ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Vascular and mesenchymal factors during heart development: a chronological study

I. D. MARINAŞ[1)], RALUCA MARINAŞ[2)], IONICA PIRICI[3)], L. MOGOANTĂ[4)]

[1)]*Cardiology Center, Craiova*
[2)]*Emergency County Hospital, Craiova*
[3)]*Department of Neurology*
[4)]*Department of Histology*
University of Medicine and Pharmacy Craiova

Abstract

As heart development is an incomplete described area, and likewise an important source of intra- and post-partum morbidity and mortality, we have aimed at analyzing both vascular and cytoskeletal factors during early heart development in humans. The distribution of CD105, CD31, α-SMA, vimentin and desmin have been studied on a series of normal human heart tissues varying between five and 33 weeks of gestational age, utilizing both enzymatic single immunohistochemistry, as well as double immunofluorescence. We showed here that CD105 is already expressed at five weeks of gestational age in the future endocardium, and that between 9 and 10 weeks it shows clear-cut formed vessels. CD31 was also present diffusely at five weeks in the myocardium, while beginning with seven weeks, endocardium and vessels were clearly positive. Contrary to what it might be expected for striated muscle cells, cardiomyocytes were α-SMA positive between 9 and 20 weeks, a time window during which the marker showed clear-cut sarcomer formation. Desmin was first detected at nine weeks lining the cardiomyocyte plasma membrane, and after 17 weeks it showed the adult-like striated pattern of the protein. As expected, vimentin was already present in the mesenchymal cells from the first investigated time point, retaining a perivascular localization only towards higher ages. This is the first study that describes these vascular, muscular and mesenchymal factors on a large series of sequential human tissues, in an attempt to shed more light on the development of heart.

Keywords: sarcomere formation, α-SMA, desmin, vimentin, vascular factors, heart development.

Introduction

The heart is one of the first organs that begin its shaping during embryo's development in mammals [1, 2].

Intermediate filament cytoskeletal proteins (IF) have important functional and structural roles in any tissue, even from embryonic stages. Desmin forms a scaffold for future contractive filaments in both cardiac and skeletal striated muscle cells, bridging the edges of Z lines together, and maintaining their connection with mitochondria, nuclei, and sarcolemma [3]. Vimentin on the other hand, is found in fibroblasts and other cell types, close to the cytoplasmic surface of membrane-to-membrane contact points between cells, maintaining the stability of mesenchymal cells and their precursors [4].

Besides other cytoskeletal proteins, actin is a fundamental protein supporting the contractile apparatus in muscle cells [5]. In mammals, the muscle actin family is composed of six distinct isoforms. Out of these isoforms, the β-cytoplasmic and γ-cytoplasmic actins are ubiquitous cytoplasmic proteins, while α-skeletal actin (α-SKA), α-cardiac actin (α-CAA), α-smooth muscle actin (α-SMA) and γ-smooth muscle actin (γ-SMA) are found in muscle tissue [6]. In normal myocardium, α-CAA, α-SKA and α-SMA isoforms are co-expressed at levels depending on the species, developmental stage, or pathological alterations [6–8]. Experiments on animal models showed that during heart formation, α-SMA is expressed by cardiomyocytes during their differentiation, and during later moments in development it is replaced by α-SKA and α-CAA [6, 9]. Although the importance of this phenomenon is not known, the study of α-SMA dynamics on human heart for different stages has not yet been done.

Vascular development and maturation is an important step in the development of any organ, and especially in such high energy-dependent organs like the heart. CD31 (PECAM-1), is a single chain type 1 transmembrane protein, which mediates adhesive interactions between adjacent endothelial cells as well as between leukocytes and endothelial cells [10]. Although studies on developing mammalian and bird cardiovascular system had been done using lectins and antibodies raised against factor VIII in order to identify endothelial cells, these detected mostly carbohydrate residues present on mature endothelial cells, but not so much on developing endothelial cells [11]. Data in the literature shows that PECAM-1 is one of the earliest adhesion molecules expressed by developing endothelial cells, but an extensive study in human developing heart was not

reported yet [12, 13]. Endoglin (CD105) is a transmembrane glycoprotein, mainly expressed by the endothelial cells in newly formed vessels in the state of active angiogenesis [14, 15], and absent in most other vessels in mature tissues [16]. Endoglin has been also shown to play an important role in development of the vascular and cardiac system [17]. For example, heterozygosity for deleterious mutations in the endoglin gene are known to led to the occurrence of vascular malformations [18].

Although intermediate filaments have been studied in heart development for different mammals [19, 20], not so many studies have approached this in conjuncture with assessing the vascular development [21].

The aim of this paper was to attempt to describe the localization of both desmin and vimentin in the developing human heart between five and 33 weeks of gestation, in combination with the above two mentioned markers for vasculature and actin.

Materials and Methods

All the human embryos needed for this study were collected following miscarriages in the Department of Gynecology, Emergency County Hospital, Craiova (Table 1). The gestational ages were calculated as the time passed from the last menstruation until the moment of harvesting the tissue.

Table 1 – ***Number and ages of the embryos' hearts for this study***

Embryo's age [weeks]	5	7	9	10	14	15	17	20	33
No. of hearts available	2	3	1	2	4	3	2	2	3

The whole embryos were fixed in 10% neutral buffered formalin for four days, and then were dissected and the hearts isolated and routinely processed for paraffin embedding (Department of Histology, University of Medicine and Pharmacy of Craiova). Serial sections were cut on a rotary microtome (Microm), and collected on poly-L-lysine-coated slides. Macroscopical and histological analysis showed that the embryos selected for our study did not have heart malformations.

For single immunohistochemistry, serial sections were stained alternatively for CD105, CD31, desmin and vimentin antibodies (Table 2).

Table 2 – ***The antibodies utilized in this study***

Antibody	Species	Specificity	Dilution	Antigen retrieval	Source
CD105	Proliferating vessels	Rbb, polyclonal	1:100	Citrate buffer, pH 6	LabVision
CD31	Endothelium	Ms, IgG1k	1:50	Citrate buffer, pH 6	Dako
Desmin	Muscle cells	Ms, IgG1k	1:50	Citrate buffer, pH 6	Dako
α-SMA	Smooth muscle actin	Rbb, polyclonal	1:100	Citrate buffer, pH 6	LabVision
Vimentin	Stromal cells	Ms, IgG1k	1:100	Citrate buffer, pH 6	Dako

Briefly, after citrate buffer antigen-retrieval, sections were cooled to room temperature and incubated for 30 minutes in a 1% hydrogen peroxide solution. The sections were next washed in PBS, followed by a final blocking step of 30 minutes in 3% skim milk. The primary antibody was added, and the slides were incubated overnight at 4^0C. Next day, slides were washed and the signal amplified utilizing the peroxidase–EnVision polymer based species-specific secondary detection system (Dako, Medicalkit, Craiova, Romania), and then detected with 3,3'-diaminobenzidine (DAB) (Dako). Finally, the slides were coverslipped after a light Hematoxylin staining.

For double immunofluorescence for CD31/SMA, after antigen retrieval and skim milk blocking, both antibodies were added on the slides in a mix of their respective dilutions (Table 2). Next day, after thorough washing, a mix of goat anti-mouse Alexa 594 and goat anti-rabbit Alexa 488 secondary antibodies was added on the slides (1:400, Invitrogen, Medicalkit, Craiova) for 30 minutes. Lastly, the sections were counterstained with DAPI (Invitrogen) and coverslipped in fluorescent anti-fading medium (Dako).

All intermediate washing steps were done in 0.1 M PBS, pH 7.2, and all antibodies were diluted in PBS with 1% BSA (Sigma-Aldrich, Medicalkit, Craiova, Romania). All incubation times were kept constant for all the slides included in the present study.

All sections were imaged with a Nikon Eclipse 90i microscope (Nikon, Apidrag, Bucharest, Romania) equipped with a 5-megapixel Nikon CCD camera and with narrowband fluorescent filters centered for Alexa 594, Alexa 488, and DAPI excitation and emission wavelengths. Images were grabbed with 40× and 60× apochromatic objectives, as uncompressed TIF files utilizing the Image ProPlus AMS 7 software (Media Cybernetics, Inc., Buckinghamshire, UK).

Results

CD105 and CD31 expression patterns in the developing heart

Immunohistochemistry for endoglin showed that the reactivity begins to occur in human heart at around five weeks, in the future endocardial layer (Figure 1A). While at this age there were no positive vessels in the myocardium itself, immunoreactivity begins to occur in endothelial cells during the weeks 9–10 (Figure 1, B and C). After this, the signal could be strongly detected in endocardium, myocardial and epicardial vessels.

Already at five weeks of development, anti-CD31 immunostaining detected scattered fully stained endothelial cells and vessels in the myocardium, while the endocardial layer was not yet fully expressing this marker (Figure 1, I and J). By seven weeks, more vessels could be noted, and endothelial layer was expressing CD31 on its complete lining (Figure 1K). At the embryo age of 10 weeks, large vessels are observed in the epicardial layer (Figure 1L). The number

of vessels was relatively constant until around the age of 20 weeks when their density increased rapidly (Figure 1, O and P). Overall, there was an increasing expression of both CD105 and CD31 with age of the embryo, mature vessels seeming to retain an endoglin positive phenotype.

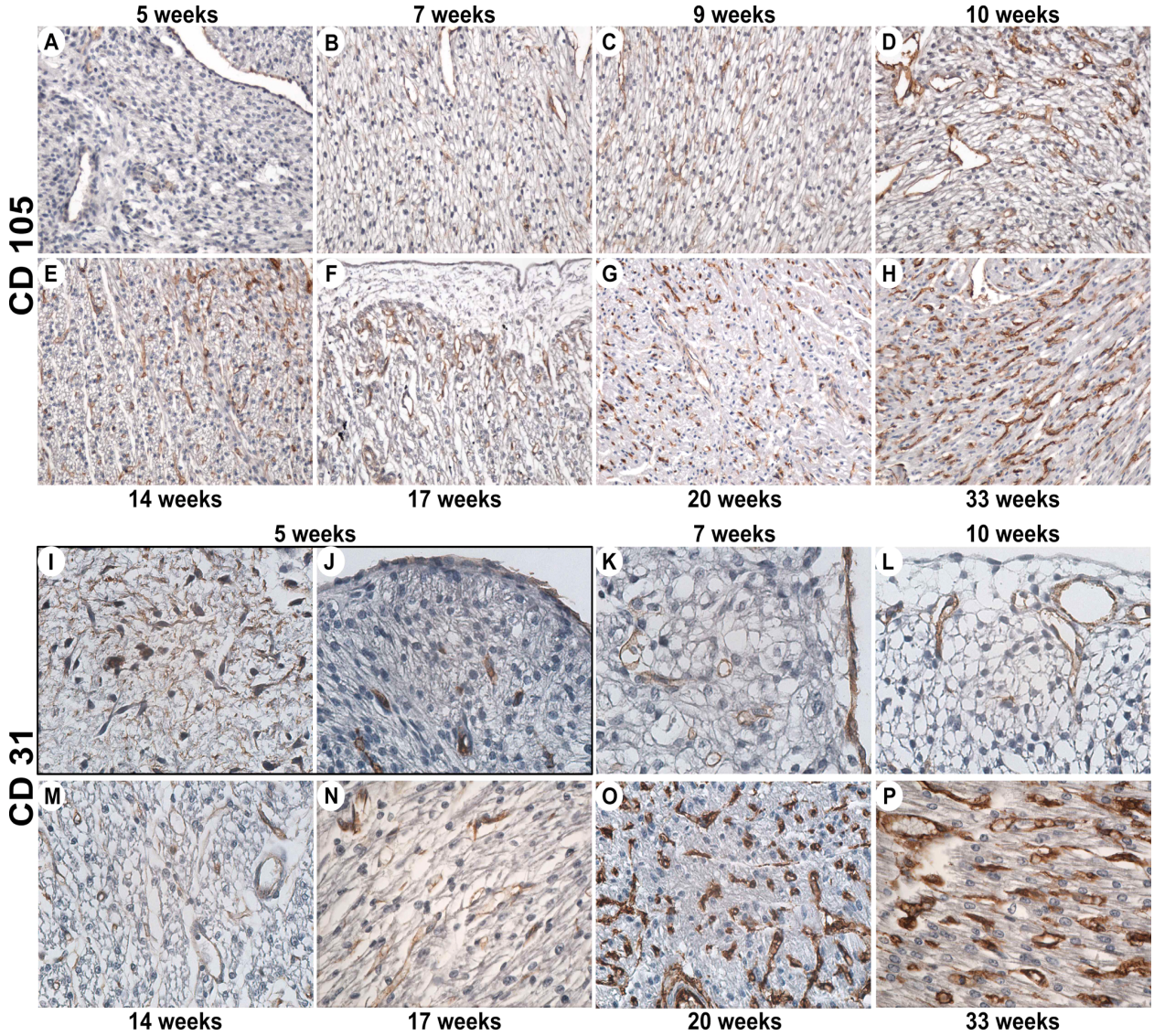

Figure 1 – ***Immunohistochemistry for endoglin and CD31 in human heart at different developmental stages. (A) CD105-positivity occurs around five weeks of age, in the endocardium. (B–D) Around 9–10 weeks in the endothelium, and later on (E–H) in all structures of the future heart. (I) CD31 was already present in five weeks old endothelial cells in the myocardium, while the endocardial cells did not show full positivity (J). All vascular structures show increasing expression of CD31, and the expression increased rapidly after 20 weeks (K–P). A–H, ob. 20×; I–P, ob. 40×.***

Desmin and vimentin expression in the developing heart

Analysis of the slides immunostained for desmin, showed that reactivity begun to appear at the age of nine weeks (Figure 2, A–C), with increasing intensity and area staining from 10 weeks onward (Figure 2, C and D).

Desmin was present only in cardiomyocytes. At stages of initial detection point, desmin seemed to be lining the cardiomyocyte plasma membrane (Figure 2, C–E). The staining pattern seemed to be stronger in the immediately sub-epicardial layer compared to myocardium itself (Figure 2F). Beginning with 17 weeks of gestational age, desmin was also localized in the myofibril striations in the cytoplasm of the cardiomyocyte (Figure 2G). This feature reached the pattern of the mature striated muscle cell beginning with 17 weeks of age onwards (Figure 2H).

Vimentin was already present in the mesenchymal cells from the first investigated time point (i.e. five weeks, Figure 2I). As the blood vessels lumens become more clearly visible, vimentin showed also a perivascular localization (Figure 2J). Vimentin was heavily expressed in aorta smooth muscle cells, while desmin could not be detected in this compartment (Figure 2K). Endocardium lining at the level of future atrio-ventricular valves was also positive for vimentin (Figure 2L). With increasing age of the embryo, vimentin expression diminished in the myocardium itself, remaining present around the vessels and to a higher extent in the sub-epicardial vessels (Figure 2, M–P).

Alpha-SMA sequential expression in the developing heart

After composing the α-SMA/CD31 double images, in the non-vascular compartment, we also observed a gradual variation of α-SMA expression during different developmental stages. Thus, except a slight tissue autofluorescence, no mesenchymal cells were α-SMA-positive below the age of seven weeks (Figure 3). After this age, α-SMA began to be expressed in the future myocardium cells, showing a sub-membranous expression pattern somehow related with the expression of desmin presented above. Examining these patterns, on week 15 we observed for the first time the formation of striate-like sarcomeric structures, suggesting a role for α-SMA in temporary scaffolding the myofibrils in cardiac striated muscle. In the later weeks, SMA expression revealed the typically striated structure of the sarcomeres, but with an abrupt decrease between the 20th and the 33th weeks of development. Finally, at 33 weeks, no α-SMA could be detected in the cardio-myocytes' compartment, like in the post-partum and adult heart tissue (data not shown). On parallel, forming endothelial cells show a dotted express of CD31, besides individual and well-defined vessels. Beginning with 20 weeks, CD31 expression is clearly present only as clear-cut vessel walls.

In the vascular compartment, CD31 was first detected both with a granular appearance in endothelial cells, some of them not having yet clear-cut lumens; but also in different sized vessels, some of them large enough to pose a proper sheet of smooth muscle cells (Figure 4). Beginning with 20 weeks of age, larger vessels clearly presented an α-SMA positive tunica media and an independent and non-granular endothelial layer.

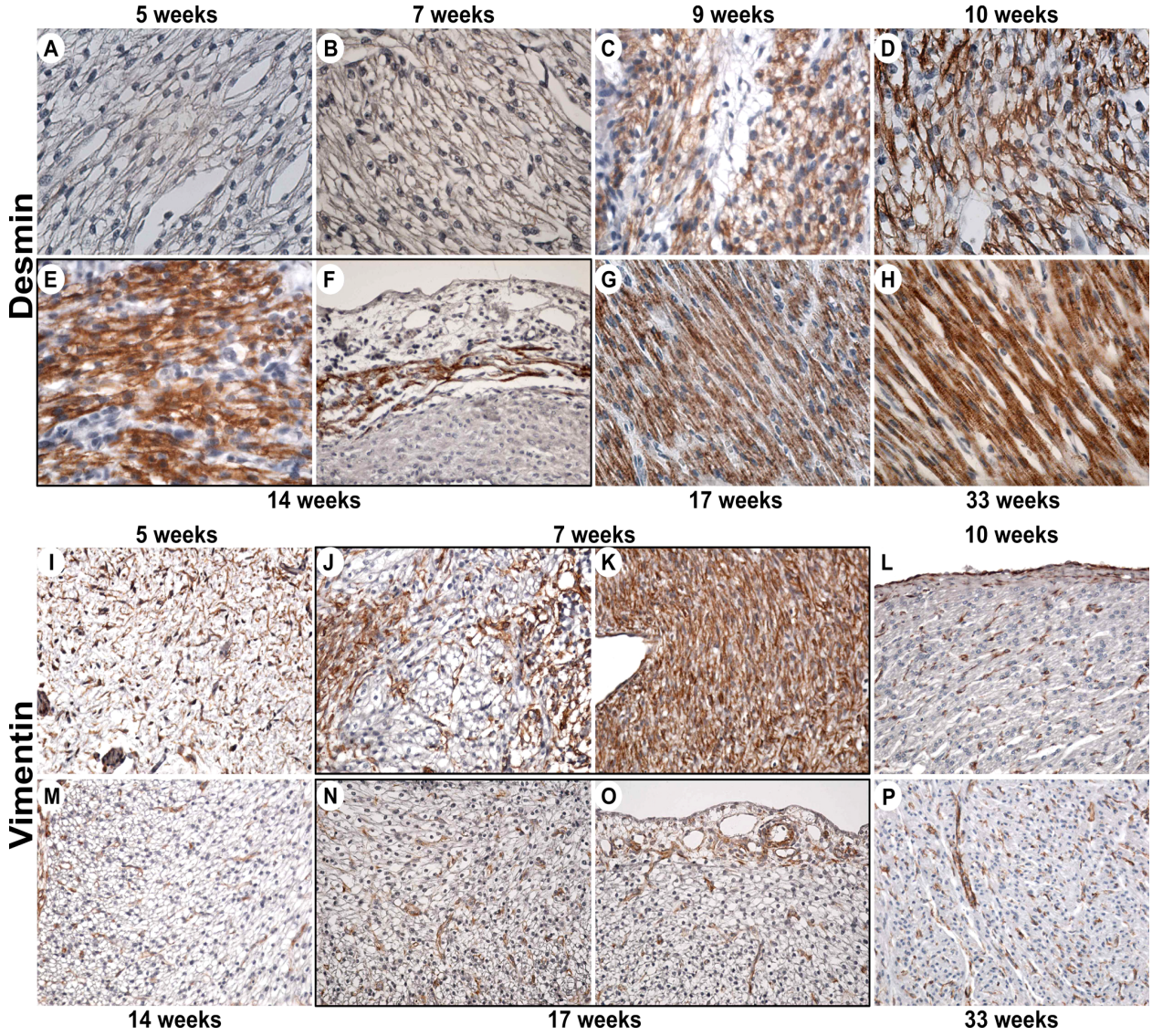

Figure 2 – ***Immunohistochemistry for desmin and vimentin in human heart at different developmental stages. Desmin reactivity begins to be present at nine weeks (A–E), especially in the sub-epicardial muscle layer (F). Myofibril striations were discernable beginning with the 17 weeks gestational age (G and H). Vimentin was present in mesenchymal cells throughout the investigated time points (I–K). In larger vessels it was also localized perivascular (J), or in the large vessels' tunica media (K). At 10 weeks, endocardium became positive (L), and later on the expression in the myocardium itself diminished, being retained only around vessels (M–P). A–H, ob. 40×; I–P, ob. 20×.***

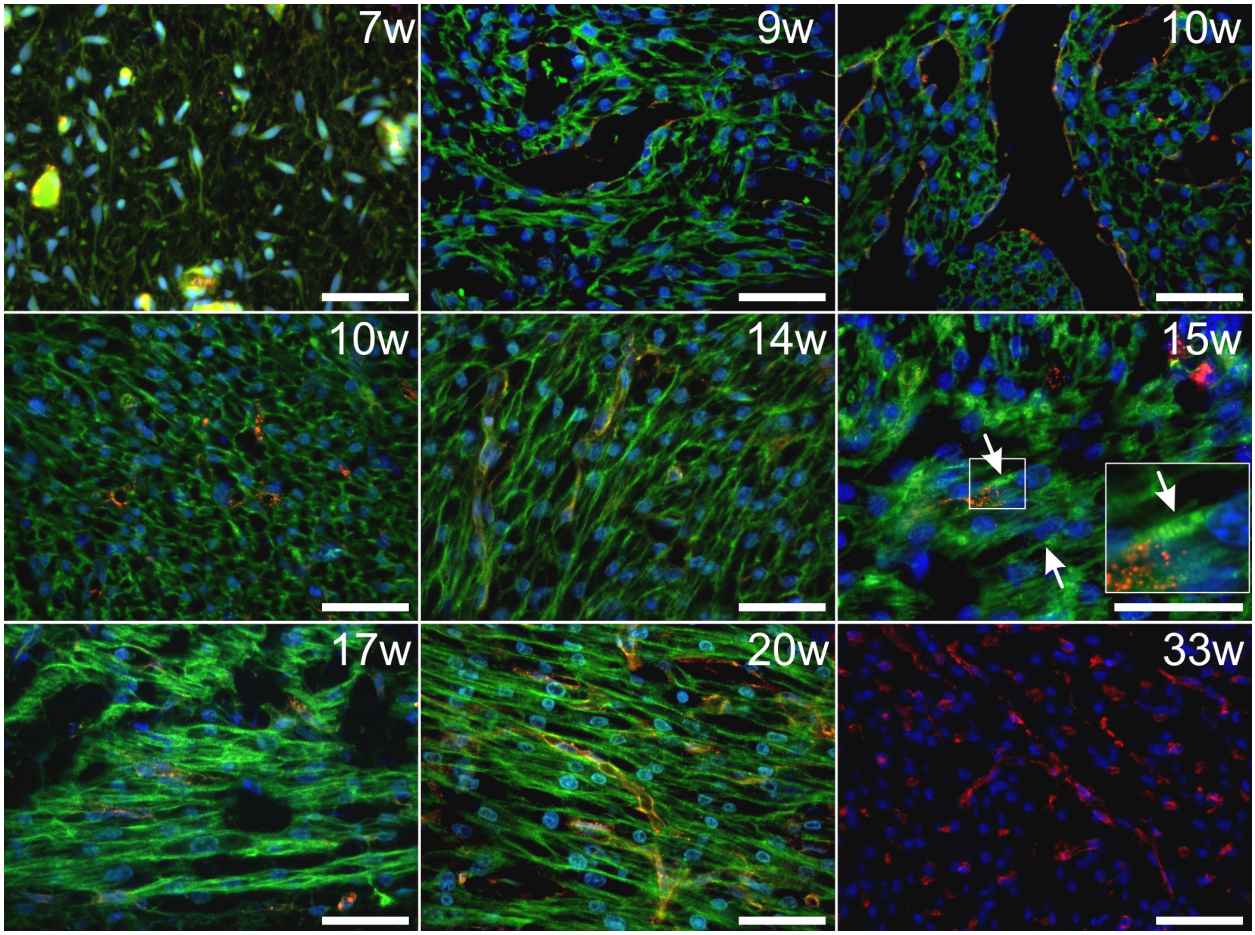

Figure 3 – ***Gradual expression variation of α-SMA (green) and CD31 (red) in the developing heart. α-SMA begins to be present in the cardiomyocytes, around nine weeks, and shows first sarcomeric striations around 15 weeks. By 17 weeks, clear-cut striations are visible. Between 20–33 weeks, the sarcomeres lose completely the α-SMA expression. CD31 expression evolves from a dotted-like appearance to mature-like vessels above 20 weeks of gestation. Scale bars represent 50 µm.***

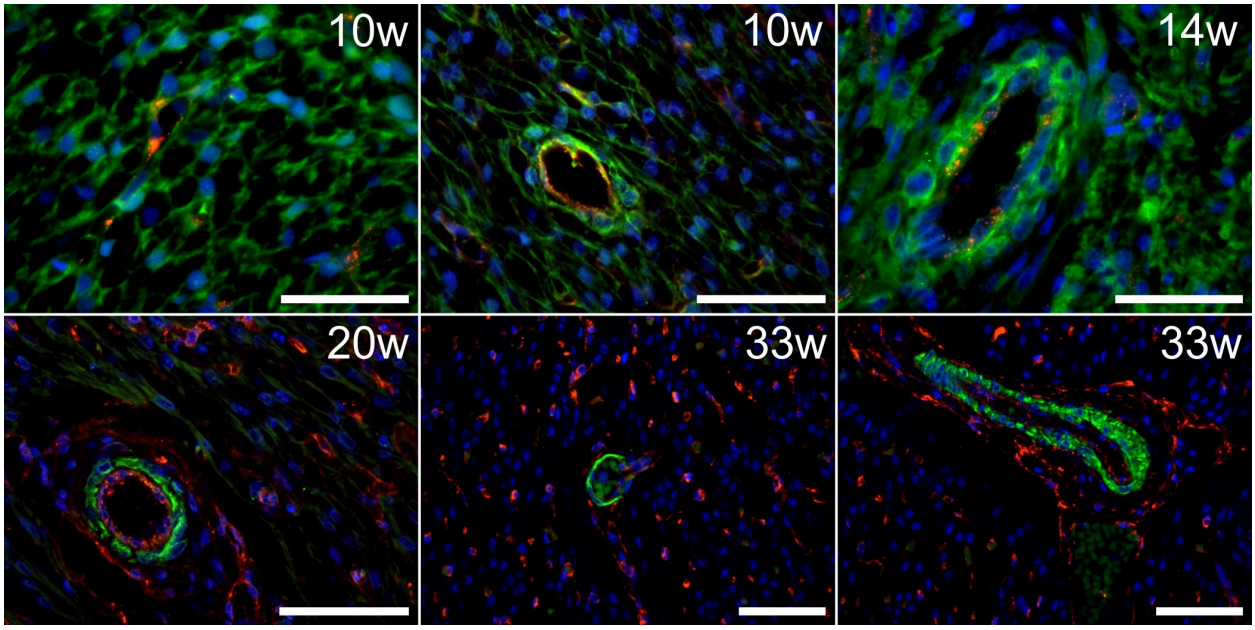

Figure 4 – ***α-SMA (green)/CD31 (red) double immunofluorescence shows gradual increase of CD31 consistency and the retraction of α-SMA distribution only towards the tunica media of larger vessels. Scale bars represent 50 µm.***

Discussion

In this study, endoglin, CD31, desmin, vimentin and α-SMA immunohistochemical expressions were evaluated in the hearts of human embryos with ages ranging from five to 33 weeks. All markers are related to the development of the vasculature and the myocytes

starting from the mesenchymal compartment, and have never been studied together for different time points in the human developing heart.

For the endoglin study, we have showed that tissue reactivity was present around five weeks of gestation in the future endocardial layer. This is in line with other studies describing its presence as early as four weeks in the human embryos, in the endocardial layer, but not in the myocardium itself [20]. In the mouse embryo, endoglin is known to occur after 8.5 days in the primitive heart tube, and later on in endothelial cells throughout the developing vascular system [10]. In fact, this early appearance of endoglin seems an important player during heart and vascular development process, as it has been showed that mice lacking endoglin develop arterial-venous malformations [22], and could also be the basis of hereditary hemorrhagic telangiectasia [14]. More, an increased altered pattern of endoglin expression is associated with ventricular septal defects, underlining once again the importance of equilibrium in its expression levels [20]. Later on, during development, endoglin expression pattern included myocardium itself and epicardium, a sequence of events confirmed by other studies on human [20] and chick embrios [15]. Between 9 and 10 weeks, CD105 immunoreactivity revealed clear-cut vessels in the myocardium itself. This is a crucial event as it is known that heart vascularization begins approximately around this period of development [17], and endoglin seems to exert at this point an important role in angiogenesis [14].

The study of CD31 (PECAM-1) molecule reveals the dynamics of maturing vessels and endothelial cells during the studied time interval. It is a known fact that PECAM-1 is one of the earliest adhesion receptors expressed by vasculogenic cells [18]. It is expressed in the yolk sac rapidly after implantation, and later on in the embryo itself [13]. In line with these data, we have also observed a strong immunostaining in cells scattered on the area of the future ventricular myocardium already at five weeks of life. It was interestingly to note that at this age no staining could be observed in either luminal, vascular-like structures or the endocardium itself. At seven weeks of development, endocardium and clear-cut vessels were already expressing CD31 strongly. Progressing with the age, a proportional increasing number of vessels was recorded, and this increase had a peak at around the age of 20 weeks. Large epicardial vessels seemed to start expressing CD31 only after the molecule was already well detectable in endocardium and myocardium. Even though CD31+ cells occur very early in the heart development time line, without forming clear-cut tubular structures, it seems that these cells are already differentiated and have lost their pluri-potent properties, as it was shown that cells expressing stem cell antigen sca-1 do not co-express CD31 [23]. On the other hand, epicardium is known to play a crucial role in heart vascularisation, as it is a mesothelial tissue and a source of coronary vascular precursor cells by epithelial-to-mesenchymal transition [24–26]. Other studies have showed that these migrating cells can give rise to vascular smooth muscle and endothelial cells as well as perivascular fibroblasts [27]. Our observations show that sub-epicardial vessels do not seem to develop earlier than myocardial vessels, and this could reflect that although derived from the mesothelium, these cells need other factors to mature into adult endothelial cells [28].

We have next derived the study of α-SMA for both vascular maturation and cardiomyocytes per se. We have thus determined that during human heart embryogenesis, α-SMA is transiently expressed between the weeks 9–33. Previous studies performed on birds or non-primates showed that there is a sequential activation of the SMA genes during early cardiogenesis [6, 9, 29]. Also, these studies showed that α-SMA is downregulated in chick developing heart until hatching [29], or soon after birth in rats [9]. The present study completes this image showing that before the week 33, α-SMA is also down-regulated in the human heart [9]. The significance of this transient expression of α-SMA during the development of striated muscle is not completely known. Since its expression upregulation coincides with the beginning of myofibril formation, it may suggest it could have a function in sarcomere organization and contractility [6, 9, 30]. Also, it decreases as the sarcomeres become more organized, in the latter developmental ages [9]. Inhibition of the α-SMA expression using siRNA blocked the heart differentiation process [6]. Like other studies, the present paper also shows a heterogeneity of the α-SMA expression for different cardiac areas, suggesting that the function of this actin isoform might not be common to all the cardiomyocytes. It was in fact showed that future sino-atrial node cells lose lastly their α-SMA expression, pointing out to a specific function towards the differentiation of pacemaker cells. Although a complete analysis in this line is not yet done, α-SMA-null mice have been created and apparently did not show any cardiac problems leading to premature death [31].

Desmin is a type III IF (intermediate filament) protein characteristic of myogenic cells, as a main structural element of the Z lines of the myofibril. We have detected desmin in embryos' heart beginning with the age of nine weeks, and the reactivity increased in intensity and area towards higher ages. In the earliest cells expressing it, it seems that the expression pattern was restricted especially to the sub-plasmalemmal domain. This correlates well with other studies showing on EM a dotted pattern of desmin being located on the inner side of the plasmalemmal membrane [32]. Markwald RR [33] studied myofibril formation thoroughly in rat and hamster embryos and reported that the majority of the first myofibrils are formed associated with dense amorphous material located as plaques along the inner side of the plasma membrane of the cardiac muscle cells. He and his colleagues proposed that these dense plaques serve as the centers for the assembly of myofibrils. In skeletal myofibrillogenesis, similar structures were also studied in more detail in a larger number of reports [34]. Vimentin is also a type III IF protein characteristic for cells of mesenchymal

origin, as endothelial cells and fibroblasts [4]. In the present study, vimentin could be detected early in the mesenchymal tissue fibroblasts showing also a peri-vascular localization. With age, its expression decreased, but it was still present around blood vessels at 33 weeks of life, and this especially around the sub-epicardial vessels. We have also noted that vimentin was expressed in the endothelium lining the endocardium of the future heart valves [32], and in the aorta smooth muscle cells but not desmin, as opposite from the other vessels' smooth muscle cells [35]. In endothelial cells, the cytoskeleton has been suggested to drive and be driven by the functional varieties resulting from different localizations. Thus, cytoskeletal differences between endocardial and vascular endothelial cells might be related for example to differences in shear stress between the laminar flow from a vessel lumen compared to the flow around the cardiac valves [36]. Data in the literature showed that vimentin intermediate filaments in endocardial endothelial cells form a filamentous network which has distinct cytoplasmic and juxtanuclear vimentin bundles [36]. Our study describes too such differences, namely vimentin expression is present in the endocardial cells of the future heart valves, while the rest of the endothelium does not show this high expression pattern. It seems very plausible that as other studies suggest, vimentin is mainly involved in parenchymal tissue support rather than cardiomyocyte formation and proliferation [21].

Conclusions

In conclusion, our study compared for the first time the expressions of CD31 and CD105 in the analysis of heart vasculature development, together with three wide distributed cytoskeletal filaments. We showed that there are not only time-point differences in the expression of these markers, but also structure-related differences. Thus, we show that while CD105 occurs in the endocardium, CD31 is present much earlier in the future myocardium area. Regarding the cytoskeletal elements, α-SMA and desmin seem to be close related to myofibril formation as they both start at the periphery of the myocyte, while vimentin seems to be mainly driving the integrity of the supporting tissue. As the dynamics of heart formation and disease is driven by the balance between the vascular, myocytic and stromal components, understanding their interactions will improve our knowledge in both directions.

References

[1] Icardo JM, *Heart anatomy and developmental biology*, Experientia, 1988, 44(11–12):910–919.

[2] Bockman DE, Redmond ME, Kirby ML, *Altered development of pharyngeal arch vessels after neural crest ablation*, Ann N Y Acad Sci, 1990, 588:296–304.

[3] Mogoantă L, Popescu CF, Georgescu CV, Comănescu V, Pirici D, *Ghid de tehnici de histologie şi imunohistochimie*, ediția a 2-a, Ed. Medicală Universitară, Craiova, 2007, 233–247.

[4] Steinert PM, Roop DR, *Molecular and cellular biology of intermediate filaments*, Annu Rev Biochem, 1988, 57:593–625.

[5] Pollard TD, Cooper JA, *Actin and actin-binding proteins. A critical evaluation of mechanisms and functions*, Annu Rev Biochem, 1986, 55:987–1035.

[6] Clément S, Stouffs M, Bettiol E, Kampf S, Krause KH, Chaponnier C, Jaconi M, *Expression and function of alpha-smooth muscle actin during embryonic-stem-cell-derived cardiomyocyte differentiation*, J Cell Sci, 2007, 120(Pt 2): 229–238.

[7] Carrier L, Boheler KR, Chassagne C, de la Bastie D, Wisnewsky C, Lakatta EG, Schwartz K, *Expression of the sarcomeric actin isogenes in the rat heart with development and senescence*, Circ Res, 1992, 70(5):999–1005.

[8] Winegrad S, Wisnewsky C, Schwartz K, *Effect of thyroid hormone on the accumulation of mRNA for skeletal and cardiac alpha-actin in hearts from normal and hypophys-ectomized rats*, Proc Natl Acad Sci U S A, 1990, 87(7):2456–2460.

[9] Sawtell NM, Lessard JL, *Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the alpha-vascular but not the gamma-enteric isoform in differentiating striated myocytes*, J Cell Biol, 1989, 109(6 Pt 1):2929–2937.

[10] Jonker L, Arthur HM, *Endoglin expression in early development is associated with vasculogenesis and angiogenesis*, Mech Dev, 2002, 110(1–2):193–196.

[11] Coffin JD, Harrison J, Schwartz S, Heimark R, *Angioblast differentiation and morphogenesis of the vascular endothe-lium in the mouse embryo*, Dev Biol, 1991, 148(1):51–62.

[12] Buck CA, Baldwin HS, DeLisser H, Mickanin C, Shen HM, Kennedy G, Chen A, Edelman JM, Albelda SM, *Cell adhesion receptors and early mammalian heart development: an overview*, C R Acad Sci III, 1993, 316(9):838–859.

[13] Baldwin HS, Shen HM, Yan HC, DeLisser HM, Chung A, Mickanin C, Trask T, Kirschbaum NE, Newman PJ, Albelda SM *et al.*, *Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, funct-ionally distinct isoforms expressed during mammalian cardiovascular development*, Development, 1994, 120(9): 2539–2553.

[14] Bourdeau A, Faughnan ME, Letarte M, *Endoglin-deficient mice, a unique model to study hereditary hemorrhagic telangiectasia*, Trends Cardiovasc Med, 2000, 10(7):279–285.

[15] Vincent EB, Runyan RB, Weeks DL, *Production of the transforming growth factor-beta binding protein endoglin is regulated during chick heart development*, Dev Dyn, 1998, 213(3):237–247.

[16] Bellone G, Solerio D, Chiusa L, Brondino G, Carbone A, Prati A, Scirelli T, Camandona M, Palestro G, Dei Poli M, *Transforming growth factor-beta binding receptor endoglin (CD105) expression in esophageal cancer and in adjacent nontumorous esophagus as prognostic predictor of recurrence*, Ann Surg Oncol, 2007, 14(11):3232–3242.

[17] Valeria B, Maddalena G, Enrica V, Onofrio T, Gaetano B, *Endoglin (CD105) expression in the human heart throughout gestation: an immunohistochemical study*, Reprod Sci, 2008, 15(10):1018–1026.

[18] Pardanaud L, Dieterlen-Lièvre F, *Emergence of endothelial and hemopoietic cells in the avian embryo*, Anat Embryol (Berl), 1993, 187(2):107–114.

[19] Coujard R, Poirier J, Racadot J, *Précis d'histologie humaine*, Masson, Paris, 1980, 667–691.

[20] Qu R, Silver MM, Letarte M, *Distribution of endoglin in early human development reveals high levels on endocardial cushion tissue mesenchyme during valve formation*, Cell Tissue Res, 1998, 292(3):333–343.

[21] Kim HD, Kim DJ, Lee IJ, Rah BJ, Sawa Y, Schaper J, *Human fetal heart development after mid-term: morphometry and ultrastructural study*, J Mol Cell Cardiol, 1992, 24(9):949–965.

[22] Sorensen LK, Brooke BS, Li DY, Urness LD, *Loss of distinct arterial and venous boundaries in mice lacking endoglin, a vascular-specific TGFbeta coreceptor*, Dev Biol, 2003, 261(1):235–250.

[23] Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, Colucci WS, Liao R, *CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation*, Circ Res, 2005, 97(1):52–61.

[24] Männer J, Pérez-Pomares JM, Macías D, Muñoz-Chápuli R, *The origin, formation and developmental significance of the epicardium: a review*, Cells Tissues Organs, 2001, 169(2): 89–103.
[25] Pérez-Pomares JM, Phelps A, Sedmerova M, Carmona R, González-Iriarte M, Muñoz-Chápuli R, Wessels A, *Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs)*, Dev Biol, 2002, 247(2):307–326.
[26] Olivey HE, Compton LA, Barnett JV, *Coronary vessel development: the epicardium delivers*, Trends Cardiovasc Med, 2004, 14(6):247–251.
[27] Vrancken Peeters MP, Gittenberger-de Groot AC, Mentink MM, Poelmann RE, *Smooth muscle cells and fibroblasts of the coronary arteries derive from epithelial-mesenchymal transformation of the epicardium*, Anat Embryol (Berl), 1999, 199:367–378.
[28] Wagner N, Wagner KD, Theres H, Englert C, Schedl A, Scholz H, *Coronary vessel development requires activation of the TrkB neurotrophin receptor by the Wilms' tumor transcription factor Wt1*, Genes Dev, 2005, 19(21):2631–2642.
[29] Ruzicka DL, Schwartz RJ, *Sequential activation of alpha-actin genes during avian cardiogenesis: vascular smooth muscle alpha-actin gene transcripts mark the onset of cardiomyocyte differentiation*, J Cell Biol, 1988, 107(6 Pt 2): 2575–2586.
[30] Marinaş ID, Marinaş R, Mogoantă L, *Light microscopy study on myofibril formation and mitotic potential in human myocardocytes*, Studia Universitatis "Vasile Goldiş" Seria Stiinţele Vieţii (Life Sciences Series), 2011, 21(3):519–524.
[31] Schildmeyer LA, Braun R, Taffet G, Debiasi M, Burns AE, Bradley A, Schwartz RJ, *Impaired vascular contractility and blood pressure homeostasis in the smooth muscle alpha-actin null mouse*, FASEB J, 2000, 14(14):2213–2220.
[32] Virágh S, Challice CE, *The development of the early atrio-ventricular conduction system in the embryonic heart*, Can J Physiol Pharmacol, 1983, 61(8):775–792.
[33] Markwald RR, *Distribution and relationship of precursor Z material to organizing myofibrillar bundles in embryonic rat and hamster ventricular myocytes*, J Mol Cell Cardiol, 1973, 5(4):341–350.
[34] Ferreira PJ, L'Abbate C, Abrahamsohn PA, Gouveia CA, Moriscot AS, *Temporal and topographic ultrastructural alterations of rat heart myofibrils caused by thyroid hormone*, Microsc Res Tech, 2003, 62(5):451–459.
[35] Virágh S, Szabó E, Challice CE, *Formation of the primitive myo- and endocardial tubes in the chicken embryo*, J Mol Cell Cardiol, 1989, 21(2):123–137.
[36] Lamers WH, Wessels A, Verbeek FJ, Moorman AF, Virágh S, Wenink AC, Gittenberger-de Groot AC, Anderson RH, *New findings concerning ventricular septation in the human heart. Implications for maldevelopment*, Circulation, 1992, 86(4): 1194–1205.

Corresponding author
Ionel Marinaş, MD, PhD student, Department of Histology, University of Medicine and Pharmacy of Craiova, 2–4 Petru Rareş Street, 200349 Craiova, Romania; Phone/Fax +40251–523 654, e-mail: ionel_marinas@yahoo.com

Received: November 10th, 2011

Accepted: February 15th, 2012