Our analyses suggest that it is also the case at earlier stages (E2, HH11 up to HH14). This likely suggests that intussusceptive growth (Djonov et al., 2000) in which the capillary plexus expands by insertion of numerous transcapillary mesodermal tissue pillars is playing an important role, besides sprouting angiogenesis.

Mesoderm Grows Between Static Ecto and Endodermal Layers

Tissue pressure builds up in the mesoderm, because we assume it is growing between static endo- and ectodermal layers. At stage HH26/27 (E5) (Sheng, 2010), the area vitellina, which contains only the ecto- and endoderm, covers most of the yolk. At the same time, the area vasculosa, the part of the yolk sac in which the mesoderm has entered, reaches the equator of the yolk. This suggests that from this stage on, the mesoderm is indeed growing in between the static endo- and ectoderm. We think it is reasonable to assume that the mesoderm also grows at earlier stages between the static endo- and ectodermal layers. However, the ectoderm and endoderm are possibly still growing in the area vasculosa. In that case, tissue pressure should still build up due to friction of the endoderm with the yolk, and of the ectoderm with the vitelline membrane.

Blood Transport Takes Place in the Capillary Plexus That Reaches Out to the Periphery of the Area Vasculosa

Independent marking at stage HH10 of globin in the blood cells, the blood precursor cells, and endothelial cells together with the blood cells (Fig. 2 in Sheng, 2010), reveals that blood cells are present close to the embryo, but not yet in the periphery, which

we also observe in our experiments. However, endothelial cells are already present up to the periphery of the area vasculosa. Thus, at the start of our experiment (at HH11) blood can be transported to the periphery through the capillary plexus.

Production of Free Flowing Blood, g_x

Free blood is produced by “liquefaction” of the blood aggregates: red blood cell dissociation from the aggregates that are carried away by newly produced plasma (Sabin, 1920). Blood aggregates are distributed in a U shaped region in the area vasculosa of the yolk sac. Progressive smaller blood islands are observed close to the embryo and expression of hemoglobin occurs initially in the more distal blood islands (Sabin, 1920; Sheng, 2010). This is proposed to reflect the progressive phase of differentiation (Sabin, 1920; Eichmann et al., 2005; Sheng, 2010). We also observe such a gradient in our experiments (Figs. 2A2, 4A2). Therefore, a more realistic description might include a spatio-temporal dependence of free flowing blood production, g_x . In addition, g_x might also depend on shear forces, which could contribute to the dissociation of red blood cells from the aggregates. Finally, g_x might be different in control and incised embryos. However, in the absence of quantitative data, we settled for straightforwardness with a homogeneous and constant production of free flowing blood.

Width of the Sinus Vein as a Measure for Volume Accumulation Due to Tissue Growth

We calibrate the production of free flowing blood (g_x) with the width of the sinus vein of the incised embryo after 7.5 hr of growth (see Fig. 5A2). We are aware that the observed width is not perfectly reflecting the amount of blood accumulated in the periphery of the area vasculosa. Besides the subjective measurement of the width, the grey level along the sinus vein is not constant, indicating that not the same amount of blood is accumulated at all positions in the sinus vein. However, sensitivity analyses of the model (Fig. 6A) show that even when we are 50% mistaken with our estimation of the observed sinus vein width, calibration of g_x would yield a 50% increase / decrease as well, thus remaining within the same order of magnitude.

Using the same values for all parameters except for the experimentally measured tissue growth rate, our model predicts a width of 0.24 mm for the control after 7.5 hr of growth. In the control experiment, we measure a width of 0.18 mm (Fig. 5A). It should be noted, however, that the simulated width is the width predicted without taking into account the pressure build up by heart contractions and the circulating blood. At $t = 7.5h$ blood started already to circulate (Fig. 2A4). Therefore, the previously accumulated blood is flowing elsewhere in the circulation, and shear forces remodeling the sinus vein contribute to the establishment of the sinus vein width. The simulated width of the sinus vein reflects thus the volume accumulation as if there was only tissue growth pressure.

Initial Conditions

At the start of our simulations we set the fraction of free flowing blood to zero ($\alpha(r,0) = 0$). This suggests that the endothelial cells of the capillary plexus tightly enclose the blood aggregates. That is to say the endothelial cells of the blood islands have just anastomosed. Blood islands anastomose at stage HH9–HH10 (Sheng,

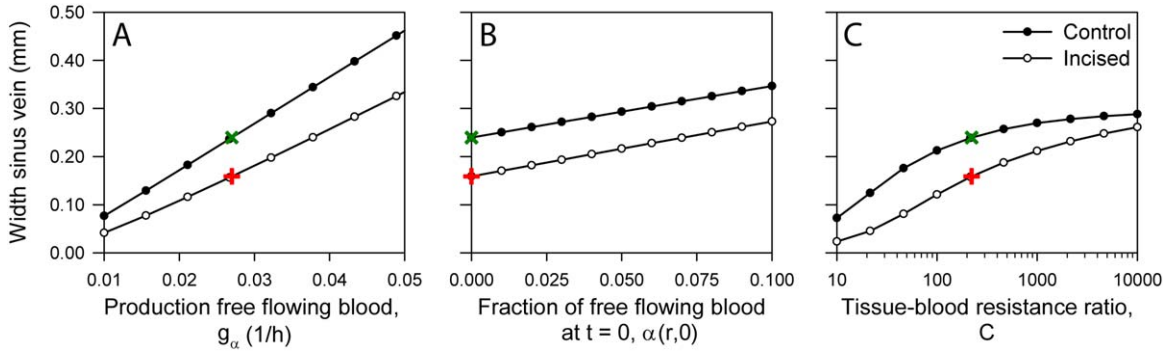


Fig. 6. Sensitivity analyses for the production of free flowing blood, g_x (A), for the fraction of free flowing blood at $t=0$, $\alpha(r, 0)$ (B), and for the tissue-blood resistance ratio, C (C). Filled circles show simulations with the control growth curve and open circles show simulations with the growth curve for the incised embryo. g_x is calibrated to the measured width of the sinus vein of the incised embryo (red +). Using the same parameters except for the growth rate, the predicted width, a measure for blood volume accumulation solely due to tissue growth, for the sinus vein in the control is higher (green X).

2010). At the onset of our experiments (HH11), we expect that mesodermal tissue growth and production of free flowing blood has already contributed to the accumulation of blood in the periphery of the area vasculosa. In the absence of precise data concerning the presence of free flowing blood ($\alpha(r, 0)$) at the onset of our experiments, we carried out a sensitivity analysis for both control and incised embryos (Fig. 6B). It suggests that increasing $\alpha(r, 0)$ requires adjusting our calibration of g_x , yet within a relatively small range (Fig. 6A). Similarly for the initial value for the accumulated peripheral volume expressed in sinus vein width. Initial values in the order of tenth of micrometers would require only a small adjustment of g_x .

Resistance to Tissue Flow vs. Blood Flow

The parameters related to resistance to tissue flow (Ω_t) comprise the velocity profile ratio, A , the total height of the mesoderm that encounters shear, h , the mesoderm viscosity in the shear layers at the ecto- and endoderm, η_t . To our knowledge, no measurements are available in the current literature that allow us to estimate the values for these parameters. Therefore, we tuned the tissue resistance such that the temporal average of tissue pressure at $r=R_{ZP}$ is equal to the diastolic pressure in the vitelline artery when the heart starts to contract at HH12 (Hu and Clark, 1989). At this stage, the heart is still a tube composed of a three- to five-cell myocardial mantle surrounding a cuff of cardiac jelly and a one-cell layer of endocardium (Hu and Clark, 1989).

At HH12, Hu and Clark measured in the vitelline artery a diastolic pressure of 0.03 kPa and a systolic pressure of 0.04 kPa. The diastolic and systolic pressure increase up to 0.05 kPa and 0.07 kPa resp. at stage HH14. Because the vitelline arterial pressure is the sum of the tissue pressure and the pressure build up by the heart, we assumed that the tissue pressure during our experiment (HH1–HH14) should not be larger than the vitelline artery diastolic pressure at HH12. If we would assume a parabolic velocity profile, with $A=2/3$ and $h=H$, the estimated global tissue viscosity would be 43 Pa·s. The viscosity of the mesoderm would then be more than 2 orders of magnitude lower than the viscosity (10⁴ Pa·s) estimated for cell aggregates of the heart, liver, and neural retina of 3.5–6 days chicken embryos (Forgacs et al., 1998).

However, at low pressure gradients, the mesoderm is likely not sheared over the full height and the velocity profile is more flat. That is, the mesoderm is more rigid because the friction within

the mesodermal layer is larger than at the ecto and endodermal interfaces. In case of a perfect plug flow, with $A=1$ and $h \rightarrow 0$, the pressure gradient diverges for a given velocity and viscosity. Thus, we can assume that the friction between the mesodermal layer and the ecto- and endodermal membranes is very small, with a viscosity in the thin shear layers small enough to reach a tissue pressure near the embryo of 0.03 kPa. Possibly, the thin shear layers solely consist of low viscous extracellular matrix.

Alternatively, we could have modeled the mesoderm as a solid growing disc with a small friction between the ecto- and endodermal layers. This would result in a tissue stress gradient, which is analogous to the tissue pressure gradient applied in our viscous approach.

The parameters related to the resistance to blood flow are the blood viscosity η_b and the effective value for the pore size b . As described in the Supplementary Materials, the values for blood viscosity are quite insecure because direct measurements lack at these early developmental stages.

In the model, blood transport depends on the ratio C between the two resistances. Yet, Figure 6C shows that the width of the sinus vein is not dramatically affected when this ratio is modified, even by a factor 10 and even when the mesoderm approaches a plug flow ($h \rightarrow 0$) and C becomes very large.

The porous model assumes a constant effective value for the pore size b , being the smallest length scale in which the blood flows, which we assume to be the capillary diameter. The pore size b could depend on the transmural pressure set by the tissue pressure, p_t , therefore it is likely that a more stressed region will have less space for flow. This feedback might have interesting consequences for the remodeling process. Indeed, a gradient of b would suggest a gradient of hydrodynamic resistance in the yolk sac, thus a higher resistance in the proximal region and a lower resistance in the distal region. This would be in agreement with previous observations of higher vascular densities in the distal yolk sac (Al-Kilani et al., 2008). It might also explain why blood flow does not shunt the distal yolk sac, going directly from the cardiac vitelline outputs to the cardiac cranial and caudal inputs through the proximal yolk sac (Al-Kilani et al., 2008).

Thickening of the Mesodermal Tissue

In the model, we neglect the thickening of the mesodermal tissue. Measurements on the evolution of the thickness lack. If the

TABLE 1. Variables and Parameters Used in the Model^a

	Symbol	Relation	Value
Radial coordinate	r		
Time	t		
Tissue velocity	$u_t(r,t)$		
Tissue pressure	$p_t(r,t)$		
Blood velocity	$u_b(r,t)$		
Free blood volume fraction in the mesentery	$\alpha(r,t)$		
Transport space fraction in the mesentery	$\psi(r,t)$	$=\alpha(r,t)$	
Tissue growth rate	$g(t)$		Fig. 2D / 4D
Yolk sac radius at zona pelucida at $t=0$	$R_{ZP}(0)$		2.1 mm
Yolk sac radius at sinus vein at $t=0$	$R_{AV}(0)$		4.0 mm
Resistance to tissue flow	Ω_t	$= \frac{8\eta_t}{Ah^2}$	$4.3 \cdot 10^5 \frac{\text{Pa}\cdot\text{s}}{\text{m}^2}$
Tissue velocity profile ratio	A	$= \frac{3H-h}{3H}$	$[\frac{2}{3}, 1]$
Yolk sac mesodermal thickness	H		35 μm
Height of the mesoderm on which there is a shear	h		$\langle 0, H \rangle$
Tissue viscosity in the shear layers	η_t		$\langle 0, 43\text{Pa}\cdot\text{s} \rangle$
Permeability	κ	$= \frac{\psi b^2}{96}$	
Effective pore size	b		10 μm
Blood viscosity	η_b		$2 \cdot 10 \text{ Pa}\cdot\text{s}$
Tissue-blood resistance ratio	C	$= \frac{\Omega_t b^2}{96\eta_b}$	222
Free blood volume fraction at $t=0$	$\alpha(r, 0)$		0
Free blood creation rate	g_z		0.027 h^{-1}

^aThe values or ranges of values of the parameters are discussed in the Discussion section and in the Supplementary Materials.

tissue becomes thicker, it would suggest more growth than that we account for in our 2D approach. Due to transverse resistance, this extra growth would result locally in a larger tissue pressure. Assuming that the tissue close to the embryo would grow thicker than at the periphery would create a larger pressure gradient than expected solely from the 2D approach. In the same time, with a transverse parabolic flow profile, increasing tissue thickness would decrease the resistance to tissue flow, decreasing the pressure gradient. However, we expect that this latter effect is negligible when the tissue flow approaches a plug profile.

Conclusion

While these remarks show the quantitative limits of the theoretical model, we believe that it provides, together with the experiments, a proof of principle that blood accumulation at the periphery before perfusion is driven by a gradient of mechanical stress caused by tissue growth. Altogether, the results presented here tend to draw a global picture of vascular morphogenesis in the chicken yolk sac: before perfusion, tissue pressure caused by growth initiates a radial blood movement, and blood accumulates at the yolk sac periphery. Upon perfusion, increased shear leads to the remodelling of the capillary plexus into a hierarchised network. Obviously, the peripheral accumulation of blood stops as soon as the venous return is established, because blood can flow back to the heart. The radial pressure gradient might also cause a permeability gradient, which might prevent the blood flow to shunt the distal regions of yolk sac.

It also provides a general temporal picture: The capillary plexus forms and free flowing blood starts to be produced while the tissue is still growing. The pressure gradient due to growth pushes

the blood outward, and starts the accumulation in periphery of the area vasculosa of the yolk sac. These movements can be taken over by the increasing efficiency of the heart, to allow blood to circulate, while finally the yolk sac growth stops and the associated pressure gradient vanishes. The timing of both events has to be well tuned as in a successful relay race. Such a precise timing of development of peripheral vascular networks and its connection to the heart might be a general developmental strategy.

In conclusion, this work constitutes a first step toward the understanding of how tissue growth pressure can drive blood flow in the yolk sac mesoderm. It introduces basic ideas, directions, and tools for future investigations. It also illustrates that the mechanical environment beyond mere hemodynamics might play a role in patterning the primitive vascular system, and in particular that tissue growth can cause pressure gradients large enough to drive blood flows. While the model system chosen here has a very basic geometry and, therefore, simple flow patterns, such a mechanical feedback between growth and vascular morphogenesis is likely to underlie, in more subtle geometries, the formation of complex vascular patterns.

Experimental Procedures

Experimental Preparation

Fertile chick eggs (Lohmann Brown eggs) were obtained from CAIF (47 rue d'Aulnay - Moulin Fourcon - 91180 Saint Germain Les Arpajon - France) and stored at 15 °C. After 50 hr of incubation at 37 °C a window in the egg shell was cut and the egg was placed in a mini-incubator (minitube HT300 controller with two heating plates) for timelapse observation (macrofluvo Leica Z16 equipped with a Fastcam SA3, Photron camera). Images were

taken using epi-illumination with a blue filter (Schott KL 1500 LED) with a 1-min interval. For the incised embryos, heart development was arrested by incision of the embryo before placing the egg in the mini-incubator. Before incision we verified if the embryo was normally developed up to stage HH10–HH11. At this stage the heart is still a single tube and not yet fully developed. In our experimental settings, we cannot distinguish the primary heart, but with fine surgical scissors we cut the embryo in a transverse plane in two pieces at the level of where the heart would develop, leaving the yolk sac around intact. Control embryos were left intact.

Data Analysis

To measure the velocity field of the yolk sac mesoderm, we used the moving blood islands and vascular bifurcations as markers. In the first image (at $t=0$), for both the control and the incised embryo, the blood islands in the yolk sac are marked manually using the point selection tool of imageJ (Rasband, 1997–2016). dPIV imageJ plugin (Cardoso, 2013) was used to obtain the position of our markers in each subsequent image (the time interval between images equals 1'). Blood islands were tracked until $t=15h59'$ for the incised embryo and until $t=8h34'$ for the control embryo. In the yolk sac of the control embryo, there is a transition from blood islands to the vascular network. Therefore, we marked manually the bifurcations at $t=6h41'$ and used the dPIV imageJ plugin to determine the position of the bifurcations in each subsequent image up to $t=18h56'$.

To estimate the growth rate $g(t)$, we first computed the distance l at time t between each pair of markers. Then we calculated the distance increase rate dl/dt at time t on a $\Delta t=30'$ window: $dl/dt \simeq \Delta l/\Delta t = [l(t+15) - l(t-15)]/30$. Growth g at time t was finally estimated from the slope of the linear regression line between $l(t)$ and dl/dt , using Equation 3 in the Results section. In addition to the slope, the coefficient of determination (r^2) of the regression line was calculated. This was repeated for all time points throughout the time lapse observation.

To double check that homogeneous growth is a good approximation, we manually marked several points on the moving front of the mesoderm (the border between the area vasculosa and area vitellina). Assuming that the center of mass of these points moves the same as the center of mass of the dPIV trajectories, we could, by using Equation [3] in the Results section and our estimate of the growth rate $g(t)$, predict the position of the moving front of the mesoderm at all time points in both control and incised embryos (see Supplementary Movies S1 and S2).

The width of blood accumulation at the periphery of area vasculosa was measured for both control and incised embryos. This is illustrated for a small section in Figure 5A. The coordinates of the inner and outer boundaries were obtained by eye (yellow dotted lines in Fig. 5A) using the polygon tool of imageJ (Rasband, 1997). With Matlab, the shortest distance from each point on the outer polygon line to the inner polygon line was obtained (green bars in Fig. 5A). Width was then calculated averaging the shortest distances.

Model

The transport model for α writes:

$$\frac{\partial \alpha}{\partial t} = g_\alpha - \text{div}(\alpha(\vec{u}_b + \vec{u}_t)). \quad (11)$$

$C = \frac{b^2 \eta_t}{12 A h^2 \eta_b} = \frac{\Omega_t b^2}{96 \eta_b}$, the ratio of the resistance against tissue flow vs. the resistance against blood flow. We have $u_t = gr/2$ and $u_b = C\alpha gr/2$. Therefore, the transport equation writes:

$$\frac{\partial \alpha}{\partial t} = g_\alpha - \frac{1}{2} \text{div}((\alpha + C\alpha^2)gr\vec{e}_r) \quad (12)$$

which leads to:

$$\frac{\partial \alpha}{\partial t} = g_\alpha - (\alpha + C\alpha^2)g - \left(\alpha C + \frac{1}{2}\right)gr \frac{\partial \alpha}{\partial r} \quad (13)$$

which we solve numerically on growing boundaries, $R_{ZP}(t)$ and $R_{AV}(t)$. Boundaries displacements are simply fixed by the tissue velocity u_r . All parameters can be found in Table 1.

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