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Development of Morphology and Function of Neonatal Mouse Ventricular Myocytes Cultured on a Hyaluronan-Based Polymer Scaffold

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ABSTRACT

In recent years cardiac tissue engineering has emerged as a promising field aimed at developing suitable techniques to repair the infarcted myocardium with a combination of cells, biomaterials, and regulative factors. In particular it could stand for an alternative strategy to simple in situ cellular implantation. In the present study our purpose was to analyze the interaction between a hyaluronan-based mesh (HYALONECT®) and neonatal murine ventricular myocytes (NMVMs). Specifically, we investigated morphological and functional characteristics of cardiomyocytes cultured on HYALONECT® in view of its employment in heart repair. Both living and fixed cells analysis was performed on in toto scaffolds with confocal microscopy. NMVMs adhesion on HYALONECT® was studied by tracking sarcomeric α-actinin immunofluorescence staining. The structural features of NMVMs adherent onto HYALONECT® were investigated at 24, 48, 72 h, and 7 days of culture by immunofluorescence for sarcomeric α-actinin and connexin-43. We observed a progressive morphological organization of the cells inside the biopolymer, with both clear sarcomeric arrangement along the scaffold fibers and gap junctions development between adjacent cells. Finally, in vivo intracellular calcium measurements performed using calcium fluorimetric confocal imaging revealed the presence of spontaneous calcium transients and contractile activity of NMVMs adherent onto HYALONECT® up to 48 h from seeding, indicating a progressive differentiation of the cells toward the adult phenotype. In conclusion, our results demonstrate that HYALONECT® allowed NMVMs to adhere to the fibers and to develop functional properties, displaying suitable features as a scaffold to perform heart tissue engineering. J. Cell. Biochem. 113: 800–807, 2012.

KEY WORDS: NEONATAL MOUSE VENTRICULAR MYOCYTES; CARDIAC TISSUE ENGINEERING; CALCIUM TRANSIENT; HYALURONAN-BASED POLYMER

Loss of contractile myocardium is the outcome of several cardiac diseases and a major mortality cause. While in general, improved therapy has prolonged the life and it has been widely debated as an approach aimed at increasing the normally poor self-reparation capabilities of the heart, for many patients cardiac transplantation is the only available life-saving option. Heart transplant has, however, limited epidemiological impact given the low number of available compatible donors. A complementary promising approach is the replacement of damaged cardiac muscle with new contractile cardiomyocytes and vessels obtained through stem cell-based regeneration. Recent research has focused on inducing hetero- or autologous stem cells of different origin and nature into becoming cardiac cells before or after injection. However, despite some positive results in early, small, non-randomized trials, and later larger randomized trials, the in vivo tracking of the intracoronary and intramyocardially injected cells in animals [Hill et al., 2003] and humans [Kraitchman et al., 2003] revealed that, within hours of their delivery, 90% of the cells died due to the hostile environment around the infarcted tissue [Hofmann et al., 2005; Freyman et al., 2006]. Moreover, the transdifferentiation potential of mesenchymal stem cells toward the cardiac phenotype is not supported by our current knowledge [Gallo et al., 2007; Rose et al., 2008; Deuse et al., 2009]. The approach based on stem cells in vitro oriented into cardiomyocyte lineage and selected still suffers from the limited ability of the implanted cells to adopt a mature ventricular cardiac physiology and could correlate with the occurrence of arrhythmias, as introduces ectopic pacemaking foci.

A different promising treatment option is the engineering of new heart tissue that can be implanted into damaged areas. This approach of regenerative medicine currently combines traditional

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engineering techniques, such us the employment of biomaterials, bioreactors and the controlled release of substances from the material with molecular and cellular biology techniques [Wang and Guan, 2010]. In this field, physical and mechanical properties of the substrate are peculiar, as they must provide a suitable environment for the neo-tissue in order to favor cell attachment, growth, proliferation, diffusion of nutrients and waste products, and a correct 3D organization.

Among the various polymers that are commonly used in regenerative medicine, hyaluronan-based materials have important biocompatible characteristics, because their primary component and degradation product hyaluronic acid (HA) naturally occurs in the extracellular matrix of many tissues, including the heart.

In particular the hyaluronan benzyl ester HYAFF® (Anika Therapeutics, Bedford, MA) is characterized by the total esterification with benzyl alcohol of the native hyaluronan backbone which confers it high hydrofobicity. This biopolymer has already been successfully employed for repairing many tissues such as skin, cartilage, adipose tissue, gland tissue reconstruction, and peripheral nerve regeneration [Vindigni et al., 2009].

In the context of the cardiovascular regenerative medicine, a recent report [Pasquinelli et al., 2009] investigated whether human early endothelial progenitor cells (eEPCs) could be efficiently cultured in this hyaluronan-based material. The authors showed that eEPCs adhered to the scaffold, maintained a good level of pre-endothelial differentiation, and produced angiogenic factors, speculating that the eEPCs containing mesh could be transplanted in vivo to improve neovascularization.

Moreover, Lionetti et al. [2010] lately showed that hyaluronan mixed esters rescued infarcted rat hearts by improving vascularization, cardiomyocytes survival, and tissue function without the need of stem cell transplantation.

Following these encouraging results, the in vitro evaluation of attachment, survival, and differentiation of myocardial cells in hyaluronan scaffolds is basic to advance future studies of implantation on infarcted heart models.

Therefore, the present study was undertaken to explore the interaction between HYAFF® knitted mesh (HYALONECT®) and neonatal murine ventricular cardiomyocytes (NMVMs). Specifically, our goal was to follow the behavior of the cells in terms of morphological and functional features, by studying multiple parametric assessments.

MATERIALS AND METHODS

BIOMATERIAL

The biomaterial used in this study was the hyaluronan-based scaffold HYAFF®, developed by Anika Therapeutics (Bedford, MA). HYAFF® is derived from the 100% esterification with benzyl alcohol of the free carboxyl groups along the sodium hyaluronate backbone. The experiments have been performed on a knitted HYAFF® construct (HYALONECT®). The chemistry of the biomaterial here employed is detailed elsewhere [Campoccia et al., 1998].

ANIMAL CARE AND SACRIFICE

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and in accordance with the Italian ethical guidelines (DL 111, 27 January 1992). The local ethical committee approved the project.

NEONATAL MURINE VENTRICULAR CARDIOMYOCYTES (NMVMs) ISOLATION AND CULTURE

Newborn CD1 mice (0.5–1 day old) were killed by decapitation, hearts were quickly excised and transferred into ice-cold phosphate buffer saline without Ca^{2+} and Mg^{2+} (PBS w/o Ca^{2+}, Mg^{2+}, Sigma, St. Louis, MO). After removing the atria, ventricles were transferred into ice-cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS w/o Ca^{2+}, Mg^{2+}, Gibco, Invitrogen, Carlsbad, CA) and then quickly minced in a solution of HBSS w/o Ca^{2+}, Mg^{2+} and Trypsin-EDTA 0.05% (Invitrogen). The fragments were then submitted to mechanical and enzymatical digestion in Trypsin-EDTA 0.5% (Invitrogen). Myocardial cells in the supernatants were resuspended in DMEM/M199 medium supplemented with 20% fetal bovine serum (FBS, Biowhittaker, Verviers, Belgium) and pre-plated for 1 h at 37°C, in order to reduce the rate of fibroblasts and endothelial cells into the final cardiomyocytes suspension. Cells were counted by Trypan Blue exclusion to calculate the dissociation yield, centrifuged (100*g*, 20°C, 10 min) and then resuspended in DMEM/M199 complete medium (5% FBS, 10% Horse Serum, HS, Invitrogen). Cells were seeded at the density of 2×10^6 cells/cm^2 and 4×10^6 cells/cm^2 onto 5×5 mm or 10×5 mm HYAFF®11 patches, placed into a 96-well plate. The cells were given 2 h to attach to the scaffold and then complete medium was added to all the samples.

After 24 h the medium was replaced with DMEM/M199 supplemented with 0.5% FBS and 1% HS and from then on it was changed every day.

Bright field images of freshly isolated ventricular cells have been taken using a digital camera (E-P1, Olympus, PA) mounted to a cell culture microscope (Zeiss, Germany).

CELL ADHESION ONTO HYALONECT®

After 24 h of culture, samples in toto were fixed for 30 min in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3, permeabilized for 20 min with 0.3% Triton and bovine serum albumin (BSA, 1:100, Sigma) in PBS and stained overnight at 4°C with primary mouse cardiac sarcomeric α-actinin antibody (monoclonal, 1:800, Sigma), in order to recognize NMVMs from other eventually residual non-cardiomyocytes. The meshes were subsequently incubated 1 h at room temperature with the secondary antibody Alexa Fluor 568 (anti-mouse IgG, Invitrogen) and mounted with DABCO solution on standard slides using SecureSeal™ Imaging Spacers (Grace Bio-Labs, OR). Samples were then analyzed with confocal microscopy (inverted microscope Olympus IX70 linked to a Fluoview 200 confocal head with an Ar/Kr laser, excitation wavelength: 568 nm, 20× magnification). For each experiment we randomly selected three $512 \times 512 \times 64$ volumes. Cell counting was performed using ImageJ® software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2010) and data were represented with Kaleidagraph (Synergy Software, PA) as the percent of cells adhering to the mesh with respect to the initial seeding density.

MORPHOLOGICAL AND FUNCTIONAL CHARACTERIZATION OF SCAFFOLD-ADHERED NMVMs

For the morphological analysis, after 24, 48, 72 h, and 7 days of culture the scaffolds in toto were fixed in 4% paraformaldehyde in 0.1 M PB, permeabilized with 0.3% Triton and BSA (1:100) in PBS, and stained overnight at 4°C with the primary antibodies mouse sarcomeric α-actinin (monoclonal, 1:800, Sigma) and rabbit connexin-43 (Cx-43, polyclonal, 1:400, Sigma). The meshes were subsequently incubated 1 h at room temperature with the secondary antibodies Alexa Fluor 568 (anti-mouse IgG, Invitrogen) and Alexa Fluor 488 (anti-rabbit IgG, Invitrogen) and mounted with DABCO solution on standard slides using Secure-Seal™ Imaging Spacers (Grace Bio-Labs). HYALONECT® patches were observed under confocal microscope (excitation wavelength: 488 nm for Cx-43 and 568 nm for α-actinin, 10×/60×/100× magnification). Image processing and analysis were performed with ImageJ. Functional characterization of adherent NMVMs was assessed by fluorimetric measurements of intracellular calcium oscillations after 24, 48, 72 h, and 7 days, using the high affinity calcium indicator Fluo-3 AM (Invitrogen): The meshes were incubated with the cell-permeant dye for 25 min at 37°C, at a final concentration of 4 μM. The samples were then carefully placed onto 35 mm glass-bottom wishes (Willco Wells B.V., The Netherlands) and covered with a CoverWell™ Perfusion Chamber (Grace Bio-Labs): The thin chamber created between the glass-bottom and the device was filled with DMEM/M199 supplemented with 0.5% FBS and 1% HS. Rapid in vivo confocal microscopy experiments (excitation wavelength: 488 nm, 60× magnification) were performed with XYT scan mode to obtain a trace of the intracellular calcium oscillations time course. Briefly, after choosing a suitable XY focal plane, several acquisitions were obtained with as frequency of scan either 0.4 or 0.2 s. Images were then analyzed with ImageJ, defining a ROI and calculating the mean fluorescence for each acquisition; Kaleidagraph was used to obtain a plot of calcium peaks in function of time.

SUPPLEMENTARIES

Supplemental movie S1 represents the 3D reconstruction of a Z-axis confocal acquisition of NMVMs spreaded onto HYAFF® fibers, made using OsiriX software (Open Source™, http://www.osirix-viewer.com/). In particular, the sample was obtained by rotating the 3D projection of the sarcomeric α-actinin staining of the cells.

Supplemental movie S2 was acquired using a digital camera (E-P1, Olympus) mounted to a cell culture microscope (Zeiss), and processed with ImageJ.

RESULTS

NMVMs MORPHOLOGY SHORTLY AFTER ISOLATION

Freshly isolated NMVMs displayed an elongated shape (Fig. 1) and a quiescent phenotype, resembling small-scale adult ventricular myocytes. It is important to notice that after the pre-plating step all the neonatal ventricular myocytes assumed a round morphology and a subsequent flattened shape with autorhythmic behaviour (data not shown), therefore starting a process of dedifferentiation.

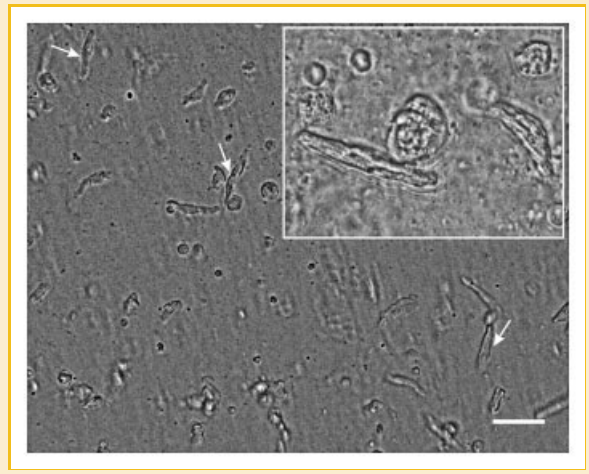

Fig. 1. Freshly isolated NMVMs morphology. Bright field images representing a suspension of freshly isolated neonatal murine heart cells containing spindle-shaped ventricular cardiomyocytes (arrows and inset). Magnification 20×, scale bar: 50 μm.

NMVMs ADHESION ONTO HYALONECT® MESHES

The first step in our study was to evaluate the percentage of cardiomyocytes adherent to the scaffold fibers after 24 h of culture. Following sarcomeric α-actinin immunofluorescence staining on in toto fixed scaffolds and subsequent analysis with confocal microscopy, we observed a good adhesion of NMVMs on the HYALONECT® fibers at the two seeding densities used (Fig. 2, panels A and B). By counting the stained cells we estimated the percentage of adhesion, respectively $4.87 \pm 0.56\%$ for 2×10^6 cells/cm^2 and $4.58 \pm 1.30\%$ for 4×10^6 cells/cm^2 (Fig. 2, panel B); the low retention rate of NMVMs was independent from the seeding density and should be attributed to the mesh ultrastructure which leaves large empty spaces between its knittings (Fig. 2, panel A, asterisks on the bright field image).

MORPHOLOGICAL ANALYSIS OF NMVMs CULTIVATED ONTO HYALONECT®

The structural features of NMVMs that adhered onto HYALONECT® scaffold were investigated at 24, 48, 72 h, and 7 days of culture by immunofluorescence staining for sarcomeric α-actinin and Cx-43. As shown in Figure 3, we observed a progressive morphological organization of the cells inside the biopolymer: After 24 and 48 h of seeding, the majority of the cells remained round and less organized covering only a little portion of the mesh (Fig. 3, panels A and B), while after 72 h a major spreading onto the fibers could be observed (Fig. 3, panel C). At day 7 almost the totality of the adherent NMVMs were spindle-shaped, following the direction of the fibers and covered a larger part of the scaffold (Fig. 3, panel D).

Based on these findings, the sarcomeric organization and the appearance of cell contacts inside the mesh were then investigated following α-actinin and Cx-43 staining after 72 h and 7 days, respectively, from seeding. Figure 4 shows a detail of NMVMs on the HYALONECT® fibers: The adherent cells have a spreaded shape that follows the longitudinal course of the fibers and present a well-organized sarcomeric structure, highlighted by α-actinin

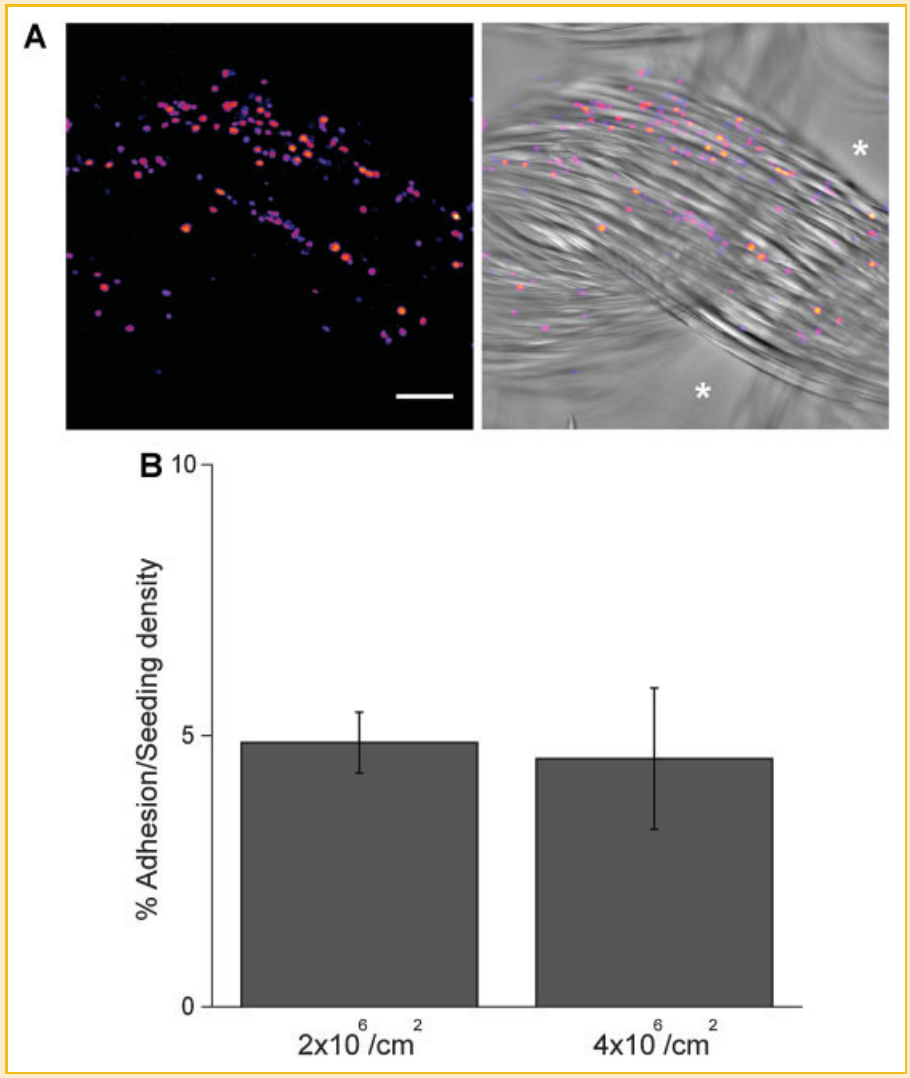

Fig. 2. NMVMs adhesion onto HYALONECT® scaffolds after 24 h of culture. Adhesion was investigated by immunofluorescence staining directed against sarcomeric α-actinin and confocal microscopy analysis of the samples, as described in Materials and Methods section. Panel A: Representative confocal immunofluorescence image of NMVMs adherent to the biopolymer after 24 h of culture (left picture: Fluorescence signal, Fire color look up table; right picture: Merge of the fluorescence signal and the correspondent bright field Nomarski image). Panel B: Resuming histogram of percent adhesion rate respect to initial seeding densities 2×10^6 cells/cm^2 and 4×10^6 cells/cm^2. Values are expressed as mean ± SEM. Magnification 20×, scale bar 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

organization (Fig. 4 red staining) and cytosolic, perinuclear (Fig. 4, asterisks), and intercellular (Fig. 4, arrowheads in the subsets) Cx-43 staining.

Moreover, cardiac myocytes could also create 3D structures inside the mesh, as they were able to wrap around the surface of hyaluronan fibers (Fig. 5 and Supplemental video S1).

FUNCTIONAL AND CONTRACTILE FEATURES OF NMVMs ADHERENT TO HYALONECT® MESHES

Well-organized structure and arrangement of NMVMs spreaded onto HYALONECT® fibers might support some functional activity of these cells. In this perspective, we performed in vivo fluorimetric analysis of intracellular calcium oscillations, which revealed a spontaneous contractile activity of NMVMs adherent onto the fibers of the scaffold (Fig. 6). Although cells after 24 h of culture had a low degree of organization, they already presented quite regular spontaneous intracellular calcium oscillations (Fig. 6, panel A); the autorhythmic activity was visualized also in NMVMs cultured on hyaluronan meshes for 48 h (Fig. 6, panel B), while it decreased after 72 h (Fig. 6, panel C) and was almost completely lost after 7 days (Fig. 6, panel D). The latter important observation is not a sign of culture degradation, as confirmed by cell morphology (Fig. 3, panels C and D, Fig. 4), but instead shows the progression of differentiation of the cells toward the adult phenotype, that requires the absence of automatic contractility in ventricular cardiac myocytes.

While we have not directly studied gap junction functionality, the propagation of electrical signal can be inferred by the development of collective contractions from the first days of culture: of note, several clusters of contractile myocytes transferred their motion to the fibers of the mesh (Supplemental video S2), indicating

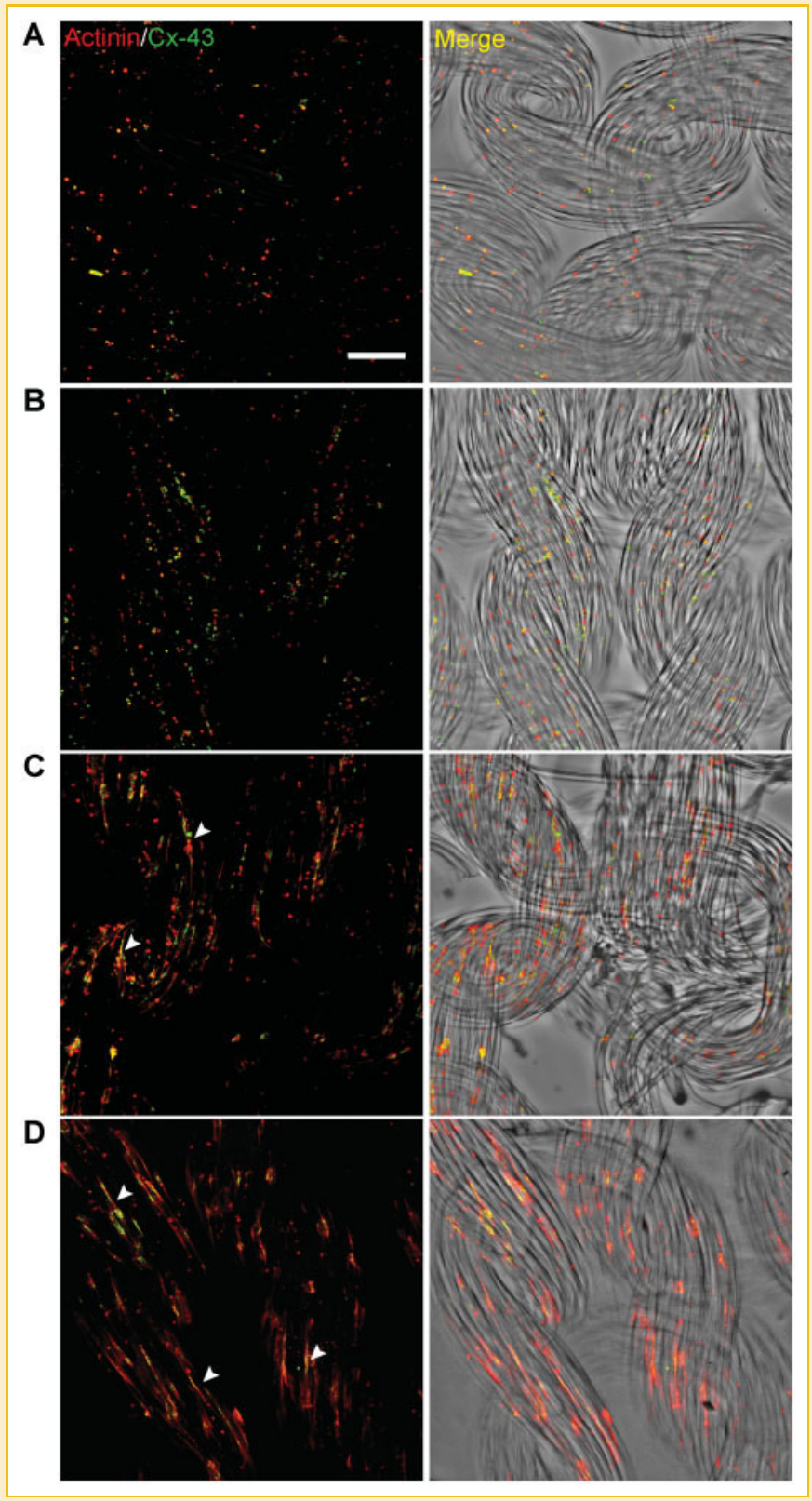

Fig. 3. Morphological organization of NMVMs adherent onto HYALONECT® meshes. Confocal microscope images of fixed cells from typical cultures at 24 (panel A), 48 (panel B), 72 h (panel C), and 7 days (panel D); superposition of sarcomeric α-actinin (red), Cx-43 (green) staining and bright field microscopy (Nomarski). Arrowheads in panels C and D focus on the spreading of cardiac cells onto the fibers of the mesh. Magnification 10×, scale bar 200 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

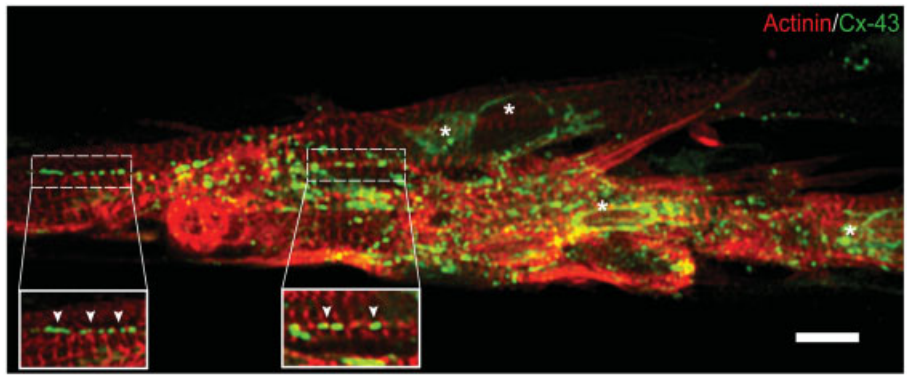

Fig. 4. Sarcomeric structure and Cx-43 organization of NMVMs cultivated onto HYALONECT® scaffolds. Confocal microscope images of fixed cells from a typical culture at 72 h, superposition of sarcomeric α-actinin (red), and Cx-43 (green) stainings. Asterisks refer to perinuclear and cytosolic localization of Cx-43, while arrowheads in the subsets underline the presence of patches of Cx-43 between adjacent cells. Magnification 100×, scale bar 10 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

that in static culture conditions the hyaluronan scaffold is able to support intrinsic tensile strength without adding other adhesion substrates.

DISCUSSION

In this study we showed that the environment created by HYAFF® knitted fabric supports survival and maturation of NMVMs in vitro, as evidenced by the progressive myofibrillar organization of the cells onto the fibers and by the development of spontaneous activity during the culture time followed by quiescence.

The final goal of cardiac tissue engineering in repairing cardiac lesions should be the achievement of full differentiation of myocytes, from both injected or resident cells, and their good working and engraftment in the healthy tissue, without creating potential arrhythmic supporting foci. In particular it is capital to

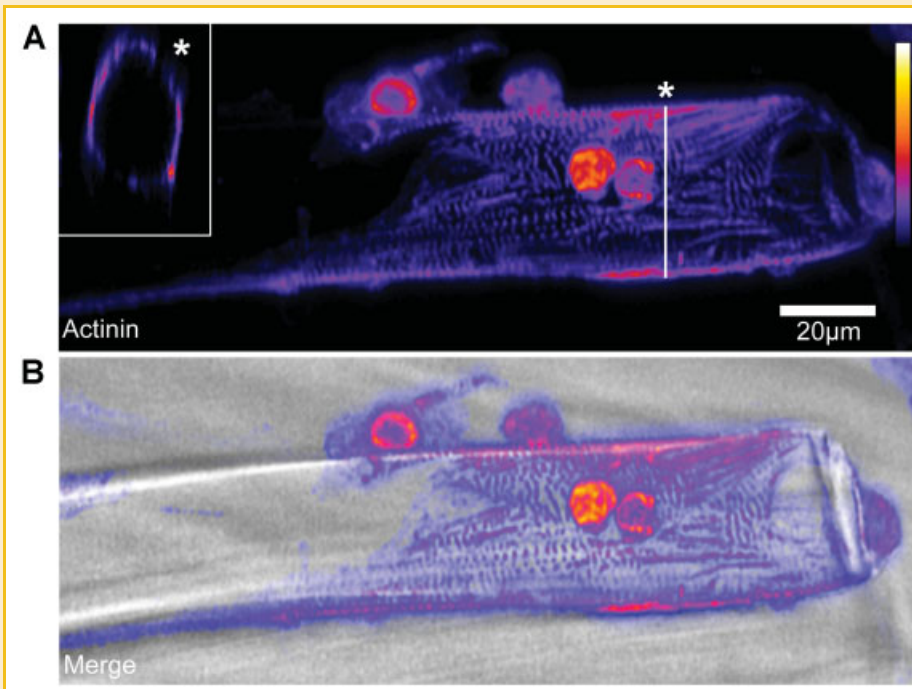

Fig. 5. 3D organization of NMVMs adhering onto HYALONECT® fibers. Confocal microscope images of fixed cells from a typical culture at 7 days; panel A shows α-actinin staining of NMVMs adherent onto a fiber of HYALONECT® (staining presented with Fire color lookup table); in the inset (indicated by the asterix) a transverse section clarifies that the cells completely cover the diameter of the fiber. Panel B is the superposition of the α-actinin staining with the bright field microscopy, to elucidate the structure of the fiber. Magnification 60×, scale bar 20 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

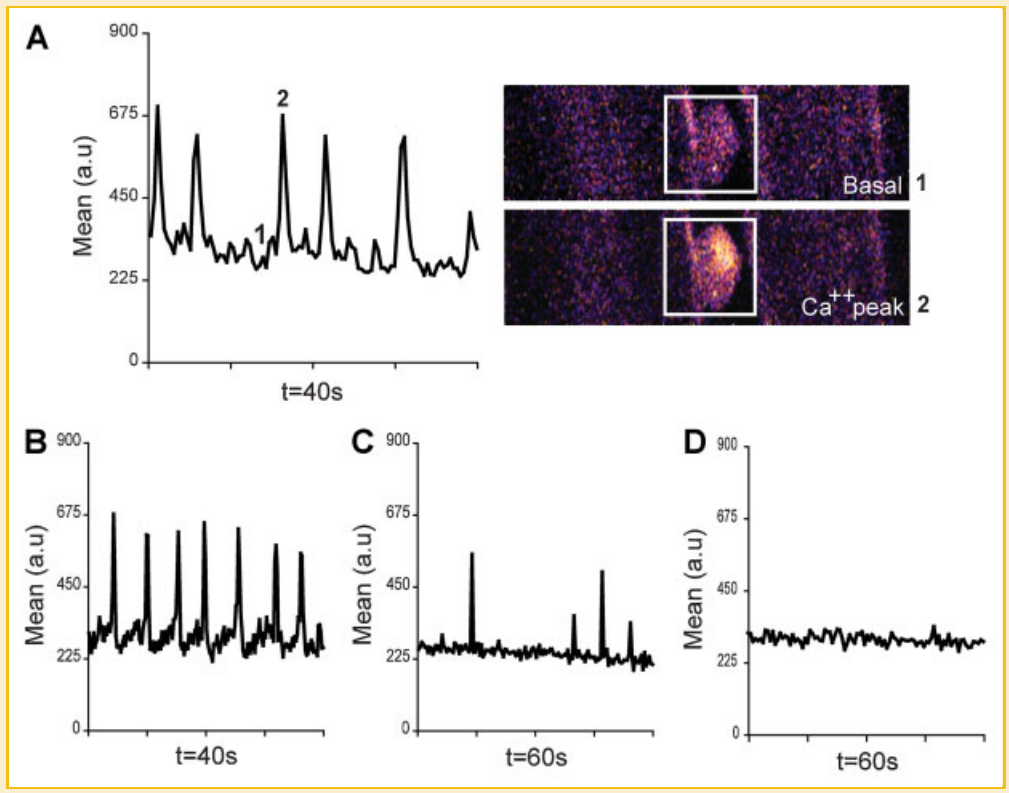

Fig. 6. Spontaneous intracellular calcium transients of NMVMs adherent onto HYALONECT® meshes. Panel A: The upper plot represents the time course of the intracellular calcium (Ca^{++}) variations at 24 h of culture. Spontaneous Ca^{++} transients are clearly featured by a Ca^{++} peak (2) from the basal intracellular Ca^{++} (1). The matching bottom pictures show the fluorescence images from a single cell adherent to the scaffold (Fire color lookup table), corresponding to the basal intracellular Ca^{++} (1) and to the peak of a Ca^{++} transient (2). Panels B, C, and D represent the mean Ca^{++} fluorescence in function of time respectively at 48 (panel B), 72 h (panel C), and 7 days (panel D) of culture. Magnification 60×. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

show that it can induce to terminal differentiation into a non-spontaneously beating but excitable and contractile phenotype.

We have used NMVMs as a model of this critical passage, as these cells in 2D culture conditions rapidly dedifferentiate because of the detachment from their native matrix, loosing the optimal biochemical and mechanical environment for their complete maturation. It is indeed well-known from the early literature on cultured cardiac cells that cardiomyocytes in monolayer networks and sheets revert back to an earlier embryonic state [Sperelakis, 1978]. In particular for neonatal cardiomyocytes, clear morphological differences were evident by comparing images from freshly isolated cells [Li et al., 1996] and culture flask plated cells [Claycomb, 1981].

As we referred to the elongated and quiescent phenotype of NMVMs immediately after isolation (Fig. 1), we therefore wish to lay emphasis on the capability of the biopolymer in reversing this process of dedifferentiation.

Firstly, we obtained a good colonization of the hyaluronan knitted mesh (Fig. 2) without adding other substrates that simulate the features of basal membrane, as previously reported [Boublik et al., 2005], thereby excluding the insertion of factors that could be potentially immunogenic.

Although the scaffold ultrastructure determined a low retention rate after 24 h of culture (Fig. 2), the study of cellularized scaffolds in toto revealed that neonatal myocytes progressively organized themselves and spreaded inside the biopolymer, as after 72 h and 1 week of culture they covered larger parts of the samples (Fig. 3).

Moreover, with high-resolution analysis of the samples in toto we were able to clearly elucidate morphological and structural organization of NMVMs inside HYALONECT®. In particular we show that, without any chemical or genetical manipulation, the adhesion to a structured matrix allows neonatal myocytes to recover the elongated phenotype and to develop a well-organized sarcomeric structure following the longitudinal course of the fibers (Figs. 3 and 4). In the postnatal and adult mouse heart Cx-43 is expressed in the working myocardium of atria and ventricles and in the distal ventricular conduction system [Eckardt et al., 2006], usually overlapping the gap junctions at the intercalated disks. However, in our model the high magnification used to visualize intracellular structures revealed that Cx-43 seems to distribute in dispersed patches both along the plasma membrane of single cells and between connected myocytes, besides a perinuclear and cytosolic localization (Fig. 4). This particular organization might depend on two factors: (1) During postnatal growth working cardiomyocytes undergo profound rearrangement in junctional electrical connectivity, with a progressive accumulation of the gap junctions in intercalated disks, while at birth they are uniformly dispersed across myocytes membranes [Angst et al., 1997]; (2) connexins expression and distribution are linked to an intracellular

trafficking that involves the synthesis of the polypeptides mainly in membrane-bound ribosomes, the co-translational integration into the endoplasmic reticulum, where their transmembrane topology is achieved, and the rapid calmodulin-dependent oligomerization into homo- or heteromeric connexons that are trafficked by the Golgi apparatus to the plasma membrane [Martin and Evans, 2004].

Functional experiments, performed on NMVMs adhering onto the fibers both on the surface and inside the knitted mesh, elucidate the suitability of the environment created by HYALONECT® for rapid maturation of the myocytes, as after 72 h (Fig. 6, panel C) of culture we recorded the decrease of spontaneous calcium oscillations, while after 7 days this contractile activity was almost absent (Fig. 6, panel D). Of note, the time slot (72 h–7 days) defined by the decrease in spontaneous calcium transients corresponds to the morphological transition from a round to a spindle shape (Fig. 3, panels C and D), suggesting a synchronicity in the morpho-functional cellular arrangement. These data are in accordance with a recent study showing that the neonatal mouse heart is capable of self-renewing after surgical resection through cardiomyocytes proliferation and that such regenerative potential is lost within the first week of postnatal life [Porrello et al., 2011].

As a further basic point of our study, spontaneous beating NMVMs adherent onto the scaffold (24 and 48 h) were able to transfer their contractile force onto the fibers of the mesh (Supplemental video S2), indicating that the biopolymer is able to support intrinsic tensile strength even in the absence of other adhesion substrates.

Taken together, our results provide for the first time high resolution data of cardiomyocytes organized inside the HYALONECT® in in vitro samples, highlighting sarcomeric structure, Cx-43 localization, calcium oscillations, and functional behavior at the single cell level.

Interestingly, HYAFF® has been shown a promising candidate as a vehicle for eEPCs for regenerative medicine applications and studies has been proposed to evaluate the neoangiogenic properties of HYAFF®-eEPCs constructs in a myocardial infarction model after the formation of the fibrous scar [Pasquinelli et al., 2009].

In this perspective, our results proposing the ''cardiocompatibility'' and the differentiative potential of the hyaluronan scaffold is of prime interest in cardiac tissue engineering. It could therefore be established as a ''golden standard'' to evaluate the potential of candidate replacement cell models to differentiate and mature in vitro.

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