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Embryonic Development of the Pharyngeal Arch Arteries in Mammals

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Abstract

The pharyngeal arch arteries (PAAs) in mammals undergo asymmetric remodeling to give rise to the major blood vessels. The first and second PAAs form rudimentarily in mammalian embryos and eventually regress as the third, fourth, and sixth PAAs predominate. Cell migration, proliferation, and apoptosis drive the remodeling process, stimulated by a number of underlying molecular mechanisms. Sonic hedgehog, Hox genes, Tgfβ2, Tbx1, and a number of transcription regulators all influence PAA morphogenesis. Tbx1, which is found in the deleted region of Chromosome 22 in DiGeorge Syndrome patients, forms anterior-to-posterior and medial-tolateral gradients in the developing PAA system to promote remodeling. The transient and rudimentary presence of the first and second arteries may help establish the Tbx1 gradient, recruit cardiac neural crest cells, contribute to formation of the other arches, and advance craniofacial development.

Keywords

pharyngeal arch arteries, aortic arches, heart development, rudimentary structures, Tbx1, DiGeorge Syndrome

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I. Introduction, History, and Evolution

Early work on the pharyngeal arch arteries (PAAs) in the developing human embryo is often credited to E.D. Congdon. In the early twentieth century, scientists held conflicting views on the significance of mammalian PAA formation. Some believed the PAAs, resembling gills of the mammal's evolutionary ancestors, formed according to recapitulation theory. Others suggested that the PAAs hardly resemble mammalian evolutionary history at all (Congdon 1922). Congdon criticizes even earlier PAA work from the mid-nineteenth century by Rathke, whose diagram of the mammalian arch system apparently does "more harm than good" due to its incongruity (Congdon 1922). Congdon, from his own research, believed that the embryonic environment—the surrounding organs and tissues—influenced cardiovascular and PAA development. He observed how remodeling of the embryonic pharynx prompted the heart's descent into the thorax and hypothesized that the new posterior environment altered the branchial system to promote cardiovascular rearrangement (Congdon 1922). At a time when little was known about the central dogma of biology, cell signaling, and regulation, Congdon knew that

communication within the embryonic environment somehow contributed to PAA morphogenesis.

Early evolutionary debate aside, the pharyngeal gill slits are a synapomorphy among the chordates and an early characteristic of the deuterostomes (Gillis et al. 2012). Pharyngeal development in vertebrates is rather complex but ultimately stems from epithelial-mesenchymal tissue interactions (Gillis et al. 2012). The number of arches also vastly differs across the various vertebrate classes. Jawless hagfish, lampreys, and teleost fish each have 15, 8, and 5 pairs of arches, respectively. In the amphibians, PAA development in larval salamander stages closely resembles that of adult teleost fish, but the salamander's arteries later regress to form the third, fourth, and sixth arches (Bamforth et al. 2012). Mammals exhibit arches 1, 2, 3, 4, and 6 with the first and second arches forming rudimentarily. The fifth PAA supposedly never forms in mammals, reptiles, or birds (Bamforth et al. 2012).

II. Developmental Progression

A multitude of processes including cell migration, proliferation, and apoptosis affect development of the PAAs. Vertebrate pharynx formation begins when the pharyngeal endodermal outpockets arise from the foregut and fuse with the body wall ectoderm to form slits. The pharyngeal arches, filled with mesenchyme, form between these slits (Gillis et al. 2012). The pharyngeal arches are bilateral bulges, each containing a nerve, bone, muscle, and artery (Bamforth et al. 2012). While all three germ layers—endoderm, ectoderm, and mesoderm—contribute to arch formation, a layer of mesenchyme derived from mesodermal and neural crest cells surrounds the artery component—the pharyngeal arch artery (Mao et al. 2019; Nie et al. 2011). The PAAs symmetrically arise from the aortic sac and undergo species-specific

asymmetric remodeling to form the major blood vessels during embryogenesis (Fujita et al. 1992; Lindsey et al. 2015). Apoptosis drives the remodeling process, which involves the rudimentary regression of the first and second PAAs in mammals (Lindsey et al. 2015).

With striking similarity, the mouse often serves as a model organism for human cardiovascular morphogenesis (Geyer and Weninger 2012). Hiruma et al. (2002) outlines the progression of PAA formation in mice: the first and second PAAs form from the foregut between 9-9.5 days of gestation (DG). Between 9.5-10 DG, the third and fourth arches begin to form while the first arch thins and regresses into the first pouch. Next, at 10 DG, the first PAA transforms into the mandibular artery, while the second PAA regresses to form a capillary plexus. Simultaneously, the sixth PAA starts to form, connecting the aortic sac to the dorsal aorta. Between 10.5-11 DG the second PAA regresses and distally transforms into the hyoid artery and proximally joins with the remnants of the first PAA to partially form the exterior carotid artery. Together the first and second PAAs also form the stapedial artery. At this point, the third, fourth, and sixth PAAs have fully developed. Hiruma et al.'s (2002) outline provides a nice basis for mammalian PAA development. 3D reconstructions of mouse embryos by Bamforth et al. (2012) also show regression of the first and second PAAs as the third, fourth, and sixth PAAs start to form (Figure 1A-C).

For over one hundred years a great debate has presided over the presence of a fifth arch artery in mammalian embryos (Bamforth et al. 2012). In his early research, Congdon (1922) recognized the controversy over the fifth PAA but stated further research would be needed to elucidate its presence. Recent studies, such as that by Hiruma et al. (2002), fail to observe the fifth PAA in mice while research by Geyer and Weninger (2012) contradicts these findings (Figure 1D). On the other hand, research by Bamforth et al. (2012) indicates rudimentary

formation and regression of the fifth PAA in human embryos (Figure 1E-F). This study describes the fifth PAA as a transient and inconspicuous mass of mesenchyme just above the pulmonary arch (Bamforth et al. 2012), which forms after the sixth arch artery develops (Geyer and Weninger 2012). The debate continues.

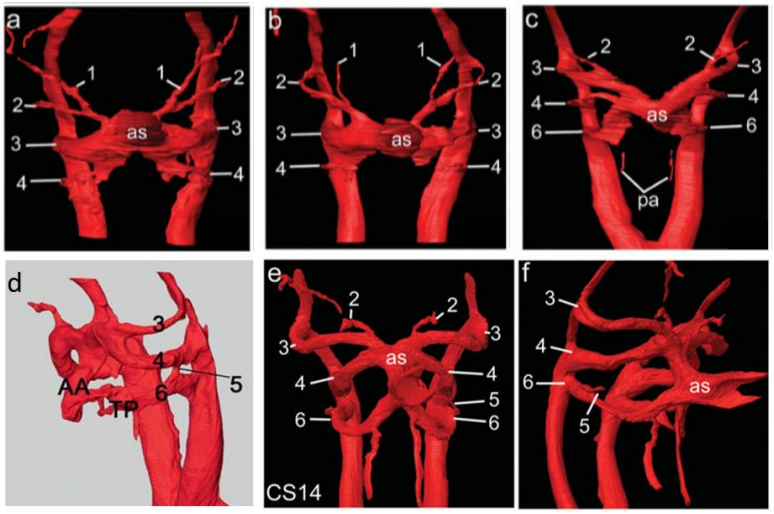

Figure 1. Morphogenesis of the Pharyngeal Arch Arteries in Mice and Humans. A-C: Ventral view of a 3D reconstruction showing regression of the first and second PAAs and morphogenesis of the third, fourth, and sixth PAAs in mice at 10.5 days of gestation. AS = aortic sac and PA = pulmonary arteries (Bamforth et al. 2012). D) Left view of a 3D reconstruction showing evidence of a unilateral 5th PAA in mice. AA = Aorta ascendants and TP = Truncus Pulmonalis (Geyer and Weninger 2012). E-F) Ventral (E) and right (F) 3D reconstructions showing evidence of a fifth PAA in a human embryo at Carnegie Stage 14 (Bamforth et al. 2012).

In humans PAAs 1, 2, 3, 4, and 6 form symmetrically. The first and second PAAs form rudimentarily and eventually regress. The first arch develops into parts of the jaw, teeth, ear, and cranial soft tissue (Yamagishi et al. 2005). It also contributes to the mandibular and maxillary arches although, in contrast to mice, the mandibular artery is vestigial in humans (Hiruma et al. 2002). As seen in mice, the second PAA also forms the hyoid artery in humans (Silbergleit et al. 2000). According to Silbergleit et al. (2000), the mandibular and hyoid arteries eventually contribute to the third PAA, suggesting the first and second PAAs function rudimentarily to form the contributors of the future and more prominent PAAs. Moreover, the stapedial artery forms

from the hyoid artery, and therefore from the second PAA. The stapedial artery extends cranially to form the craniofacial vasculature in the ears (Silbergleit et al. 2000). The third, fourth, and sixth arch arteries contribute to the formation of the major vessels (Figure 2). The third PAAs form the common carotid arteries. The right fourth PAA forms the right subclavian artery while the left fourth PAA contributes to the aortic arch. The left sixth PAA contributes to the formation of the fetal ductus arteriosus (Rana et al. 2012). A seventh intersegmental artery contributes to the left subclavian artery, but this seventh artery is not considered part of the PAA system (Rana et al. 2012).

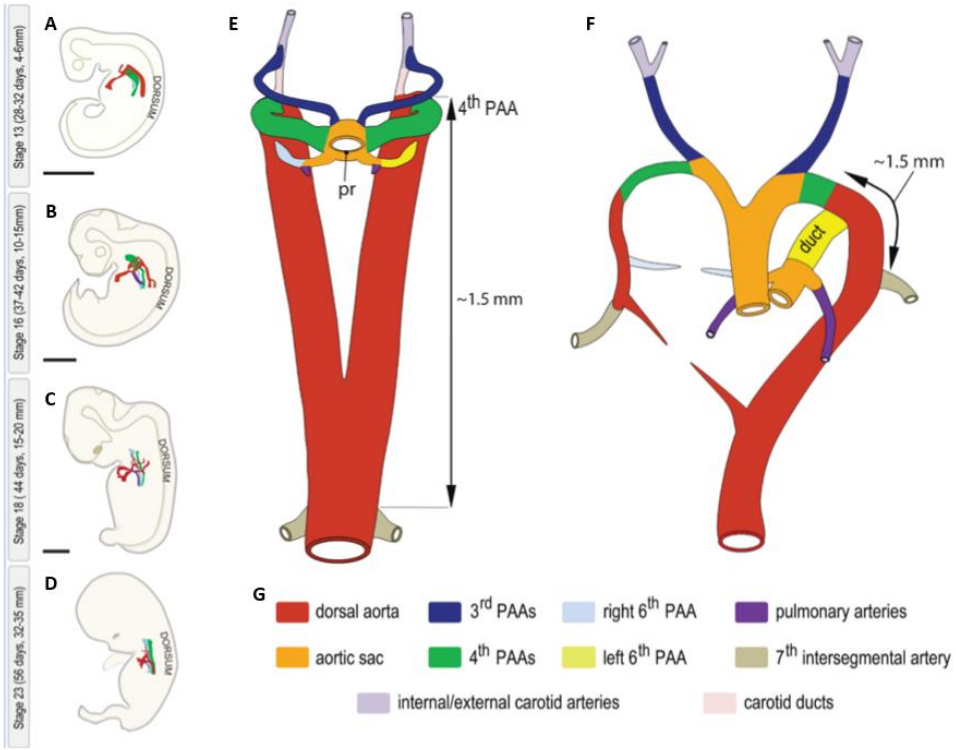

Figure 2. A-D) Morphological overview showing the relative position and descent of the aortic arch system in the developing human embryo between 28 and 56 days of development. Red = PAAs, Green = pharynx, Blue = trachea/bronchi, Purple = pulmonary arteries (Rana et al. 2012). E-F) Contributions and morphogenesis of the human embryonic arch arteries to the adult system between embryonic days 28 (E) and 44 (F). The third PAAs contribute to the common carotid and internal carotid arteries. The fourth PAAs contribute to the dorsal aortae and the right subclavian artery, which is not shown in this diagram. The left sixth PAA contributes to the ductus arteriosus (Rana et al. 2012). G) Color code for E-F (Rana et al. 2012).

III. Developmental Molecular Mechanisms

A number of molecular mechanisms underlie PAA remodeling. To begin, sonic hedgehog affects formation and regression of the PAAs in combination with fibroblast growth factor (Fgf) signaling molecules. Sonic hedgehog (Shh) is expressed in the arch epithelium, and disruption of its expression has resulted in craniofacial and organ abnormalities in mice (Yamagishi et al. 2006). These mutant mouse embryos had smaller heads and severe hypoplasia, or underdevelopment, of the first PAA. Moreover, the first PAA did not separate into two bilateral components and rather remained connected at the midline above the second PAAs (Yamagishi et al. 2006). This study also showed that Shh promotes mesenchyme survival in the first PAA, as downregulation of Shh led to increased apoptosis in this artery, thus leading to the observed hypoplasia (Yamagishi et al. 2006). As Shh contributes to mesenchyme survival, it also aids in epithelial-mesenchyme signaling via Fgf8 pathways in the first PAA (Yamagishi et al. 2006). Sonic hedgehog likely acts concurrently with other signaling molecules and transcription factors to regulate growth of the first PAA.

Like sonic hedgehog, transcription factors Hrt1 and Hey1 are also expressed in the pharyngeal arch ectoderm. Hrt1/Hey1 null mouse embryos did not reveal any changes in cellular processes of the cardiac neural crest cells (NCCs) or in expression of other signaling molecules, such as Tbx1 and Fgf8 (Fujita et al. 2016). Hrt1/Hey1 mutations most significantly affected remodeling of the fourth PAA, although they also affected morphogenesis of the third and sixth PAAs (Fujita et al. 2016).

Many studies on the pharyngeal arches, such as the research done by Fujita et al. (2016) focuses on remodeling of the fourth arch, as that is where many deleterious effects are often observed. The fourth PAAs are also extremely important in development, since they eventually

become part of the aorta. I will return to this idea later to discuss a potentially valuable relationship between the first, second, and fourth PAAs.

The homeobox (Hox) genes also play a significant role in PAA development. According to Roux et al. (2017), Hoxa1 and Hoxb1 help direct PAA morphogenesis in a dose-dependent manner. In knockout experiments, homozygous mice for either Hoxa1-/- or Hoxb1-/- deletions showed abnormalities of the fourth PAA. The same result occurred when a mouse was heterozygous for both Hoxa1-/+ and Hoxb1-/+ (Roux et al. 2017). Downregulation of Hoxa1 and Hoxb1, such as in a mutant mouse homozygous for Hoxb1-/- and heterozygous for Hoxa1-/+, also caused improper migration of cardiac neural crest cells and thus negatively impacted development of the third, fourth, and sixth PAAs (Roux et al. 2017). The Hox genes help specify and direct the migration of NCCs, which invade the outflow tract and the mesodermal core of the pharyngeal arches (Roux et al. 2017). The NCCs recruit smooth muscle cells necessary for remodeling the PAAs (Roux et al. 2017). Roux et al. (2017) does not address the effects of Hoxa1 and Hoxb1 downregulation on remodeling of the first and second PAAs.

Tgfβ2 and its role in apoptosis further promotes PAA morphogenesis and remodeling. The transforming growth factor beta (Tgfβ) family of cytokines functions in a number of biological processes including inflammation, repair, immunity, and signaling (Clark and Coker 1998). Apoptotic cells reside in the mesenchyme of the PAAs, most notably in the regressing first and second PAAs (Molin et al. 2002). Homozygous mutant Tgfβ2-/- mice showed a number of defects in the systematic remodeling of the PAAs. These mice showed condensed mesenchyme, underdevelopment of the aortic arch, and lack of apoptotic cells (Molin et al. 2002). Even though there were fewer apoptotic cells, the rate of apoptosis actually increased in all PAAs of the mutant mice with the most detrimental effects visible in the fourth PAA. This

study suggests that proper remodeling of the PAAs occurs when apoptotic and mitotic processes counterbalance each other (Molin et al. 2002). If, however, deletion of Tgfβ2 also increases apoptosis in the first and second PAAs, does Tgfβ2 contribute to their regression in wild-type mice? Perhaps Tgfβ2 eventually becomes downregulated in the first and second PAAs to promote apoptosis and subsequent regression during development. On the contrary, additional molecules involved in Tgfβ2 pathways may alter expression to stimulate apoptosis.

Together BMP4 and Tbx1 also affect PAA remodeling. BMP4 is a bone morphogenic protein that binds to receptors on target cells to initiate signaling through Smad and kinase pathways, but it also affects PAA morphogenesis due to its overlapping expression with Tbx1 in the pharyngeal mesenchyme (Nie et al. 2011). In mutant mice, inactivation of BMP4 in the Tbx1 expression domain resulted in defects of outflow tract separation. Initially, the outflow tract connects the right ventricle to the PAAs. Without proper separation, the right outflow tract cannot adequately connect the right ventricle to the pulmonary arteries, and the left outflow tract fails to connect the left ventricle and aortic outlets (Nie et al. 2011). Interestingly, PAA formation was not significantly affected, but inactivation of BMP4 in the mesenchyme negatively impacted PAA remodeling (Nie et al. 2011). Like the Hox genes, BMP4 helps recruit smooth muscle cells during remodeling, but BMP4 inactivation did not impact NCC migration (Nie et al. 2011). Since inactivation of BMP4 impaired recruitment of smooth muscle cells and also increased apoptosis, remodeling could not effectively take place (Nie et al. 2011). Clearly, BMPs like BMP4 are requisite players in PAA morphogenesis, but their relationship with Tbx1 may further elucidate the process. While these molecular mechanisms are vast and diverse, they each play a unique role in remodeling the pharyngeal arch arteries.

IV. The Role of Tbx1

The T-box transcription factor 1, or Tbx1, gene plays an important role in the development of the pharyngeal arch system. Early in mouse development, Tbx1 is expressed in the endoderm and mesoderm of the first pharyngeal arch but not in NCCs. Later, Tbx1 is expressed in the third pouch. As development of the arches progresses, Tbx1 is expressed in the most posterior arches, establishing an anterior-to-posterior gradient (Vitelli et al. 2002). Tbx1 expression also follows a medial-to-lateral gradient (Vitelli et al. 2002). Tbx1 is required for remodeling the PAAs. To illustrate, in homozygous mutant mice for Tbx1, the third, fourth, and sixth PAAs do not form, and even in heterozygous mutants, the fourth PAA shows significant hypoplasia (Vitelli et al. 2002). Tbx1 also functions to remodel the outflow tract, likely in concert with BMP4 signaling, since Tbx1 mutants showed defects in the proximal regions and septa of the outflow tract (Vitelli et al. 2002).

Tbx1 is expressed as orthologs in other vertebrates with multiple paralogs like Tbx2 and Tbx3 within species, as well (Figure 3). The orthologs between human and mouse unsurprisingly show the closest relationship, as these animals are both mammals. The frog and chicken also show an evolutionary relationship with these mammals in Tbx1 and Tbx3 and in Tbx2 for the frog only (Figure 3). While the starlet anemone also shows evidence of a Tbx1 gene, this outgroup is much more distantly related to the vertebrates. Since this invertebrate lacks a circulatory system, Tbx1 may function in other processes for this organism.

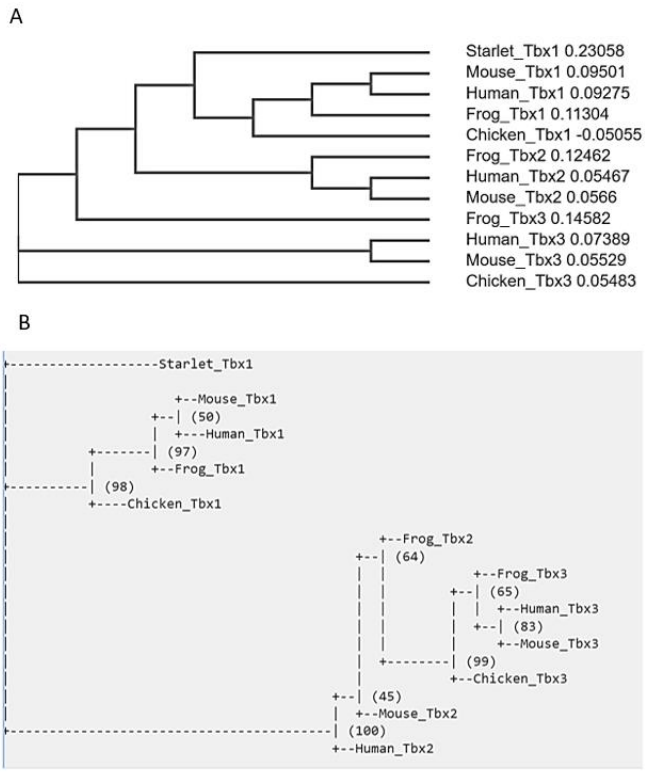

Figure 3. Phylogenetic reconstruction of protein family Tbx1, Tbx2, and Tbx3. Panel A is a Neighbor-Joining Tree and Panel B is a Maximum Likelihood Tree from 1000 bootstrap replicates. Parentheses in B are bootstrap values for individual nodes. Both trees show evolutionary relationships, particularly between the mammals, and Panel B shows monophyly of conserved orthologs of Tbx1 and Tbx3 (Hoang et al. 2017; Kalyaanamoorthy et al. 2017; Nguyen et al. 2015).

Tbx1 assumes a multitude of other functions in humans in addition and complementary to PAA remodeling. As a transcriptional regulator involved in protein dimerization and RNA Polymerase II transcription factor activation, Tbx1 is also involved in processes such as angiogenesis, heart development, cell proliferation, mesenchymal cell apoptosis, artery/blood vessel morphogenesis, PAA and outflow tract morphogenesis, neural crest cell migration, and craniofacial and ear morphogenesis (UniProtKB). The involvement of Tbx1 in development is made possible through its interactions with many other molecules and cellular components (Figure 4). Notably, Tbx1 interacts with various BMPs, RIPPLY, EYA, and SIX, all of which

are transcriptional regulators. Disruptions to these interactions would likely interfere with transcription of gene products necessary to perpetuate the PAA remodeling process.

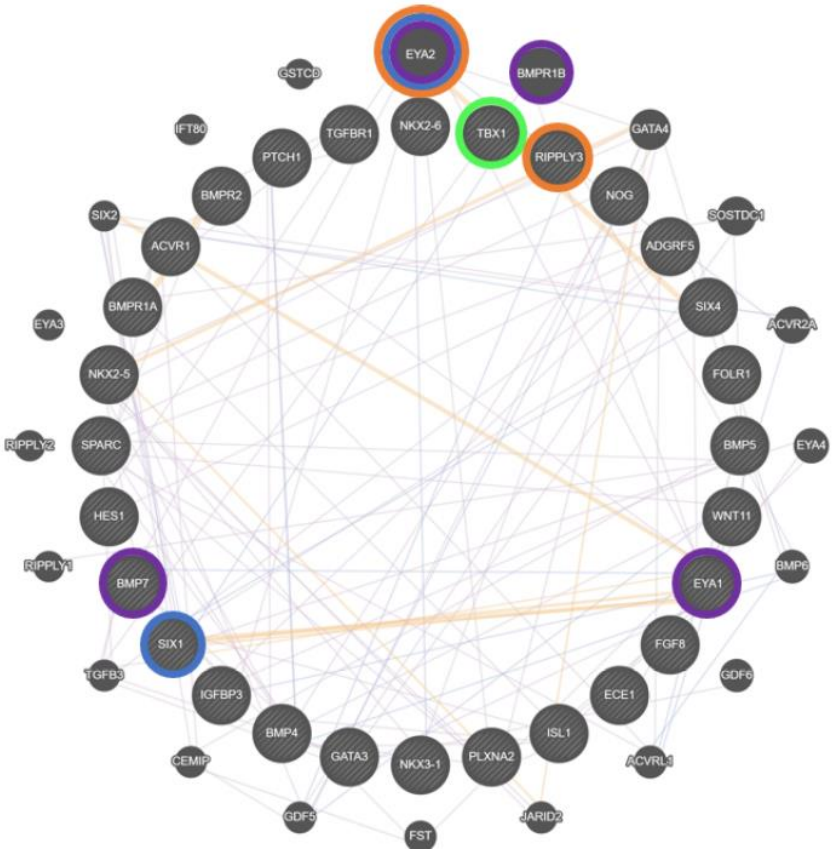

Figure 4. Genemania network analysis for representative genes from Uniprot GO-Biological processes category "pharyngeal system development". Network colors indicate purple: co-expression; orange: predicted; blue: co-localization. Direct relationships to Tbx1, circled in green, are circled in respective colors. Modification of this network is likely involved in the formation and regression of the pharyngeal arch arteries. Several of these interactions are also involved in other developmental or physiological processes. Loss of these network components may interfere with development of the embryonic pharyngeal arches, causing malformations, hypoplasia, DiGeorge Syndrome, and/or embryonic lethality. Therefore, retention of these network interactions may account for the retention of the pharyngeal arch arteries.

V. Hypotheses, Future Directions, and Medical Significance

Based on the information presented above, I propose several hypotheses to explain the formation and subsequent rudimentary regression of the first and second PAAs in mammalian development. To begin, the first and second PAAs may help recruit cardiac neural crest cells to

the aortic sac. Since the PAAs are surrounded by a layer of NCCs, their regression might assist in transporting those cells to the aortic sac, where the NCCs can influence remodeling of the other PAAs. Although Roux et al. (2017) does not address the effects of downregulating Hoxa1 and Hoxb1, which are expressed in the NCCs, in the first and second PAAs, it is possible that Hox gene inactivation also impedes their regression. This study observed defects in third, fourth, and sixth PAA remodeling, which may have resulted from improper NCC migration from Hox downregulation in first and second PAAs. On the other hand, the NCCs may not affect remodeling at all, as one study found active mitotic cells in the second arch despite absence of NCCs (Gavalas et al. 2001). An additional study showing the impact of Hoxa1 and Hoxb1 knockout at earlier embryonic stages would provide greater insight into the role of the PAAs in NCC migration.

Next, the first and second PAAs may influence the formation of the other PAAs, especially the fourth PAA. A great number of studies focuses on remodeling the third, fourth, and sixth PAAs, since these arteries give rise to the major vessels. Perhaps transferring cellular processes and resources from the first and second PAAs to the developing third, fourth, and sixth PAAs provides some sort of energetic benefit. In order to test this hypothesis, one may have to calculate energetic costs of tissue maintenance, regression, blood flow diversion, and remodeling of the main PAAs. This work would be tedious but may provide insight into the physics of cardiovascular development.

The formation and regression of the first and second PAAs may also be necessary for craniofacial development. In the study performed by Yamagishi et al. (2006), disruption of sonic hedgehog expression in mouse embryos resulted in underdevelopment of the first arch, as well as the head. Although sonic hedgehog might have different roles in craniofacial and cardiovascular

development, the positioning of the first and second arches within mammalian embryos provides evidence for a possible correlation. Figure 2A-D shows how the arch system is initially positioned more anteriorly in the embryo before descending into the thorax, as observed by Congdon (1922). Early in development, the first and second PAAs would then have access to the head to provide blood supply for craniofacial morphogenesis. When they are no longer needed for head development, the PAAs would then regress as the entire system moves posteriorly and settles into the thorax. In humans, as suggested by Silbergleit et al. (2000), the first PAA becomes the regressing mandibular artery while the second artery becomes the hyoid artery, which later morphs into the stapedial artery and eventually transforms into a multitude of cranial arteries. This evidence further supports the idea that the first and second PAAs contribute to craniofacial development even if they do so indirectly. This hypothesis could also be tested using human and mouse embryos at earlier stages to monitor cranial positioning of the first and second arteries before they regress.

Finally, the first and second PAAs may function to establish the gradient of Tbx1. The expression of Tbx1 begins in the first arch and extends posteriorly to the third, fourth, and sixth arches. Since Tbx1 is a transcription factor, it is unlikely that this molecule physically moves to the other arches. Instead, the presence of the first and second arches may spatially contribute to the gradient and promote other signaling activation of Tbx1 farther down the gradient. Perhaps the regression of these arches also helps establish the medial-to-lateral gradient of Tbx1 expression. In order to test these hypotheses, studies should carefully examine how the gradient of Tbx1 changes over the course of PAA remodeling. Observation of Tbx1 expression could be accomplished using RNA in situ hybridization, as suggested by Vitelli et al. (2002). Determining

the spatial and temporal formation of the Tbx1 gradient may further elucidate transcription regulation and molecular pathways involved in PAA morphogenesis.

Studying early embryonic development of the cardiovascular system can provide further insight into the pathogenesis of DiGeorge Syndrome. At the molecular level DiGeorge Syndrome is caused by a heterozygous deletion on Chromosome 22, and the deleted region includes the Tbx1 gene (Vitelli et al. 2002). Downregulation of Tbx1 expression is linked to the malformations and characteristic phenotypes associated with this disease (Ohnemus et al. 2002). Babies born with DiGeorge Syndrome exhibit cardiac malformations, pharyngeal and aortic arch defects, and craniofacial abnormalities (Nie et al. 2011). Taken together, studying the pharyngeal arch arteries and the role of associated molecular mechanisms, such as those of Tbx1, in mammalian development can shed light on evolutionary history and the implications of congenital diseases.

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