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LIGHT MICROSCOPY STUDY ON MYOFIBRIL FORMATION AND MITOTIC POTENTIAL IN HUMAN MYOCARDOCYTES

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ABSTRACT
Myocardocyte myofibril formation has been extensively studied in animal models, and in vitro, for both its importance during embryogenesis and for its putative role in a possible treatment for severe heart diseases accompanied by myocardocyte loss. However not much is known about the actual stages during the embrio life, when the myofibrils can first be observed, or how is their mitotic potential related to this functional maturation.
In the present work we performed a 1 week time step sweep analysis on the myofibril formation and mitotic activity of human myocardial tissue between <3 weeks to 20 weeks of life. Thus we found out that myofibrils began to be observed at around 15 weeks of life and the myocardocytes retain their mitotic potentials until later after the myofibril formation has started.
Key words: myofibril formation, myocardocytes, PCNA, embryonic life

INTRODUCTION

The heart is the first functional organ that occurs in the developing embryo (Lyons, 1996). The tubular heart begins to form from splanchnic mesoderm, and the future myocardium appears to the posterior side of the tube - forming the inflow region; and to the anterior side - forming the outflow tract (Sedmera, 2011). The **intracardiac mesenchyme** forms also myocardium of the outlet and atrioventricular septa (van den Hoff et al., 2001). Mesothelial cells lining the pericardial cavity transform into cardiomyocytes (Viragh and Challice, 1973). At the venous pole of the tube, myocardium formation continues in the **extracardiac mesenchyme**, which lines the cava and pulmonary veins (van den Hoff et al., 2001). This process leads to the formation of cava and pulmonary myocardium. Thus, after formation of the linear heart tube, myocardium formation takes place in the intracardiac and extracardiac mesenchyme. Two cellular mechanisms are responsible for myocardium formation: (i) myocardialization as the growth of existing mesenchyme cardiomyocytes, and (ii) recruitment and differentiation of non-cardiac muscle cells into cardiac muscle cells (Kruithof et al., 2003).

Many studies have been done on different animal models in order to observe the time frames for the formation of mature functional myocardocytes. For example, in chick embryos (which fully develop in 21 days), contractions of cardiomyocytes can be observed after only 36 hours from the fecundation (Tokuyasu and Maher, 1987), and blood flow through the heart begins at 2 days after conception (Sissman, 1970). Sparsely distributed myofibrils begin to be visible in the myocardocytes at around 30 hours post-fecundation, then the first Ca2+ - Mg2+ ATP-ases become active, and around the 1,5 days stage the first action potentials occur and precede the sustained contractions (Jorgensen and Bashir, 1984; Fujii et al., 1981). Cultured myocardocytes have been also studied for the dynamics of fibrillar and pre-fibrillar assemblies of actin and myosin filaments.

Despite this, not much is known regarding the actual stage of sarcomere formation in human heart in vivo. At the approximate age of 3-4 weeks, the human heart starts to contract, but to what extent the sarcomeres are structurally developed it is not really known (Mercola et al., 2011).

In the present paper we have investigated the histological aspects of the human embryonic hearts with ages ranging from less than 3 weeks to more than 20 weeks post-fecundation. We have showed that sarcomeres become readily identifiable on classical histology slides at around 15 weeks, with a complete mature aspect at around 16 weeks of life. Together with this functional maturation, these cells also seemed to loose the mitotic capacity that characterize the mesenchymal tissues.

MATERIALS AND METHODS

All the embryos needed for this study were collected following miscarriages in the gynecology department of the Emergency County Hospital 1, Craiova. The gestational ages were calculated as the time passed from the last menstruation till the moment of harvesting the tissue.

The whole embryos were fixed in 10% neutral buffered formalin for 4 days, and then were dissected and the hearts isolated and routinely processed for paraffin embedding (department of Histology, University of Medicine and Pharmacy Craiova). Serial sections were cut on a rotary microtome (Microm), and collected on poly-lysine - coated slides (Sigma). Macroscopical and histological analysis showed that the embryos selected for our study did not have heart malformations (**Table 1**).

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

Embryo's age (weeks)	<3	5	8	9	10	12	14	15	16	17	20
No. of hearts available	2	2	3	1	2	3	4	3	3	2	2

Sections were stained with standard Harris hematoxylin and a modified Heidenhain's iron hematoxylin as follows. After deparaffination and hydratation through a graded series of alcohols, the sections were incubated in a mordant solution of 5% iron aluminum for one hour at room temperature. After rinsing in distillate water, the sections were incubated for another hour in Harris hematoxylin. The slides were next washed in running tap water and differentiated in the 5% iron aluminum solution with alternate rinses in tap water. The degree of differentiation was verified under the microscope. In the end the sections were washed in running tap water for 10 minutes, dehydrated, cleared and mounted with DPX (Fluka). The result was a dark blue shade for the nuclei, and black for striated muscle striations.

For immunohistochemistry, serial sections following the ones colored with Heidenhain's iron haematoxylin were utilized. 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 1% skim milk. Anti-PCNA primary antibody was added (Dako, dilution as 1:100), and the slides were incubated overnight at 4°C. 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). 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. Finally, the slides were coversliped after a light Hematoxylin staining.

All sections were imaged with a Nikon Eclipse 90i microscope (Nikon, Apidrag, Bucharest, Romania) equipped with a 5-megapixel CCD camera and images were grabbed with an 40× apochromatic objective, as uncompressed TIF files utilizing the Image ProPlus software (Media Cybernetics). All nuclear countings were done by manual tagging in Image ProPlus and reporting the results in Excel sheets. As the cell volumes were increasing during the heart maturation process, we quantified PCNA-positive nuclei as the percentage from the total counted nuclei rather than absolute values. All values are expressed as average ± standard deviation (± SD).

RESULTS

On the youngest developing heart in the study, histology investigation identified mesenchymal cells covering thorough the cardiac vesicle areas. These cells were mostly stelate, with ramified arms joining together with the neighbouring cells to form a cellular net.

Beginning with 3-4 weeks embryo heart, the cells began to look more distinct, with enlarged and clear cytoplasm, the nucleus being most frequently round, hyperchromatic and situated central. The same aspect was constant throughout the week 14, with the ventricle areas having denser cells compared to the future atrial areas. Beginning with this age, the cells began to have a more elongated shape as mature cardiomyocytes.

On Heidenhain's haematoxylin, in the week 15, the first sarcomeres began to be visible, probably reflecting a previous state of myofibril existence and aggregation, and now were dense enough to be visible on light microscopy (**Figure 1**). In all three embryo hearts available for this gestational age, the first light microscopy visible sarcomeres appeared at the edge of the cell, just beneath the plasma membrane. This arrangement was constant all around the membrane, with no clear interruption, and constant again for ventricular and atrial areas. It was not possible to narrow the phenomenon in finer time steps, as our embryos had at least a 1 week age intervals.

On PCNA immunohistochemistry, as expected, we noted a decreasing nuclear staining with incr easing embryonic age (**Figure 2**). In very young hearts (<3 weeks), practically all mesenchymal cells' nuclei strongly expressed PCNA. At this age we counted around 25,4% (±8,1%) positive nuclei. The nuclear positivity decreased with a relative constant slope at 21,5% (±7,6%) for 12 weeks and to 18,3% (±11,7%) for 15 weeks.

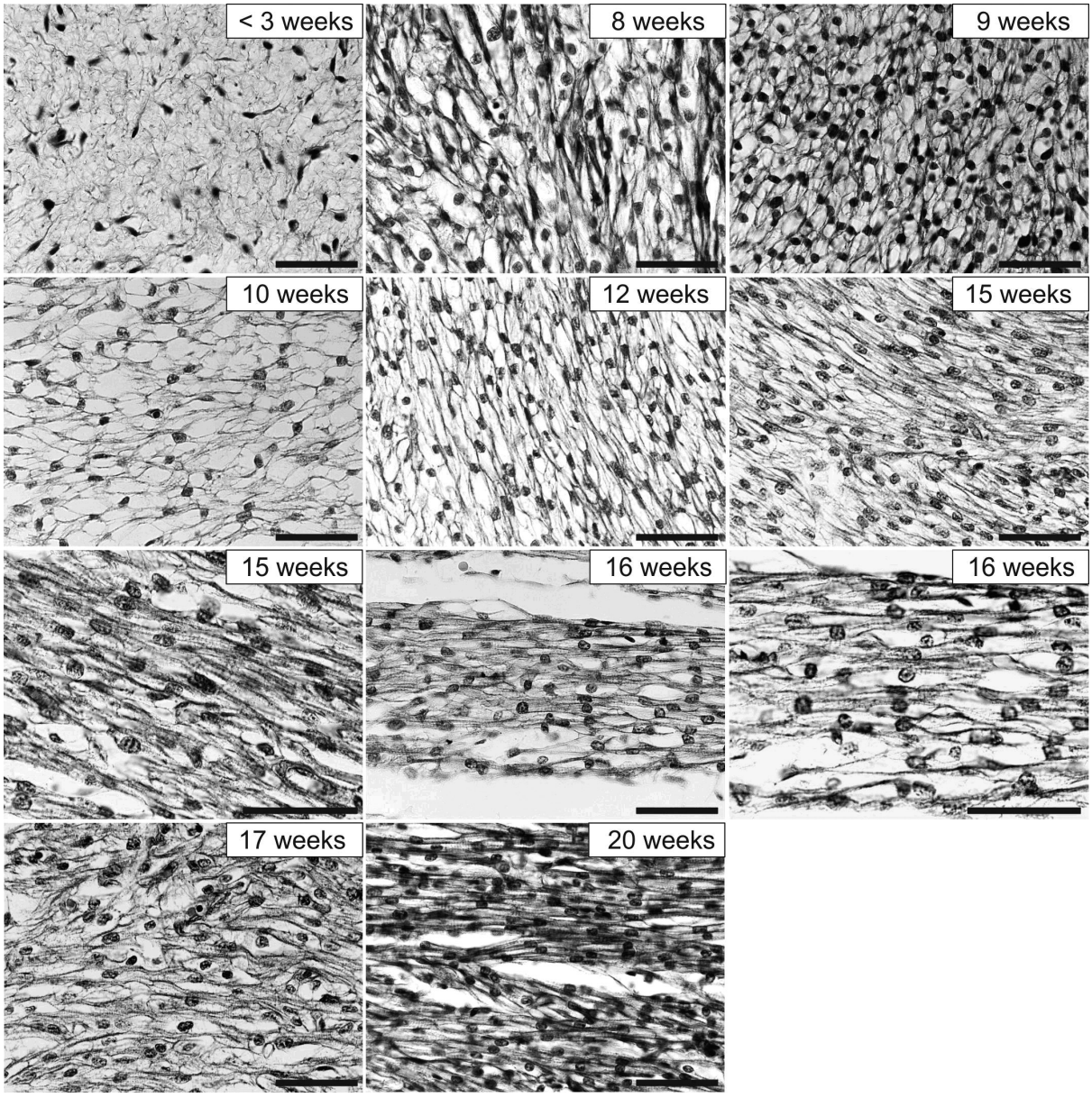

Figure 1. Heidenhain's hematoxylin stained embryonic hearts of different ages. Myofibril occurrence is observed first at 15 weeks embrios, under the plasma membrane. Scale bars represent μm.

After 16 weeks of age however, the nuclear positivity dropped-out drastically at 3,3% (±1.6%) at 17 weeks, and respectively at 1,8% (±1,8%) for 20 weeks of age. After this age there was an almost constant PCNA staining percentage in myocardocytes.

Paralleling the formation of the first light microscopy visible sarcomeres and the described drop-out in PCNA-nuclear positivity, we observed that the two phenomenon did not coincide and in all studies cases of respective ages, formation of the first visible sarcomeres was followed by a two week interval after which the nuclear maturation drastically increased (as revealed by lower PCNA-staining).

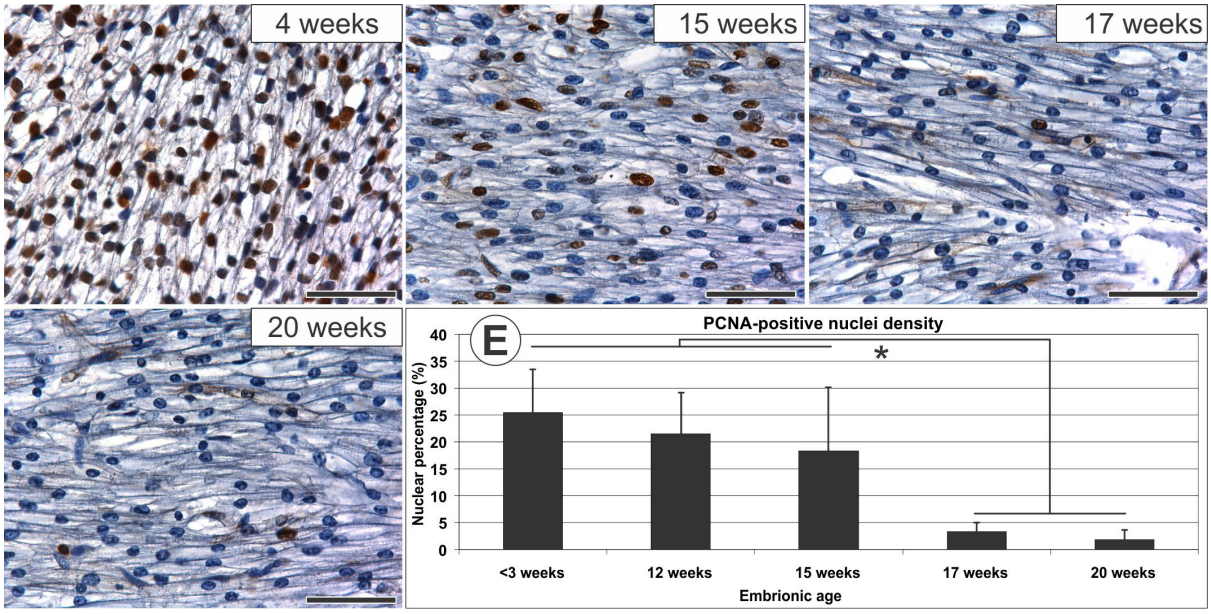

Figure 2. Anti-PCNA staining reveals decreasing nuclear mitotic capacity. Between 15th and 17th weeks tere is a suddent drop in PCNA staining. Error bars represent standard deviation, * significant difference based on student t test ($p<0.05$).

DISCUSSIONS

It is overall accepted that myocardocytes became rapidly functional and the human embryonic heart begins beating approximately 21 days after conception, typically with a rate near the mother's frequency. This implies that the sarcomeres in the human striated cardiac muscle become fully functional in a very early stage in order to be able to support this function.

Although myofibril formation in the heart has been extensively studied in animal models, not many studies approached the human embrio from this point of view. Transmission electron microscope studies on embryonic lobsters showed that thick and thin myofilaments appear first at the cell periphery near the sarcolemma (Burrage and Sherman, 1979). They align in parallel in a sequential fashion to form consecutive sarcomeric units. Well-defined A and I bands appear before any trace of a Z line is present. The initial sarcomeres are anchored to the sarcolemma by the insertion of thin myofilaments into a region of electron dense material associated intimately with the sarcolemma. Myofibrils grow outward in several planes away from these electron-dense regions of membrane that seem to serve as focal points for myofibril formation. A different theory coming from cultured myocardocytes proposed that so-called premyofibrils would exist as precursor structures during myofibril assembly, being composed of minisarcomeres which contain non-muscle isoforms of contractile proteins (Rhee et al., 1994). Later on this theory was infirmed by in vivo studies, thus supporting once more the studies on human embryonic tissue (Tullio et al., 1997).

Markwald (Markwald, 1973) 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. However, these structures were observed in the chick embryo of stage 10 and in the 10-day rat embryo (Markwald, 1973), which are both in the stage of the first myocardial contractions (van der et al., 1987). Thus, these studies did not provide a clear answer to the question of whether such plaques are generated before or nearly in coincidence with the formation of myofibrils. In skeletal myofibrillogenesis, similar structures were also studied in more detail in a larger number of reports (Ferreira et al., 2003). The elucidation of the molecular nature of this ultrastructural entity is important in understanding the process of cardiac myofibrillogenesis.

CONCLUSIONS

In this conjuncture, our study showed that myofibril formation in human cardiomyocytes begins to be visible on light microscopy at embryonic age 15, beneath the cellular membrane. This parallels in vitro or animal model studies describing the dense plaques that seem to initiate myofibril formation and which seem to be anchored on the inner side of the plasma membrane. Moreover, we have found a sudden nuclear maturation between weeks 15th and 17th, with a rapid loss of nuclear cell division protein PCNA. Interestingly, this maturation phenomenon occurred after the fist myofibrils begun to be observed on light microscopy. Given the fact that electron microscopy might in fact detect these structures

earlier in their evolution, this time gap is even larger than 2 weeks. Thus it seems that myocardocytes retain their mitotic capacity until after they begin myofibril synthesis. For the future, a better understanding of how these processes are linked might offer new hopes for adult myocardocyte regeneration in heart diseases.

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