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Systematic analysis of the development of the ductus venosus in wild type mouse and human embryos☆

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ABSTRACT

Background: Doppler flow velocities of the ductus venosus are increasingly used to assess fetal increased nuchal translucency, growth-restriction and monochorionic twins, and might contribute to screening for cardiac defects. It is disputed whether a sphincter at the ductus venosus inlet actively regulates blood flow.
Aims: This study aims to define the morphogenesis of the developing mouse and human ductus venosus and to address the existence of a sphincter.
Study design: The presence of endothelium, smooth muscle, elastic fibers and nerves in the ductus venosus of E10.5–15.5 mouse embryos and in three corresponding human embryos (CS16, CS19 and CS23) was examined using immunohistochemistry. Three-dimensional reconstructions of the ductus venosus of E11.5–15.5 mouse and CS14–23 human embryos were generated and examined.
Results: The ductus venosus lumen was narrowed from ventral–caudal to dorsal–cranial in E13.5–15.5 mouse and CS16–23 human embryos. Mouse embryos showed positive endothelial Pecam1 expression from E11.5–15.5 and smooth muscle actin staining in the ventral–caudal part of the ductus venosus from E12.5–15.5. At all developmental stages, elastic fiber and nerve marker expression was not detected in the ductus venosus (Fig. 2). In human embryos endothelial Pecam1 and smooth muscle actin expression was found in the ductus venosus from CS16 and CS19 onwards. Elastic fiber and nerve marker expression was not detected in all stages (Fig. 4). Morphogenesis and staining results of the ductus venosus were similar in both species.
Conclusions: The ductus venosus lacks a sphincter at its inlet as no accumulation of smooth muscle cells, elastic fibers or nerve innervation was found in mouse embryos from E11.5–15.5 and in human embryos from CS14–23.
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1. Introduction

The ductus venosus is a trumpet-like shunt connecting the intra-abdominal part of the umbilical vein to the inferior vena cava. The inlet or isthmic part of the ductus venosus has the narrowest diameter [1–3]. Due to this narrowing, blood flow acceleration towards the heart is established. In fetal circulation, the function of the ductus venosus is to ensure that highly oxygenated blood from the placenta is transported directly to the fetal heart and brain, bypassing the fetal hepatic circulation.

Doppler flow velocity waveforms of the ductus venosus are included into daily used assessment of fetal well-being [4,5]. These flow velocity waveforms reflect the pressure gradient between the umbilical vein and the cardiac atria [2]. A low forward, absent or reversed flow velocity in the ductus venosus during atrial contraction (a-wave) is suggested as a sign of fetal distress or cardiac failure [6–8]. However, changed flow velocities are also described in human fetuses with increased nuchal translucency (NT) without cardiac defects or signs of cardiac decompensation, like pleural or pericardial effusion, ascites or edema [9,10]. Furthermore, according to previous studies [11–14], the fetus has developed a mechanism to adapt to different physiological and pathological conditions (e.g. hypoxemia, stress) by varying the extent of shunting of umbilical blood through the ductus venosus using an active sphincter at the ductus venosus inlet. However, the morphology of the fetal ductus venosus and the presence of such a sphincter are controversial [15].

The genomes of mice and human show extensive similarities [16] and the development of the ductus venosus in both species is quite similar [17], making this a favorable experimental model. Also, generating transgenic mice carrying a human genetic disease can be used to gain insights into genetic (congenital) defects.

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The aims of this study are to define the morphology of the developing mouse and human ductus venosus and to assess the existence of a sphincter.

2. Methods

2.1. Embryos

Wild type mouse embryos were analyzed from E10.5–15.5, approximately corresponding to Carnegie Stage (CS)14–23 and human embryos were investigated at CS16, 19 and late 23 (corresponding to 10 weeks of gestation). A total of three human embryos were analyzed, one human embryo was examined for each CS. These stages coincide with cardiac and lymphatic development and (increased) NT. Wild type mouse embryos were provided by the Department of Anatomy, Embryology & Physiology, Academic Medical Center (AMC), Amsterdam, The Netherlands. Guidelines for care and use of animals approved by this department were followed.

Human embryos were provided by the Human Developmental Biology Resource, Newcastle, UK, and approved the use of these embryos. The Medical Ethical Committee of the AMC approved the use of these embryos for the project 'Three-dimensional model of human heart development for developing morphological and molecular staging system' (Appendix A). Embryos were fixed in 4% formaldehyde at 4 °C overnight, dehydrated and embedded in paraffin. Serial transverse sections of 7 μm in mouse and 10 μm in human embryos were made and every 5th section was mounted on the slide. Slides were dried at 37 °C for at least 24 h.

2.2. Histological staining

Elastic fibers were visualized by Lawson van Gieson (LvG) staining. Deparaffinized slides were stained with Lawson (Klinipath) for 60 min, then differentiated in ethanol 96% for ±10 s. Slides were rinsed briefly with bidistilled water and stained in Van Gieson's picrofuchsin (5% fuchsin in 100 ml picric acid with 0.25% hydrochloric acid) for ±8 min. Slides were rapidly dehydrated through a graded series of ethanol and xylene, followed by mounting using Entellan (Merck).

2.3. Immunohistochemical markers

In mouse embryos an antibody for smooth muscle actin (SMA, mouse monoclonal antibody clone 1A4 (1:4000), Sigma-Aldrich, St Louis, USA), for nerves NeuN (neuronal nuclear protein; mouse monoclonal antibody clone MAB377 (1:1000), Chemicon, Temecula, USA) and Ncam1 (Neural Cell Adhesion Molecule 1; rabbit polyclonal antibody clone AB5032 (1:1500), Chemicon, Temecula, USA) and for endothelium (Platelet endothelial cell adhesion molecule-1 (Pecam1); goat polyclonal antibody clone SC-1506 (1:200); Santa Cruz Biotechnology, Santa Cruz, USA) was used.

In human embryos staining was performed as stated above without using NeuN and including von Willebrand factor, an additional antibody for endothelium (von Willebrand factor (VWF) rabbit polyclonal antibody clone AB7356 (1:2000), Chemicon, Temecula, USA). Slides were deparaffinated using a xylene to ethanol series. Subsequently, slides were incubated for 30 min in a solution of 0.3% H_2O_2 in PBS (phosphate buffered saline: 150 mM NaCl, 10 mM NaPi, pH 7.4)/50% ethanol to eliminate endogenous peroxidase activity and rinsed twice with PBS for 5 min. With the exception of PECAM1, slides were placed in 200 ml 1% Antigen Unmasking solution (Vector Laboratories, Burlingame, USA) in a rack and cooked for 5 min at 1000 W in a high pressure cooker. The rack was cooled in bidistilled water and once the pressure cooker was depressurized, placed on ice for ±20 min. Slides were rinsed with PBS for 5 min and blocked in Tris–sodium buffer (1 M Tris, 1.5 M NaCl, adjust to pH 7.4 using HCl) with 0.5% blocking reagent for 30 min. All slides were incubated overnight with the first specific antibody. The next day, slides were rinsed three times with TNT (0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween-20) for 5 min, followed by 30 min incubation with the second antibody (Envision+ HRP anti-mouse or -rabbit depending on the first antibody). Slides were rinsed three times with TNT followed by ±10 min DAB (Dako kit), depending on background staining intensity. The reaction was stopped in bidistilled water. Counterstaining was performed by dipping slides in 50% Mayer's-Hematoxylin for 30 s. Slides were rinsed for 10 min with running tap water, dehydrated to xylene, mounted using Entellan (Merck) and analyzed by microscopy using Leica DFC 320.

2.4. Three-dimensional reconstruction

Three-dimensional reconstructions of the ductus venosus were generated using E11.5–15.5 wild-type mouse embryos and CS14–23 human embryos. For mouse embryos digital images were made of serial sections stained with SMA. For human embryos digital images of serial sections stained with Hematoxylin–Eosin obtained from the Carnegie Institute (Washington D.C., USA) were used, as described by de Bakker et al. [18]. Digital images were scanned, aligned, analyzed and converted to create a three-dimensional reconstruction by using Amira software version 5.1.

3. Results

3.1. Development of the mouse ductus venosus

The earliest embryonic stage investigated was E10.5 (see Table 1). At this stage the ductus venosus has not developed yet. Two vitelline veins were located paired in the embryo and entered the septum transversum at the junction with the intra-hepatic venous sinusoids. The two umbilical veins were positioned lateral of the vitelline veins. Both vitelline veins and the left umbilical vein were connected to the intra-hepatic venous sinusoids and via the cardinal vein (precursor of the vena cava) to the sinus venosus of the heart. The right umbilical vein continued directly to the cardinal vein and sinus venosus.

At E11.5 the ductus venosus evolved from the left umbilical vein, which lost its connection to the sinus venosus of the heart at this stage. The ductus venosus was located centrally in the liver; the ventral–caudal part was continuous with the umbilical and portal veins, and the dorsal–cranial end was connected to the proximal part of the inferior vena cava, continuous with the right atrium of the heart (see Fig. 1). The right vitelline vein has developed into the portal vein, positioned centrally in the embryo between the caudal lobes of the liver and the stomach. The cranial end of the portal vein was connected to the junction with the ductus venosus and umbilical vein, the caudal part was connected to the portal sinus. The ductus venosus showed a wide lumen over the entire length. The endothelial cells of the ductus venosus were positive for Pecam1 expression. SMA staining was absent from the ductus venosus but positive in the umbilical and portal veins at this stage. LvG staining was absent from the ductus venosus. No expression of neural markers was observed in the ductus venosus: Ncam1

Table 1
Number of wild-type mouse embryos examined per embryonic day.

Embryonic day	Number of mouse embryos used for: Histological and immunohistomechical stainings	Three-dimensional reconstruction
10.5	2	1
11.5	4	1
12.5	4	1
13.5	4	1
14.5	4	1
15.5	5	1

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