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Date deposited: 22nd July 2013

Version of file: Author final (peer-reviewed)

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Clarification of the identity of the mammalian fifth pharyngeal arch artery

Original communication

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8 figures, 1 table

Abbreviated title: The fifth pharyngeal arch artery

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ABSTRACT

The remodeling of the pharyngeal arch arteries is a complex process that occurs across vertebrates, although the specific number of arteries varies across species, with six in fish, but only five in birds and mammals, although they are numbered one through four, and six. The existence of a fifth arch artery in mammals has been debated for more than a century. Although some have doubted, and continue to doubt, its existence, several cardiovascular malformations can be explained only on the basis of its presence. We have analyzed the developing pharyngeal arch arteries in mouse and human embryos, using high-resolution episcopic microscopy. We have then created three-dimensional models, allowing us to identify any structures that would satisfy the descriptions of fifth arch arteries. This detailed examination revealed collateral channels connecting the fourth and sixth pharyngeal arch arteries in approximately half of the mouse embryos examined. Such collateral channels were seen in only one human embryo of eight examined by high-resolution episcopic microscopy, although we had previously identified such collateral channels using wax plate reconstruction. An extra vessel, occupying a discrete component of the pharyngeal mesenchyme, and therefore resembling a true fifth pharyngeal arch artery, was observed in one CS14 human embryo. The pharyngeal mesenchyme in the human, therefore, can contain a fifth arch, with a contained artery, albeit transiently. Persistence of this structure, and the observed collateral channels, provides mechanisms to explain the congenital cardiovascular malformations described as persistent fifth aortic arch, and double-barreled aorta.

Keywords: heart development; pharyngeal arch arteries; embryology

INTRODUCTION

The aortic arch arteries of the mammal are derived from the arteries that develop within the embryonic pharyngeal arches. These pharyngeal arches are transient structures that develop as a cranial-to-caudal series of paired bulges, with each arch containing a nerve, bone, muscle and artery component (Graham, 2001, 2003). In mammals, the arteries are initially symmetrical in appearance, but then undergo a complex process of remodeling, resulting in the characteristic asymmetric pattern seen in the adult (Congdon, 1922; Hiruma et al., 2002). The resulting system is comprised of five arteries, numbered one through to four and six, allegedly with the fifth artery being absent. The process of remodeling comprises regression of the first and second arteries by mid-embryogenesis, retention of the third arteries bilaterally to form the carotid arteries, with the right fourth artery contributing to the brachiocephalic artery, and the left fourth artery forming part of the aortic arch. The right sixth artery regresses, while the left sixth artery becomes the arterial duct (ductus arteriosus).

The evolution of the pharynx and the cardiovascular system reveals a reduction in the number of pharyngeal arches, and hence the number of blood vessels within them. For example, the jawless hagfish has 15 aortic arches, whereas the lamprey has eight (Kardong, 2008). From the jawed fishes, such as the teleosts, and in a range of vertebrate species, the developing arteries are modeled on the same pattern of six paired arches (Kardong, 2008). The teleosts have 5 pharyngeal arches following the loss of the first, with the arch arteries numbered three to six servicing the gills. Within the amphibians, the salamander, a terrestrial urodele, retains a similar pattern, with the most caudal arteries supplying blood to the gills in the larval form. In the adult, following the loss of the carotid duct between the third and fourth arch arteries, they supply blood to the heart and body. In the adult frog, however, only the third, fourth and sixth arteries persist, providing separate systemic and pulmonary circulations (Kardong, 2008). In higher vertebrates, such as reptiles and birds, and in

mammals, current dogma suggests that the fifth artery is never seen, even during development (Hiruma et al., 2002; Knackstedt and Mangold, 1997).

The presumed absence of the fifth artery in mammals, nonetheless, has been the subject of debate for well over one hundred years, with early researchers either making claims for its existence (Locy, 1907; Reinke, 1910; van Bemmelen, 1886), or else arguing against it (Lewis, 1906). Congdon (1922) reviewed the situation admirably when commenting as follows: "The so-called fifth arch is described by several authors as arising later than the pulmonary. In the human embryo, at least, it will require further data to determine the time relation between the two vessels. The difficulty lies in the lack of a precise period at which we may regard an arch as coming into existence, owing to the gradual nature of its development from a plexus. Nothing is known on the manner in which the so-called fifth arch disappears. Certainly it does not retain its individuality long, since it has not been described in older mammalian embryos." Irrespective of the subsequent lack of appearance of further data in the developing human, presence at some stage of the alleged fifth arch artery has been invoked on numerous occasions to explain congenital cardiovascular malformations that are otherwise difficult to understand (Donti et al., 1997; Gerlis et al., 1989; Herrera et al., 1987; Izukawa et al., 1973; Lawrence and Stiles, 1975; Van Praagh and Van Praagh, 1969). With the ongoing need for further data in the human, we have now studied a number of human embryos using high-resolution episcopic microscopy and three-dimensional (3-D) reconstructions (Geyer et al., 2009; Weninger et al., 2006; Weninger and Mohun, 2002; Weninger and Mohun, 2007), comparing the findings with those obtained from a larger series of murine embryos.

MATERIALS AND METHODS

Mouse embryos

We analyzed the datasets relating to a total of 37 wild-type mouse embryos of the NIMR Parkes strain from the high-resolution episcopic microscopy collection available at www.embryoimaging.org. The stages and quantity selected for detailed analysis were: seven embryos at embryonic day (E) 10.5, 19 at E11.5, ten at E12.5 and one at E13.5. All embryos were staged by somite number according to Kaufmann (Kaufmann, 1992), and subsequently by the extent of remodeling of the outflow tract and pharyngeal arch arteries. This staging method was employed to avoid the possibility of a lack of concordance between somite number and the degree of remodeling observed. The embryos were fixed in 4% PBS-buffered formaldehyde.

Human embryos

We obtained eight human embryos from the MRC/Wellcome-Trust funded Human Developmental Biology Resource at Newcastle University (HBDR, <http://www.hdbr.org>), with appropriate maternal written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee. The HDBR is regulated by the UK Human Tissue Authority (HTA; www.hta.gov.uk) and operates in accordance with the relevant HTA Codes of Practice. The embryos, one at Carnegie Stage (CS) 13 (equivalent to mouse E10.0), three at CS14 (E10.5), two at CS15 (E11.0), and one each at CS16 (E11.5) and CS17 (E12.0), were fixed overnight at 4°C in 0.1M phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA; Sigma Aldrich, Poole, UK). An additional model of a CS14 human embryo heart was prepared using the classic wax plate method (Born, 1883).

High-Resolution Episcopic Microscopy

The procedure has been described in detail elsewhere (Geyer et al., 2009; Weninger et al., 2006; Weninger and Mohun, 2002; Weninger and Mohun, 2007). The embryos were dehydrated in methanol, embedded in JB4 methacrylate resin (Polyscience) containing

fluorescent dyes (eosin and acridine orange) and sectioned with a Leica SM2500 microtome. After each 2 μ m section was cut, the block face was imaged using a Jenoptik C14+ or Hamamatsu Orca HR CCD camera and Olympus MVX optics with GFP excitation and emission filters. Each stack of intrinsically aligned serial images was appropriately sub-sampled and converted into a volume data set. Amira software (Visage Imaging) was used for segmentation to create 2- and 3-dimensional (3-D) images. The pharyngeal arch arteries were manually outlined using the label field function of Amira and surface rendered to produce the 3-D images.

RESULTS

Development of the pharyngeal arteries and collateral channels in murine embryos

Mouse embryos with 32 somites have arteries extending through the first four pharyngeal arches in bilaterally symmetrical fashion (Fig 1a & b). By the 40-somite stage, sixth arch arteries are also evident, along with the developing right and left pulmonary arteries (Fig 1c). At this stage, the aortic sac, from which the arteries arise, is undivided. We found no evidence, at this stage, of any additional channels on either side, either between the terminations of the arch arteries themselves, or between the arteries and the dorsal aorta in any of the seven embryos studied at this stage.

During E11.5 (somites 41-50), there is extensive remodeling, which results in loss of symmetry, with regression of the dorsal part of the right sixth artery, and diminution in size of the dorsal part of the right fourth artery (Fig 2a-d). The carotid duct, a segment of the embryonic dorsal aorta between the third and fourth arch arteries that eventually regresses, is also apparent at this stage. In some, but not all, embryos, collateral channels now connect the fourth and sixth arteries dorsally, bilaterally in some, and unilaterally in others (Fig 2a-c; Fig 3; and Table 1). These channels, with varying diameters, were seen bilaterally in five E11.5 embryos, unilaterally and left-sided in five embryos, and unilaterally on the right in one (Table 1). There was no correlation between number of somites and the presence or absence of the collateral channels (Fig 4).

The system continued to be remodeled during E12.5 (somites 51-57), which involved ongoing regression and disappearance of the dorsal part of the right fourth artery, and regression and virtual disappearance of the right dorsal aorta (Fig 5). Collateral dorsal channels between the fourth and sixth arteries were seen in three of the ten embryos examined (Table 1 and Fig 3 & 5). Some embryos also demonstrated spurs, which we interpreted as representing closing collateral channels (Fig 3).

Evidence of a fifth arch and artery in human embryos

In human embryos, the overall remodeling of the system parallels the changes seen in the mouse embryos (Fig 6). The arteries are bilaterally symmetrical at CS13, comparable to mouse E10.0, with the sixth arteries not yet fully formed (Fig 6a). By CS14 through to CS16, equivalent to mouse stages E10.5 - E11.5, the aortic sac remodels to form separate aortic and pulmonary channels (Fig 6b - d). By CS17, equivalent to mouse E12.0, the carotid duct has almost disappeared bilaterally, the right sixth has regressed completely, and the right fourth artery is also regressing (Fig 6e). A unilateral collateral channel between the dorsal parts of the left fourth and sixth arch arteries was seen in one CS14 human embryo (Fig 7a-d). A more extensive channel, running through a discrete part of the pharyngeal wall, was found in another CS14 embryo (Fig 7e-h). The vessel is seen running parallel to the fourth and sixth arteries. Most importantly, it is contained within a block of mesenchyme not seen in the other CS14 embryo (compare Fig 7i-k with Fig 7l-n). We interpret the presence of a spur on the right side as indicating that the channel was initially bilateral (Fig 7f). Bilateral collateral channels connecting the fourth and sixth arch arteries were also observed in a CS14 human embryo heart reconstructed using the classic wax plate technique (Fig 8).

DISCUSSION

In this study, we have used high-resolution episcopic microscopy and 3-D reconstructions to visualize the developing pharyngeal arches and their contained arteries in man and mouse. We have identified collateral channels connecting the fourth and sixth arteries, as well as an artery in a human embryo which is contained within a discrete block of pharyngeal mesenchyme situated cranial to the pulmonary arch. Such a fifth arch artery has rarely been observed. Indeed, its very existence has been questioned (Lewis, 1907), while many no doubt continue to doubt its existence. In our human embryo at Carnegie Stage 14, the artery was almost complete, and ran through a discrete segment of pharyngeal mesenchyme, its course indicating that it had initially extended from the aortic sac to join the left-sided dorsal aorta. It is comparable to the extensive arterial channel extending through the pharyngeal mesenchyme reconstructed from a pig embryo by Reinke (1910).

Debate concerning the presence or absence of the fifth pharyngeal arch arteries in mammals originated in the 19th century (van Bemmelen, 1886). By the turn of the 20th century, Locy (1907) had summarized the various anatomic patterns that could be considered to represent fifth arch arteries. Potential candidates included an artery taking origin from the fourth arch artery and returning to it, a channel joining the fourth and sixth arch arteries, an artery running through the pharyngeal mesenchyme from the aortic sac to the sixth arch artery, irregular vascular elements joining the fourth and sixth arch arteries, and a solitary vessel extending from the sixth arch artery to an unknown ventral connection. Congdon (1922) endorsed the possibilities proposed by Locy (1907), emphasizing that Tandler (Tandler, 1902) had described two such arteries in developing human embryos, but as we indicated in our introduction, he also emphasized the need for additional data from developing humans.

We have now identified collateral channels connecting the caudal margin of the fourth arch artery, and the cranial margin of the sixth arch artery, in 14 of 37 mouse embryos examined

within the range of 30 to 60 somites, or E10.5 to E13.5. No collateral channels, nor developing plexuses, were observed in the embryos from E10.5, with 30 to 40 somites. At this stage, the gap between the developing fourth and sixth arteries is relatively large when compared to the E11.5 stage. This suggests that the collateral channels must appear after the sixth artery has formed, when the process of remodeling brings the cranial surface of the sixth arch into close proximity with the caudal surface of the fourth arch. It could be, therefore, that the channels are plexiform entities, but we visualized them only as collateral channels. The incidence of such collateral channels in two-thirds of the embryos examined from E11.5 shows them to be common occurrences at this stage. That the connections do not appear to be correlated precisely to somite number, and do not persist for long, with a number of spurs being observed which we interpreted as closed channels, suggests them to be necessary yet transient connections. As remodeling of the system progresses during later development, the frequency of collateral channels is markedly reduced, with no collateral channels seen after 54 somites.

Two recent studies in mice have identified similar structures to the collateral channels described in our study. The first study, investigating cardiovascular defects in a mouse model of Down's syndrome (Lorandau et al., 2011) identified the structures in E11.5 wild type mouse embryos. These collateral channels were patent to ink, and occurred in over half of the embryos examined at this stage. The authors referred to the channels as fifth arch arteries, and also as vascular rings. As discussed above, we have opted to classify these vessels as collateral channels, rather than true fifth arch arteries, as they do not connect the dorsal aorta to the aortic sac, as do the other arch arteries. The second study identified collateral channels in 76% of mouse embryos at E12.5 by high-resolution episcopic microscopy (Geyer and Weninger, 2012). These embryos were the inbred Him:OF1 strain. With the Down's syndrome mouse model being on an outbred background (Lorandau et al., 2011), this suggests that genetic background does not play a significant role in changing the variability in the configuration of the arch arteries. Further analysis of the collateral channels

and spurs observed in the developing mouse embryos will need to be conducted to confirm the endothelial identity of these vessels. This can be achieved by using molecular markers for the endothelium such as antibodies against CD31/Pecam1.

We have also identified such a collateral channel unilaterally in one CS14 human embryo, as well as bilateral collateral channels in a human wax plate reconstruction. More importantly, in one of our human embryos we identified a channel extending almost the entirety of the distance from the aortic sac to the distal aorta. Of equal significance was the fact that it was contained within a block of pharyngeal mesenchyme not seen in our other human embryos. We submit that our finding begins to answer the question posed by Congdon (1922) with regard to the existence of the fifth aortic arch in man, although we recognize that we will need to study more human embryos fully to resolve the situation. The structure contained within the pharyngeal bulge, nonetheless, was markedly different from the collateral channels, which do exist in the developing human, albeit at a more reduced incidence compared to mouse.

Pediatric cardiologists have long invoked the presence of the fifth aortic arch artery so as to account for vascular channels difficult to explain on any other basis, such as double lumen aorta (Van Praagh and Van Praagh, 1969), or systemic-to-pulmonary arterial collateral channels (Gerlis et al., 1989). Persistence of the dorsal collateral channels accounts well for double-barreled aortas. It requires persistence of a rarer true arterial channel extending from the aortic sac to the dorsal aorta, however, to provide a realistic explanation for the existence of a systemic-to-pulmonary collateral channel comparable to the arterial duct. Those who continue to doubt the existence of the fifth arch artery, or persistence of the collateral channels, however, will now need to provide reasonable alternative explanations for these congenital entities.

ACKNOWLEDGEMENTS

We thank Professor Susan Lindsay for the human embryos and Diane Spicer for photographing the wax model. This research was financially supported by a British Heart Foundation Intermediate Basic Science Research Fellowship to SDB (FS/08/016/24741). The human embryo material was funded by the Medical Research Council (G0700089) and Wellcome Trust (GR082557). The high-resolution episcopic microscopy material was funded by the Wellcome Trust (WT087743MA) and supported by the Medical Research Council.

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Tables

	Embryo #	Stage	Somites	Collateral channel	
				On left	On right
1	SID1843	E10.5	30	No	No
2	SID1912	E10.5	30	No	No
3	SID1904	E10.5	31	No	No
4	SID1911	E10.5	31	No	No
5	SID1908	E10.5	32	No	No
6	SID1910	E10.5	32	No	No
7	SID1530	E10.5	40	No	No
8	SID1715	E11.5	44	Yes	Yes
9	SID1718	E11.5	44	(No)	Yes
10	SID1722	E11.5	45	Yes	No
11	SID1745	E11.5	45	(No)	No
12	SID1845	E11.5	45	Yes	No
13	SID1872	E11.5	45	(No)	(No)
14	SID1528	E11.5	46	Yes	Yes
15	SID1550	E11.5	46	No	No
16	SID1869	E11.5	46	No	(No)
17	SID1693	E11.5	46	Yes	Yes
18	SID1720	E11.5	46	Yes	No
19	SID1841	E11.5	47	No	(No)
20	SID1844	E11.5	47	(No)	No
21	SID1868	E11.5	47	No	No
22	SID1527	E11.5	48	Yes	No
23	SID1838	E11.5	48	Yes	(No)

24	SID1842	E11.5	48	Yes	Yes
25	SID1675	E11.5	49	Yes	No
26	SID1678	E11.5	50	Yes	Yes
27	SID1672	E12.5	51	(No)	No
28	SID1605	E12.5	53	Yes	Yes
29	SID1887	E12.5	53	(No)	No
30	SID1633	E12.5	54	(No)	No
31	SID1640	E12.5	55	No	No
32	SID1905	E12.5	55	Yes	No
33	SID1893	E12.5	55	No	Yes
34	SID1638	E12.5	56	No	No
35	SID1876	E12.5	56	(No)	No
36	SID1639	E12.5	57	No	No
37	SID1606	E13.5	60	No	No

Table 1. Mouse embryos examined in this study. Episcopic datasets of wild-type mouse embryos were retrieved from www.embryoimaging.org and either directly examined through the website or the data imported into Amira for segmentation. (No) denotes that no connecting collateral channel was observed, but a spur was seen which possibly relates to a forming or regressing collateral channel.

Figures

Figure 1. Reconstructions of murine embryos at E10.5. The arrangement of the pharyngeal arch arteries is typical for this stage of development. **a, b,** The first and second arch arteries are still visible at 32 somites, and the sixth arch artery has yet to form. **c,** By the 40 somite stage the first and second arch arteries have regressed, the sixth is fully formed, and the pulmonary arteries (pa) have begun to appear in the pharyngeal mesenchyme. The numbers show the pharyngeal arch arteries arising from the aortic sac (as).

Figure 2. Reconstructions of murine embryos at E11.5. Representative embryos from 3 stages at E11.5 are shown and staged by outflow tract development. **a**, Early (SID1715, 44 somites), **b**, middle (SID1693, 46 somites) and **c**, late (SID1678, 50 somites). By the middle of E11.5, the aortic sac (as) has divided into its aortic (at) and pulmonary (pt) components. Bilateral collateral channels (cc) are now seen dorsally between the fourth and sixth arch arteries in all 3 embryos presented. **d**, A reconstruction of a mid-stage E11.5 embryo (SID1745, 45 somites), without visibly connecting collateral channels, has also been included. The carotid duct (cd) is obvious on the left side in this embryo. Pharyngeal arch arteries are indicated by number.

	right view	left view
SID1718	44s
SID1722	45s
SID1720	46s
SID1675	49s
SID1672	51s
SID1633	54s

Figure 3. Sequential series of murine embryo reconstructions. The reconstructions show selected higher power views of the junction between the pharyngeal arch arteries and the dorsal aorta on the right side (left hand column) and the left side (right hand column). The embryos were selected to illustrate increasing age, going from top to bottom, with somite numbers ranging from 44 to 54. The collateral channels are seen in various stages of patency or regression. Note also the regression of the dorsal right sixth arch artery. The numbers indicate the identity of the various arch arteries.

Figure 4. Graphical representation of embryo somite number and number of collateral channels seen. The somite number of each embryo examined (SID###; x-axis) was plotted on the left y-axis (line) along with the number of collateral channels seen for each embryo on the right y-axis (bars). This reveals that there is no apparent correlation between the number of somites and presence or absence of collateral channels seen.

Figure 5. Reconstructions of murine embryos at E12.5. Two embryos are shown to represent early (**a**) and late (**b**) stages at this time point. **a**, In the 53 somite embryo, the dorsal right sixth arch artery has almost regressed. This embryo has a small collateral dorsal channel (cc) on the right, and a much larger one on the left. Pharyngeal arch arteries are indicated by number. **b**, By late E12.5, in an embryo with 57 somites, the remodeling has been extensive. Abbreviations: bc, brachiocephalic; rva, right vertebral artery; psc, primitive subclavian; rct, right cervical trunk; ra, right axillary artery; rtd, right thoracodorsal artery; lva, left vertebral artery; ltd, left thoracodorsal artery; pa, pulmonary arteries; cd, carotid duct; rda, right dorsal aorta (regressing); lda, left dorsal aorta.

Figure 6. Reconstructions of human embryos. The remodeling of the aortic arch system is shown in a developmental series of human embryos at **a**, Carnegie stage (CS) 13; **b**, CS14; **c**, CS15; **d**, CS16; and **e**, CS17. The changes are comparable to those shown for the developing mouse. Pharyngeal arch arteries are indicated by number. as, aortic sac; at, aortic trunk; cd, carotid duct; pt, pulmonary trunk.

Figure 7. Evidence of a collateral channel and a fifth pharyngeal arch artery in human embryos. a-h, 3-D reconstructions of the pharyngeal arch arteries of 2 human embryos at CS14. **a-d,** In embryo SID1260 a unilateral collateral channel (cc) can be seen connecting the fourth and sixth arch artery on the left side. **e-h,** In embryo SID1238 a vessel, potentially a fifth arch artery, can clearly be seen on the left extending from the dorsal part of the sixth arch artery and extending in parallel to the fourth and sixth arch artery towards the aortic sac. **f,** On the right a spur of a vessel is seen emanating from a similar position on the right sixth arch artery. **i-n,** 2-D sections (coronal, i, l, and sagittal, j, k, m, n) of the CS14 embryos reconstructed in a-h. **i-m,** Embryo SID1238, possessing a fifth pharyngeal arch artery, has an extra pharyngeal arch to accommodate the extra artery (marked with * on the right, and “5th” on the left). Pharyngeal arch arteries are indicated by number. dAo, dorsal aorta.

Figure 8. Wax plate reconstruction of a developing human embryo. Bilateral collateral channels between the dorsal components of the fourth (4) and sixth (6) arch arteries are visible on the right (a) and left (b). The collateral channels, shown in the insets, are colored brown.