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Forum Review

Role of Hypoxia in the Evolution and Development of the Cardiovascular System

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ABSTRACT

How multicellular organisms obtain and use oxygen and other substrates has evolved over hundreds of millions of years in parallel with the evolution of oxygen-delivery systems. A steady supply of oxygen is critical to the existence of organisms that depend on oxygen as a primary source of fuel (*i.e.*, those that live by aerobic metabolism). Not surprisingly, a number of mechanisms have evolved to defend against oxygen deprivation. This review highlights evolutionary and developmental aspects of O_2 delivery to allow understanding of adaptive responses to O_2 deprivation (hypoxia). First, we consider how the drive for more efficient oxygen delivery from the heart to the periphery may have shaped the evolution of the cardiovascular system, with particular attention to the routing of oxygenated and deoxygenated blood in the cardiac outlet. Then we consider the role of O_2 in the morphogenesis of the cardiovascular system of animals of increasing size and complexity. We conclude by suggesting areas for future research regarding the role of oxygen deprivation and oxidative stress in the normal development of the heart and vasculature or in the pathogenesis of congenital heart defects. *Antioxid. Redox Signal.* 9, 1339–1352.

EVOLUTION OF OXYGEN REQUIREMENTS, HYPOXIC STRESS RESPONSES, AND RESPIRATORY STRUCTURES

The early oxygen environment

OXYGEN BEGAN TO ACCUMULATE on earth several billion years ago in photosynthetic reactions in which energy from the sun was used to convert carbon dioxide and water into sugar, with oxygen as a waste product. The accumulation of large amounts of oxygen was likely initially toxic, leading to what is termed the Oxygen Catastrophe, also called the Oxygen Crisis or Great Oxidation (40). This increase in environmental oxygen ~2.4 billion years ago is postulated to have caused a massive die-off in obligate anaerobic organisms (bacteria) and may have provided the opportunity for expansion of organisms that could both defend against oxidative stress and use oxygen. Over time, O_{2x} came to be highly abundant in the earth's air (~21% O_2/79% N_2) and water (solubility of ~5 ml/L), with the O_2 content dependent on environmental factors such as atmospheric pressure, temperature, and water salinity.

Evolution of biochemical/metabolic responses to oxygen deprivation

That a continuous O_2 supply was critical to the survival of the simple multicellular eukaryotic aerobic organisms that first evolved is suggested by the presence of a phyletically ancient gene-based system to defend against changes in O_2 availability. The primary biochemical pathway for gene-based defenses against O_2 deprivation is the induction of the hypoxia-inducible transcription factor (HIF) [reviewed in (87)] and elsewhere in this series]. The accumulation of the HIF-α subunit (-1, 2, 3) is suppressed under normoxic conditions by its hydroxylation by the prolyl 4-hydroxylases (PHD), a reaction in

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which O_2 serves as a cofactor. The O_2-dependent posttranslational modification of HIF-α protein leads to binding of the von Hippel–Lindau (VHL) factor followed by proteasomal degradation. As O_{2x} concentrations decrease, PHD activity is reduced, leading to accumulation of HIF-α, which, through its dimerization partner HIF-β (ARNT), binds DNA and regulates the transcription of multiple hypoxia-responsive genes.

The constituents of this pathway were originally identified in mammalian cells [reviewed in (62, 87, 95) and elsewhere in this series]. Orthologues of HIF, PHD, and VHL have subsequently been identified throughout the animal kingdom, including the nematode worm *Caenorhabditis elegans* (30, 48). Adult *C. elegans* are only 1 mm in length and several cell layers thick. They have become a favored model organism because of their simplicity at maturity (<1,000 cells) combined with evidence for conservation of life processes that are relevant to more-complex animals. Oxygen delivery is accomplished by diffusion from the surrounding medium. The presence in the worm of a hypoxia/HIF-inducible transcriptional program (30, 48) suggests that it originally evolved as a cell autonomous system for defense against O_2 deprivation. Unlike larger and more complex animals, *C. elegans* can survive at ambient O_2 concentrations as low as 0.1%. Under O_2 deprivation, C. elegans reduces energy expenditure by limiting motility, feeding, and metabolism (70, 71) .This strategy of energy conservation in the setting of reduced O_2 availability is used by worms and even some lower vertebrates that are termed "hypoxia tolerant." This may be viewed as analogous to hibernation in more-complex animals, as well as the downregulation of energy expenditure in the face of reduced energy supplies that occurs in the myocardial cells and other cell types of more-advanced organisms (9, 19, 24, 111). In *C. elegans* and cells of other organisms, the alternative strategy of increasing energy supply through a switch to anaerobic metabolism (glycolysis) in response to O_2 deprivation is also used (Pasteur effect). This is dependent on the hypoxia/HIF-dependent upregulation of glucose transporters and glycolytic enzymes. Because of the accumulation of lactic acid as the byproduct of glycolysis, the switch to anaerobic metabolism by itself is self-limiting. Deletion of the HIF gene from *C. elegans* limits survival in severe hypoxia (0.1% O_2) (48, 90), suggesting that the evolutionarily early function of HIF was to promote cell survival under conditions of oxygen deprivation, at least in part through induction of stress-response genes and changes in cellular metabolism.

In *C. elegans*, as in almost all cells tested, hypoxia results in the induction of the prolyl hydroxylases, serving as a negative-feedback regulator of the hypoxic signal. In *C. elegans*, as in other animals, some genomic responses to hypoxia are still evident after HIF ablation (90), suggesting HIF-independent mechanisms for gene-based responses to hypoxic stress. Whether these represent more-generalized stress responses, or are specifically induced in response to O_2 deprivation in a manner analogous to HIF, is not yet clear.

Evolution of respiratory systems for oxygen acquisition

As larger, multicellular animals evolved, simple diffusion became inadequate as a method of O_2 delivery to tissues (Fig. 1). The size at which a convective system for oxygen delivery is

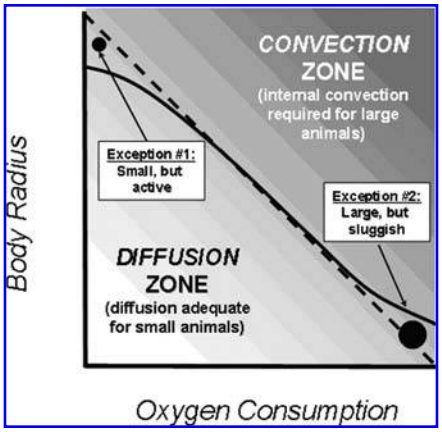

FIG. 1. The relation between body size, metabolic rate, and the ability of simple diffusion to provide adequate oxygenation of small animals. Generally, animals falling in the "diffusion zone" on this figure can supply all of their oxygen needs by diffusion, but as body size or metabolic rate or both increase, the need for an internal convection of body fluids increases. Exceptions are large animals with unusually low metabolic rates, or small animal with unusually large metabolic rates. (From Burggren and Reiber, 2007).

required can be estimated based on the rate of diffusion of O_2, the O_2 gradient driving diffusion, and O_2 consumption. At sea level, the partial pressure of O_2 in the atmosphere is ~150 mm Hg, and assuming an anoxic core and Krogh's constant of oxygen diffusion of 0.045 μmol/cm/sec/mm Hg, the maximum size for effective O_2 uptake by diffusion is ~2 mm (15). Clearly, if animals were to evolve into larger and more-complex organisms, new systems for oxygen and substrate delivery and metabolic waste removal would have to be developed. This is known as the environmental gatekeeper theory for molecular oxygen, which suggests that the Cambrian era explosion in biologic diversity came about as animals evolved systems for more-effective delivery and utilization of oxygen [reviewed in (55)].

The fruit fly *Drosophila* has an intermediate level of complexity, with developed cardiac and respiratory systems. The presence of these more-simplified organ systems, combined with their ease of rearing and genetic manipulation *via* insertional mutagenesis, has made the fruit fly a favored model organism for experimental study. The orthologues for the HIF-dependent hypoxia signaling system have been described in a number of invertebrates, including *Drosophila* and other insects, and is thus thought to be common to arthropods and bilaterians [reviewed in (41)]. The cardiovascular systems of insects and other arthropods do not contain O_2-binding pigments (hemoglobin in higher eucaryotes), and thus the circulating hemolymph is thought not to play a role in oxygen transport. Instead, O_2 delivery to the cells in these animals occurs primarily *via* its distribution in gas through the tracheal system [reviewed in (41)]. Tracheal cells are specified at midgestation *Drosophila* under the control of the bHLH/PAS family member TRACHEALESS, a binding partner for the HIF-1α/ARNT orthologue TANGO [reviewed in (41)]. These cells then migrate to regions where the tracheal system forms under the control of the fibroblast growth factor (FGF) ligand and receptor ortho-

logues BRANCHLESS and BREATHLESS, respectively. FGFs also play a role in a number of morphogenic processes of the respiratory and cardiovascular systems of vertebrates (102); it is not known whether this represents some degree of evolutionary conservation of function. Although some of the details remain to be worked out, a role for hypoxia/HIF in the establishment and repair of the tracheal O_2 delivery system of arthropods seems likely (4). Oxygen may also control other fly body characteristics (*e.g.*, body size), through an effect on insulin/PI3K/TOR signaling (92).

EVOLUTION OF CARDIOVASCULAR SYSTEMS IN VERTEBRATES

Increasing size and complexity as a driver of cardiovascular development

As body size increased beyond the ability of diffusion to serve it, internal cardiovascular systems evolved to provide internal transport of respiratory gases, nutrients, and metabolic wastes. The evolution of vertebrates with increasing energy demands required high-pressure/high-output systems for O_2 delivery to meet the increasing O_2 and nutrient demand. One driver of the increasing demand was likely body size itself, with the ratio of surface area to mass being a key determinant of an organism's resting metabolic rate. This, along with large, specialized organs with high energy demands, such as the heart and brain, dictated the need for a delivery system that was capable of greater O_2 delivery and not subject to the whims of environmental change. Perhaps the most significant development in the increasing demand on cardiovascular function in vertebrates was the evolution of endothermy and the emergence of warm-blooded birds and mammals. Endotherms, whose activity is not dependent on environmental temperature, have an O_2 consumption typically 10-fold greater than that of the ectotherms, such as reptiles and amphibians. Correspondingly, birds and mammals require a constant internal convective supply of O_2 to meet the metabolic demands of the tissues.

The basic vertebrate circulatory plan

The basic cardiovascular plan of vertebrates comprises a muscular pump, the heart, ejecting blood into the arterial vascular network. Oxygen is off-loaded from hemoglobin into respiring cells. The deoxygenated blood returns toward the heart through the venous vascular network, and the cycle of oxygenation of blood and its recirculation is repeated. The general conservation of this system through the vertebrate classes, as well as evidence for conserved transcriptional pathways that direct cardiac morphogenesis, has led to the hypothesis that the cardiovascular system of more-complex vertebrates represents the modular addition of repetitive elements onto the basic single-chamber heart pump [reviewed in (69)]. In this review, we explore the hypothesis that, subsequent to the formation of the basic template, the development of increasing complexities of the vertebrate heart was a series of adaptations to optimize O_2 delivery to tissues with increasing yet variable metabolic demands. First, we describe these arrangements in the different taxa, starting with the more phyletically ancient animals, the fishes, and moving on to more energetically demanding warm-blooded birds and mammals. A comprehensive comparative anatomic and physiologic review of these systems is beyond the scope of this review, and the reader is directed to other sources [see (17, 57)]. Here we focus on specific aspects of heart and vascular development that are likely to be shaped by oxygen demand.[1]

Fish

The circulatory system of a hypothetical ancestor of all of the vertebrates was one in which a structurally simple heart pumped blood *via* a simple, valved outflow tract (OFT) into a single, ventrally located aorta. From there, blood passed through a large number of cephalic aortic arches in proximity to the gills, where oxygenation of the blood would occur. The oxygenated blood then passed into the dorsal aorta and was distributed to the tissues. The heart received deoxygenated blood from the tissues, and its ejection into the ventral aorta would reinitiate a new cycle.

In this circulation plan, blood is pumped to the oxygenating organs (gills and, to a lesser extent, skin) and then to the periphery and thus represents a single-pump "in-series circulation" (Fig. 2). The circulations of both cartilaginous (elasmobranch) and bony (teleost) fish have retained the series arrangement with four specialized chambers: the sinus venosus and atrium, which receive the blood; the muscular ventricle, which pumps the blood; and an outlet chamber, with variable morphology, designated as either the bulbus or conus arteriosus, which is thought to aid in the expulsion of blood into the main artery through a "windkessel" effect (17, 76, 85). Aside from the characteristics of an "in-series circulation" (discussed later), two unique traits of the fish heart morphology exist that we concentrate on in this thesis that O_2 requirements played a major role in shaping the development of the heart. The first trait involves the heart's outlet tracts. The development of air-breathing organs (not only true lungs, but other bladder-like respiratory organs) followed by the migration onto land, provided both a rich source of O_2 and release from the tyranny of potential daily or seasonal aquatic hypoxic exposure. However, the evolution of an air-breathing organ in fishes that retained gills (as do almost all air-breathing fishes) required a more-elaborate vascular structure that would minimize if not eliminate the intracardiac mixing of oxygenated blood returning from the air-breathing organ with deoxygenated blood returning from metabolizing tissues (and possibly poorly oxygenated blood draining gills that were ventilated with hypoxic water). Achieving this separation required significant changes to the outlet tract of the heart, including new vessels, spiral valves within existing vessels, *etc.*, as well as the development of separate

[1]Each of the major vertebrate groups—fishes, amphibians, reptiles, birds, and mammals—has generated considerable diversity as they adapt to extreme environments. Given this, the dictum that "ontogeny recapitulates phylogeny" is not accurate in a strict sense (*i.e.,* the fish cardiovascular system is not ancestral to that of birds and mammals). Nonetheless, these taxa shared common ancestors, and comparisons of their organ systems is useful in considering how these systems may have adapted and evolved to various pressures, considered here in the context of O_2 availability and demand.

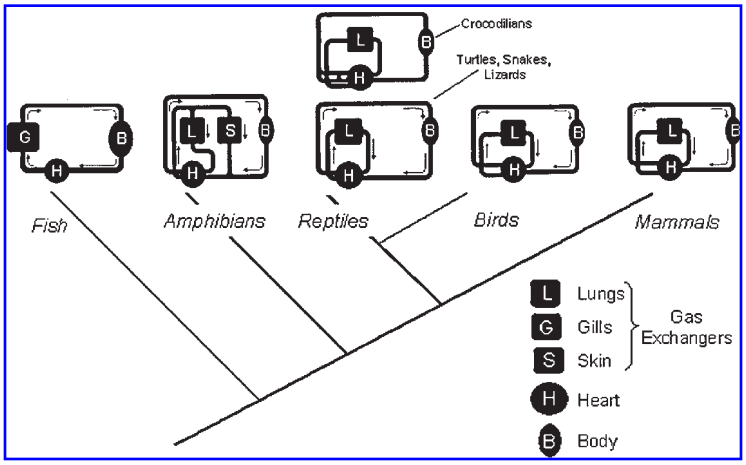

FIG. 2. General schematics of the vertebrate circulatory patterns, showing the relations between the heart and the gas-exchange organs(s) of each circulation. From Burggren and Reiber (2006) after Hicks (2002).

venous structures carrying oxygenated blood back to the heart (analogous, if not homologous, with a pulmonary vein) (Fig. 3). Moreover, the variability in gas-exchange organs (skin, gills, lungs, nonpulmonary air bladders, even specialized regions of the gut) has also likely contributed to the great variability in cardiac OFTs between classes.

Increased body size and metabolic requirements also required increased work of the heart to meet these demands. This required a dedicated supply of well-oxygenated blood to the heart tissue itself. In some fishes (*e.g.*, salmonid fishes), a dense compact layer of myocardium is supplied with oxygenated blood through dedicated coronary arteries, whereas an inner trabecular (spongy) layer is bathed in relatively deoxygenated venous blood (17, 32, 85). However, in many fishes, coronary arteries are absent or rudimentary, and a highly trabecular myocardium is supplied with O_2 primarily by diffusion from the O_2-poor venous blood moving in and out of the trabecular pockets in the heart wall.

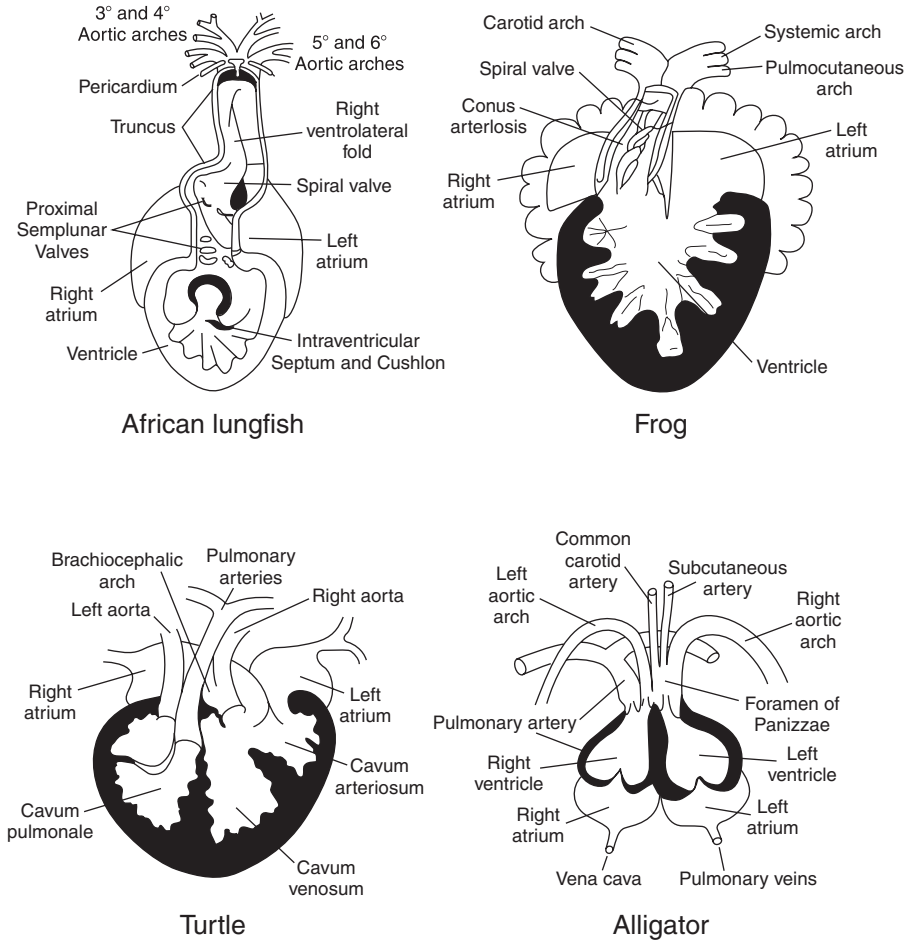

FIG. 3. Gross morphology of the outflow tract(s) of the heart of representative lower vertebrates. The patterns of flow of oxygenated and deoxygenated blood through each of these hearts is surprisingly complex, involving both spatial and temporal components to maintaining separation of bloodstreams. (For description of blood-flow patterns and literature sources, see Burggren *et al.*, 1997).

Amphibians

The amphibians, such as frogs and salamanders, demonstrate a rather interesting arrangement in which two potential sites of O_2 uptake, the lungs and the skin, are arranged in parallel (Fig. 2) (14, 17). Deoxygenated blood returning from the systemic circulation mixes in the right atrium with relatively oxygenated blood returning from the skin. Oxygenated blood returns from the relatively large, saccular lungs to the left atrium. Both bloodstreams enter a single ventricle and are ejected *via* a single OFT into a single-outlet structure (Fig. 3). However, the heavily trabeculated ventricle and the spiral valve that runs longitudinally down the proximal part of the outflow vessel direct primarily oxygenated blood to the arterial arches and on to the systemic circulation and direct primarily deoxygenated blood into the pulmocutaneous circulation for subsequent oxygenation. The ratio of blood flow between the systemic and pulmocutaneous circuits (and indeed between the cutaneous and pulmonary arteries) is regulated in the short term depending on oxygen demands of the tissues and the pattern of pulmonary ventilation (89). The absence of complete septation of the OFT and ventricle that would force two separate, parallel circuits of identical volume, as occurs in birds and mammals, is thus advantageous in amphibians, allowing switching between cutaneous and pulmonary O_2 sources depending on O_2 availability in the lungs of these intermittent breathers.

Reptiles

Reptilians also have an incompletely divided systemic and pulmonary circulation, representing a functionally (though not structurally) intermediate position in terms of bloodstream separation (see Fig. 2). Turtles, nonvaranid lizards, and snakes have two separate atria emptying into a complexly structured ventricle best described as having three separate cava (see Fig. 3). Not surprisingly, intracardiac blood-flow patterns in noncrocodilian reptiles are complex, but still yield both left-to-right (systemic bypass) and right-to-left (pulmonary bypass) shunts. Among the reptiles, only the crocodilians have a structurally separate left and right ventricle, reflecting the avian and mammalian condition in which no intracardiac mixing of oxygenated and deoxygenated blood occurs. However, in crocodilians, the presence of a left aorta arising from the left ventricle, and a foramen of panizzae allowing connection between the aortic bases, still provides the capability of a right-to-left shunt. As in the case of the amphibians, it is likely that the intra- or extracardiac shunting of blood is a highly effective adaptation for an intermittently breathing, diving animal with waxing and waning pulmonary oxygen stores (16, 45).

Birds and mammals

Whereas birds and mammals are thought to have evolved separately from reptile-like ancestors, their cardiovascular systems bear much in common, probably reflecting the similarities of high metabolic rate and correspondingly high O_2 demand. In adult birds and mammals, mixing of oxygenated and deoxygenated blood is prevented by an in-series circulation powered by two separate pumps within the heart (see Fig. 2). Deoxygenated blood enters the right atrium and flows on to the right ventricle, where it is ejected into the pulmonary artery, destined for the lungs. As blood passes through the pulmonary alveoli, hemoglobin in the red blood cells becomes saturated with O_2, and HCO_3^- in plasma and within erythrocytes is reconverted to molecular CO_2, which diffuses out into alveolar gas. Oxygenated blood returns to the heart *via* the pulmonary veins into the left atrium, flows into the left ventricle, and is ejected into the aorta and the systemic circulation. Thus, the aortic and pulmonary arteries (cardiac outlet) and the right and left ventricles are completely separated by septae. An extensive coronary arterial system directly provides O_2 (saturating the red blood cell hemoglobin) and nutrients to the working myocardium of the left and right ventricles. A significant failure of functional separation of pulmonary and systemic circulations in humans and other mammals can result from congenital defects in cardiac septation or abnormal positioning of the great vessels, and other OFT anomalies (33, 58, 105). The ejection of partially oxygenated blood into the systemic circulation as occurs in what are collectively termed *conotruncal heart defects* (tetralogy of Fallot, double-outlet right ventricle, transposition of the great arteries, and persistent truncus arteriosus, Fig. 4), along with the increased hemodynamic burden, is usually poorly tolerated, resulting in reduced functional capacity, and, if not surgically corrected, limited survival.

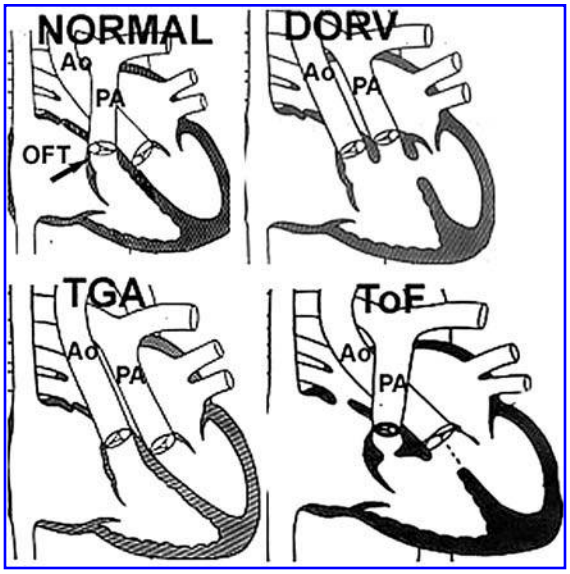

FIG. 4. Congenital defects in the human cardiac outlet structures (conotruncus) that result in right-to-left shunting of deoxygenated blood. These defects are postulated to arise from malrotation of the OFT or myocardial infundibular defects or both. DORV, Double-outlet right ventricle. Both great vessels (Ao, aorta; and PA, pulmonary artery) connect *via* the myocardial infundibulum to the right ventricle. TGA, Transposition of the great arteries. The Ao connects *via* the infundibulum to the RV, and the PA directly connects to the LV. ToF, Tetralogy of Fallot. Pulmonic stenosis is secondary to infundibular hypoplasia, and the aorta is malrotated and thus partially overrides the right ventricle. These defects are associated with ventricular septal defects, which provide a necessary conduit for blood flow. Although shown as individual entities, these dysmorphologies represent a spectrum of defects in the conotruncal structures.

We have now reviewed the evolution of the cardiac structures and particularly the cardiac OFT. We have done this in the context of the common assumption that the dual-series circulation, in which deoxygenated blood transits the pulmonary circuit and oxygenated blood transits the systemic circuit, evolved to deliver oxygen more efficiently to the tissues in warm-blooded birds and mammals. We next consider the necessary corollary that tissue hypoxia is a driver of the development of the dual in-series circulation in the developing avian and mammalian embryo.

INFLUENCE OF HYPOXIA IN CARDIOVASCULAR DEVELOPMENT

All embryos begin as a single cell created by the fusion of a sperm and egg. The growing zygote must then obtain a source of O_2 and other substrates that will provide the energy needed for continued development and growth. That tissue hypoxia and subsequent HIF-dependent transcription is required for normal development is strongly suggested by the results of gene-inactivation studies in mice. In experiments in which HIF family members ARNT (HIF-1β) or HIF-1α are inactivated in the germline, the mouse embryo fails to develop past mid-gestation [reviewed in (92)]. Here we consider the evidence that O_2 deprivation and the resultant transcriptional response plays a key role in the development of specific heart and vascular structures of the vertebrate embryo.

Development of placenta and chorioallantoic membrane

Based on studies using avian models, the early endotherm embryo initially derives its O_2 and nutrient supply by simple diffusion, falling within the "diffusion zone" (based on size and oxygen consumption) indicated in Fig. 1, much as in its simple, avascular primitive ancestors. However, as the embryo rapidly grows, its O_2 demand outstrips diffusional oxygen delivery, and the internal circulation (which has already begun to form and propel blood) takes on an increasingly important function in convective gas transport (13, 15). In mammals, concurrent with the elaboration of the embryonic circulation, the placenta forms as a necessary interface for gas and nutrient exchange with the maternal circulation. The placenta forms by the invasion of cytotrophoblasts from the embryo into the uterine wall in an oxygen-dependent process, with striking resemblance to tissue invasion in the process of angiogenesis. The behavior of the cytotrophoblasts is oxygen dependent: these cells proliferate under the severely hypoxic conditions ($\sim$2% O_2) that mimic the uterine environment before placentation (36, 114). When exposed *in vitro* to higher O_2 concentrations, as would occur as the invading cells approach the uterine arterioles, the cytotrophoblasts differentiate to form the syncytium that makes up the uteropacental unit for exchange of gas and nutrients (37). Inactivation of the *ARNT* (HIF-1β) gene in the germline results in defects in the placenta and early demise of the embryo, likely due to defective hypoxia-dependent differentiation of the trophoblastic cells (2, 60). That the placentation is finely regulated by O_2 levels and transcriptional responses is indicated by the defects in placentation that also are caused by HIF gain of function induced by germline inactivation of PHD2 (99).

In the egg-bound embryos of birds, respiration is achieved throughout all but the last hours before hatching by the extensive vessels of the chorioallantoic membrane, with perfusion supplied by the vitelline arteries and veins of the yolk sac (100, 101). The yolk sac in birds and mammals is also the site of origin of the hemangioblast, the primitive common precursor of both blood and endothelial cells. Studies of these and other stem cells suggest that their development also is oxygen sensitive (75), a topic beyond the scope of this review. The formation of the vitelline circulation on the yolk sac is evident within 1 day after the formation of the pigmented blood islands [*i.e.,* at $\sim$48 h of development in the chicken embryo (reviewed in ref. 6)]. According to the more-precise staging of Hamburger and Hamilton (43), this would be approximately stage 15. At this early stage, the heart has begun to beat, and a functional closed circulation is developed. This is much earlier than the mammalian embryo, in which in the mouse, the placental circulation is first established, followed by a closed functional embryonic circulation, at days 10–12, or $\sim$50% of prenatal development. In the chicken embryo, the allantois fuses with the chorionic membrane, forming the chorioallantoic membrane (CAM) at about day 8, well after the heart has formed, and then serves as the primary site of gas exchange for the remainder of the incubation. Thus, the timing of the maturation of the mouse (mammalian) gas exchanger and cardiovascular system is both different and compressed relative to that of the chicken embryo, which must support itself from the early stages of development. Despite these differences, both models will continue to prove useful to understand vertebrate cardiovascular development.

Oxygen delivery to the developing embryo and fetal tissues

To discuss the role of tissue O_2 in embryonic cardiovascular morphogenesis, we must first consider O_2 delivery to the embryo. The mammalian placenta takes many different forms [reviewed in (29, 110)]. In all cases, O_2 readily passes through the placenta by simple diffusion, as governed by Fick's law of diffusion,

$$dm/dt = DF(C1 - C2)/b$$

where dm/dt is the amount of gas transferred per unit time, D is the diffusion coefficient, F is the area of exchange, and (C1 $-$C2) is the partial pressure gradient between maternal and fetal circulations. It is estimated that the partial pressure of O_2 traveling from the placenta to the fetus *via* the umbilical vein is $\sim$30 mm Hg, providing 80% saturation of the blood hemoglobin. In the mammalian fetal circulation, blood is shunted at three levels: the ductus venosus, bypassing the liver, and foramen ovale and ductus arteriosus, providing a right-to-left shunting of blood away from the pulmonary circulation and the lungs, which do not function in gas exchange in the fetus. This arrangement is more reminiscent of a parallel circulation of a lower vertebrate as compared with the series circulation of the adult human (Fig. 2). A full consideration of fetal blood shunting is beyond the scope of this review. However, of relevance is that

the mixing of blood with differing O_2 contents occurs at multiple sites, resulting in O_2 saturations of hemoglobin in the systemic arteries of 50–60%, well below the 95% saturation in the adult. Oxygen content of the blood and its release to the tissues also depends on the hemoglobin (red blood cell) concentration (the amount of O_2 dissolved in plasma is relatively negligible, ~0.3 ml/100 ml, but can account for 10% of total oxygen in the early embryo when blood hemoglobin concentrations are low). Also important to oxygen content and release are the characteristics of the fetal hemoglobins, which have higher O_2 affinities and greater sensitivity to CO_2 and protons compared with adult mammal hemoglobin [reviewed in (11, 110)]. To summarize, the fetal mammalian blood carries less O_2 than that of the adult, giving rise to the generalization that the embryo and fetus develop in a relatively hypoxic environment.

Similar principles apply to the oxygen content in developing avians. The partial pressure of oxygen in the chicken embryo systemic arteries, 40–50 mm Hg, tends to be higher than that of the developing mammalian embryo, but the calculated hemoglobin saturation of 37% is similar [reviewed in (6, 64)]. Thus, the O_2 content of the early chicken embryo's arterial blood is very low, 1–2 μl O_2/100 μl blood, as compared with adult values of ~15 ml/100 ml blood. The pO_2 values in venous blood and in tissues are also very low, suggesting (a) that the tissues are consuming much of the available O_2, and (b) that little O_2 reserve remains in the system.

Indicators of O_2 deprivation within cells and tissues

The adequacy of the O_2 supply to the developing tissues is dependent on the O_2 delivery versus the O_2 demand of the tissues. Direct measurement of O_2 demand and O_2 concentrations within intact cells and tissues *in vivo* is problematic. Anaerobic metabolism, as evidenced by lactate production, is one surrogate indicator of tissue hypoxia. So too is the nitroimadazole class of chemicals, such as 2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) and pimibendazole, which form adducts in cells in a chemically reducing environment [*i.e.*, their binding is logarithmically inversely proportional to O_2 concentration (31)]. These chemicals are thus used as indicators of O_2 gradients among cells, but because of differences in binding activities between cells, comparisons between different cell types are problematic. A second indicator of hypoxia is HIF-1 itself. Expression of the HIF-1β (ARNT) subunit is constitutive. The HIF-α subunits are regulated by hypoxia through the action of PHDs (discussed earlier). Accumulation of HIF-α protein indicates relative hypoxia, with the caveat that HIF-α protein may be induced by O_2-independent mechanisms. A related and potentially very useful indicator was recently developed in which the oxygen degradation domain (ODD) of HIF-1α was fused to the luciferase reporter. In these mice, the readily measurable luciferase serves as a reporter for O_2 gradients (84). A third indicator of tissue hypoxia is the accumulation of hypoxia/HIF-dependent transcripts and the processes that they control. A large number of such genes have been identified, based on mostly *in vitro* studies [for example, (28, 61) and a few *in vivo* studies (59) of tissue hypoxia]. Many more studies have examined the effects of tissue ischemia, in which reductions in blood flow result in oxygen and substrate deprivation and accumulation of metabolic wastes, a topic beyond the scope of this review. The hypoxia-dependent transcriptional program may control cell autonomous hypoxic responses, such as glycolysis and cell death and survival [reviewed in (44, 72)], and noncell autonomous responses such as angiogenesis and erythropoiesis, to restore O_2 delivery to meet oxygen demands. We now consider how relative tissue hypoxia, acting through an HIF-dependent transcriptional program, may drive specific morphogenic processes in the heart.

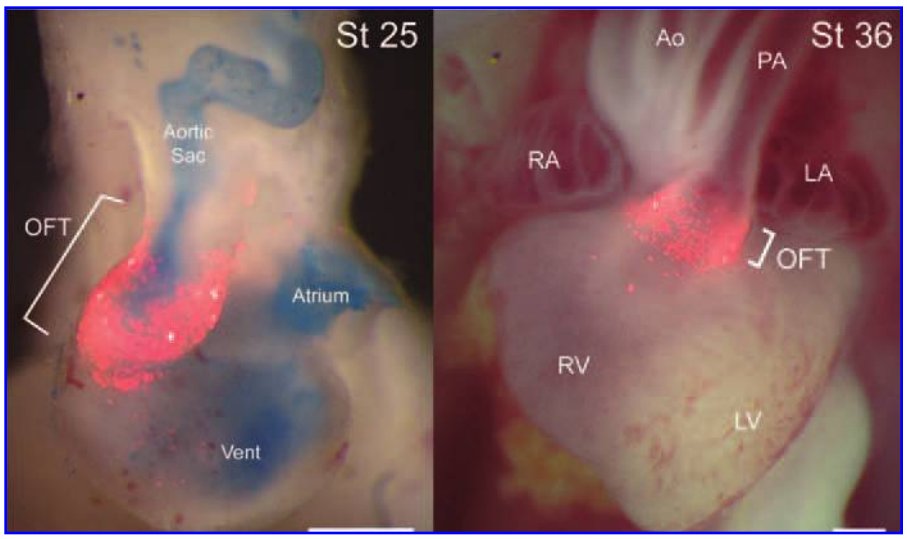

FIG. 5. Avian heart development: transition from the more-primitive single circulation (stage 25, ED5) to the mature dual in-series configuration (stage 36, ED10). Development and remodeling of the embryonic chick cardiac OFT (**A**). The OFT myocardium of the stage 25 (ED5) and stage 36 (ED10) chick heart is labeled with red fluorescent protein expressed from recombinant adenovirus. The lumens of the hearts and vessels are filled with blue ink in (**A**) and blood in (**B**). In this developmental window (1), the OFT myocardium shortens and commits to the right heart, forming the subpulmonic infundibulum. The OFT rotates, placing the aorta in a posterior position to connect with the LV; (2) the OFT, ventricles, and atria are septated to divide the left and right circulations; (3) the coronary arteries form to supply the working myocardium with well oxygenated blood; (4) the cushion mesenchyme is remodeled to from the valves and other structures (not shown). [Reprinted with permission from *Trends in Cardiovascular Medicine* (98)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

Development of the heart and outflow tract

Role of tissue hypoxia in cardiac outflow tract development. The outflow portion of the heart has developed considerable complexity through evolution, as noted earlier. For a review of its development in higher vertebrates see (1, 39, 52, 79, 81, 98). In birds and mammals, myocardium is added to the arterial pole of the heart from a secondary or anterior heart-forming field, well after the primary heart tube has formed. In Fig. 5, the completed OFT myocardium of the stage 25 embryonic chick heart is labeled with a red fluorescent protein tag. At this stage of development, the avian heart (and mammalian heart at a comparable stage of development) resembles the more-primitive single-chamber configuration discussed earlier, in which blood flows into a single ventricle through a single atrial chamber and is pumped out through an unseptated OFT into the aortic sac. This outflow portion of the heart subsequently undergoes complex remodeling in the transition to a dual in-series circulation. The remodeling includes

1. the shortening of the OFT myocardium, and the rotation of the aorta to a posterior position where it will directly connect with the left ventricle (stage 25 vs. 36; see Fig. 5). The shortening and rotation of the OFT in avians requires the elimination of OFT cardiomyocytes by programmed cell death (107, 108) (Fig. 6);
2. the septation of the OFT and "aortic sac" into pulmonic and systemic (aortic) vessels, a process dependent on invasion by cells from the neural crest (54);
3. the recruitment of endothelial progenitor cells (see Fig. 6), which will be organized to form the coronary arteries (50, 96) [reviewed in (103)]; and
4. the remodeling of the cushion mesenchyme to form the semilunar (aortic and pulmonary) valves and part of the ventricular septum in mammals.

We hypothesized that tissue hypoxia may play a role in the remodeling of the OFT in chicken embryos based on two observations: (a) the high incidence of programmed cell death (PCD) in the cardiomyocytes of the OFT during its remodeling (107), and (b) the recruitment of endothelial progenitor cells to the OFT and their organization into a coronary vascular plexus approximately synchronous with the PCD-dependent remodeling of the OFT (82). Because cell survival/death and vasculogenesis both may be responses to tissue hypoxia [reviewed in (8)], O_2 gradients were examined by using the surrogate markers EF5 and HIF-1α (96–98, 109). EF5 was injected into the vitelline vein of chicken embryos and detected with a Cy3-conjugated monoclonal antibody. HIF-1α protein was detected with a polyclonal antibody and TSA signal amplification. Good correspondence was found between EF5 and HIF-1α signals (Fig. 7). Each was first detected in the OFT myocardium at stage 25, increased to a peak at stage 30, and then subsided to background levels by stage 32. This staining was restricted to the OFT myocardium (see Fig. 7) and several other locations (see later) and showed the appropriate responses when the eggs were incubated under hypoxia (7.5% O_2 for 6 h) or hyperoxia (95% O_2 for 24 h). These results suggest that the chicken embryo OFT myocardium is hypoxic relative to the other chamber myocardium specifically at the stages of PCD-dependent remodeling of the OFT in the transition to a dual circulation.

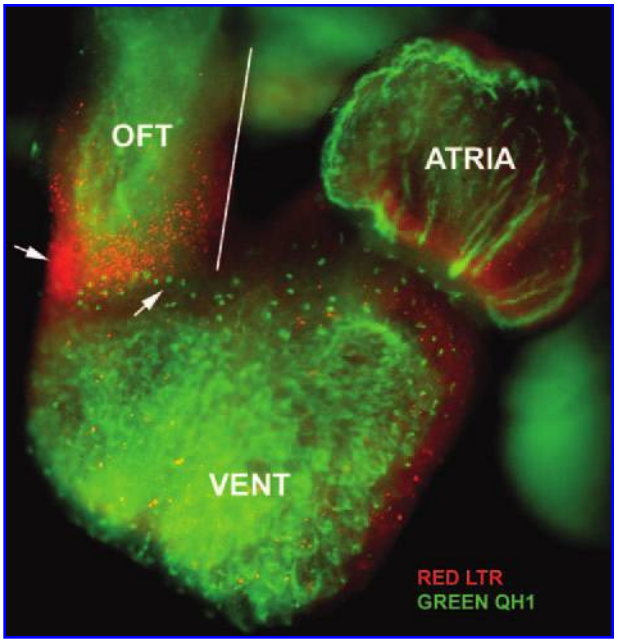

FIG. 6. Key cellular processes in avian heart morphogenesis. OFT cardiomyocytes are eliminated by apoptosis, as indicated by LysoTracker Red (LTR) staining (*arrow*), observed in this whole-mount picture of a stage 25 quail heart. Endothelial progenitor cells, detected with an anti-QH1 antibody, are observed within the epicardial tissue covering the ventricle (*arrow*) and are recruited to the OFT, where they will be organized into a vascular network. The chamber endocardial endothelium is also QH1 positive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

Why specifically the embryonic chicken OFT myocardium is relatively hypoxic is suggested by anatomic considerations. The coronary arterial system that is dedicated to supplying O_2 to the myocardium does not become functional until after stage 32 (106). Before this, O_2 supply to the myocardium occurs by diffusion from the blood within the chamber, resembling the arrangement in the more-primitive vertebrate hearts reviewed earlier. The ventricles of the embryonic chicken heart are highly trabeculated with a relatively thin myocardium. The high degree of trabeculation increases the surface area/mass ratio of the myocardium, and thus facilitates O_2 and substrate diffusion into the myocardium. The OFT myocardium does not become trabeculated and instead is adjacent to a thick endocardial mesenchyme, visible in Fig. 7, that will subsequently be remodeled to form the semilunar valves and other structures. The thickness of this mesenchyme, several hundred micrometers, likely represents a significant barrier to O_2 diffusion to the adjacent myocardium, given the estimates of the limits of O_2 diffusion through respiring tissues of ~100 microns in thickness (73). O_2 diffusibility varies between tissues and cell types, depending on the O_2 gradient produced by tissue O_2 consumption and on the presence of proteins such as myoglobin in cardiac (and skeletal) muscle that serve as a significant O_2 store. In summary, the binding of EF5 and accumulation of HIF-1α in the stage 25–32 OFT myocardium suggest that the O_2 supply is inadequate and

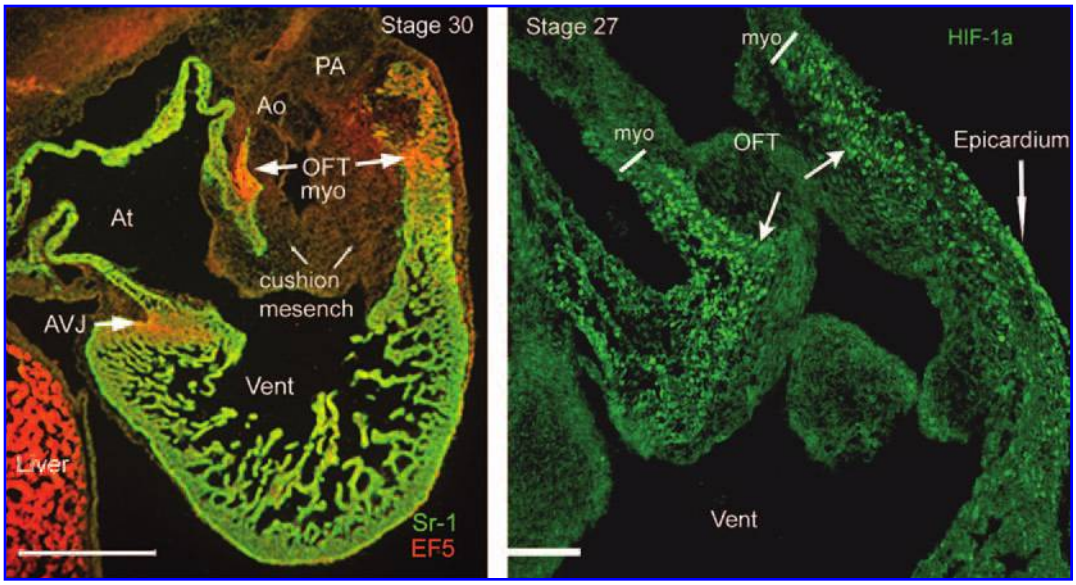

FIG. 7. The nitroimidazole EF5 and HIF-1α protein indicate hypoxic regions of the stage 30 (ED6) embryonic chicken heart. After infusion of EF5, binding was detected with a Cy3-conjugated anti-EF5 monoclonal antibody. The section was co-stained with Sr-1, an antibody against sarcomeric a-actin, to delineate the myocardium. HIF-1α was detected with a polyclonal antibody followed by TSA amplification. Good agreement is seen in the distribution of the signals for EF5 and HIF-1α, indicating relatively hypoxic regions of the developing heart in the myocardium of the OFT and AV junction. Note that the liver is also intensely EF positive. Bars, 500 μm. PA, pulmonary artery; Ao, aorta; Vent, ventricle; At, atrium; myo, myocardium; OFT, outflow tract. [Reprinted with permission from *Developmental Dynamics* (109) and *Developmental Biology* (97)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

a reducing environment is present within these cells, likely due to the unique structural arrangements of this region of the heart.

That O_2 is critical to the developmental remodeling of the avian cardiac OFT development is supported by experiments in which the O_2 supply is altered. Of note, in very early stages of development, the dependence on diffusion for a supply of O_2 appears not to be a limitation to continuing growth and development. Chicken embryo development (and development of other vertebrates) can proceed normally in the complete absence of blood convection until eventually the embryonic metabolic rate outstrips diffusional mechanisms for oxygen and nutrient supply (13, 18). At the later stages of development (HH 25–32), reduction of ambient O_2 concentrations to 5–7.5% (from 21%) results in demise of the embryo because of circulatory arrest within hours (96, 97). In contrast, more-modest degrees of hypoxia (12–15%) are tolerated by the embryo, but have little effect on O_2 supply, likely because of adaptations to maintain O_2 supply to the tissues (7). Incubation of the eggs under high concentrations of O_2 (95%), thereby blunting the hypoxia in the OFT myocardium, results in conotruncal (*i.e.*, OFT) heart defects, suggesting that hypoxia in the OFT myocardium is necessary for the proper remodeling of the OFT. However, these experiments must be interpreted with caution because (a) the entire egg is exposed to increased O_2, and (b) the increased O_2 could result in the generation of toxic reactive oxygen species (*i.e.*, have an effect independent of hypoxic blunting).

A mechanistic understanding of how hypoxia may regulate the PCD-dependent remodeling of the OFT requires the targeting of HIF and the hypoxia-dependent transcriptional program specifically in the cells of the OFT. Genes can be specifically targeted to the OFT cardiomyocytes of the chicken embryo by using an adenoviral gene-delivery system (34, 35). Recombinant replication-defective adenoviruses are injected into the pericardial space of the stage 15–17 chick embryo *in ovo* when the epicardial surface of the heart is a bare myocardium. Using the CMV enhancer and promoter specifically drives gene expression in the OFT cardiomyocytes, providing a way to target these cells specifically. Adenoviral-mediated forced expression of a constitutively active HIF-1α construct [described in (51)], or short exposures to moderate degrees of global hypoxia (5–7.5% O_2) that do not cause the demise of the embryo, do not result in increased programmed cell death of the OFT cardiomyocytes. Hypoxia may trigger cardiomyocyte cell death in other settings [reviewed in (8)], and perhaps the moderate degree of hypoxia in this model is not sufficient to trigger a cell-death response. Preliminary studies suggest that the forced expression of HIF-1α actually protects the cardiomyocytes of the OFT from programmed cell death (data not shown). One potential prosurvival target of HIF-1α in this context is the VEGF receptor (VEGFR2). VEGFR2 is selectively expressed by the cardiomyocytes of the OFT during the stages of tissue hypoxia, and the survival of the OFT cardiomyocytes is dependent on the activity of a kinase downstream of VEGFR2 termed Akt (96). VEGFR2 has also been shown to be upregulated in mature muscle and other parenchymal cells in response to O_2 deprivation/ischemia (49, 68, 80). These observations suggest that VEGF/VEGFR2 signaling may have originally evolved as an autocrine mechanism for cell survival under hypoxic stress. It

is now better known as a paracrine signal to stimulate angiogenesis in ischemic tissues and is also a vascular permeability factor. A similar scenario has been suggested for erythropoietin (EPO)/EpoR signaling, which is required in the embryo for hematopoiesis and thus O_2 delivery and cell/embryo survival (112), but also seems to function in an autocrine cell-survival pathway (113). Hypoxia signaling through HIF and other mechanisms may regulate the expression of a number of prosurvival and prodeath genes (3, 25, 26, 56, 78, 93). The elucidation of the role of the broader array of the HIF/hypoxia-dependent gene program in the death or survival of the OFT cardiomyocytes awaits further investigation.

Several other processes are required for the remodeling of the OFT in the transition to the mature dual in-series circulation. One is the septation of the OFT into distinct pulmonary and systemic arteries, a process dependent on migration of cells from the neural crest into the OFT [reviewed in (47)]. The invasion of the heart by the neural crest cells is dependent on neuropilins and semaphorins (12), signaling proteins related to the VEGF family. VEGF also has been suggested to regulate negatively the formation of AV mesenchymal cushions (27), a tissue that will be remodeled to form the valves. Similarly, the recruitment of the endothelial progenitor cells and formation of the coronary arteries at the base of the OFT appears to be under the control of VEGF (103). Thus, it is tempting to speculate that the O_2 deprivation in the embryonic OFT has a field effect, leading to its remodeling into the adult structures, a hypothesis that may be tested with targeted gain- and loss-of-function experiments. A number of other signaling pathways have also been shown to play a role in these various processes in the OFT, and the role of hypoxia will have to be considered within the broader context of these signaling pathways and cell–cell interactions.

Vasculogenesis and angiogenesis. The formation of new blood vessels, either by de novo assembly of endothelial progenitor cells (vasculogenesis), or by extension from preexisting vessels (angiogenesis), is the prototypic hypoxic response to restore oxygen supply to a tissue (21, 67, 91). Not much investigation has been done on the extent to which tissue hypoxia, or oxygen gradients, arising during normal development, drive blood-vessel formation in the embryo. Inactivation of a number of hypoxia-responsive genes in the mouse germline cause mid-gestation death (see later), consistent with a potential role for tissue hypoxia. However, because most hypoxia-responsive genes are also regulated by other factors, the observed defects in these embryos cannot with confidence be attributed to an effect of tissue hypoxia. In the developing chicken heart, at the same time that the OFT cardiomyocytes are experiencing hypoxic stress, EPCs are recruited to the cardiac OFT *via* the proepicardial tissue (97), form a peritruncal vascular plexus (50, 97), and are reorganized to form the coronary vessels [reviewed in (77)]. These cells are also VEGFR2 positive, and VEGFR2 is essential to the process of developmental vasculogenesis (see later). Whether the processes of PCD-dependent OFT remodeling and coronary vasculogenesis are mechanistically linked, particularly in relation to hypoxia, or merely coincidental, requires further investigation. An increasing gradient of EF5 binding and HIF-1α protein is also observed in the ventricular myocardium from endocardium to epicardium (97, 109); any relation of this gradient to the coverage of the epicardial surface with epicardial mesenchyme containing vascular progenitors, and the formation of the vessels within the ventricular myocardium, will require further study. Increased EF5 binding is also observed (a) in the myocardium of the atrioventricular junction (109) (Fig. 7), suggesting that hypoxia could also play a role in the developmental remodeling of this structure and its underlying mesenchyme; and (b) in the ventricular septum, particularly at the site of formation of the AV node/bundle of His. As yet no data exist as to whether hypoxia plays a role in the development of the specialized cardiac conduction system, another relatively late development in the evolution of the cardiovascular system.

Mammalian heart development. The indicators of relative tissue hypoxia, EF5 binding, and HIF-α accumulation show similar distributions in the mouse embryo (5), as described for the chick earlier. Thus, the OFT myocardium and ventricular septum are EF5 and HIF-1α positive at ED11–14, the period of remodeling of the OFT in the transition to a dual circulation in the mouse embryo that corresponds to stage 25–32 (ED5–8) of chicken development. This is also the developmental period in the mouse for the formation of the coronary vessels. One observation from mouse development that was not noticed in the chicken embryo was the striking accumulation of EF5 in the forming aorticopulmonic septum. Whether this represents relative hypoxia of this tissue, and whether this may also be true in the chicken embyro, requires further study.

No analysis exists of the developing mammalian heart and vascular structures of a possible correlation of the hypoxic regions of the heart with the expression of hypoxia-inducible genes, or the extent to which these genes may be regulated by hypoxia/HIF. This will be important to establish to interpret the results of mouse gene gain- and loss-of-function studies in the context of the proposed model of hypoxia-dependent cardiac morphogenesis. A number of gene loss-of-function, and fewer change-of-function or gain-of-function experiments, have been performed in the mouse with hypoxia-responsive genes. The deletion of HIF-1α or β genes in the mouse germline results in death at ED10–14 [reviewed in (88, 92)], when the embryonic cardiovascular system is beginning to function in O_2 and substrate delivery to the tissues. The cell or tissue type for which deletion of the hypoxia-inducible HIF-α is catastrophic to the embryo has yet to be clearly defined, and any defect in the vascular system, including the placenta, with resultant effects on oxygen delivery, is likely to have pleiotropic effects on the embryo. Similarly, the germline inactivation of other HIF-related and hypoxia target genes, such as PHD2 (reviewed earlier) and the Von Hippel–Lindau protein, which like PHD2 is required for the degradation of HIF-α under normoxia [reviewed in (42)] also results in the death of the embryo between ED10 and 14, almost certainly due to the embryo's growing need for O_2 and substrates at this stage of development.

The early lethality of embryos with deletion of HIF-α, as well as the likely pleiotropic effects of vascular (placental) insufficiency, confounds the study of the role of hypoxia in organ morphogenesis and vasculogenesis by using germline gene inactivation. A more targeted strategy for gene inactivation has been developed in which the gene is inactivated in specific cell types. This is the Cre-Lox technology, in which a portion of

the gene of interest is flanked by lox recombination sites (86). Cre recombinase is expressed from a tissue-specific promoter driving expression in the cells of interest. A mouse carrying the Cre transgene is then crossed with a mouse with the floxed alleles, resulting in tissue-specific inactivation of the gene with varying efficiency. This approach has been used to alter the expression of a limited number of genes in the hypoxia signaling pathway within specific cell types in the developing heart and vascular system.

Deletion of a floxed HIF-1α gene restricted to the embryonic ventricular myocardium by using the MLC2vCre driver did not affect viability and produced a mild (~15%) reduction in the vascularity of the mature myocardium (46). Although one could conclude that no major role for HIF exists in the embryonic ventricular myocardium, confounding factors in gene-inactivation studies may include family member redundancy, cell and physiologic compensations and adaptations within the embryo, issues in the timing of gene inactivation relative to morphogenesis, and efficiency of inactivation of the gene and its protein product. In a study using a different floxed HIF-1α allele, neural-specific inactivation of HIF-1α by using nes-Cre resulted in a much greater reduction in vessel density (80%) in the brain's ventral cortical plate associated with apoptosis of the neuronal cells (104). As a reduction in VEGF was found in the brains of these mice, this suggests that brain parenchymal hypoxic induction of HIF-1 and VEGF is required to generate a vascular supply to this organ. Alternatively, or in addition, these results could also be consistent with VEGF promoting survival of the neuronal cells through autocrine signaling.

We now turn our attention to the gene targets of HIF. Inactivation of VEGF or the VEGF receptor (VEGFR2) in the germline is embryonic lethal [reviewed in (23)]. Inactivation of floxed VEGF alleles in the ventricular myocardium by using the MLC2vCre driver resulted in nonviability with partial penetrance, myocardial thinning, and 50% reduction in myocardial capillary density (38). The large epicardial coronary arteries were normal. These results suggest that VEGF transcribed in the embryonic ventricular cardiomyocytes is necessary for the formation of the microcirculatory vessels, similar to a conclusion reached in experiments with germline inactivation of HIF-1α (83). A similar conclusion was reached when VEGF was inactivated in the brain neuronal cells by using the nes-Cre driver, in which guidance of capillary growth to the brain parenchyma was defective (74). VEGF gain-of-function, induced by insertion of a LacZ transgene at the 3′ end of the transcript, resulted in lethality at ED12–14 with thinned myocardium, dilated epicardial vessels, and defects in OFT septation (65). At least four isoforms of VEGF-A are produced by alternative splicing of exons [reviewed in (22)]. VEGF change of function, produced by inserting a specific isoform into the VEGF genomic locus, also caused lethality and cardiovascular defects. Mice that only expressed the 120-kDa or 188-kDa isoforms of VEGF had OFT defects, including a spectrum of defects in the ventriculoarterial connections and in the remodeling of the aortic arches (94). Some of these defects resembled those seen in congenital human heart defects such as tetralogy of Fallot, persistent truncus arteriosus, and DiGeorge syndrome (see Fig. 4). These studies demonstrate that the developing cardiovascular system is exquisitely sensitive to changes in VEGF activity, and also suggest that alterations in hypoxia-dependent morphogenesis in the developing cardiovascular system could play a role in congenital human heart defects. Another signaling system that is induced by hypoxia and recruits endothelial progenitors to sites of hypoxia is the SDF-1 ligand/CXCR4 receptor signaling system (20). SDF-1 expression in the developing heart (63) appears to overlap with regions of tissue hypoxia, although a direct comparison has not been made. Germ-line deletion of SDF-1 results in embryonic or neonatal lethality and a membranous ventricular septal defect (66), suggesting that this signaling system could be involved in heart septation or other morphogenetic processes.

Erythropoietin, as its name implies, is the primary hormone responsible for erythropoiesis throughout life, and its expression is highly regulated by hypoxia and HIF. In addition to its function in stimulating erythropoiesis, erythropoietin also has an autocrine function to promote cell survival under hypoxic stress (10, 53, 113), a striking analogy to the VEGF proposed paracrine and autocrine functions reviewed earlier. The germline deletion of Epo or the Epo receptor results in midgestation lethality. Although these embryos are severely anemic, it has been suggested that part of the lethality is due to the abrogation of the autocrine growth effect of Epo on the parenchymal cardiomyocytes (112) and neuronal cells (113). Given that these embryos have reduced O_2 delivery secondary to anemia, the sorting out of the extent to which the defects in heart and brain development are primary or secondary will require tissue-restricted gene-inactivation experiments.

CONCLUSIONS

The cardiovascular system has evolved over millions of years to optimize its function in O_2 and substrate delivery matched to the metabolic demands of the tissues that it serves. As animals became larger and more complex, with greater energy demands, the demand for O_2 increased. Key developments that facilitated increased O_2 delivery were pigments to increase O_2 carrying capacity and the separation of oxygenated and deoxygenated blood in a dual in-series circulation. A number of lines of investigation suggest that the adaptations of the developing avian and mammalian embryo to O_2 gradients in a relatively hypoxic environment may play a critical role in the formation of specific heart and vascular structures, such as the OFT, coronary vessels, and placenta.

Although a great deal is known about OFT formation in vertebrate hearts, many pressing questions remain. We believe that the following topics are ripe for investigation:

- What are the molecular mechanisms by which hypoxia may trigger the remodeling of the embryonic cardiac OFT?
- Does a direct or indirect role exist for tissue hypoxia in the morphogenesis of other cardiovascular structures where EF5 staining/HIF-1a is observed, such as the atrioventricular junction, the cushion mesenchyme, and the conduction system?
- What might be the role of developmental tissue hypoxia in the phylogenetic differences in the cardiac outlet and other structures?
- Do mutations in the genes in the hypoxia signaling pathway relate to congenital structural heart disease?

- Could hypoxia-dependent morphogenesis be particularly susceptible to disruption by oxygen deprivation or oxidant stress?

Answering these and related key questions should rapidly advance our understanding of heart and OFT morphology, evolution, development, and disease.

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ABBREVIATIONS

Akt, protein kinase B; Ao, aorta; Cre-Lox, Cre recombinase and LoxP sites; ED, embryonic day; EF5, 2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide; EPC, epithelial cell; Epo, erythropoietin; HIF, hypoxia-inducible transcription factor; LV, left ventricle; OFT, cardiovascular outflow tract; MLC2vCre, myosin light-chain 2v Cre; PA, pulmonary artery; PCD, programmed cell death; PHD, prolyl 4-hydroxylases; RV, right ventricle; VEGF, vascular endothelial growth factor.

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