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ORIGINAL ARTICLE

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Morphogenesis of the primary arterial trunks of the forelimb in the rat embryos: the trunks originate from the lateral surface of the dorsal aorta independently of the intersegmental arteries

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Abstract It has been believed that the primary arterial trunk of the mammalian forelimb is derived from the 7th intersegmental artery. Here we examined the early morphogenesis of the arteries and nerves in the forelimb region by adopting a method that combined intravascular dye-injection with nerve staining to whole mounted rat embryos. The study was carried out on greater numbers of specimens at smaller intervals of embryonic stages and from earlier stages than those in previous reports. We report that: (1) The multiple primary arterial trunks in the forelimb region (primary subclavians) originate directly from the lateral surface of the dorsal aorta independently of the intersegmental arteries, previous to the formation of limb buds. (2) The tips of the 8th (and the 9th) primary subclavians that originate from the aorta near the origin of the 8th (or the 9th) intersegmental artery bend cranially and/or caudally. With the formation of limb bud, they extend to form the longitudinal trunks in the presumptive axillary region. The primary arteries in the free arm region branch off from this longitudinal trunk, and one of them develops into the axial artery. (3) The origins of the primary subclavians shift their positions on the surface of the dorsal aorta and approach the origins of the neighboring intersegmental arteries to join them, and then replace the latter. Consequently, the primary subclavians appear to be "the lateral branches of the in tersegmental arteries." (4) The 8th primary subclavian is dominant at first, but is replaced by the 7th primary subclavian, which develops into the definitive subclavian artery. (5) With the brachial nerve plexus formation, the axillary arterial plexus derived from the longitudinal trunk develops to form two stems of the axillary artery.

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Introduction

In recent textbooks and reports the mammalian subclavian artery has been described as being generally derived from the 7th intersegmental artery (7th ISA; Patten 1953; Hamilton et al. 1959; Langman 1975; Carlson 1988; Williams et al. 1989). The same artery is called the 6th cervical intersegmental (Elze 1907; Padget 1954; Vitums 1969), the 6th segmental (Müller 1903, 1904) and the 7th segmental (Göppert 1910; Evans 1912; Congdon 1922; Woollard 1922; Heuser 1923). However, the level at which the axillary artery penetrates the brachial plexus varies (Müller 1903, 1904; Adachi 1928; Aizawa et al. 1996). Concerning the formation of the axillary artery, Müller (1903, 1904) speculated that the mammalian axillary artery originated from a hypothetical primary arterial network, the "plexus axillaris arteriosus," which might be composed of some lateral branches penetrating the plexus from the intersegmental arteries (ISAs) and two longitudinal anastomoses between the lateral branches aligning medially (proximally) and laterally (distally) to the plexus, and the variation in the level at which the axillary artery penetrates the brachial plexus might depend on the intersegmental origin of the lateral branch that is selected as a route penetrating the brachial plexus. In contrast to Müller, Göppert (1910) reported that such a primary network as advocated by Müller (1903, 1904) could not be observed in mouse embryos, and concluded that the lateral stem of the axillary artery, which might be derived from the 7th ISA, was replaced by the medial stem originating from the anastomosis between the secondary branches from the lateral stem at a later stage. This inconsistency between the two concepts of the formation of the arterial system of the anterior limb has been known as the "Göppert-Müller dispute," which was partially summarized in Woollard (1922).

The axillary artery in adult rats and mice runs medially to the medial cord of the brachial plexus without penetrating the plexus, showing the same pattern as observed in the human case for the C-type brachial plexus (Adachi 1928). We have macroscopically revealed that the axillary artery in the case of the human C-type plexus is formed from a local anastomosis between the proximal part of the lateral thoracic artery and the end of the middle third part of the normal axillary artery, and that the level variation of the axillary artery penetrating the brachial plexus may not be a consequence of a segmental variation of the subclavian artery (Aizawa et al. 1995, 1996).

The above dispute on the formation of the arterial system of the anterior limb may be mainly due to the limitations in the reconstruction method adopted by Müller and Göppert, which can not represent the network of thin primary peripheral vessels and is applied to hardly sufficient numbers of specimens to demonstrate a fine representation of the embryonic stages. Some investigators have attempted the dye-injection method to examine the morphogenesis of the vessels, but the method requires live embryos, and also requires a high level of technical proficiency. Although a few investigators (Woollard 1922; Shearer 1933) adopted the dye-injection method to examine the morphogenesis of the vessels in the mammalian forelimb, they could not clarify the detail of the process of the early morphogenesis of the limb vessels. Ura (1944, 1956) investigated the early morphogenesis of several vessels in various vertebrates by the dye-injection method. Continuing Ura's research, Isogai reported on the early morphogenesis of the intestinal vessels in the rainbow trout by the Berlin blue-injection method (Isogai and Horiguchi 1997) and of the renal vessels in urodels by the acrylic-resin-injection method (Isogai and Horiguchi 1994, 1996). These studies showed that the injection methods might also be very useful in examining the early morphogenesis of the mammalian vessels. However, the early morphogenesis of the peripheral nerves could not be examined by the injection methods alone.

Therefore, to study both the fine vascular and nerve plexuses in the same specimen, we combined two methods: the Berlin blue-injection method for the vascular system and the neuro-filament-staining method for the nerve plexus in this study. A large number of rat embryos were obtained at finely divided developmental stages at intervals of 0.1 day or shorter.

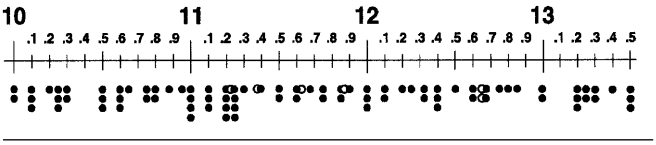
Materials and methods

Animals and embryos

The "Guidelines for animal experimentation" as laid down by the Animal Research Committee of Iwate Medical University School of Medicine in 1997 were followed in this study.

Parents rats were 50 female and 17 male SPF (Specific Pathogen Free)/VAF (Virus Antibody Free) Wistar white rats aged 9–10 weeks, weighing between 186 and 210 g in females (some lots of litters), 294 and 340 g in males, and purchased from Charles River Japan. They were kept under fixed room conditions (temperature, humidity, and time of lights on/off: 6:30 a.m/8:30 p.m.). After acclimatization to the above conditions for at least a week, female rats were checked daily for their external appearances, their body weights, their feedings, and their estrus cycles with vaginal smears. To regulate the estrus cycles of female rats and the developmental stages of embryos, the times for these checks and feedings were fixed, so that rats from the same litter displayed a tendency to have a synchronous estrus cycle.

Table 1 Stages of sampled embryos. Each dot indicates the stage of a litter of embryos (5–12) that is contained in a half of the uterus



Mating

A female rat at the end of proestrus was mated with a male rat from 0:00 to 6:00 a.m. We assumed that the E0 point (the starting point of development) would be at 9:00 a.m. in the day when we found the vaginal plug and the sperms in the vaginal smear, because it was estimated that ovulation of the rutting rats on the synchronized favorable conditions might occur about 6 h after the lights-out (2:30 a.m.) and that fertilization might occur about 6 h after ovulation (from 8:30 to 11:00 a.m.).

Extraction of embryos

Pregnant rats (aged 13–17 weeks) were intramuscularly injected with 10 mg/kg of xylazine to induce anesthetization, then anesthetized with 12 mg/kg of ketamine *via* the caudal vein. After the laparotomy, one of the paired uterine horns was removed, and immediately put in cool saline (Tyrode solution or phosphate-buffered saline at 4°C) to extract embryos (5–12). After about 0.1 day (the shortest significant interval based on the fluctuation of the fertilization time), the same preparation was used for the other horn. Such short intervals can be significant, not when the mating time is limited, but only when the environment surrounding the parent rats is strictly controlled, because in rats and mice the ovulation time is regulated not by the coition time but by estrus cycle and light-out.

The stages of embryos are presented in Table 1.

Dye injection

To recover the active heart beat, embryos were transferred into saline at 37°C just prior to the following injection (Ura 1944, 1956).

About 2/3 of embryos of each litter were injected with 0.5% Berlin blue solution in distilled water *via* the heart by a glass needle. Specimens for vascular analysis were fully injected to observe both the arterial and venous vascular systems (dense dye injection), whereas for the arterial and nervous analysis the injection was stopped before the veins showed clearly in the specimens, because the dye in the veins interfered with the observation of deep arteries and nerves (weak dye injection).

Fixation

In the specimens at stages earlier than E12 (the 12th embryonic stage, 12 days past E0), the whole body was fixed by 4% paraformaldehyde fixative in 0.1 M phosphate buffer (pH 7.4) at 4°C. In those at later stages (E12–13.5), the body cavity and the brain vesicle were opened in the fixative. After the fixative had been changed more than three times, the specimens were stored in the fixative at 4°C.

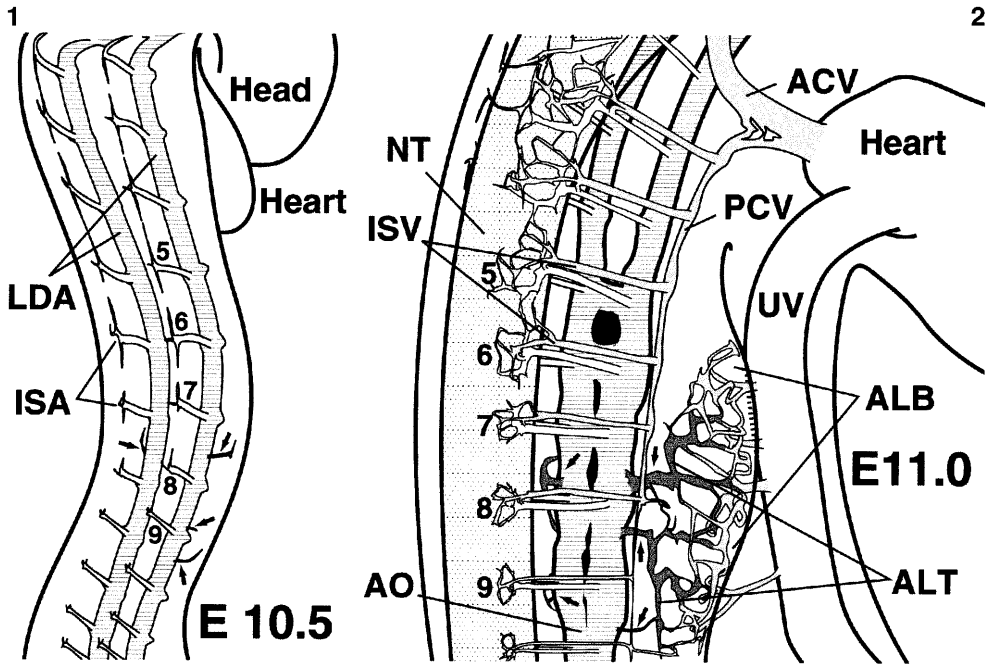


Fig. 1 Drawing showing the dorsal view of the rat embryo at E10.5. No limb buds have developed yet. Many pairs of intersegmental arteries (*ISA*) originate from the dorsal surface of the paired lateral dorsal aortae (*LDA*, *hatched*). A few sprouts of the primary arterial trunks of the forelimb (*arrows*) that originate from the lateral surface of the lateral dorsal aorta independently of the intersegmental arterie s are observed in the middle portion of the body (*Arabic numerals* segmental levels of the intersegmental arteries

Fig. 2 Drawing showing the dorso-lateral view of the right forelimb region at E11.0. The anterior limb bud (*ALB*) is recognized as a slight swelling of the lateral body wall. The primary arterial trunks of the forelimb (*dark gray*) elongate and form the primary network in the ALB. These primary trunks bend cranially and/or caudally and elongate longitudinally in the basal area of the ALB to form the axillary longitudinal trunk (*ALT*). The proximal transverse parts of the primary trunks (*arrows*) are called "primary subclavians." The primary network of the vessels in the ALB is drained mainly by the umbilical vein (*UV*) and slightly by the posterior cardinal vein (*PCV*). Veins, except the UV, are indicated by *light gray*. The lateral dorsal aortae fuse to make the dorsal aorta (*AO*) just at the forelimb level. The PCV, which runs dorsally to the primary subclavian, develops to drain the intersegmental veins (*ISV*; *Arabic numerals* levels of the intersegmental arteries and veins, *ACV* anterior cardinal vein, *NT* neural tube)

Neurofilament staining of whole mount embryos

The weak dye-injection specimens were rinsed with 0.9% saline to remove the fixative, dehydrated in a cooled methanol series (at 4°C), and stored in 100% methanol with 20% dimethyl sulfoxide (DMSO) at –20°C until the next treatment. They were next transferred to a solution of 100% methanol with 20% DMSO and 3% H_2O_2 at 4°C overnight. Then they were treated with 0.15% Triton X100 in TRIS buffer (TST) with 5% DMSO (TST-DMSO) for 3 h (the solution was changed every 1 h), and by 5% non-fat dry milk in TST-DMSO (TSTM-DMSO) overnight at room temperature. They were next incubated with the anti-neurofilament antibody 2H3 (mouse monoclonal IgG_1 to rat 165 kDa neurofilament: Developmental Studies Hybridoma Bank of the University of Iowa) in TSTM-DMSO with 0.1% sodium azide for more than 1 day at room temperature. After washing the primary antibody with TST-DMSO for 6 h (the solution was changed every 2 h), they were incubated with the HRP-labeled secondary antibody (goat anti-mouse-IgG(H+L): catalog No. A106PU, American Qualex) in TSTM-DMSO for more than 1 day at room temperature. After washing by TST-DMSO, they were stained by the DAB-HRP reaction or the Nickel-DAB-HRP reaction. The above staining procedure followed the protocol of Kuratani (personal communication in 1997, modified from Kuratani et al. 1997).

For the observation under the light microscope, the specimens were dehydrated through a graded glycerol series, and then immersed in a mixture of pure glycerol and starch syrup (1:1) to enhance the light-permeability without damage to the specimens (Isogai et al. 1997).

Counting method of intersegmental arteries

The segmental level of the ISAs was determined by their relation to the cervical nerves, which were well identified at stages later than E11.2; i.e., an ISA between the hypoglossal nerve and the C1 nerve was determined as the 1st one. It was estimated by comparison to the status of E11.2 at the earlier stages.

Results

E10.0–E11.0

Parallel dorsal aortae (the lateral dorsal aortae) ran caudad to the tail end, where they fused to form the umbilical artery and the vitelline artery. The ISAs ("dorsal branches") had already branched off dorsally from the dorsal aortae at E10.0.

Although the limb bud was not recognized yet, the sprouts of the primary arterial trunks of the forelimb began to grow laterally from the lateral surface of the dorsal aorta at E10.5 (Figs. 1, 7). However, in some specimens, the first eruption occurred somewhat later, or occurred only in one half. From E10.5 to E10.75, such arteries were directly given off from the lateral surface of the dorsal aorta, ranging from the 6th to the 10th interseg-

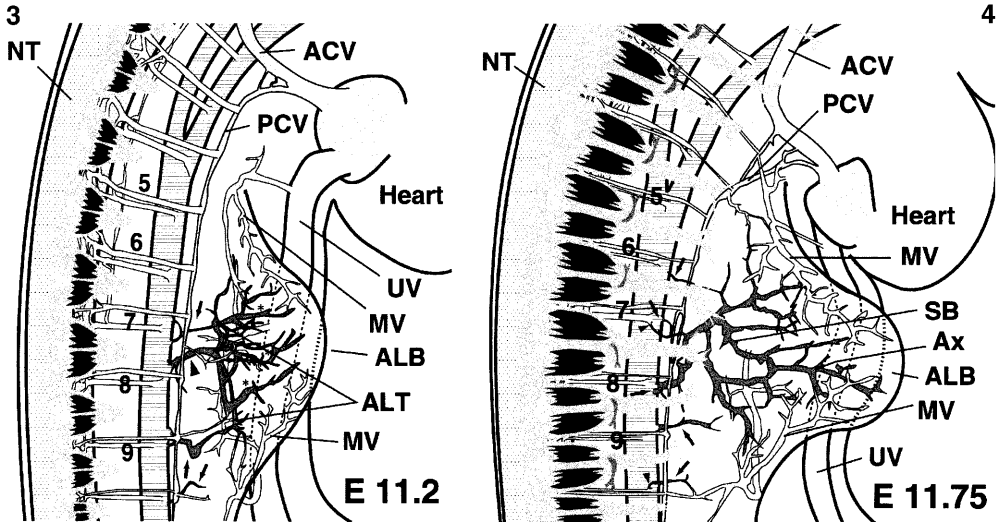


Fig. 3 Drawing showing the dorso-lateral view of the right forelimb region at E11.2. The spinal nerves (*darker gray*) with ganglia (*black*) develop near the neural tube (*NT*), but do not reach the anterior limb bud (*ALB*) yet. The origins of the primary subclavians (*arrows*) with some rootlets move toward the vicinity of the origin of the neighboring ISA. In the primary subclavians, the 8th (*arrowhead*) is the most dominant and forms the axillary longitudinal trunk (*ALT*), from which the main arteries (*asterisks*) in the free arm develop transversely. Veins in the ALB join to form two marginal veins (*MV*), each of which runs along the cranial or the caudal margin and flows into the umbilical vein (*UV*). *White* and *black Arabic numerals* show the levels of the spinal nerves and pairs of the intersegmental arteries and veins, respectively

Fig. 4 Drawing showing the dorso-lateral view of the right forelimb region at E11.75. The spinal nerves reach the base area of the anterior limb bud (*ALB*). Each of the rootlets that constitute the original part of each primary subclavian (*arrows*) begins to anastomose with the original part of the ISA (*arrowheads*). The 7th primary subclavian (and sometimes the 6th) gradually participates in the network of limb arteries, but at this stage, it is still thin. In the axillary region where the tip of the 7th spinal nerve approaches, the arteries begin to form an axillary arterial plexus. The main trunk in the free arm gradually forms the axial artery (*Ax*), which is derived from the 8th primary subclavian. The thick superficial brachial artery (*SB*), which gradually degenerates at later stages, is observed. Following the descent of the heart and the expansion of the interval between the ALB and the umbilical vein (*UV*), the drainage routes from the limb vessels gradually change from the UV to the posterior cardinal vein (*PCV*; *MV* marginal veins)

mental levels, without any relation to the origins of the ISA. The total number of the arteries varied, but gradually increased as development proceeded and eventually became between three and five on each side. These arteries were neither branches ("lateral branches") of the ISA nor even "intersegmentally situated" branches. Such lateral branches, which were given off from the lateral surface of the dorsal aorta, were not observed except in the forelimb region at these stages. As the limb bud had not yet appeared in these stages, the presumptive forelimb region was recognized only by the existence of these branches.

The tip(s) of the earliest developing primary arterial trunk(s) of the forelimb, which generally arose from the aorta near the origin(s) of the 8th (or the 8th and the 9th) ISA, bent cranially and/or caudally at the middle portion between the aorta and the lateral body wall (Figs. 1, 7).

The anterior limb bud first appeared at the end of the 10th day. The bent tip(s) of the primary arterial trunk(s) began to extend craniad and/or caudad to form the longitudinal trunk(s) in the presumptive axillary region at the base of the anterior limb bud (Figs. 2, 3, 9, 10). In contrast to this axillary longitudinal trunk (ALT), the proximal transverse parts of the primary arterial trunks were tentatively called "primary subclavian" in the following descriptions. From the ALT, some primary arteries in the limb bud branched off transversely, then their distal parts formed the primary vascular network in the free arm region that drained into the umbilical vein (Figs. 2, 8, 9). Then some primary subclavians that were delayed in their development, anastomosed with the ALT.

E11.0–E11.75

The fusion of the dorsal aorta reached the level of the forelimb region at E11.0 (Figs. 2, 8). Then the level of the fusion of the dorsal aorta reached the most cranial point near the 3rd ISA at E11.5, but proceeded gradually caudad following the descent of the heart at later stages.

The peripheral nerves appeared for the first time only in the cranial region at E11.0, then in the cervical and thoracic regions at E11.1, and some accumulations of nervous tissue forming the sympathetic ganglia appeared along the lateral margin of the dorsal aorta at E11.2 (Fig. 10). However, tips of the cervical nerves did not reach the forelimb region until E11.75.

The primary subclavians running across the ventral side of the posterior cardinal vein thickened (Figs. 2, 3, 9, 10). Each of them arose generally from the lateral surface of the dorsal aorta by 2 or 3 rootlets (Figs. 2, 3, 9) that began gradually shifting their locations toward the vicinity of the origin of the neighboring ISA. The thickest primary subclavian was generally located near the or-

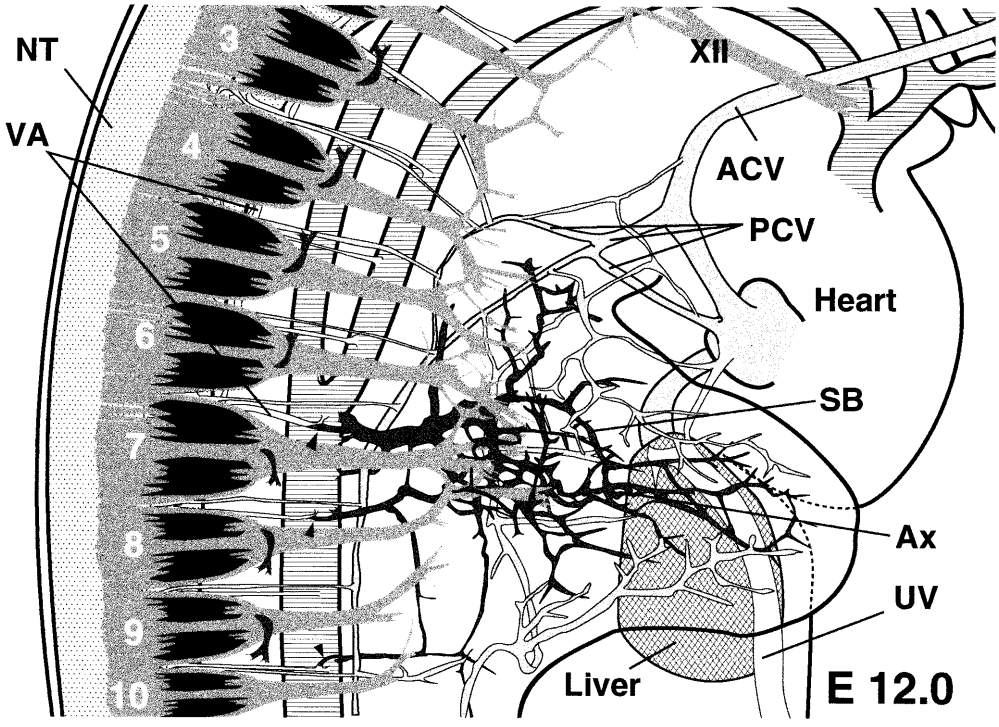


Fig. 5 Drawing showing the dorso-lateral view of the right forelimb region at E12.0. Tips of spinal nerves begin to form the brachial plexus, but the 9th one dose not reach the plexus yet. Following the descent of the heart, the fusion of the dorsal aorta also descends to the 6th segmental level and the posterior cardinal vein (*PCV*) loses its connection with the upper intersegmental veins. The PCV gradually changes its route from dorsal to ventral to the primary subclavians. The 7th primary subclavian gradually becomes thick to take the place of the 8th, which is still visible at this stage. The primary subclavians anastomose with the nearby ISAs by their rootlets (*arrowheads*) and the anastomosing rootlets display a tendency to become thick and take the place of the original parts of the ISAs. In accordance with the brachial nerve plexus formation, the axillary arterial plexus becomes very complicated, but displays a tendency to be divided into two parts: the proximal one, which is located proximal to the brachial plexus, and the distal one. The axial artery (*Ax*) also becomes very plexiform (*SB* superficial brachial artery, *UV* umbilical vein, *VA* vertebral artery)

igin of the 8th ISA (not the 7th) at this stage and was tentatively called the 8th primary subclavian (Figs. 3, 4, 10–12). However, the more cranial one near the origin of the 7th ISA (the 7th primary subclavian) grew thicker to replace the 8th to become the main subclavian at later stages. In addition, there were some variations in this shift of the main subclavian.

In the anterior limb bud, the vascular network gradually developed. Some primary main arteries of the free arm and two (cranial and caudal) marginal veins were recognized (Figs. 3, 4, 10). The most dominant artery in the forelimb bud originating from "the 8th primary subclavian" (not the 7th) developed into the axial (or central) artery (the prersor of both the brachial artery and the interossean artery) later at this stage (Figs. 4, 12). The marginal veins drained into the umbilical vein at this stage (Figs. 2, 3), but in accordance with the descent of the heart, the drainage routes to the posterior cardinal vein were gradually developed (Figs. 4, 5).

E11.75–E12.1

The origins of the primary subclavians from the lateral surface of the aorta approached those of the neighboring ISA by the shift of rootlets, and in some primary subclavians one of the rootlets anastomosed with the origin of the ISA (Figs. 4, 5, 13, 14). Then, the initial part of the ISA gradually degenerated and was compensated for by the rootlet anastomosing with a primary subclavian. As a consequence of this process, the primary subclavians and the ISAs appeared to be "the lateral branches" and "the dorsal branches" of "the intersegmental arteries," respectively. After that, a rootlet of the 7th primary subclavian gradually became dominant and the anastomosing rootlet with the 7th ISA became the initial part of the vertebral artery, whereas the other rootlets of the "7th primary subclavian" and the other primary subclavians gradually degenerated (Figs. 5, 14). However, these degenerating rootlets of the multiple primary subclavians remained until the middle of the 12th day.

The lower cervical nerves reached the base of the forelimb region at E11.75 (Figs. 4, 12), then began to form the brachial plexus. As soon as the nerve plexus was formed, the axillary longitudinal trunk contacting the plexus changed into the complicated axillary arterial plexus around the nerve plexus (Figs. 4, 5, 12–14). The axillary arterial plexus showed a tendency to be divided into two parts, the proximal and the distal, which were located proximal or distal to the brachial nerve plexus, respectively (Figs. 5, 14).

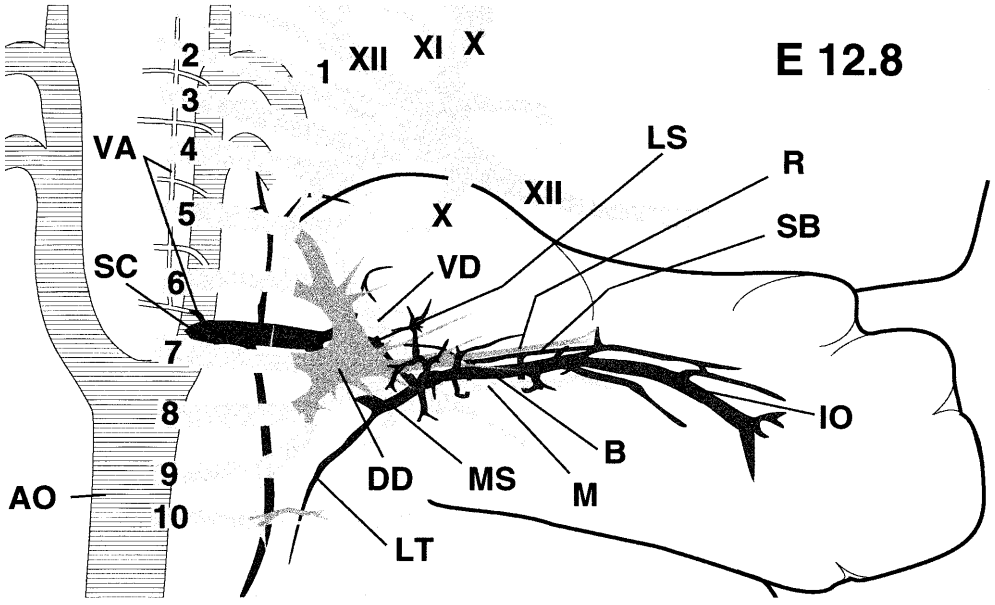


Fig. 6 Drawing showing the dorsal view of the main arteries and nerves in the right forelimb at E12.8. The subclavian artery (*SC*) originates just above the fusion of the dorsal aorta (*AO*), which is not derived from the 7th ISA but from the 7th primary subclavian (*black*). The initial part of the vertebral artery (*VA. black*) is also derived from the 7th primitive subclavian, whereas the rest of the VA (*white*) is derived from the ISAs. The brachial plexus is divided into the dorsal division (*DD, darker gray*) and the ventral division (*VD, lighter gray*), from which some main nerves as the radial nerve (*R*) and the median nerve (*M*) begin to develop. The axillary arterial plexus between the SC and the brachial artery (*B*) are gradually simplified into two stems, the lateral and the medial stems (*LS* and *MS*) of the axillary artery. The LS penetrates the VD but the MS passes caudally to the VD. The former is thicker than the latter at E12.7, but the relation is reversed at this stage; then the former degenerates and the latter develops as the definitive axillary artery at later stages. The development of the latter stem is closely related to the formation of the lateral thoracic artery (*LT*; *Roman numerals* the numbers of the cephalic nerves; *IO* anterior interossean artery, *SB* superficial brachial artery)

The axial artery in the free arm region, being derived from the "8th primary subclavian," was established previously to the formation of the axillary arterial plexus, and grew thick and plexiform (Figs. 5, 13).

As the heart descended to reach the level near the forelimb region, plexiform terminals of the veins from the forelimb lost drainage pathways to the umbilical vein and flowed into the thin posterior cardinal vein or the thick anterior cardinal vein, the proximal part of which was also plexiform and variable (Figs. 4, 5). In accordance with those changes, the trunk of the posterior cardinal vein changed its route from passing dorsally to the primary subclavians to ventrally (Figs. 4, 5).

E12.1–E13.5

At E12.5, the level of the fusion of the dorsal aorta shifted down just to the level of the origin of the 7th primary subclavian, then the origin of the artery became located on the lateral dorsal aorta that formed a part of the aortic arch (on the left side) or a part of the brachio-cephalic trunk (on the right side) at later stages. The brachial plexus was almost completed at E12.7, i.e. the roots of the 9th (Th1) spinal nerve joined the plexus; the ventral and the dorsal divisions of the plexus and short fascicles of the anlagen of five major nerves were detectable, but their branches had not yet developed (Fig. 6).

The 7th primary subclavian grew thick to develop into the definitive subclavian artery and gave off such main branches as the vertebral artery and the internal thoracic artery, whereas the other primary subclavians degenerated (Fig. 6).

Two stems (lateral and medial) of the axillary artery deriving from the axillary plexus passed through the ventral division of the brachial plexus and connected the terminal of the subclavian artery and the origin of the axial artery (the brachial artery; Figs. 6, 15). The lateral stem penetrated the brachial plexus between C6 and C7, whereas the medial stem passed caudally to the plexus without penetrating and took a route like that observed in the human axillary artery with the C type brachial plexus (Adachi 1928; Figs. 6, 14, 15). The medial stem was thinner than the lateral stem at first, but thickened and gradually replaced the lateral stem to become the definitive axillary artery, although both coexisted until the middle of the 14th day. The precursor of the lateral thoracic artery was closely related to the formation of the medial stem.

Both the stems of the axillary artery and the axial artery were very plexiform by the early half of the 12th day, but they gradually grew thick and less plexiform. The basic patterns of the major arteries in the forelimb were fundamentally laid down by E13.5, except for the formation of their branches. Although many variations were observed in the formation of the primary trunks in the early stages, the rat embryos finally built up a very uniform pattern of arteries in the limb region.

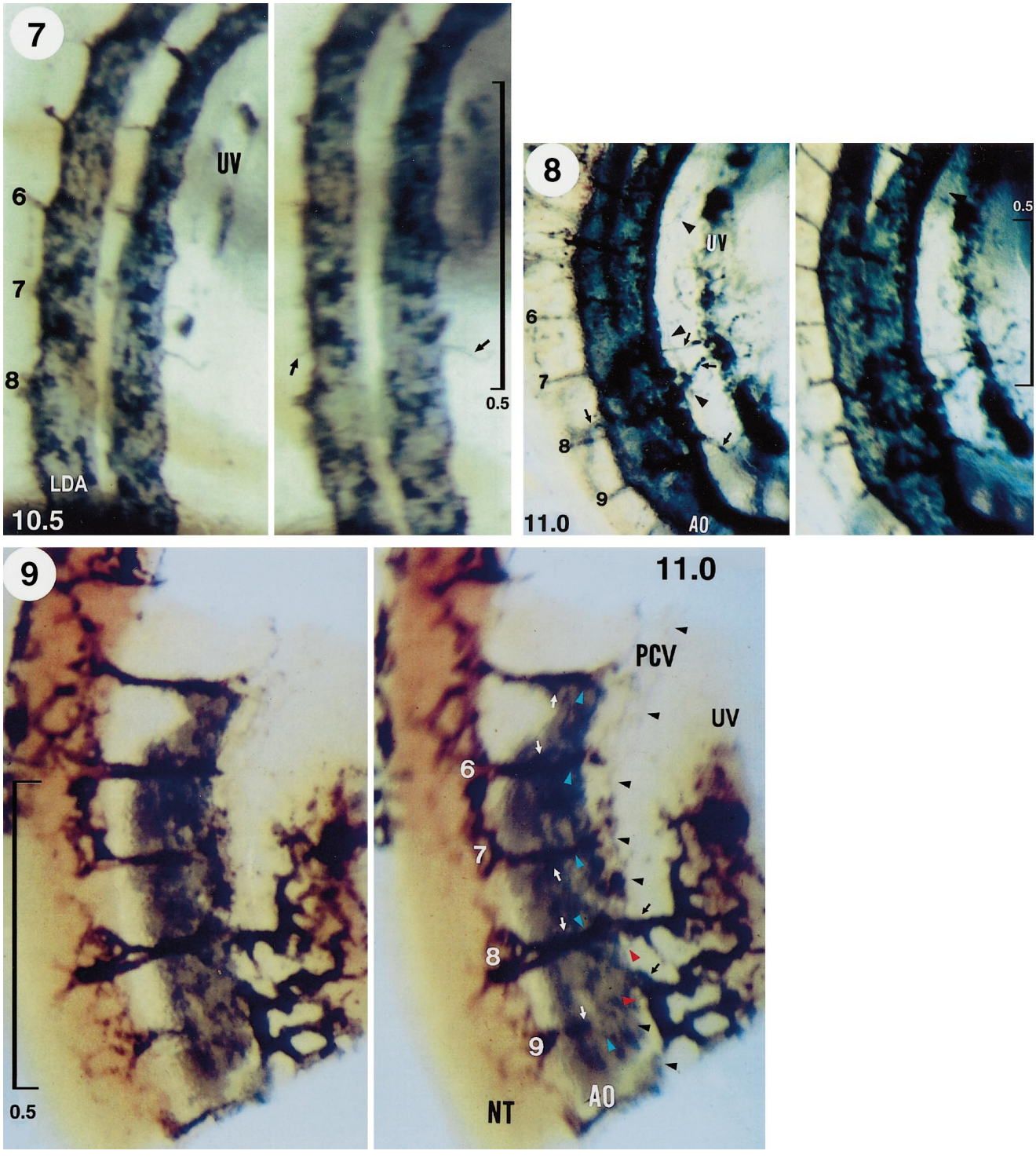


Fig. 7 Photographs for cross vision stereo showing dorsal view of a rat embryo at E10.5. Many pairs of intersegmental arteries originate from the dorsal surface of the paired lateral dorsal aortae (*LDA*). Despite the absence of the limb bud, a few sprouts of the primary arterial trunks of the forelimb (*arrows*) already originate from the lateral surface of the lateral dorsal aorta independently of the intersegmental arteries. Bar 0.5 mm

Fig. 8 Photographs for cross vision stereo showing dorsal view of the right limb region of a rat embryo at E11.0. The lateral dorsal aortae fuse to make the single dorsal aorta (*AO*) just at the forelimb level (*arrows* the primary subclavians, *arrowheads* the posterior cardinal vein, *UV* umbilical vein); *Bar* 0.5 mm

Fig. 9 Photographs for cross vision stereo showing dorso-lateral view of a separated body of an embryo with the right anterior limb bud at E11.0. The neural tube (*NT*) without any peripheral nerves, the dorsal aorta (*AO*) and the anterior limb bud are observed. Origins of the 8th and the 9th primary subclavians (*black arrows*) with accessory rootlets *(red arrowheads*) are obviously separated from the origins of the intersegmental arteries (*white arrows*). The posterior cardinal vein (*PCV* and *black arrowheads*), which is rather hard to recognize because of the weak injection of dye, drains the intersegmental veins (*blue arrowheads*) and runs dorsally to the primary subclavians. *Bar* 0.5 mm

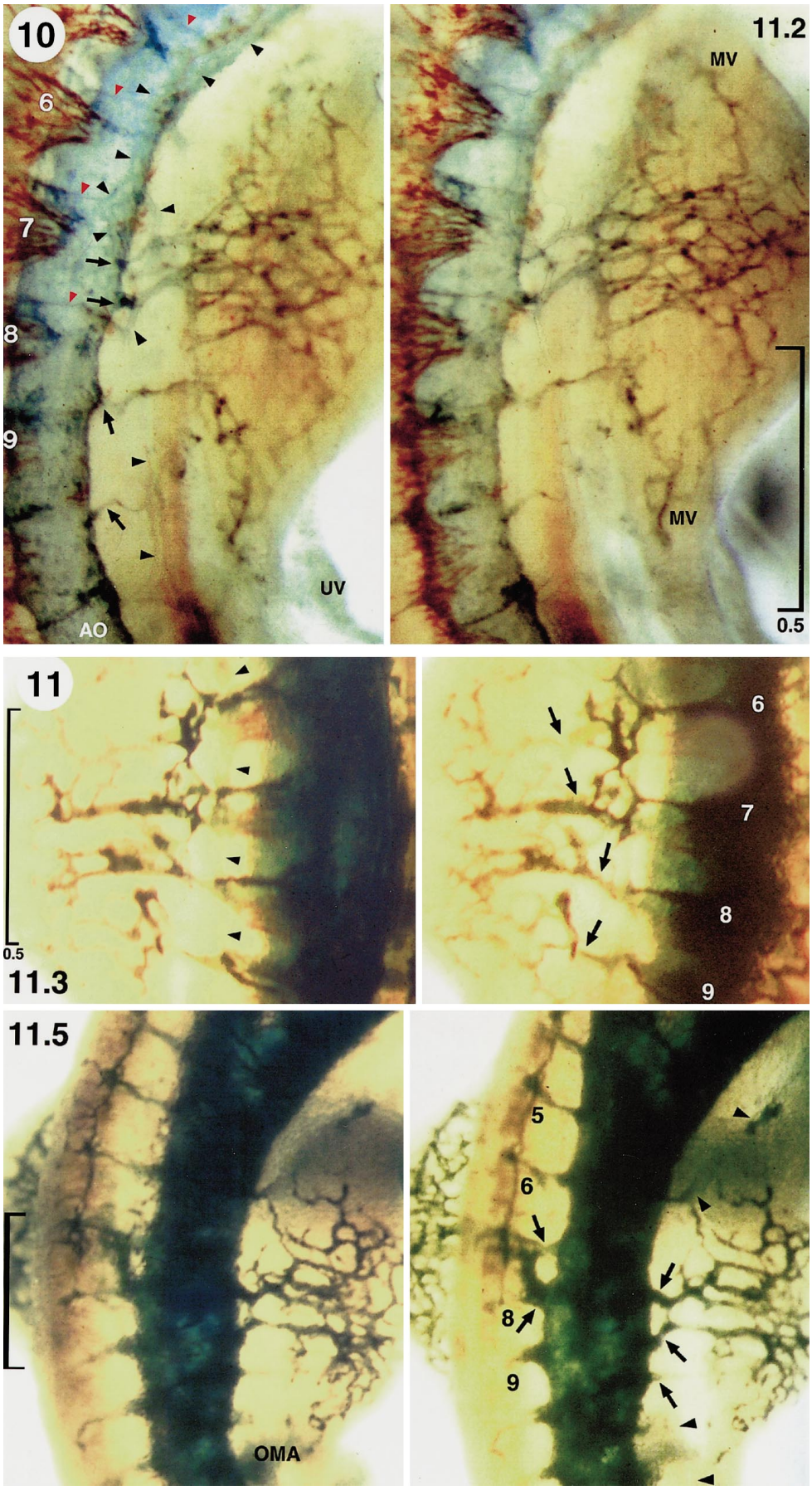
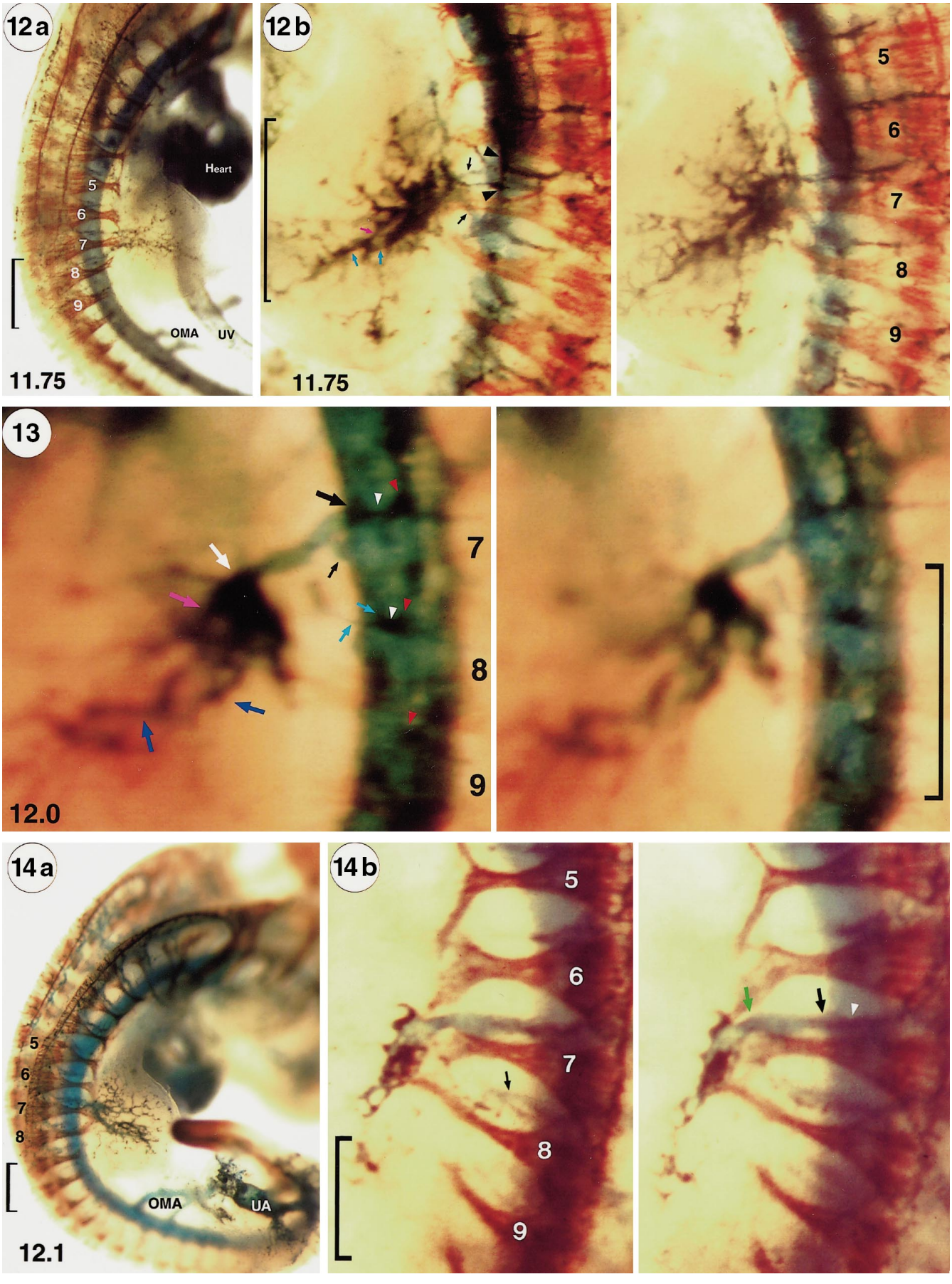


Fig. 10 Photographs for cross vision stereo showing dorsolateral view of a right anterior limb bud at E11.2. The brownish stained spinal nerves are observed to develop but do not reach the limb bud. Four primary subclavians (*arrows*) are located near the 7th to 10th intersegmental levels, respectively. The primary network in the limb bud, the axillary longitudinal trunk at the base of the limb bud and the marginal veins (*MV*) are observed. Brownish particles along the lateral surface of the dorsal aorta are clusters of sympathetic ganglion cells (*AO* dorsal aorta, *black arrowheads* posterior cardinal vein, *red arrowheads* intersegmental veins). *Bar* 0.5 mm

Fig. 11 Photographs for cross vision stereo showing a variation observed on the origins of the primary subclavians (*arrows*). **Upper** Dorsal view of a left anterior limb bud at E11.3. Four primary subclavians (the 6th, 7th, 8th and 9th) develop in the limb bud. **Lower** Dorsal view of both sides of the limb bud at E11.5. The 8th and two 9th primary subclavians on the right side, and only the very thick 8th primary subclavian on the left side are observed, but the very thin 7th anastomoses with the bent part of the left 8th behind the 7th ISA (not visible). In spite of this variation, the main trunk in the free limb is generally derived from the 8th (*arrowheads* posterior cardinal vein, *OMA* omphalomesenteric artery). *Bar* 0.5 mm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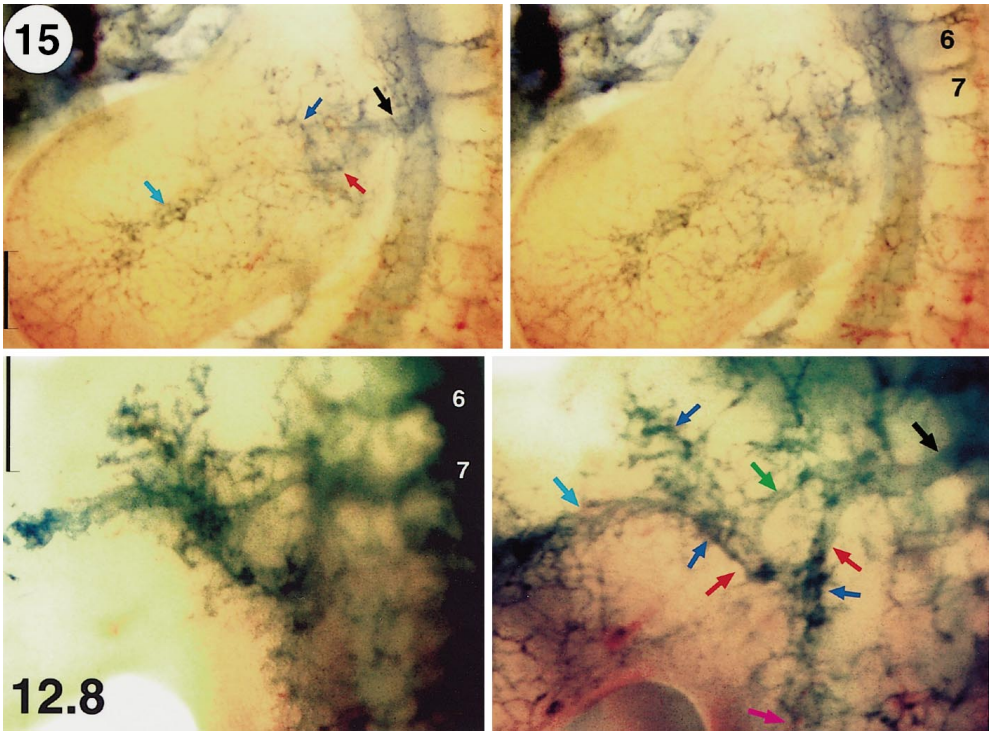

Fig. 15 Cross vision stereo photographs showing dorsal view of the left limb bud of two cases of embryos at E12.8. **Upper** Whole limb bud to show the arterial network (lower magnification). As the fusion point of the dorsal aorta descends to the 8th intersegmental level, the subclavian artery (*black arrow*) originates from the lateral dorsal aorta. **Lower** Higher magnification photograph showing the axillary region. The lateral stem (*green arrow*) and the medial stem (*red arrows*) of the axillary artery are seen. The former gradually becomes thinner and the latter gradually develops to become the dominant axillary artery (*black arrows* subclavian artery, *light-blue arrows* axial artery, *blue arrows* deep arteries in the subscapular region, *purple arrow* lateral thoracic artery). *Bars* 0.5 mm

◀ **Fig. 12A, B** Photographs of two cases of embryos at E11.75. **A** Dorso-lateral view of the right side of the body to indicate the nerve developing (lower magnification). **B** Cross vision stereo photographs showing lateral view of the left anterior limb bud (higher magnification). The spinal nerves reach the base of the anterior limb bud. The 8th primary subclavian (*lower black arrow*) is thick, runs slightly craniad near the tip of the 7th nerve, and then bends caudally. The 7th primary subclavian (*upper black arrow*) anastomoses with the arterial plexus at the bent point of the 8th, but the former is still thin. The arteries near the tip of the nerves gradually form the axillary arterial plexus. The main trunk in the free limb region gradually develops to become the axial artery (*light-blue arrow*). Thick superficial brachial artery (*purple arrow*) is also formed (*arrowheads* posterior cardinal vein). *Bars* 0.5 mm

Fig. 13 Cross vision stereo photographs showing dorsal view of the left limb bud in an embryo at E12.0. The primary subclavians anastomose with the intersegmental arteries (*red arrowheads*) by their rootlets (*white arrowheads*). The 7th primary subclavian (*large black arrow*) with accessory rootlets (*small black arrow*) becomes dominant, taking the place of the 8th, but the latter (*light-blue arrows*) still remains (*white arrow* axillary arterial plexus, *blue arrows* axial artery, *purple arrow* superficial brachial artery). *Bar* 0.5 mm

Fig. 14A, B. Photographs of two cases of embryos at E12.1. **A** Dorso-lateral view of the right side of a whole embryo. **B** Cross vision stereo photographs showing proximal part of the left limb bud. The brachial plexus is gradually formed. The 8th primary subclavian (*small arrow*) barely remains, but the thick 7th primary subclavian with a single root (*large arrow*) almost forms the definitive subclavian artery. The anastomosis with the 7th ISA (*white arrowhead*) grew thick to form the initial part of the vertebral artery and the original part of the 7th ISA almost degenerates. Two parts, (the proximal and the distal) of the axillary arterial plexus, and the lateral stem of the axillary artery (*green arrow*) that connects them and passes between the 6th and the 7th cervical nerves, are observed. *Bars* 0.5 mm

Discussion

In many reports and textbooks, the arterial trunk of the tetrapod forelimb was described as being derived from the ISA (Hochstetter 1891, 1901; Zuckerkandl 1894, 1895; de Vriese 1902; Elze 1907; Svensson 1908; Sabin 1917; Wetterdal 1920; Congdon 1922; Woollard 1922; Shearer 1933; Arey 1954; Padget 1954; Blechschmidt 1961; Hamilton et al. 1969; Vitums 1969; Carlson 1988), and the only exception was said to be the avian primary subclavian artery. It was known that the primary arteries in the avian forelimb region were randomly and independently given off from the lateral surface of the dorsal aorta, but that they were soon replaced by the secondary subclavian artery, which originated from the 3rd branchial artery (Rabl 1906; Evans 1908, 1909a, b; Bakst and Chafee 1928; Suzuki 1987). In mammals, such primary arterial trunks, as independently given off from the lateral surface of the dorsal aorta, had only been observed in mouse embryos by Göppert (1908, 1910) and in mouse, rabbit, and human embryos by Evans (1908, 1912). Göppert observed that many trunks were given off from the dorsal aorta independently of the ISAs and that some of them crossed ventrally to the posterior cardinal vein in the early mouse embryo. However, he considered that those trunks showed a kind of variational state of the lateral branches of the ISAs. Evans called such arteries "segmental subclavians," and reported that the 7th one remained as the definitive subclavian artery.

Despite the descriptions by Göppert (1908, 1910) and Evans (1908, 1912), the mammalian primary arterial trunks of the forelimb have been considered as being given off from the segmental arteries (Woollard 1922; Shear-

er 1933; Arey 1954; Hamilton et al. 1959; Blechschmidt 1961) because both Woollard and Shearer reported that such primary trunks directly given off from the aorta, as observed by Göppert (1908, 1910), were not detected. However, the earliest stages observed by them were too late, as the limb bud and the primary arterial trunks with the primary vascular network in the limb region had been already well-developed in their specimens. Therefore, they did not observe any process concerning the formation of the primary trunks in the limb region.

By adopting a reconstruction method, Müller (1903) examined the process of arterial formation in the forelimb in human embryos and Göppert (1908, 1910) did so in mouse embryos. Although their ideas are well-known, there are some problems associated with the reconstruction method, which can treat only a limited number of specimens. Müller (1903, 1904) examined neither the early formation of the primary arterial trunks nor that of the arteries in the proximal part of the limb region. His famous concept is almost entirely based on the speculation from the synthesis of the macroscopic observations of the axillary arteries in several adult animals and those in human fetuses. Woollard (1922) and Shearer (1933) applied the carbon ink injection method, but they did not examine the early embryos, and the intervals between the stages of the specimens in their examinations were not short enough. Therefore, we tried to examine many specimens of rat embryos at many stages, with short intervals. Thus, we could observe the details of the process of arterial formation in the forelimb region at every short interval for the first time.

We observed in detail that each origin of the primary subclavians was situated on the dorsal aorta with some rootlets independent of the ISA at first, and gradually shifted its position on the dorsal aorta and approached the origin of the ISA. Then one of the rootlets anastomosed with the origin of the ISA, and then this anastomosing rootlet grew thick and the origin of the ISA gradually degenerated. Thus, the initial part of the ISA was replaced by the origin of the primary subclavian and the so-called "lateral branch of the intersegmental artery" was established. A similar shift in the primary trunk of the forelimb with similar rootlets was also observed in avian embryos (Bakst and Chafee 1928).

The primary arterial trunks of the forelimb of rat embryos crossed ventrally to the posterior cardinal vein at first, but later shifted dorsally. The shift might be a consequence of a shift of the venous route correlated with the descent of the heart, which also caused a shift in the venous drainage of the forelimb, i.e., the umbilical vein at first, the posterior cardinal vein next, and the anterior cardinal vein last. The positional difference between the ISA and the primary subclavian relative to the posterior cardinal vein in the early stages suggests that these arteries might fundamentally belong to the different lineage of arteries.

We determined that the primary trunks and the primary network of the forelimb were formed at a very early stage, and the primary arteries in the free arm region were formed prior to the formation of the nerve plexus. In contrast to the traditional concept that the main arteries in the arm (the median artery, the subscapular artery, etc.) secondarily develop from the axial artery (de Vreise 1902; Shearer 1933), these arteries may be formed from the preceding primary network in the limb bud. The details of the changes in the thin network in the limb bud were not made clear because the network was too complicated and some parts of the vessels were too thin. The traditional view of the arterial formation is only based on the observation of thick vessels, but it is necessary to also examine the changes in the primary thin vessels using the other methods of revealing the fine detail.

The axial artery was also reported as being derived from the 7th ISA (Woollard 1922; Shearer 1933; Hamilton et al. 1959). However, we observed that it was generally derived fro m the 8th primary subclavian.

Müller (1903, 1904) considered that the primary axillary arterial plexus was formed from the longitudinal anastomoses between the lateral branches of the intersegmental arteries. However, our study revealed that the ALT was formed by the longitudinal extension(s) from the earliest developing (the 8th, or the 8th and the 9th) primary subclavian(s) previous to the participation of the delayed primary subclavians. Furthermore, the transverse primary arteries in the free arm region were also generally given off from the ALT previous to the development of the delayed primary trunks excepting a few cases (Fig. 11; upper).

From our results, the formation of the axillary artery and the variation of the passing point through the brachial plexus by this artery are considered to depend on route selections in the axillary arterial plexus around the brachial plexus. However, the origin and the features of the axillary plexus of the rat embryo are very different from those proposed in Müller's concept. The mechanism of the variation in the passing point of the axillary artery through the brachial plexus remains to be understood. Although the "Göppert-Müller dispute" concerning the formation of the axillary artery was the initial motivation for our study, it has now become irrelevant in the light of our results.

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