3D atlas of human embryology

New insights in human development

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CHAPTER 2.2 - AN INTERACTIVE THREE-DIMENSIONAL DIGITAL ATLAS AND QUANTITATIVE DATABASE OF HUMAN DEVELOPMENT

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Supplementary material is available online at http://www.3d atlasofhumanembryology.com

"The greatest progressive minds of embryology have not looked for hypotheses; they have looked at embryos."

Jane Oppenheimer, 1955
Abstract

Current knowledge about human development is based on the description of a limited number of embryonic specimens published in original articles and textbooks, often more than 100 years ago. It is exceedingly difficult to verify this knowledge, given the restricted availability of human embryos. We created a three-dimensional digital atlas and database spanning the first 2 months of human development, based on analysis of nearly 15,000 histological sections of the renowned Carnegie Collection of human embryonic specimens. We identified and labeled up to 150 organs and structures per specimen and made three-dimensional models to quantify growth, establish changes in the position of organs, and clarify current ambiguities. The atlas provides an educational and reference resource for studies on early human development, growth, and congenital malformations.

Introduction

The basic human body plan is laid down during embryonic development. Insight into the formation of this plan has been shown to provide rational explanations for the relative positions of organs in the adult, as well as for the origin of congenital malformations. Congenital defects have an incidence of 3% in the human population (Organization 2014) and cause up to one-quarter of all neonatal deaths (WHO 2009). Knowledge of normal human development is therefore of great clinical interest, particularly for pediatricians and clinical geneticists. Despite modern approaches such as three-dimensional (3D) reconstruction, it remains difficult to map the intricate morphogenesis of the developing human body. Current textbooks (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015) on human development are usually based on the articles and textbooks of various stellar embryologists (Broman 1927; Foster and Balfour 1874; Gasser 1975; Heuser 1932; Heuser, Rock, and Hertig 1945; His 1880, 1882, 1885; Keibel and Mall 1910, 1912; Keith 1933; O’Rahilly and Müller 1987; Prentiss and Arey 1917; Streeter 1942; Streeter 1945, 1948; Streeter 1949; Streeter 1951), often published more than 100 years ago. However, it is almost impossible to independently verify the information presented in these textbooks, or even to assess whether this information is derived from studies on human or animal material.

By visualizing development, normal embryogenesis and even malformations can be better understood. In this study, we provide an atlas and database spanning the entire embryonic period of human development, covering early organogenesis based on human embryonic specimens from the Carnegie Collection (Table S1). The Carnegie Collection consists principally of serially sectioned normal human embryos in the first 8 weeks of development. It was started by the Carnegie Institution of Washington’s Department of Embryology in 1914.
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Our interactive atlas allows the user to directly link the annotated organs in the 3D reconstructions with the underlying histological sections of the Carnegie Collection, thereby enabling independent verification and further analyses. The atlas identifies differential growth and the changing relative positions of organs and structures during the first 8 weeks of human development in a quantitative fashion. Initial analyses provide new insights into these relationships.

Data generation and reconstruction pipeline

The degree of detail required for 3D reconstructions of distinct organ systems in early development is currently impossible to obtain with noninvasive techniques such as magnetic resonance imaging (Matsuda et al. 2003; Yamada et al. 2012). Moreover, with a few exceptions, the scarcity of human embryos does not permit tissue identification based on specific immunostaining of the individual organs. Because of these limitations, we chose to analyze histological sections from the Carnegie Collection, with manual identification and labeling of every organ and structure, followed by knowledge-driven modeling to prevent loss of essential detail.

A flowchart of the methods used to generate the 3D reconstructions and morphometric contents of the database is presented in Fig. 1. We imaged and analyzed 17 embryonic stages, with two embryos per stage, spanning the first 2 months of development; Table S1. We analyzed stages CS7 (15 to 17 days of development), CS8 (17 to 19 days), CS9 (19 to 21 days), CS10 (21 to 23 days), CS11 (23 to 26 days), CS12 (26 to 30 days), CS13 (28 to 32 days), CS14 (31 to 35 days), CS15 (35 to 38 days), CS16 (37 to 42 days), CS17 (42 to 44 days), CS18 (44 to 48 days), CS19 (48 to 51 days), CS20 (51 to 53 days), CS21 (53 to 54 days), CS22 (54 to 58 days), and CS23 (56 to 60 days). Although analysis of two specimens per stage is insufficient to estimate variance in development, the extensive series of embryonic stages allows generalization of continuous patterns of growth. Images were acquired from about 15,000 histologically stained sections, and up to 150 organs and structures per specimen were manually segmented and spatially reconstructed. Structures were identified on the basis of anatomical and histological characteristics and named in accordance with the international standard of embryonic terminology, the Terminologia Embryologica ((FIPAT) 2013). The morphological reconstructions were prepared with Amira and Blender software (see supplementary materials). The 3D reconstructions were made in such a way that the original sections can be placed within the reconstruction, permitting independent verification of the identification of organs.
The variability of the organ volumes measured by different observers ranged from 0.3% to 2% for simple and complex structures (Fig. S1). A smooth and easily recognizable organ, such as the otic vesicle, is considered a simple structure, whereas a small and tangled structure, such as the mesonephros and mesonephric duct, consisting of different types of tissue, is termed a complex structure. This small interobserver variation underscores the reproducibility of the segmentation and reconstruction process.

Because organ morphology gradually and consistently changed both qualitatively and quantitatively in the embryonic series studied, we conclude that no grossly abnormal embryos were included in the study and that the staging of the embryos was accurate.

Fig. 1. Flowchart of three-dimensional reconstruction, model generation, and data analysis. Histologically stained sections of the embryos from the Carnegie Collection were imaged, aligned, and segmented. Three-dimensional models were created in the 3D reconstruction package Amira; surfaces were smoothed, without loss of essential details, by knowledge driven modeling in Blender. The resulting 3D models were then incorporated into interactive 3D-PDF files. Data analysis was performed on the segmented sections and the 3D models. The resource database contains the aligned images, the segmented Amira models, the interactive 3D-PDFs, and tables and graphs of the quantitative, topographic, and developmental data of all structures in every specimen. Green arrows indicate quality checks; red and blue arrows indicate the flow of data toward the contents of the resource database. Stage 20 specimen 462 was used to illustrate this flowchart.
The applied reconstruction protocol has dual outputs: (i) a series of 14 3D reconstructions that covers the entire embryonic period in an interactive format that can be viewed on different computer types and smartphones (3D-PDF format), and (ii) a set of tables and figures that provides quantitative information about the growth of the distinct structures, as well as demonstrates the changing position of structures relative to the vertebral column. The reconstructions provide spatial information, including observations about the complex and changing relationships between different structures in the developing embryo (supplementary 3D-PDFs). The information in the tables and figures allows interpretation of organ growth relative to the growth of the embryo (Excel S1) and enables the choice of reference points for analysis of specific organs (Excel S2).

**Fig. 2.** Three-dimensional model of a stage 20 human embryo (specimen 462 of the Carnegie Collection, 7.5 weeks of development). A: Lateral view of the original embryo before sectioning. B: Lateral view of all reconstructed organs and structures, except for the skin. C: Three-dimensional view of the reconstructed embryo highlighting the skeleton and neural tube. The sagittal plane cuts through the digitized image stack. D: Cranial view on the transverse section from C through the shoulder region. E: A detail of a transverse section through the lungs, as presented in Amira. Note the colored outline of each annotated structure. The neural tube is represented in green, the skeleton in off-white; the transparent body cavities enable inspection of the liver (brown). Scale bars, 2.5 mm [A to C], 1 mm [D and E].
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Interactive three-dimensional models of early human development

The supplementary 3D-PDFs comprise 14 interactive 3D-PDF files, covering the first 2 months of human development, and illustrate the complex morphological changes that occur during development. With the interactive version, it is possible to focus on a specific organ, or on the system related to the organ of interest. An example of a 3D model of a CS20 embryo (51 to 53 days) is shown in Fig. 2. This view shows the nervous system in relation to the developing skeleton. Note that the vertebral arches are not yet closed and that the vertebral column and the spinal cord are still of equal length; the relative ascent of the latter has yet to occur.

To illustrate the scientific potential of these models, we analyzed the development of the vasculature in detail, summarized the results in schematics (Fig. 3, table S2, and fig. S2, A to O), and tabulated notable differences from the literature (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015; Gasser 1975; Gilbert 2010; Hamilton, Boyd, and Mossman 1972; Tuchmann-Duplessis and Haegel 1974) (Table S3). Some conspicuous differences from the literature are in the connections of the umbilical arteries with the aorta, the origin of the intestinal arteries, the sprouting of the pulmonary arteries, the origin of the external carotid artery, and the absence of the fifth pharyngeal arch artery in all embryos (Table S3). It is difficult to trace when and why the descriptions in textbooks started to deviate from reality in the human embryo, because it is impossible to recover the original sources of these texts.

Fig. 3. Development of the vascular system. The developing vascular system is presented in a Carnegie stage 9 embryo (19 to 21 days; left) and a stage 13 embryo (28 to 32 days; right). Dashed lines represent developing vessels; dotted lines represent regressing vessels. See fig. S2, A to O, for a complete schematic overview of the developing vascular system between stage 9 (19 to 21 days) and stage 23 (56 to 60 days). Changes in the vascular system are summarized per stage in tables S2 and S3. Abbreviations: a., artery; ao., aorta; p.a.a., pharyngeal arch arteries; v., vein. Scale bars, 250 mm (stage 9), 500 mm (stage 13).
The organization of embryonic growth

The growth rate of the embryo, as derived from interpolation of the volumes of the series of embryos, is remarkably constant in the first 2 months of development. During this period, the embryo grows exponentially; its volume increases 25% per day and reaches a volume of 2790 mm³ at 60 days of development, or CS23 (Fig. 4, A and B). Nonetheless, there are substantial differences among the growth rates of different organs, which lead to differential relative growth between organs and between developmental phases (Fig. 4C and excel S1). The liver initially grows substantially faster than does the entire embryo, but at stage 15/16 its growth rate tapers off to match the overall growth rate (Fig. 4C, blue line). The notochord shows an exponential decline in relative volume, whereas the metanephros grows faster than the total embryo (Fig. 4C, green and red line, respectively). The neural tube (minus neural canal) and its derivatives, in contrast, grow at a rate similar to that of the entire embryo throughout the period analyzed and thus show a constant relative volume of 10% (Fig. 4C, yellow line).

We tabulated the first appearance, and sometimes disappearance, of the different organs and structures within the human embryo for each stage, and compared the findings with the corresponding data for mouse and chicken embryos (Fig. S3 and excel S3). This analysis is of particular importance in teratological studies that apply data from experimental animals to the human situation. The order of appearance of the distinct organs in these species agrees largely with the order reported by Butler and Juurlink (Butler and Juurlink 1987), who based their staging exclusively on the exterior characteristics of the complete embryo. However, we found a consistent difference of one to two stage equivalents when comparing matched mouse and human or chicken developmental stages (Fig. S3 and excel S3). Thus, our data show that mouse embryonic day 9.5 (E9.5) corresponds to CS12 rather than CS10, and so on. However, we also found different timing of the appearance of some internal organs, such as the choroid plexus, which is first recognizable in mouse stage E11.0 but only five equivalent stages later in human (CS18) and chicken (HH27 to 28) (Excel S3). Similarly, the regression of the stalk of the pharyngeal hypophysis is completed six stages earlier in mouse (E12.0) than in human (CS21) and chicken (HH33 to 34) (Excel S3). The adrenal gland, in contrast, starts to develop in the same stage in human (CS18) and mouse (E13.5) embryos, but four equivalent stages later in chicken embryos (HH35) (Excel S3). In the current literature, the data for the timing of development of structures within the human embryo are anecdotal (O’Rahilly and Meyer 1979; Sissman 1970), and textbooks are inconsistent and lack references (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015; O’Rahilly and Müller 1987; Hamilton, Boyd, and Mossman 1972; Tuchmann-Duplessis and Haege 1974; Larsen 1993). Overall, our tabulated human data are consistent with those presented in the atlas of human development by O’Rahilly and Müller (O’Rahilly and Müller 1987).
Fig. 4. Growth of the human embryo during the first 2 months of development. A: Length of the embryo between Carnegie stages 7 and 23. Stages 7 to 12 are enlarged. Note the large round yolk sac (y) in stage 8. Drawings (left to right) are of specimens 8752, 8671, H712, 6330, 6784, 8505A, 0836, 8314, 3512, 6517, 8521, 8524, 2114, 462, 7258, 895, and 9226. B: Increase in body volume with respect to days after conception (days a.c.) and Carnegie stages (x axis). Embryo volumes are plotted on a logarithmic scale (left y axis; blue dots) and on a linear scale (right y axis; red dots). The linear relation between log(volume) and days of development indicates a constant growth rate of the embryos in this development period. C: Relative volume of organs as percentage of embryonic volume (y axis) with respect to days after conception and Carnegie stages (x axis). Neural tube (not including the neural canal), liver, metanephros, and notochord are shown as examples (see excel S1 for other organs). The neural tube accounts for a constant relative volume of ~10% of the total volume, whereas the relative volume of the liver first increases, after which it also reaches a steady relative volume of 8%. The relative volume of the notochord decreases exponentially, whereas the relative volume of the metanephros increases exponentially after its first appearance.
Fig. 5. Assessment of organ position during development. A: To enable determination of the position of organs, planes perpendicular to the notochord were generated from the intersection of the developing vertebrae with the notochord. Left: Reconstruction of the individually labeled vertebrae and notochord. Center: The notochord was skeletonized to a line and masked with the colors of the vertebrae. Right and far right: The skeletonized notochord was expanded to form a cylinder. Each colored disc corresponds to a vertebra, and the planes of these discs, perpendicular to the notochord, now serve as rulers. The user can increase the diameter of the discs to determine which discs intersect with the organ of interest. The most cranial and caudal intersections of an organ with the planes of the discs then give the position of an organ relative to the vertebrae. Stage 17 (specimen 6521) and stage 20 (specimen 462), with annotated liver (brown) and kidneys (orange), are illustrated. Note the caudal shift of the liver from stage 17 (C7-Th10) to stage 20 (Th2-S5), whereas the kidneys remain at the same cranial position (L1), in contrast to descriptions in textbooks. B: Cartoons based on 3D reconstructions. The relation of the metanephros (orange), the gonad (purple), and the inferior mesenteric artery (IMA; red) is indicated in stages 16, 17, and 20. See the text for their positional changes. C: Margins of the position of the metanephros (orange band), the inferior mesenteric artery (red dots), and the gonads (purple band) during development (x axis) relative to the vertebrae (y axis) (see excel S2 for other organs). Th, thoracic; L, lumbar; S, sacral; Co, coccygeal; CS, Carnegie stage. Scale bars in A, 1 mm.
Changing organ topography

Regional differences in growth and migration of the organs are usually suggested to explain the changes of the position of organs during development (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015; O’Rahilly and Müller 1987; Costa et al. 2002; Müller and O’Rahilly 2011). Perhaps the most cited example is the ascent of the primordial kidneys (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015; Larsen 1993). This ascent, in turn, has led to the notion that fusion of the kidney primordia, prior to their ascent, would account for the horseshoe lesion, the fused middle part allegedly being prevented from ascent by the presence of the midline inferior mesenteric artery, whereas the lateral parts have no impediment to their ascent (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015). The greatest weakness of these suppositions is the almost total lack of quantitative data about the relative positions of the different structures during development. Our 3D reconstructions permit us to show the position of each individual organ relative to the developing vertebrae (Fig. 5 and excel S2). The primordium of the definitive kidney, or metanephros, can be identified already at CS14, but we could first reliably relate its position to the developing vertebrae at CS16 (Fig. 5). The kidney is then positioned within the lumbar region extending from the fourth lumbar vertebra to the first sacral vertebra. Within a few days, it elongates up to the level of the first lumbar vertebra (CS17). Its cranial margin remains at this “adult” level, whereas during further development its caudal margin “ascends” from the first sacral to the fourth lumbar vertebra, owing to a relatively faster growth of the developing vertebral column. This raises the question of whether such a pattern of growth implies a real ascent of the kidneys during development.

A confounding factor that may have led to the notion of ascent of the kidneys is the hitherto unrecognized substantial difference in growth along the entire length of the aorta, which determines the relative position of the arterial branches (Fig. 5, fig. S2, H to O, and excel S2). During development, the branching point of the seventh intersegmental artery remains positioned at the level of the seventh cervical vertebra and that of the umbilical arteries at the level of the third to fourth lumbar vertebrae, whereas the relative positions of the three major intestinal arteries change considerably. From CS16 to CS23, the branching point of the celiac trunk and the superior mesenteric artery “descends” from the sixth and seventh thoracic vertebrae, respectively, to the first lumbar vertebra; the inferior mesenteric artery “descends” only slightly from the first to the third lumbar vertebra. As a consequence, the position of this latter artery changes from the cranial border of the kidney at CS17 to its caudal border from stage 20 onward. Therefore, taking this artery as the point of reference gives the erroneous impression that the kidneys ascend.

Another example is the developing gonads, which are generally assumed to descend during development (Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015; Hamilton, Boyd, and Mossman 1972). Our data show that this is not the case. During the embryonic period, the caudal margin of the developing gonads remains approximately at the level of the fifth lumbar or first sacral vertebra.
The cranial margin, however, does not grow proportional with the vertebral column, and thus it “descends” from the level of the fifth thoracic vertebra in CS16 to the second lumbar vertebra in CS23 (Fig. 5). Obviously, this does not imply that the gonad, as an organ, truly descends in this period, but merely that it shortens relative to the growing vertebrae. Analysis of 34 specimens of the first 2 months of human development thus enabled us to elicit the information required to describe the changes in the position of internal organs relative to one another (Excel S2).

**Fig. 6. Schematic overview of the development of the notochord.** A: The notochord develops from stages 8 to 12. Three representative transverse sections per stage are shown along with a schematic representation of the developing notochord, showing the position of the sections and the partitions of the developing notochord. The notochordal process and plate develop in craniocaudal direction, whereas formation of the definitive notochord starts in the middle of the embryo, expanding in both directions. The caudal extreme of the developing notochord widens in stage 12 (26 to 30 days) as a result of secondary development in the caudal eminence. B: Schematic overview of the five stages of notochordal development in a transverse view. Red, gastrulation zone; green, notochordal process with notochordal canal; yellow, notochordal plate; orange, "inverted U"-shaped notochord; blue, definitive notochord; Ao, aorta; Nc, neurenteric canal; Ng, neural groove; Nt, neural tube. Presented specimens are 10175, 3709, 6330, 6344, and 8505A.
Comparative morphological analyses

In the absence of human experimental studies, medical embryologists necessarily rely heavily on data obtained from experimental animals such as chicken and mouse. A proper understanding of the development of birth defects thus requires insight into the similarities and differences between human development and that of these experimental animals. However, it is not clear whether cartoons of certain developmental processes in medical textbooks truly reflect the human situation. The data collected for the current atlas remedy this situation because they allow verification of experimental findings in the developing human. We cite two examples.

The first example is the development of the notochord, represented in textbooks as a mixture of human and animal data (Moore, Persaud, and Torchia 2016; Sadler 2015; Tuchmann-Duplessis, David, and Haegel 1982). Figure 6 shows the human sections of the relevant developmental stages in our atlas, along with cartoons indicating our observations. These data show that in humans, a group of primitive cells briefly persists after gastrulation. This transient group of cells, through which the neurenteric canal traverses, is dubbed the notochordal process. The ventral side of this cell cluster is incorporated into the endodermal roof of the foregut, and its dorsal side is closely attached to the developing neural tube. This intimate association is often ignored, even though animal studies have shown that the developing notochord induces the formation of the floor plate of the neural tube (van Straaten et al. 1988) and is attached to the developing neural system (Jurand 1962, 1974). Then the notochordal process incorporates entirely into the endoderm, forming the epithelial notochordal plate, which adopts an “inverted U” shape and remains intimately associated with the neural tube. Subsequently, the notochordal cells detach from the endoderm to form the definitive notochord, allowing the two dorsal aortas to fuse between the notochord and the roof of the foregut (Fig. 6B). Similar to gastrulation, the formation of the notochordal process and plate proceeds in the craniocaudal direction. However, in contrast to the mechanism described in several textbooks (Carlson 2014; Moore, Persaud, and Torchia 2016; Sadler 2015; Schoenwolf et al. 2015), the formation of the definitive notochord in humans starts in the middle of the embryo and then proceeds in both cranial and caudal directions, similar to what is generally assumed for the closure of the neural tube.

The second example relates to the origin of the derivatives of the pharyngeal arches. According to current theories, the hyoid body develops by fusion of parts of the second and third pharyngeal arch cartilages, and the thyroid cartilage by fusion of parts of the fourth and sixth pharyngeal arch cartilages, as described for lower vertebrates (Romer 1962). In contrast, we observed that in human development, the body of the hyoid bone develops from a single growth center, without overt contributions from the second and third pharyngeal arch cartilages. The thyroid and cricoid cartilages develop separately from mesenchymal thickenings (Fig. S4). Anatomical variations of the hyoid-larynx complex occur in up to 25% of the general population (de Santana Jr et al. 2006). Most variants are not important in the clinical setting, but they may be important in a forensic context if a fracture of the hyoid bone is suspected. In that case, it is of utmost importance to distinguish between true
fractures of the hyoid bone or the thyroid horns, and failure of these parts to fuse during development.

Discussion

The recent expansion of molecular genetic technology has brought with it an improved understanding of the regulatory mechanisms that control the development of the vertebrate building plan. Although the findings in model organisms are being extrapolated to human development, insight into the divergence and conservation of human morphogenesis, as compared to experimental animals, remains crucial to assess whether such extrapolations are justified. A global analysis of the changing position of just a few structures such as the gonads, kidneys, and arteries shows discrepancies with current textbooks and thus demonstrates the value of a 3D atlas based on human embryonic specimens. The generated 3D models are presented in interactive 3D-PDF files (Murienne, Ziegler, and Ruthensteiner 2008), which facilitates the understanding of complex 3D structures and permits the reader to develop an independent judgment about human embryology. Although new editions of biomedical textbooks update recent molecular insights, the morphogenesis is not updated and has become increasingly schematic as well as alienated from the original human substrate. This 3D atlas reinstates this link because the morphological reconstructions are connected directly to the original sections of the human embryos in the Carnegie Collection.

Our atlas can serve as an educational and reference resource for (bio)medical students, clinicians, and scientists interested in human development and development-related disease. The original images and reconstructions are available for educational use and further scientific analysis. This, in turn, will permit updates and extension of the atlas with time, in cooperation with other research groups.
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References


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Supplementary Materials and Methods

**Human specimens**

All embryos included in this study are historical specimens, which, according to available information, have been collected between 1910 and 1975, after hysterectomy (n=13), extra uterine pregnancy (Morgan 2009) or miscarriage (O’Rahilly and Müller 1987). Miscarriages were only included in this atlas if no other specimens of comparable stage and quality were available and if the histologically stained sections were of sufficient quality. Most of the embryos are from the Carnegie collection in Silver Spring, MD, USA; two specimens are from the Boyd collection of the University of Cambridge, UK and one specimen is from the collection of the Department of Anatomy, Embryology & Physiology, Academic Medical Center, Amsterdam, The Netherlands. Serial histological sections of a duplicate series of 17 embryonic stages, spanning the first 2 months of development (Carnegie stages 7 to 23) were used. No ethical approval was needed for this retrospective study.

After fixation and embedding, all specimens have been sectioned and stained with different histological staining methods. Additional information about the specimens can be found in supplementary table 1 (O’Rahilly and Müller 1987; Streeter 1942; Streeter 1945, 1948; Streeter 1949; Streeter 1951; Morgan 2009). The embryos were staged using Streeter’s original classification of embryos (Streeter 1942; Streeter 1945, 1948; Streeter 1949; Streeter 1951) and its modified version by O’Rahilly and Müller (O’Rahilly and Müller 1987). Specimen number 3709 is known in the Carnegie collection as a stage 10 (21-23 days) embryo, but because of its external features with 4 somites it is considered to resemble a late stage 9 (19-21 days) embryo. Sections of the other stage 9 embryos in the Carnegie collection were of unsatisfactory quality.

**Image acquisition and processing**

Nikon E800 microscopes with Plan Apo lenses and a Ludl motorized XY-stage (http://www.ludl.com), were used to capture digital images of the sections. Image-Pro Plus 5.1 with Scope-Pro (http://www mediacy.com) was used for capturing and tiling, resulting in image tiles with a resolution of 2000x1600 pixels. Flat field correction and stitching was accomplished using Image-Pro Plus 7 and custom routines in Matlab R2011b (http://www.mathworks.com). Some specimens were imaged using a macro setup consisting of a Canon EOS 500D camera, Canon EF 100mm f/2.8L Macro lens and EOS utility software for remote shooting (http://www.canon.com). Thumbsplus 7 (http://www.thumbsplus.com) was used for batch-wise image processing like color-to-grayscale conversion, cropping, resolution reduction and contrast enhancement. Because of the large file sizes, stitching and further image processing was performed on PCs with Windows 7 (64 bit) and 16GB RAM.

**Alignment, segmentation and visualization in Amira**

Amira (version 5.3 - 5.6, http://www.amira.com) was used for the 3D reconstruction process, consisting of alignment, segmentation and visualization (Soufan et al. 2003). Alignment of the stitched serial images was performed mainly automatically (least-squares alignment mode), but some manual adjustments were needed, especially in the cranial region of the embryos, because the rounded shape of the head hampered automatic alignment. The aligned 3D images were compared with photographs of the exterior of the original embryos and the alignment was adjusted, if necessary.

Segmentation of the various embryonic structures in more than 15,000 sections was done by trained analysts, under supervision of four experienced
embryologists of our department. The various organ systems were mostly delineated by hand, with a Bamboo tablet and pen (http://www.wacom.com), based on the images of the serial sections. Segmentation was performed on greyscale images in Amira, while the high-resolution full-color dataset was displayed on a second computer screen. Some structures, like parts of the coelomic cavity and the lumen of the neural tube, could be segmented by using a threshold, but over 95% of the tissues had to be outlined manually. Every analyst focused on a distinct region, i.e. an organ or organ system with its constituent and adjacent tissues, which then was segmented in the different stages of development, using unique labels to depict the different parts of the organ system.

From the segmentation label files, triangulated surface files were made using the SurfaceGen function, mostly resulting in very large surface files. Depending on the purpose, label resolution reduction (Resample), label interpolation (InterpolateLabels), triangle reduction (Simplify) and surface smoothing (SmoothSurface) was applied for visualization or modeling.

**Inter-observer variability**
The reproducibility of the manual segmentation of organs by the trained analysts, mainly (bio)-medical students, was evaluated by comparison of the volumes of three different annotated organs. To this end, each analyst segmented the otic vesicle (a smooth and easily recognizable organ), the mesonephros (existing of different types of tissue) and the mesonephric ducts (a small and tangled structure) in ten serial sections (supplementary Fig. S1). For each structure, the volume was calculated and variation in volumes between observers was determined. The relative standard error ranged from only 0.3% to 2% for simple and complex structures, respectively and comparison of trends revealed only a small systematic bias between observers (Friedman test: p=0.06; supplementary Fig. S1). This analysis of variability showed that the reproducibility of manual segmentation is very high and that effects of segmentation errors on observed volumes of organs and other structures can be ignored.

**3D modeling**
For visualization purposes only, the rough but highly detailed Amira models were transformed into smooth and informative models. To this end, the open source 3D modeling software package Blender (version 2.51 - 2.76, http://www.blender.org) was used to remove artifacts and minimize the complexity and thus the file size of the models (Garwood and Dunlop 2014). Artifacts in the Amira models, caused by sectioning, stretching, alignment or under-sampling errors, were pinpointed by morphological experts and corrected during this knowledge-driven modeling process. The basic technique that was used is box modeling, by which a model is created by subdividing and refining a basic shape (e.g. cube); for more complex and organic modeling, digital sculpting techniques were applied. The total number of triangles of the final Blender models is about 90-99% reduced, compared to the original Amira surface models, without loss of correct topology and essential morphological details.

In short, the reconstructed, simplified and smoothed Amira surfaces were exported as Stanford (.ply) files and imported into Blender. Manifold (“watertight”) models of every separate structure were manually created by trained game development students, using the imported models as templates. The models need to be manifold to enable solid digital sectioning. Many Blender functions, tools and add-ons were used, depending on the shape and complexity of the embryonic structure. The resulting low-poly models obtained their smooth and natural appearance by
applying a Subdivision Surface modifier (Catmull-Clark). Because many neighboring structures were modeled separately from each other, special care was taken to avoid that structures overlap after combining the models into the complete embryo. Some larger structures, such as the alimentary canal, were modeled as one structure and subdivided by using a Boolean modifier to create sharp cutting edges.

Standardized material colors and an object tree with the structure names, in accordance with the Terminologia Embryologica (FIPAT 2013), were assigned to the complete model. As a final step the quad-based models were triangulated and the number of triangles was reduced, where possible. For creating a 3D-PDF, the model was exported as an Autodesk FBX (.fbx) file, which keeps the structure tree and material colors intact.

Three-dimensional PDFs
The exported Blender models were imported into Deep Exploration (version 6.5 CSE, part of Corel DESIGNER Technical Suite X5 http://www.corel.com). After final small corrections in the object tree and material list the models were exported as Universal 3D (.u3d) file and imported in Adobe Acrobat XI Pro (http://www.adobe.com). Interactive 3D portable document format (.pdf) files containing all modeled organs and structures per embryo were created (de Boer et al. 2011). These 3D-PDFs can be viewed in a recent version of Adobe Reader® (X or higher, freeware, http://www.adobe.com) on MS Windows or MacOS systems, with javascript and playing of 3D content enabled.

We designed a user-friendly navigation panel with a structure tree, subdivided into organ systems. Combined with options to select each separate structure and the ‘show’, ‘transparent’ and ‘hide’ buttons, the user is now able to show any combination of structures from any chosen angle and in any chosen zoom. Using these interactive tools, the user is able to obtain a good understanding of the spatial relations between the various organs in the embryo. Some predefined views were created as examples to direct the attention of the user to certain structures. To provide the user with a perception of size of the specimen, an ‘actual size’ page was added to each 3D-PDF. The supplementary interactive 3D-PDFs are available via the portal (http://3datlasofhumanembryology.com).

Quality checks
Quality checks during the image acquisition and the reconstruction process are indicated with a green arrow in Fig. 1 of the main text. First, the quality of the available sectioned and stained specimens was checked. This included checks of appropriate histology of preferably transverse sections, presence of tears and artifacts in the sections, irregular or faded stains and missing sections. When one glass in a series was missing the embryo could not be used for reconstruction. The second check was performed during and after alignment of the sections in Amira. The aligned stack of sections was compared with the photograph of the original embryo before sectioning, when available. Also, a constant spacing of the sections throughout the complete stack is of utmost importance for a 3D reconstruction. The correct Z-value, i.e. sampling distance, could be calculated in this phase, based on the pixel sizes of the captured images and the photograph of the original embryo before sectioning. The third quality check was performed during and after manual segmentation by the analysts, under supervision of experienced embryologists. The segmentation was performed on gray images in Amira while the aligned color images of the same sections were used as a control. Every segmented structure in Amira had to be approved by two experienced embryologists. The fourth quality check was performed with the reconstructed
embryo in Amira. The reconstruction was compared again with the original embryo before sectioning and with the reconstruction of the other embryo within the same developmental stage and with reconstructions of previous and subsequent stages. The last quality check concerns the knowledge-driven modeling step. Every modeled structure was compared with the original Amira model, clipping-through issues were corrected and the final models were approved by the embryologists.

**Morphometry**
Measurement of the volumes of organs and structures requires knowledge on the magnification of the images used for 3D reconstruction and the distance between these images. Magnification, or pixel size, was determined by measuring the real distance between two points in the image of a histological section and dividing this distance by the number of pixels along the same line in the image. For images that were acquired specifically for this project, this could be accomplished by counting the number of pixels on the known length of a simultaneously imaged microscopic ruler. For images that were downloaded from the Carnegie collection website, the measurement tool on the EHD webpage (http://www.ehd.org/virtual-human-embryo) served to obtain the real distance. Thus, for each embryo the magnification of the images was determined.

For each embryo a systematic sample of sections, ranging from each section till one section per several sections, was used for 3D reconstruction. Most often each section was used for reconstructing. However, in some older and larger embryos a systematic sample of one section per X sections was used to prevent the Amira data file from becoming too large (max. 2.2 GB). However, section thickness and spacing of the sections was not known for each embryo. Therefore, the section, or slice, thickness to be used in the calculation of volumes was determined using the length and width ratio that could be measured from the photograph of the exterior of the embryo before fixation and the width that could be measured in the section at the same position. Slice thickness can then be calculated as length times the width in a section divided by the width in the photograph. Note that this use of the original photograph in the estimation of voxel size corrects for differences in shrinkage between embryos.

Volumes of organs and structures are determined by counting the number of pixels within the manual outline of the structure, summing this number for all sections and multiplying by the voxel volume. The volumetric measurements thus follow the principle of Cavalieri which states that, in a systematic sample of sections, an unbiased estimate of volume can be obtained as the product of summed area times distance (Chieco et al. 2013).
Topography
To determine the relative position of developing organs and structures, the vertebral column was used as ruler by which the levels of the organs were compared between Carnegie stages 16 (37-42 days) to 23 (56-60 days). To this end, all vertebrae were individually labeled in Amira. Thoracic vertebrae were identified by the presence of ribs; from there the cervical vertebrae were identified in cranial direction and the lumbar, sacral and coccygeal vertebrae in caudal direction. To take the variable curvature of the embryo into account, the notochord was skeletonized to a line and then expanded to form a cylinder. Such a blown-up version of the notochord keeps the same curvature as the vertebral column. Masking of the skeletonized notochord with the vertebrae permits its partition into differentially colored discs, or “vertebra-domains”, each perpendicular to the notochord and corresponding to a vertebra. These discs allow the unambiguous identification of the position of an organ-of-interest by assessing which discs intersect with its most cranial and its most caudal borders. In this way, we defined the most cranial border of an organ as the most caudal vertebra-domain that still intersects with the most cranial point of the organ. Similarly, the most caudal border of an organ was defined as the most cranial vertebra-domain that still intersects with the most caudal point of the organ. In doing so, we viewed all structures from the left and the right side of the embryo to avoid bias by asymmetries that might be obscured by the expanded notochord disks. When the most cranial or caudal margin of an organ extended the borders of the blown-up version of the notochord, no position was noted. See also [Fig. 5](#) and [supplementary Excel 2](#) for further details on the location of organs compared to the vertebrae.

Chronology and heterochrony
Histological sections of human, mouse and chicken embryos of different stages have been studied for the presence or absence of several organs and structures at a particular stage. The development of these organs and structures was compared by using the atlas of Butler & Juurlink (Butler and Juurlink 1987). Besides the above-described human specimens, specimens from the collection of the Department of Anatomy, Embryology & Physiology, Academic Medical Center, Amsterdam, The Netherlands, were used to study mouse (E8.5-E18.0) and chicken embryos (24 hours-10 days). All human specimens were staged using Streeter’s classification (Streeter 1942; Streeter 1945, 1948; Streeter 1949; Streeter 1951) and its modified version by O’Rahilly and Müller (O’Rahilly and Müller 1987). Mouse embryos were staged according to Kaufman (Kaufman 1992) and the chicken embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton 1951) and Romanoff (Romanoff 1960). Two specimens per stage were studied for each species. When more variation was found, three or even four mouse or chicken specimens were studied. If additional information was necessary for studying the development of organs or structures in the human embryo, the available 3D models were used, along with the histological images. The results of the research in the three species were summarized and used to construct a schematic chronology ([supplementary Fig. S3](#)) that compared the corresponding stages according to the study of Butler & Juurlink (Butler and Juurlink 1987). A detailed listing of all results can be found in [supplementary excel 3](#).
References Supplementary Materials and Methods


———. 1951. 'Developmental horizons in human embryos: Description of age groups XIX, XX, XXI, XXII, and XXIII, being the fifth issue of a survey of the Carnegie Collection', *Publ Contrib Embryol*, 34: 165-96.
To test the reproducibility of the manual tracing of the different organs (top panel), 16 of the participating analysts were asked to draw the contours of 3 types of organs on a set of ten successive images (middle panel). The organs ranged from complex (left: mesonephric duct) to simple (right: otic vesicle). Per organ and analyst, the measured volume was calculated. To compare the reproducibility in volumes, the mean of the volumes of the smallest organ was set to 1 and all measured volumes were scaled accordingly (bar graphs). A Friedman test showed that the standard deviation of the measured volumes is independent of the measured volume (P=0.06). The complex organ has the highest coefficient of variation. To study between-operator bias, the geometric mean of the volumes per organ was set to 1 and all volumes were scaled accordingly (line graphs). The crossing lines in these graphs show that there is no systematic bias between operators.

Fig. S2A-O Stage-specific schematics of the vascular system Based on the 3D reconstructions and histological sections of two specimens per stage, stage-specific schematics of the developing vascular system were made. A dashed line represents a developing vessel; a dotted line represents a regressing vessel. Specific stage-dependent changes in the vascular system are indicated in supplementary table 2. From stage 16 onward the vertebrae are shown in fluorescent green, to indicate the change in position of the coeliac trunk, and the superior- and the inferior mesenteric artery. The 7th intersegmental artery or subclavian artery remains at the cervical 7 level and the umbilical arteries, or iliac arteries remain at the lumbar 4 level. Note the enormous shift in position into caudal direction of the coeliac trunk and the superior mesenteric artery relative to the developing vertebrae. In the cartoons, the other vessels cannot directly be correlated to a vertebral level, because they are projected in a single plane. See also Fig. 5 and supplementary excel 2 for further details on the location of organs compared to the vertebrae.
An interactive three-dimensional digital atlas and quantitative database of human development

CS09

Fig. S2A

Ventral view

Arterial pole
Primary heart tube
Venous pole

1st p.a.a.
Paired dorsal ao.

Vitelline v.
Vitelline venous plexus
Yolk sac
Vitelline arterial plexus
Vitelline a.

Umbilical v.
Umbilical a.
Umbilical cord

R

L
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Fig. S2C
CS12

- Cranial dorsal ao.
- 1st p.a.a.
- 2nd p.a.a.
- Aortic sac
- Paired dorsal ao.
- Superior cardinal v.
- Common cardinal v.
- Inferior cardinal v.
- Heart
- Sinus venosus
- Vitelline v.
- Vitelline venous plexus
- Transverse septum
- Vitelline arterial plexus
- Vitelline a.
- Fusing dorsal ao.
- Umbilical v.
- Umbilical a.
- Umbilical cord

Fig. S2D
An interactive three-dimensional digital atlas and quantitative database of human development

Fig. S2E
An interactive three-dimensional digital atlas and quantitative database of human development

Fig. S2G
An interactive three-dimensional digital atlas and quantitative database of human development
An interactive three-dimensional digital atlas and quantitative database of human development

Fig. S2K
Fig. S2L
An interactive three-dimensional digital atlas and quantitative database of human development

Fig. S2M
Fig. S2N
An interactive three-dimensional digital atlas and quantitative database of human development

Fig. S2O
**Fig. S3 Heterochrony of ear development between the human, mouse and chicken embryo.**

Boxes indicating the first appearance of an organ or structure are placed on a scale indicating equivalent stages of development of human, mouse and chicken embryos. This classification has been published by Butler & Juurlink in 1987 and applies for the complete embryo. At the organ level, we observed significant differences in the appearance of structures between these three species. Therefore, we made a comprehensive comparison of the appearance of the most prominent structures ([supplementary excel 3](#)). CS: Carnegie Stage, E: Embryonic day, HH: Hamburger & Hamilton stage.
**Fig. S4**

Developmental series of the hyoid-larynx complex.

Histological sections of six human embryonic specimens between stages 18 and 23 are presented in the right panel, each with a frontal view of its matching 3D reconstruction in the left panel. The arrows in the right panels indicate the hyoid body anlage in stage 18 to 21, which becomes fully incorporated in the hyoid bone in stage 22 and 23. The plane and level of sectioning through the embryo is shown in every right upper corner.

CS18 (44-48 days) specimen 6524.
CS19 (48-51 days) specimen 2114.
CS20 (51-53 days) specimen 462.
CS21 (53-54 days) specimen 7254.
CS22 (54-58 days) specimen 895.
CS23 (56-60 days) specimen 950.

Abbreviations: CS: Carnegie stage, 2: second pharyngeal arch cartilage, L: laryngeal orifice, T: thyroid cartilage.
### Table 5. Overview of the specimens used for the atlas

| Name                        | Formol | Formalin | Formalin | Hematoxylin and Eosin | Formalin - Zinc | Zinc hematoxylin | Zenker’s Formol | Bouin | Hematoxylin and Eosin | Phloxin | Zenker’s Hemotoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and Eosin | Zenker’s Hematoxylin and 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Table S2. Stage-specific changes of the vascular system

<table>
<thead>
<tr>
<th>CS</th>
<th>Major changes in the vascular system</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>a) Cranial part of Ao appears. b) Uvs form completely.</td>
</tr>
<tr>
<td>11</td>
<td>a) Cardinal plexus begins to form. b) Aortic sac forms. c) Both parts of dorsal Ao start to move toward each other midways.</td>
</tr>
<tr>
<td>12</td>
<td>a) 2nd Paas appear. b) Superior, common and inferior Cvs appear. c) Dorsal Ao fuses midways. d) Vit venous plexus extends into transverse septum. e) Ua form completely. f) Venous pole forms into sinus venosus.</td>
</tr>
<tr>
<td>13</td>
<td>a) 3rd Paas appear. b) 1st Paas regress. c) Cvs increase. d) Dorsal Ao fuses further caudal and cranial. e) Vit venous plexus turns into intrahepatic vessels. f) Cranial part of left Uv regresses.</td>
</tr>
<tr>
<td>14</td>
<td>a) 2nd Paas regress. b) 4th and 6th Paas appear. c) Basilar artery and first seven intersegmental arteries appear, forming a dorsal inter-intersegmental arterial plexus that later becomes vertebral artery. d) Subclavian/brachial veins appear. e) Parts of the left and right Vit veins regress. f) Ductus venous appears. g) Right Uv regresses. h) Left part of sinus venosus disconnects from the heart. i) Coeliac trunk appears. j) Sacral artery appears.</td>
</tr>
<tr>
<td>16</td>
<td>a) Pulmonary arteries appear. b) Intersegmental arteries regress between dorsal Ao and vertebral artery and continue their growth dorsal of the vertebral artery towards the neural tube (not shown).</td>
</tr>
<tr>
<td>17</td>
<td>a) Parts of dorsal Ao between 3rd and 4th Paas regress. b) Right 6th Paa. c) Aortic sac becomes ascending Ao. d) Right pulmonary artery connects to left 6th Paas. e) Caudal part of basilar artery connects to the 7Ia via the inter-intersegmental arterial plexus and becomes vertebral artery. f) The part of the left 6th Paas before the pulmonary arteries divericate becomes the pulmonary trunk. g) Right Vit vein becomes inferior vena cava. h) Caudal parts of inferior Cvs fuse and form a sacral vein.</td>
</tr>
<tr>
<td>18</td>
<td>a) Cranial parts of dorsal Ao become internal carotid arteries. b) External carotid arteries appear. c) 3rd Paas become common carotid arteries. d) Left 4th Paas becomes aortic arch. e) Part of the 6th Paas after the pulmonary arteries divericate becomes ductus arteriosus. f) 7Ia become most proximal subclavian arteries and most distal brachial arteries. g) Part of right ventral Ao before bifurcating into the common carotid artery and the subclavian artery becomes the brachiocephalic trunk. h) Parts of the inferior Cvs cranial of the external iliac veins regress. i) Subcardinal vein appears. j) Internal and external iliac arteries appear.</td>
</tr>
<tr>
<td>19</td>
<td>a) Right dorsal Ao between subclavian artery and fused dorsal Ao disappears. b) Remaining part of inferior Cvs fuses caudal. c) Subcardinal vein bifurcates to the external iliac veins and forms two sacral veins. d) Cardinal plexus begins to move cranially. e) Insertion of the pulmonary veins into the left atrium moves more to left and cranial.</td>
</tr>
<tr>
<td>20</td>
<td>a) Left brachiocephalic vein begins to form.</td>
</tr>
<tr>
<td>21</td>
<td>a) Left brachiocephalic vein forms completely.</td>
</tr>
<tr>
<td>22</td>
<td>a) Internal thoracic arteries appear. b) Renal arteries form.</td>
</tr>
<tr>
<td>23</td>
<td>a) Superior vena cava forms.</td>
</tr>
</tbody>
</table>

Descriptions are based on the 3D reconstructions and histological sections of two specimens per stage, stage-specific changes of the developing vascular system were indicated. See supplementary figures S2a-o for the accompanying illustrative stage-specific schematics of the vascular system.

7Ia: 7th intersegmental arteries, Ao: Aorta, Cv(s): Cardinal vein(s), Cs: Carnegie stage, Paq(s): Pharyngeal arch artery(arteries), Ua: Umbilical arteries, Uv(s): Umbilical vein(s), Vit: Vitelline.
### Table S3. Arterial and venous development and discrepancies with literature

<table>
<thead>
<tr>
<th>Arterial discrepancies</th>
<th>Our results</th>
</tr>
</thead>
<tbody>
<tr>
<td>The umbilical arteries acquire a second connection to the aorta and lose the original connection.</td>
<td>The umbilical arteries have just one persisting connection to the aorta.</td>
</tr>
<tr>
<td>The coeliac trunk and the superior and inferior mesenteric artery derive from the vitelline artery.</td>
<td>Just the superior mesenteric artery is a derivation from the vitelline artery.</td>
</tr>
<tr>
<td>The right 6th paa does not totally regress, but proximally contributes to the right pulmonary artery.</td>
<td>Both pulmonary arteries are new vessels that sprout very close to the aortic sac.</td>
</tr>
<tr>
<td>The 1st and/or the 2nd paa’s contribute to the external carotid artery.</td>
<td>The external carotid artery is a new vessel.</td>
</tr>
<tr>
<td>The 5th paa is present either in 50% of all cases or temporarily.</td>
<td>We did not find the 5th paa in any of the embryos.</td>
</tr>
<tr>
<td>The external carotid artery sprouts from the ventral aorta.</td>
<td>The external carotid artery sprouts from the 3rd paa.</td>
</tr>
<tr>
<td>The 5th paa is present either in 50% of all cases or temporarily.</td>
<td>We did not find the 5th paa in any of the embryos.</td>
</tr>
<tr>
<td>The external carotid artery sprouts from the ventral aorta.</td>
<td>The external carotid artery sprouts from the 3rd paa.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Venous discrepancies</th>
<th>Our results</th>
</tr>
</thead>
<tbody>
<tr>
<td>There are supracardinal veins that have an anastomosis beneath the heart and connect with the common cardinal vein.</td>
<td>The inferior cardinal vein anastomoses beneath the heart.</td>
</tr>
<tr>
<td>The inferior cardinal vein regresses totally.</td>
<td>The subcardinal vein takes over the existing caudal part of the inferior cardinal vein.</td>
</tr>
</tbody>
</table>

After studying the histological sections and reconstructed models, schematic overviews of the embryological development of the blood vessels were created (Supplementary Fig. 2A-O). In this table, the most profound discrepancies between our findings and several embryology textbooks are presented. Paa(s): Pharyngeal arch artery(arteries).
Supplementary Excel files

**Excel S1** (accessible online)
Overview of all measured organ volumes per specimen. Volumes of organs and structures were determined by counting the number of pixels within the manual outline of the structure on a digital picture of a histological section, summing this number for all sections and multiplying by the voxel volume. For further details see the Morphometry section of the supplementary methods and Fig. 4. Worksheet 1: Absolute volumes. All measured volumes for the structures present in each specimen are presented here and some examples of possible graphs are given, which allows for creation of dedicated new graphs by the user to accurately describe the volume changes of each developing structure. Note that growth as presented in these graphs is interpolated from multiple specimens. Worksheet 2: Relative volumes. Here the volumes of structures are presented relative to the total embryonic volume that has been set at 1.0. This information allows interpretation of organ growth relative to the growth of the embryo. The neural tube, for example, retains a constant relative volume of approximately 10% of the total embryonic volume throughout development. Worksheet 3: Data figure 4. In this worksheet, the calculated exponential growth by the developing human embryos and their neural tubes is presented, which served as basis for Fig. 4B and C.

**Excel S2** (accessible online)
Organ position relative to the developing vertebrae. Our three-dimensional reconstructions permit us to unambiguously show the position of each individual thoracic and abdominal organ relative to the developing vertebrae. Worksheet 1: Legend for position. The organ position was measured relative to the developing vertebrae (see the Topography section of the supplementary methods and Fig. 5 for further details). The numbers used in worksheet 2 and 3 correspond to the vertebrae as presented in worksheet 1. Worksheet 2: All organs all observations. All organ positions measured relative to the developing vertebrae in both specimens per stage are presented here. Worksheet 3: All organs average per stage. The average organ position was calculated by averaging the vertebral level numbers of both specimens from the same Carnegie stage. These average numbers serve as basis for the graphs in worksheet 4. Worksheet 4: Graph examples. Examples of possible graphs are presented. Together with worksheet 3 this sheet allows for creation of dedicated new graphs by the user to accurately describe the changes in the relative position of each organ.

**Excel S3** (accessible online)
Overview of the appearance of structures in human, mouse and chicken embryos. A comprehensive overview of the appearance of the most prominent structures in human, mouse and chicken embryos is presented. A yellow box indicates the first appearance, and sometimes disappearance, of the different organs and structures in a specimen. A green box indicates that the organ is definitely present in that specimen. Explanatory notes have been given when necessary (red triangles). The order of appearance of the distinct organs in these species agrees largely with the order reported by Butler and Juurlink (1987), who based their staging exclusively on the exterior characteristics of the complete embryo. However, we found a consistent difference of approximately 1-2 stage equivalents when comparing matched mouse and human or chicken developmental stages. For further details see the Chronology and heterochrony section of the supplementary methods and supplementary Fig. S3. Worksheet 1: Human; Carnegie stage 7 - 23 (15-60 days of development). Worksheet 2: Mouse; Embryonic day 8.5 – 18. Worksheet 3: Chicken; 24 hours – embryonic day 10.
Supplementary PDFs (accessible online)
Stage 7 specimen 8752 (15-17 days of development)
Stage 8 specimen 8671 (17-19 days of development)
Stage 9 specimen H712 (19-21 days of development)
Stage 10 specimen 6330 (21-23 days of development)
Stage 11 specimen 6784 (23-26 days of development)
Stage 12 specimen 8505A (26-30 days of development)
Stage 13 specimen 836 (28-32 days of development)
Stage 15 specimen 3512 (35-38 days of development)
Stage 16 specimen 6517 (37-42 days of development)
Stage 17 specimen 6521 (42-44 days of development)
Stage 18 specimen 6524 (44-48 days of development)
Stage 20 specimen 462 (51-53 days of development)
Stage 21 specimen 7254 (53-54 days of development)
Stage 23 specimen 9226 (56-60 days of development)

The reconstructions on which the 3D-PDFs are based, are generated from histological sections of human embryos, as described in the supplementary Materials and Methods section. Some structures could be segmented in Amira by using a threshold, but over 95% of the tissues had to be outlined manually, after which artifacts were restored by knowledge-driven modeling in Blender. Drawbacks in this process are the inability to reconstruct very thin structures and the time-consuming modeling process in Blender. Therefore, these 3D-PDFs serve solely as didactic models, whereas the morphometric measurements have been performed directly on the Amira models.

Each 3D-PDF consist of three pages; the 3D model, the actual size page and the information page. The orientation axis in the actual size page describes the body axes by the following abbreviations; Cr: cranial, Ca: caudal, V: ventral, D: dorsal, L: left, R: right. The PDF files should be viewed in Adobe Reader® X or higher, available from http://www.adobe.com/downloads/. 3D interaction is only possible on MS Windows or Mac OS. Javascript must be enabled. For more advanced selection options, right-click on the 3D model and choose: “Show Model Tree”.