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Cardiovascular Development and Angiogenesis in the Early Vertebrate Embryo

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Abstract—Embryonic cardiovascular physiology (as opposed to that of the more developed fetus) is being more closely examined by developmental physiologists to explore the onset of cardiovascular function and its regulation, as opposed to the later maturation of these processes as is typically examined in fetal mammal models. As our understanding of embryonic physiology grows, the dogma that the early embryonic heart serves the same convective bulk transport role that it does in the fetal and adult heart is being carefully evaluated. Experimental approaches have involved genetic, surgical and environmental manipulation, and have revealed that blood flow generated by the early embryonic heart is *not* required for bulk transport of respiratory gases, nutrients, and wastes. Rather, the very small size of the typical vertebrate embryo enables this critical transport function to be achieved by simple diffusion alone. Surprisingly, however, the heart begins to beat (and so expend valuable energy) well before convective blood circulation is actually required. This review postulates that angiogenesis may be a driving factor for the "early" beat of the heart. Recent experiments examining the effect of increased blood pressure and flow pulsatility on proximal blood vessel development offer initial support for the "synangiotropy" hypothesis, namely that the onset of heart beat occurs synchronously with the need for peripheral angiogenesis. Yet, the complexity of the patterns of angiogenesis (regional variations of opposite sign) suggests that we have much more to be learned about the relationship between angiogenesis and the circulation in vertebrate embryos.

Keywords—Angiogenesis, Embryo, Heart, Vasculature.

INTRODUCTION

It has been noted throughout the embryological study of vertebrates that the heart is the first organ to begin functioning in the vertebrate embryo. Indeed, in his *Historia Animālium*, Aristotle (384BC-322BC) noted the pulsating red spot at the base of the air cell of a chicken egg laid only 2 days previously. Clearly, the heart is beating and generating a blood flow well before almost any other organ has begun to function, or even begun to form in most instances. Development of the lungs, kidneys and digestive system, for example, all lag by several days behind the onset of heart beat in avian and mammalian embryos. This early activity of the heart is reflected in the development tables of common developmental models such as the zebrafish *Danio rerio*, the frog *Xenopus laevis*, the chicken *Gallus gallus*, and the mouse *Mus musculus*.

Typically, when one considers the function of the early embryonic heart, there is generally an interpolation of function(s) occurring in the adult animal back to the early embryonic condition. Semantics loom large, however, when we erroneously describe the "function" of the early embryonic heart, as if there were only a single function of blood circulation. Indeed, we know from studies of adult animals that the convection of blood results in transport of:

1. *respiratory gases* (O_2 into the animal, and CO_2 outwards)
2. *nutrients* (amino acids, carbohydrates, fats and other materials used in structural assembly)
3. *wastes* (nitrogenous and other wastes products created by cellular metabolism)
4. *regulators* (primarily hormones, but also molecular chaperones, *etc.*)
5. *heat* (at least in endothermic vertebrates—birds and mammals—an important role of blood circulation is the convective transport of heated blood to sites where heat offloading to the environment can occur.

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6. *pressure* (at least throughout the arterial side of the circulation, with pressures falling in the smaller distribution vessels and the venous side).

Does the embryonic heart perform all of these functions ascribed to the adult heart? Numerous studies have now demonstrated the peril of assuming that adult heart functions equally apply to the early embryo, and vice versa.[7,14,16,73,83] Indeed, the purpose of this review is to propose that angiogenesis—the formation of new blood vessels—is inextricably linked to early embryonic heart function, and perhaps vessel creation actually precedes the many transport functions of the heart listed above.

To provide the necessary contextual background to evaluate the assertion that angiogenesis is a key function of the early embryonic heart, it is necessary to first briefly explore the morphological and physiological development and regulation of the early embryonic cardiovascular system. Important to note is that a great deal of prenatal morphology and physiology has focused on the mammalian fetus, but the exploration of truly embryonic cardiovascular function has required the use of alternative animal models, as will be described. This review then moves on to the process of angiogenesis and finally addresses the putative role(s) of the developing heart, and the blood convection it produces, in the process of angiogenesis.

THE EMBRYONIC CIRCULATION

The general morphological and physiological development of the heart of a variety of vertebrates has been described in numerous textbooks and treatises. For most recent discussion, the reader is referred to the following studies on popular vertebrate animal models for cardiovascular development, which can provide an entry into the voluminous literature: zebrafish, *Danio rerio*[3,41,45,60]; African clawed frog, *Xenopus laevis*[49,62]; chicken, *Gallus gallus*[51,75] and see numerous articles in Ref. 10; mouse, *Mus musculus*.[64,78]

Morphological Development

The heart anlage derives from two lateral masses of splanchnic mesoderm on either side of the embryo's central longitudinal axis. These masses migrate towards the dorsal midline and subsequently merge to form the heart tube. These movements are under the control of the *Mil* gene and sphingosine-1-phosphate, which is a lysosphingolipid that acts as a *Mil* ligand.[55] Weak, irregular peristaltic contractions of the heart tube begin at this time, which is typically around 24 h in the zebrafish embryo and 42–46 h in the chicken embryo. Erythropoiesis also occurs coincident with heartbeat, as evident in small numbers of immature red cells in the dilute plasma within the forming vessels.[77] The circulation lacking discrete one-way valves at this point in development, these peristaltic movements of the forming heart walls generate a futile back-and-forth motion of the forming blood, although the developing endocardial cushion soon provides some valve-like function. Within hours the heart migrates anterio-posteriorly and the linear tube shape folds into a helically wound loop formed into an S-shaped curve.[3,80] A constriction subsequently develops at the atrio-ventricular boundary, creating the atrio-ventricular canal, and the main body of the heart continues migrating until the atrium is anterior to the ventricle. Discrete contractions of the atrium and ventricle replace peristaltic contraction as the primary mechanism for propelling blood in a one-way direction, aided by the appearance of both cardiac and peripheral vascular valves that reduce retrograde blood flow following cardiac contraction. In those vertebrates with a completely divided ventricle, the intraventricular septum arises from the floor of the single primitive ventricle and grows anteriorly to close off the ventricle into left and right chambers, congruent with the appearance of the great arches, which have arisen from neural crest tissue.[63] Division of the atrium into left and right atria (except in fishes) is a slightly more complex process than ventricular division, and involves the fusion of multiple structures, including the septa primum and secundum. The heart's conduction system is functional at this point in development, as evident from the ability to record an electrocardiogram with the essential electrical components of the adult heart, at about the same time that chamber formation of the heart is complete—for example about 50–52 h (HH 13, 20 somites) in the chicken embryo.[70,87]

Once chamber formation is complete, hyperplastic growth of the heart transitions to hypertrophic growth, and the main changes that occur are in the regulation and subsequent physiological performance of the heart, as will now be considered.

Physiological Performance and Regulation of the Embryonic Heart

Great variation exists in the "plumbing" of the cardiovascular systems of the vertebrates, and this is reflected in the physiological performance of their hearts and vasculature. The adult fish heart is essentially a two chamber heart with atrium and ventricle in series, producing a relatively high blood pressure to

which the gas exchange organ (the gills) is exposed. In amphibians and most reptiles, there are two atria and a variety of ventricular arrangements, some of which can allow for the development of high systemic pressure but low pulmonary pressure even as both "left-to-right" and "right-to-left" shunts can develop (e.g. varanid lizards and python snakes). In the crocodilians, the ventricle is completely divided, but the capability for blood shunting between pulmonary and systemic sides is preserved because of the central vascular arrangement. In the homeotherms (birds and mammals), the heart is essentially a low pressure pulmonary pump (the right side of the heart) and a high pressure systemic pump (the left side of the heart) located in series. Much has been written not only on the forms of the adult heart, but also on the presumed evolution of these arrangements, and the reader is directed to these representative reviews for an entry into the voluminous literature.[2,9,11,12,21,26,28,39,69]

Despite this fascinating evolutionary divergence of the cardiovascular plan in *adult* vertebrates, the situation is quite different when considering an embryonic perspective. The development of all vertebrate hearts follows a surprisingly similar common plan because of the highly conserved nature of the genetic regulation of the generation of the cardiac and vascular tissues from the pluripotent cells of early embryo. Thus, the basic description of a tube heart that grows into an S-fold, followed by additional condensation and chamber formation, as described in "Introduction" section above, generally holds for all vertebrate embryos. Not surprisingly, then, the *physiological performance* of the circulation of various early vertebrate embryos is also very similar. Indeed, we can meaningfully refer to the "vertebrate heart" in early embryonic stages, a construct that would be meaningless if applied to adults of any or all of the five different vertebrate classes.

How, then, does the embryonic vertebrate heart begin to function, and what general patterns can be discerned? Briefly, the rate of beat of the heart, having progressed beyond the tube heart stage, is initially quite variable between species,[15,68] ranging from less than 50 beats/min in poikilotherms ("cold-blooded" animals) at moderate temperatures (e.g. frogs) to well beyond 150–200 beats/min in birds and mammals. In many species, heart rate increases sharply in the first hours and days of beating, eventually beginning to decrease once again, perhaps reflecting standard allometric scaling relationships within the rapidly growing embryo, in which larger animals, including larger embryos, have lower heart rates than smaller ones.[15] In others, heart rate declines or stays level during early development. In fact, heart rate *per se* indicates little of cardiac function, requiring additional factors such as blood velocity, stroke volume and/or cardiac output to be assessed. Blood flow in the embryonic vertebrate heart coincides with blood pressure generation, and becomes more effective once one-way valves have formed at the base of the outflow vessels.

Blood pressure generation coincides with the onset of pulsatility, and by the formation of the compact, folded heart and its valves. Systolic pressures begin in the range of 1–3 mmHg, as measured directly either by microelectrodes inserted into the proximal vasculature or the heart itself or through cannulation of peripheral vessels—e.g. mouse[46,52]; chicken,[22,44,61,81]; frog[32,42,43] and zebrafish.[60,73]

Simultaneous measurements of both arterial blood pressure *and* cardiac output (typically by visualization of the cardiac dimensions during the cardiac cycle with subsequent calculation of cardiac volume changes—Bagatto and Burggren[3]) have been made in the very early stages of zebrafish, frog and chicken embryos, and these parameters have been calculated in early mammalian embryos. Generally, the overall level of blood pressure, cardiac output, peripheral resistance and their subsequent rate of increase during the first days of heart beat are all surprisingly similar in the zebrafish,[74] *Xenopus*,[32,42,43] the bullfrog[72] and the chicken.[10–19]

The physiological regulation of the heart and peripheral vessels is a key aspect of the overall mature of the cardiovascular system, but has been problematic to study in vertebrate embryos. In mammals, the embryo and then subsequently the fetus are well protected by, and deeply embedded within, the placental/uterine environment. This provides a highly nurturing environment for the developing mammal, but greatly confounds experimental measurements and manipulations. For this reason, most mammalian fetal physiology is focused on the latter phases of gestation which, in many instances, is beyond the first appearance of critical elements of cardiovascular regulation. Consequently, a great deal of what we know about the *onset* (as opposed to the *maturation*) of cardiovascular regulation has been derived from vertebrates that lay eggs or have free living larval forms, which not only makes the embryo more accessible to the researcher, but also imposes the need for early regulatory abilities to combat environmental fluctuations that are imposed directly on embryo lacking maternal mitigation.

Cardiovascular regulation in early embryonic stages has been explored primarily in the larvae of fishes and amphibians and in avian embryos. Typically, experiments involve the use of pharmacological agonists and agonists, which can be useful in teasing apart endocrine from neural mechanisms, and generally elucidating the onset of cardiovascular regulation. Several studies have generated embryonic "timelines" that depict the first onset and subsequent

maturation of adrenergic and cholinergic control of chronotropic and inotropic functions in vertebrate embryos.[8,10,22] Certainly general statements can be extracted from these studies—e.g. adrenergic cardiovascular regulation, as evident from cardiac neural tonus, appears before cholinergic regulation.[20,22,23] Importantly, however, the detailed ontogenetic patterns of onset appear to be species-specific. From common avian modes, for example, we can see that the general mechanisms for cardiovascular regulation occur later in the chicken (*Gallus gallus*) than the emu (*Dromaius novaehollandiae*, also referred to as *Dromiceius novaehollandiae*) (Fig. 1). Moreover, there a difference in the *order of onset* of functional baroreflexes, chemoreflexive cardiovascular control and the onset of vagal tonic regulation of the cardiovascular system of the chicken compared to the emu.

Cardiac regulation in adults occurs through three general mechanisms: endocrine, neural, and intrinsic (Frank-Starling mechanisms, otherwise known as Starling's Law of the Heart). While the onset and maturation of endocrine and neural regulation of the cardiovascular system have been investigated, as discussed above, the maturation of intrinsic mechanisms centering around Starling's Law of the Heart remain to be explored in any detail in fetal vertebrates, and remains virtually unexplored in early vertebrate embryos. Early studies on chicken embryos and amphibian larvae have examined blood volume effects on hemodynamics (see Refs. 11, 43, 88 for additional references), but a study dedicated to the maturation, as opposed to the presence, of the Frank-Starling mechanism in embryo myocytes will prove highly fruitful.

WHAT IS THE "REAL" PURPOSE OF THE EARLY EMBRYONIC CIRCULATION?

The traditional view of the early embryonic heart, to the relatively few that have actually reflected on this subject, is that it provides precisely the same functions as in the adult heart, namely to generate blood flow which in turn transports respiratory gases, nutrients, wastes, regulators, heat and pressure (see above). This view has prevailed as dogma, despite very little evidence that these well-established functions for the adult condition can or should be interpolated back to the early embryo. Indeed, evidence in the last decade or so has begun to unravel this dogma as it becomes evident not only what the early embryonic heart is for, but importantly what it *isn't* for. And what the early embryonic heart isn't needed for is the bulk transport of materials by convection! Essentially, despite the intrinsically slow nature of diffusion through biological tissues, early vertebrate embryos are so small that sufficient respiratory gases, nutrients and wastes can be exchanged by diffusion alone. This is posited from theoretical calculations of the size of an hypothetical spherical animal given a specific set of variables related to O_2 diffusion.[7,24,35] Thus, Eq. (1) specifies the necessary driving pressure for diffusion (ΔPO_2) from external environment to the interior of a spherical animal for a given tissue oxygen consumption (MO_2), Krogh's diffusion coefficient for oxygen (KO_2), and diffusion distance (r, radius):

$$\Delta PO_2 = \frac{r^2}{6}\frac{MO_2}{KO_2} \quad (1)$$

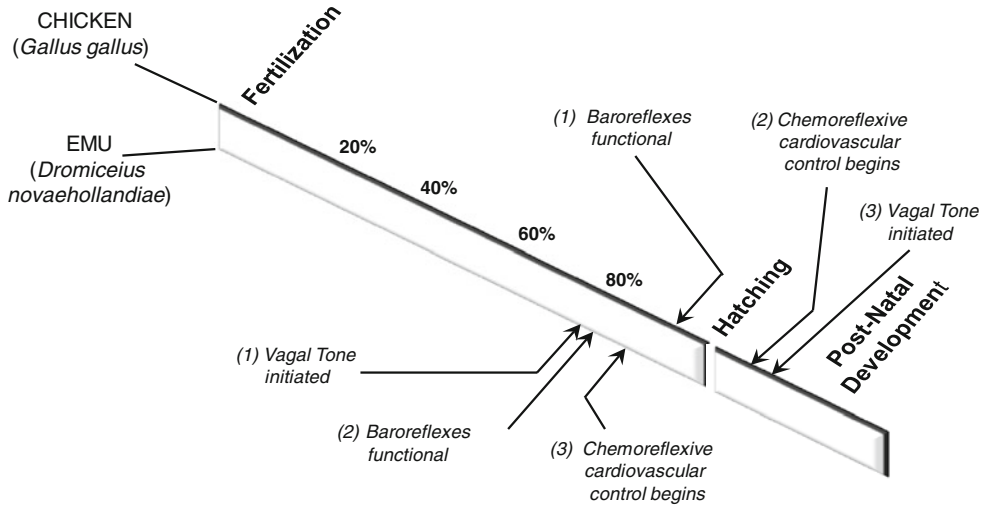

FIGURE 1. Normalized developmental timeline illustrating the onset of various mechanisms for cardiovascular control in embryos of the chicken (*Gallus gallus*) and the emu (*Dromaius novaehollandiae*—also referred to as *Dromiceius novaehollandiae*). Note that both the timing and order of onset differ between the chicken and emu. After[8].

Re-expressing Eq. (1) to solve for animal radius yields:

$$r = \sqrt{\frac{6\Delta PO_2 KO_2}{MO_2}} \quad (2)$$

Consider an ideal O_2 partial pressure gradient of 150 mmHg from external environment to the interior of the mitochondrion, a Krogh's diffusion constant of 0.045 μmol O_2 cm^{-1} s^{-1} $mmHg^{-1}$, and an average value of tissue O_2 consumption levels (μmol O_2 g^{-1} body mass s^{-1}, or μmol O_2 mL^{-1} body mass s^{-1} assuming 1 g tissue occupies I mL) that reflects "typical" rates for poikilotherms (~6×10^{-4} at 25 °C) and homiotherms (~5×10^{-3} at 37 °C). Given these values, the hypothetical spherical animals with these attributes in a fully oxygenated environment can grow to an effective *maximum* radius of ~8–9 mm for poikilotherms and ~3–4 mm for homeotherms *without any internal convective circulation for O_2 bulk transport*. How realistic are these estimates? There are, in fact many complicating factors. For example, the embryos of many poikilotherms (most invertebrates, fish, amphibians and reptiles) are enclosed within an egg capsule for some of their early development. Egg capsules serve as additional impediments to O_2 diffusion, effectively reducing the PO_2 at the embryo' surface.[6,65] Of course, mammalian embryos are implanted in the uterine walls, where they will experience "environmental" PO_2s which might be ½ or less of atmospheric PO_2. These factors will reduce the PO_2 gradient driving O_2 diffusion, and so reduce the calculated radius from Eq. (2). On the other hand, many vertebrates have their lowest rates of MO_2 at their earliest stages of development,[4,36] which would result in calculated radii beyond those predicted above. To summarize, many vertebrate embryos are substantially smaller than a few millimeters (the upper limit for gas exchange by diffusion), suggesting that they could exist solely on simple diffusion of gases, nutrients and wastes. As an embryo grows, of course, it eventually must abandon its dependency on diffusion and migrate bulk transport to internal convective blood transport.

Equation (2) can be used to provide theoretical support for the notion that the heart generates blood flow well in advance of the point where convective O_2 transport is required. Figure 2 relates the *actual* embryo size (expressed as radius) at which embryonic/larval heart beat begins, determined from normal tables of development, to the *calculated* maximum embryo size at which diffusion alone can still serve O_2 uptake needs, using Eq. (2). For this calculation, a PO_2 gradient of 150 mmHg was used for zebrafish and frog, which are exposed directly to the environment (this assumes full oxygenation of the surrounding water, which is not always the case). This gradient was also used for the chicken embryo, which early in development sits on the air cell of the egg and experiences essentially full atmospheric oxygen.[16] However, a gradient of 100 mmHg was assumed for the post-implantation mouse embryo, though the micro-environment surrounding the newly implanted embryo has not, to our knowledge, been measured. Also used was a Krogh's O_2 diffusion constant of 0.045 μmol O_2 cm^{-1} s^{-1} $mmHg^{-1}$. Finally, and importantly, the calculation of maximum radius for diffusion relied upon the very few published values of early embryo oxygen consumption that have been made at the point of blood flow onset. The sources of these oxygen consumption values are as follows: zebrafish[82]; African clawed frog[36,84]; Bibron's toadlet,[67] quaking frog[67] chicken[16] and mouse.[58] Evident from this analysis is that, broadly across the vertebrates, convective blood flow begins at animal sizes far smaller (that is, far earlier in development) than the radius at which diffusion can serve as a mechanism for O_2 transport. Interestingly, as body temperatures rise to the incubation temperatures of birds and the placental temperatures of mammals (both ~37–38 °C), metabolic rate as expressed by oxygen consumption will also increase. This input variable adjustment reduces the size at which convection will be needed for oxygen delivery to the mitochondria, according to Eq. (1), yet as evident in Fig. 2, doesn't necessarily reduce the "gap" between size at onset of blood flow and calculated size where diffusion becomes inadequate. The key question, of course, is why does the heart beat "early"?

On the face of it, the most parsimonious situation—at least from a transport function perspective—is that convective generation of internal blood flow would develop more or less concurrently with the switch from diffusion to convection to provide bulk transport functions. Burggren and Territo[13] defined this condition as ***synchronotropy*** (together-time-moving), a "just-in-time" arrangement in which the developing heart starts to beat *synchronously* with the need for convective blood flow just as diffusion becomes ineffective with continuing embryonic growth (Fig. 3). An alternative situation would be ***prosynchronotropy*** (before-together-time-moving), in which the developing heart starts to beat and generate convective blood flow *before* such flow is required for transport of gases, nutrients and wastes. Indeed, prosynchronotropy would seem to be suggested from the theoretical calculations of body sized served strictly by diffusion and the timing of onset of actually measured heart contraction and blood flow. These two concepts, which present a starkly contrasting view of the relative timing of the onset of heart beat in the developing vertebrate embryo, are portrayed in Fig. 3.

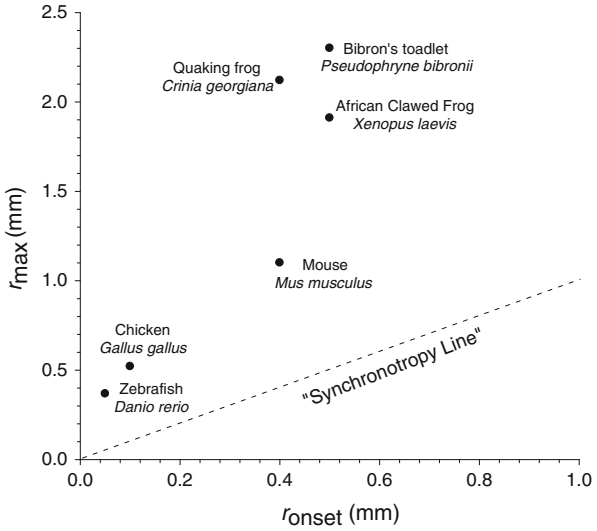

FIGURE 2. Embryo radius at onset of convective blood flow (r_{onset}) vs. calculated maximum embryo radius at which diffusion can serve O_2 uptake needs (r_{max}) for four vertebrate classes. The line of identity, or "synchronotropy line", indicates the point at which there is perfect matching between the onset of blood flow and the development of the need for blood flow for O_2 transport. r_{onset} was determined from normal tables of development for each species, while r_{max} was calculated from Eq. (2) using values drawn from the literature. See text for additional details.

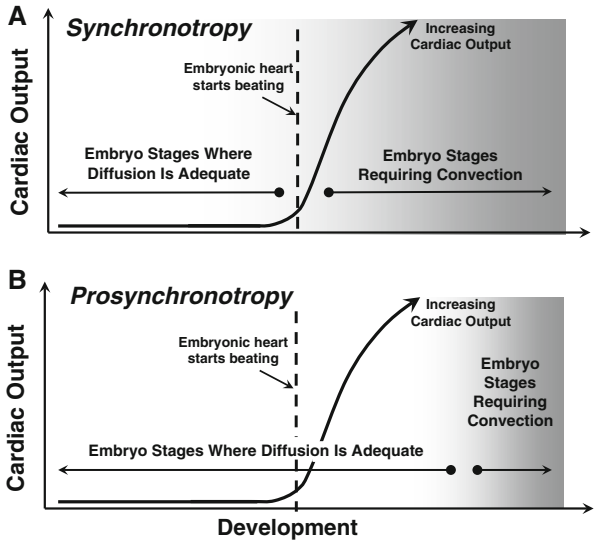

FIGURE 3. A conceptual diagram illustrating (a) *synchronotropy*, where the heart begins to beat precisely when diffusion is no longer adequate and internal convection of blood becomes required by the embryo, and (b) *prosynchronotropy*, where the heart begins to beat much earlier in the point in development where internal convection of blood becomes required by the embryo.

Theoretical calculations have been provided in Fig. 2 to support the prosynchronotropy hypothesis. What *experimental* evidence is there to lead developmental physiologists to reject the synchronotropy hypothesis in favor of prosynchronotropy? The first line of evidence is perhaps the most compelling—early amphibian larvae can survive and even grow without hearts! Either through surgical interference with presumptive heart tissue (including complete removal of such tissue), or through genetic strains whose heart fails to develop, larvae of the clawed frog *Xenopus laevis* up to 10 mm long will continue to actually grow even though they lack a heart[7,66]! Less dramatic, but equally compelling, is the existence of a genetic strain of amphibian salamander (*Ambystoma tigrinum*) whose heart, suffering a pacemaker channel defect, never even begins to beat and so, of course, generates no blood flow (see reviews by Refs. 31, 59). Another experimental approach has involved the disruption of blood O_2 transport by either full lysis of red blood cells using phenylhyrdazine in zebrafish, *Danio rerio*,[73] or by complete blockage of Hb–O_2 transport by rearing animals in levels of carbon monoxide (2%) that would be absolutely lethal to adults (*Xenopus*, Ref. 83). Even in higher vertebrate embryos with higher metabolic rates, such as the chicken *Gallus gallus*, completely elimination of cardiac output by ligation of the outflow vessel emerging from the undivided ventricle causes no disruption of normal growth and oxygen consumption for at least 24 h between day 3 and 4 of the 21 day incubation period (Fig. 4). Collectively, then, these data derived from three different vertebrate classes suggest that the early embryo does not absolutely require convective blood oxygen transport to survive, if not actually continue development at normal rates, for additional hours or days of embryonic life. Of course, these data do not say that *some* transport cannot occur through convection, but rather than it is not required (Fig. 5).

Heart contraction is a significant energy-consuming process (~2% of total whole body oxygen consumption for mammals, calculated from data in Gibbs[33]). Why, then does the heart begin to beat, expend energy, and propel blood, when it is unnecessary—at least from the perspective of a need for oxygen and nutrient transport via blood convection? An answer to this question may arise from a sometimes overlooked function of the heart—the "transport" of pulsatile pressure. Pulsatile pressure may be a key component of the development of the emerging circulation in the embryo, as we will now explore, beginning with consideration of the process of angiogenesis.

ANGIOGENESIS IN VERTEBRATE EMBRYOS

Vasculogenesis and Angiogenesis

The creation of the vasculature is among the first products of tissue differentiation in the vertebrate embryo. The process starts with "vasculogenesis", in which the heart and the first vascular plexus of the embryo differentiate from mesodermal-derived hemangioblasts.[37,71,76]

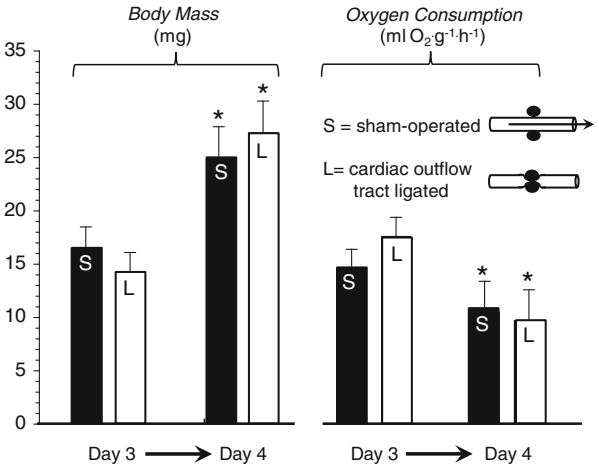

FIGURE 4. Cardiac output is unnecessary for continued growth and oxygen consumption between Day 3 and Day 4 of development in the early embryo of the chicken, *Gallus gallus*. Normal rates of body mass increase, and oxygen consumption decrease, are evident in sham-operated embryos (ligature around the cardiac outflow tract but untightened). These changes still occur even in embryos in which the cardiac outflow tract is surgically fully ligated, eliminating all cardiac output. An * indicates a significant difference at Day 4 form the condition measured at Day 3. After[16].

Vasculogenesis is a very early activity of embryonic development, typically occurring as a necessary prerequisite to the formation of the heart and vasculature, and certainly prior to the generation of significant anterograde blood flow. In the chicken embryo, for example, primary vasculogenesis occurs at the 11–14 somite stages, some 38–45 h after fertilization. The process of "angiogenesis" occurs immediately subsequent to vasculogenesis and continues throughout embryonic development, involving the growth and remodeling of this primordial vascular tissue as the vascular network forms. While angiogenesis is a vital aspect of cardiovascular development in vertebrates, this process has also long been recognized as a key component of tumor formation in mature animals.[30]

Angiogenesis occurs through one of two distinct mechanisms. Endothelial sprouting results from a series of activities starting with proteolytic actions that degrade the endothelial basement membrane. Cell migration then occurs concurrently with removal of obstructing matrix proteins, which generates a space in the cell matrix allowing the endothelial cells to generate the lumen of the emerging blood vessel.[40] A second mechanism for angiogenesis involves intussusceptive

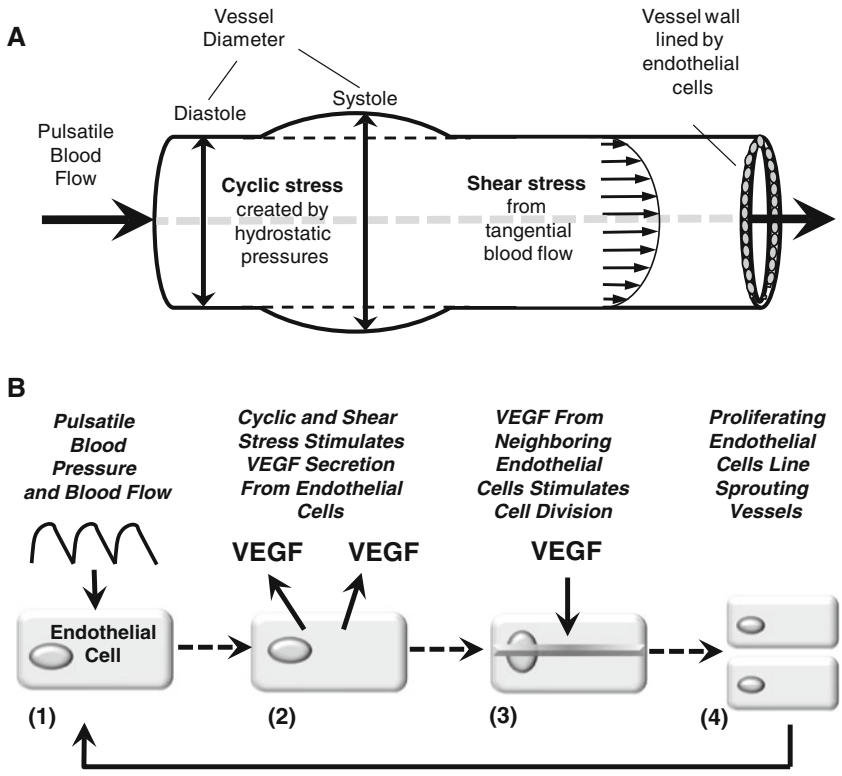

FIGURE 5. Factors affecting angiogenesis in the vertebrate embryo. (a) Hemodynamic factors generating cyclic and shear stress on the endothelium-lined walls of the vasculature. (b) Individual endothelial cells respond to these stressors by secreting vascular endothelial growth factor (VEGF), which through a paracrine effect stimulates division of adjacent endothelial cells.

microvascular growth (IMG), where tissue folds and interstitial tissue columns (intervascular tissue structures—ITSs) form in existing vessel lumens, eventually resulting in the vessel's internal division into two distinct vessels.[25,71]

Angiogenesis is regulated by both the influence of local factors (biochemical and mechanical) as well as genetically-dictated morphogenesis (for an entry into the literature see[1,17,38,40,48,50,54,57]). With respect to local factors, blood pressure and flow have major impacts on angiogenesis.[47,56] In compliant vessels both absolute transmural pressure and circumferential stretch (tension) result in *cyclic stress* on blood vessel walls, created by the rhythmic pressurization/depressurization of blood in the lumen as a natural course of the systolic and diastolic phases of the cardiac cycle. Additionally, blood flow generates a *shear stress* on the inner surface of the blood vessel. These factors are shown schematically in Fig. 4. In all vertebrates and some invertebrates, the inner layer of the vasculature, as well as the sprouting vessel tips, are lined by endothelial cells.[11] These cyclic and shear stresses directly stimulate endothelial cells to release vascular endothelial growth factor (VEGF), which in turn promotes endothelial cell division via a paracrine effect, resulting in stimulation of angiogenesis.[27,34] Growth and maturation of the capillary network and the interconnection of arteries and veins is highly dependent on this angiogenic process—failure of angiogenesis in the embryo is quickly fatal in embryos too large for bulk transport by simple convection, based on experiments with VEGF or erythropoietin knockout embryonic mice that only develop an incomplete microvasculature that is incapable of allowing adequate tissue perfusion.[18,29,53]

Cyclic and shear stresses clearly play a vital role in angiogenesis in the embryo, as discussed above, but the generation of such stresses requires a pulsatile blood flow. Thus, angiogenesis appears to be inextricably bound to the developmental emergence of pulsatile blood pressure and flow in the early vertebrate embryo. Consequently, as an extension to the paired hypotheses of synchronotropy and prosynchronotropy, we add ***synangiotropy*** (together-vessel-moving), a "just-in-time" hypothesis just like synchronotropy but, in this more narrowly defined term (really a subset of prosynchronotropy), the heart begins beating synchronously with the need for angiogenesis in the peripheral circulation of the developing embryo (Fig. 6). The remainder of this essay turns to the evidence in support of synangiotropy.

Embryonic Angiogenesis, Pulsatile Blood Flow and Synangiotropy

The only experimental test of the synangiotropy hypothesis comes from examination of angiogenesis in the early chicken embryo.[5] Chicken embryos from 48 to 72 h of development—a prime time for angiogenesis—were chronically exposed to ZD7288, a purely bradycardic drug that acts by blocking hyperpolarization-activated transmembrane currents in the cardiac pacemaker. Experimental embryos showed a heart rate reduction of ~25% throughout the ZD7288 exposure period. Reduced heart rate created lengthened diastolic periods and greater diastolic runoff in arterial pressure (i.e. increased pulse pressure). This increased pulse pressure also produced a more pulsatile stroke flow (and thus greater shear stress), even though overall cardiac output decreased.[5] Morphometric analysis was then used to determine the degree of angiogenesis (vessels density, degree of vessel branching, vessel diameter) of the proximal vessels in the chorioallantoic membrane (CAM) at intervals of 100 μm out to 600 μm from the umbilical stock by in control and experimental populations. Increased blood pulsatility from lower heart rate caused a series of complex, regionally-based changes in the CAM vasculature. Vessels between the second and fourth generations of vessel branching were significantly shorter in overall length in ZD7288-treated embryos when compared with control embryos. There was also an unexpected *decrease* in CAM vessel density beyond the second generation of branching following ZD7288 treatment.

The experimental evidence of Branum *et al.*'s study[5] is tantalizing, in that it shows that the process of angiogenesis in at least the more proximal vessels of the CAM (beyond the second order of branching) can be altered in complex ways by changes in blood pressure pulsatility and blood flow in the early developmental period of time when diffusion is still providing bulk transport to embryonic tissues. Whether even more profound changes are affected in the more distal vessels (arterioles, capillaries) when conditions

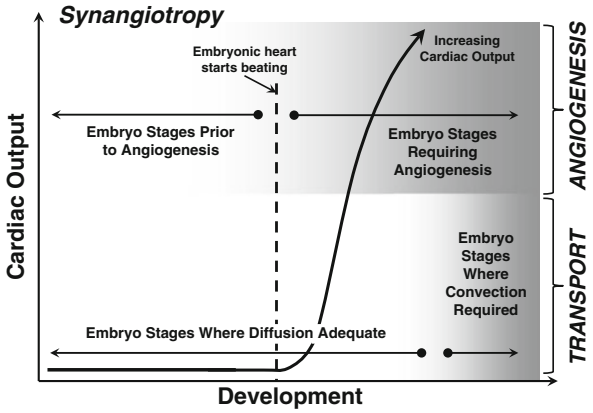

FIGURE 6. Synangiotropy, the beginning of embryonic heart beat synchronous with the onset of peripheral angiogenesis. Both the onset of the need for angiogenesis (upper panel) and the need for convective bulk transport (lower panel) are illustrated relative to the onset of heart beat.

affecting shear strain on the vascular endothelium occur awaits further investigation.

Interestingly, as a final note, in Branum *et al.*'s study[5] reduced cardiac output had no effect on developmental rate (as determined by developmental stage), but did slow embryonic growth rate (as determined by body mass), showing for the first time that development and hypertrophic growth of the early embryo have differential sensitivities to convective blood flow.

CONCLUSIONS

A great deal of attention has been applied to the fetal circulation of mammals such as the sheep, which serves an obvious modeling function for the human circulation (e.g., Refs. 85, 86 see also papers in current volume). While the mammalian in utero model has proven very useful, the placental environment presents two obstacles—difficulty of access, and maternal intervention in fetal functions. The embryos of birds and lower vertebrates (fish, amphibians, reptiles), on the other hand, can serve as useful models in their own right precisely because of the *ease* of access and the *lack* of direct maternal intervention. Moreover, the initial differentiation and growth of the circulation can be monitored through physiological as well as morphological assessments from the time of the initial heartbeat. Additionally, evidence from a wide variety of vertebrates points to the validity of the concept of the ''vertebrate embryo heart'', in which not only qualitative changes (onset of cardiac regulation) but quantitative changes (actual levels of pressure, flow, peripheral resistance) appear to follow similar patterns in the early embryo.

As embryonic (as opposed to later fetal) cardiovascular physiology continues to be examined more closely by developmental physiologists, some dogmas (e.g. the heart serving the very same convective bulk transport roles as in the fetal and adult heart), are becoming more carefully scrutinized. In the case of bulk transport, a variety of experimental approaches (heart removal, occlusion, Hb poisoning) have shown that the early embryonic heart is not required for bulk transport of respiratory gases, nutrients, and wastes, because this critical function can be achieved by simple diffusion alone. Yet, the heart begins to beat before convective blood circulation is required (''prosynchronotropy'').

It has been appreciated for some time that hemodynamic factors themselves (shear, strain) are critical in the normal development of the embryonic vertebrate heart,[34,79] though the heart will develop normally, to a point development, in the absence of the heart beat (e.g. cardiac mutations interfering with heart beat[31]). Recent experiments examining the effect of increased blood pressure pulsatility and blood flow changes on proximal blood vessel development offer initial support for the ''synangiotropy'' hypothesis, that is, the onset of heart beat occurs synchronously with the need for peripheral angiogenesis. Indeed, angiogenesis may be a driving factor for the previously presumed ''early'' beat of the heart. Yet, the complexity of the changes in patterns of angiogenesis (including regional variations of opposite sign) suggests that we have much more to be learned about the relationship between peripheral angiogenesis and the overall development of the circulation in vertebrate embryos.

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