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2014 Nanotechnology 25 065102

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Nanotechnology
Nanotechnology 25 (2014) 065102 (13pp)
doi:10.1088/0957-4484/25/6/065102

Multistructural biomimetic substrates for controlled cellular differentiation

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Received 14 August 2013, in final form 13 November 2013
Published 16 January 2014

Abstract
Multidimensional scaffolds are considered to be ideal candidates for regenerative medicine and tissue engineering based on their potential to provide an excellent microenvironment and direct the fate of the cultured cells. More recently, the use of stem cells in medicine has opened a new technological opportunity for controlled tissue formation. However, the mechanism through which the substrate directs the differentiation of stem cells is still rather unclear. Data concerning its specific surface chemistry, topology, and its signaling ability need to be further understood and analyzed. In our study, atomic force microscopy was used to study the stiffness, roughness, and topology of the collagen (Coll) and metallized collagen (MC) substrates, proposed as an excellent substrate for regenerative medicine. The importance of signaling molecules was studied by constructing a new hybrid signaling substrate that contains both collagen and laminin extracellular matrix (ECM) proteins. The cellular response—such as attachment capability, proliferation and cardiac and neuronal phenotype expression on the metallized and non-metallized hybrid substrates (collagen + laminin)—was studied using MTT viability assay and immunohistochemistry studies. Our findings indicate that such hybrid materials could play an important role in the regeneration of complex tissues.

Keywords: nanomaterials, stem cell differentiation, gold-functionalized collagen–laminin substrate, extracellular matrix

(Some figures may appear in colour only in the online journal)

1. Introduction

Recently, significant advances have been made in the area of tissue engineering and organ reconstruction by using, as therapeutic tools, a combination of 3D biomaterial scaffolds with stem cells. There is considerable interest in synthesizing substrates that are able to control cellular adhesion and regulate the cells' fate by surface-mediated signaling or by the controlled release of active molecules [1–5]. The interaction between the cell membrane and the synthesized substrate controls cellular fate [6]. There is also evidence that the mechanical environment along with the biomolecular components of the substrate strongly affect cellular signaling and cell fate [7]. Therefore, there is a great need to develop novel

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multifunctional materials with controllable properties that are able to provide a substrate with well-controlled topography, mechanical properties, and signaling molecules. A variety of such substrates have been proposed in the literature: synthetic materials, such as poly-L-lactic acid and poly-glycolic acid [8–10] or natural materials, such as collagen [11, 12], fibrin [13], arginine [14, 15] or hydrogel scaffolds [16]. Pure hydrogel matrix with laminin or a laminin-(-GGSDPGYIGSR-) sequence has been found to change the mechanical properties of the substrate and initiate neuronal differentiation [17, 18].

However, identifying a tunable biological substituent that sustains and mimics tissue function still represents a significant challenge that needs to be overcome. There is a crucial need to address all of the limitations that lead to unsuccessful differentiation. In order to develop an effective tissue-engineering therapy, it will be necessary to control the mechanical properties of the scaffolds, to decrease their immunogenicity, and to increase their efficiency in supporting cell fate [19].

In order to address these challenges, we propose the application of nano-based approaches to chorion-derived mesenchymal stem cells (Ch-MSCs). The Ch-MSCs are stem cells isolated from the chorionic villi of human term placenta, and their role is to maintain and repair the placental tissue [20]. The chorion-derived MSCs possess superior properties: they are multipotent, have low immunogenicity, and anti-inflammatory functions. Their immunosuppressive properties are explained by the fact that they do not express HLA-DR molecules. Another specific property is their ability to differentiate into several lineages, including osteocytes, chondrocytes, myocytes, adipocytes, cardiomyocytes—and even into cells of nonmesodermal origin, including hepatocytes, neurons, and insulin-producing cells [21, 22]. Moreover, owing to the unique features of nanomaterials—novel electronic, optical, magnetic, and structural properties—nanotechnology can help us to understand and control the biological signaling function of a single cell or molecule and offers a promising tool for controlling and guiding the differentiation process based on the control of surface interaction energies, topography, and mechanical properties. Holy *et al* [23] have suggested the use of multi-walled carbon nanotubes (MWNTs) to direct pluripotent stem cell differentiation, and Kim *et al* [24] have successfully differentiated MSCs into neuronal cells using carbon nanotubes. Similar, Yi *et al* [25] reported that gold nanoparticles can promote osteogenic differentiation of MSCs through the p38 MAPK pathway.

Here, we propose to underline the importance of using nanomaterials in cell differentiation, based on their capacity to control the topography and the mechanical properties of cell substrates, and to show the importance of using biomolecules as natural signaling agents. We intend to understand the mechanism through which gold nanoparticles along with biological molecules—laminin and collagen—control cellular differentiation. We should note that the topology, surface chemistry, and mechanical properties, along with biomolecule signaling, have been found to be critical in controlling the cell phenotype [26].

A novel layer-by-layer approach was used to construct the scaffold. The first layer was composed of gold–collagen and followed a second one composed of laminin. This procedure was repeated 3 times in order to obtain the desired multilayer and multistructural composite scaffold. This scaffold acts as an extracellular matrix and can be constructed with controllable topographic stability and chemistry and high mechanicobiological properties due to the possibility of driving the interconnection and further aggregation of the proteins' fibers and their gold nanoparticle cross-linking. To our best knowledge, such complex studies have not been previously described. Pre-differentiation of placental stem cells on suitable scaffolds could make them potential candidates for use in the regeneration of tissue or the treatment of various cardiac and neurological disorders.

2. Experimental procedure

2.1. Materials

Type I collagen, phosphate buffer solution (PBS pH 7.4), and laminin were delivered by Aldrich (Sigma-Aldrich Inc.). Sodium borohydride ($NaBH_4$) and tetrachloroauric acid ($HAuCl_4$) were delivered by Fluka. The collagen nanofiber solution and the $HAuCl_4$ solution were obtained as previously described [27].

2.2. Substrate preparation

For the preparation of the substrate, we coated layer-by-layer the surface of one-well chamber slides in a manner that permitted the metallized collagen layer to sustain the second layer of laminin. Collagen-based gold nanoparticles were assembled and metallized, as we previously reported, by using borohydrate as a reduction agent [27]. Laminin solution of 15 μg ml^{-1} was prepared. The dimension of the particles used for this experiment was 16 nm.

We applied the additional component of extracellular matrix (ECM)-laminin on the dried metal absorbed collagen substrate at a dilution of 1.5 μg cm^{-2}. We used 300 μl of collagen-based gold nanofiber solutions and 300 μl of laminin (15 μg ml^{-1}) per well. Afterwards, another 300 μl of solution was added. This procedure was repeated 3 times. After 5 min, the excess of the solution was discharged and an adhesive uniform layer was formed. In order to sterilize the plates, a flow of ethylene oxide was used.

2.3. Analytical characterization

The morphology and roughness of the metallized collagen + laminin substrate compared with the non-metallized substrate was investigated by AFM (Bruker DNP-S10), having a spring constant: 350 pN nm^{-1}, frequency: 50–80 kHz, radius: 10 nm. The measured parameters were as follows: spring constant 354.03 pN nm^{-1}, frequency: 65.11 kHz, Sensitivity (deflection/volt): 50.11 nm V^{-1}. For the elastic modulus, a MFP-3D AFM (Asylum Research, Santa Barbara, CA) with a tip velocity of 600 nm s^{-1} (1 Hz) was used. For the cantilever spring constant, a thermal tuning method was employed. In order to calculate the reduced elastic modulus (Er), the Hertzian contact theory was applied. Also, the metallization of the collagen fibers used in the construction of the substrate was investigated by FTIR and UV–vis spectroscopy [27].

2.4. Cell cultures

Adult stem cell isolation, differentiation protocol, immunocytochemistry studies, and metabolic and viability assays—MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and FDA (fluorescein diacetate)—were performed according to the protocol previously described [27].

2.5. Statistical analysis

In order to analyze the data, the GraphPad Prism 5 statistics program (La Jolla, CA, USA) was used. A one-way ANOVA Tukey's multiple comparison test and a two-way ANOVA Bonferroni post-test were performed in order to compare the control data with the data obtained on each substrate. (Statistical significance was set at $p < 0.05$.)

3. Results and discussions

In order to control cell differentiation, used for further regeneration of specific organs, efficient biocompatible substrates capable of inducing contact stimuli, inhibitory cues [28, 29], or signaling molecules are required [30]. We recently reported the synthesis of such substrates, and we demonstrated that the cross-linking of collagen fibers with gold nanoparticles sustained better proliferation, growth, and differentiation of MSCs into neuronal and cardiac cells, compared with collagen alone [27].

As described herein, we continued our study by predicting the mechanism through which the substrate components, such as gold nanoparticles and biological molecules, control the differentiation process. By metallization of the collagen substrate, the mechanical properties and the surface chemistry, such as roughness, elasticity modulus, and force curves, can be tuned. The contact stimuli and the signaling of the substrate are more favorable; therefore, superior cell adherence, proliferation, and differentiation are induced. Moreover, we constructed a more advanced substrate by combining two extracellular matrix proteins, collagen and laminin, with gold nanoparticles.

The capacity of some cells to adhere and proliferate on specific collagens by using these glycoproteins as a support has been established. For example, chondrocytes adhere preferentially on collagen II, while epithelial and endothelial cells prefer type IV collagen. The adhesion of the cell is dependent on temperature [31], as well as the electrostatic forces between substrate and cells [32], and is inhibited by cytochalasin B [33]. Differentiation of cells is induced in a more accelerated way on different extracellular matrix substrates compared with controls without substrates. For example, Qian *et al* [34] studied the impact of poly-D-lysine, poly-L-lysine, collagen, laminin, fibronectin, and Matrigel substrates on the growth and differentiation of MSCs. They found that all of the substrates, except poly-D-lysine, enhanced proliferation and differentiation.

The interface between nanomaterials and stem cells provides new strategies for the reconstruction of lost myocardial or nervous tissue. In this study, based on the nanoparticles' properties, along with the proteins' functions, we were able to create a substrate that acts as a cellular microenvironment in order to control *in vitro* the MSCs' behavior and functionalities by promoting increased adhesion, proliferation, and differentiation into myocardial and neuronal stem cells. AFM proliferation/viability assays and immunocytochemistry studies were used to characterize the relationship of the substrates with the stem cells' growth and differentiation efficiency.

3.1. Metallization of the collagen fibers

The metallization reaction was performed using a Fisher etherification reaction, and the linkage was demonstrated using x-ray photoelectron spectroscopy (XPS). XPS is a powerful surface analysis technique used to determine the elemental composition, chemical, and electron states of the elements of a material. The spectra are obtained by exposing the material to x-rays and measuring the kinetic energy of electrons escaping from the atoms. Depending on the chemical state of the element in a material, the binding energy may shift, which serves to reveal chemical changes in the material.

In the first spectra, figure 1(A), which is a survey scan of the sample, various peaks show the presence of corresponding elements. We indexed only the elements of interest: gold and carbon. The second spectra, shown in figure 1(B), presents a detailed scan of gold. Typically, the gold peak is a doublet with one peak around 84 eV and the other around 87 eV. This indicates that the gold was in a metallic form and did not react chemically during the synthesis of gold-decorated collagen. The third spectra, figure 1(C), shows a detailed scan of the carbon (C 1s) peak in the sample that has only collagen without gold. There are three-peaks (C–C, C–N and C–O), which are typical for collagen. The fourth spectra, shown in figure 1(D), presents carbon spectra in the gold-decorated collagen sample. Two more new peaks appear. While the C–C and C–N peaks are intact, an additional O=C–OH peak appears based on the reaction of the COOH group from the surface of gold nanoparticles with the OH group from the collagen structure [35].

The metallized collagen fibers were further used for the construction of a multilayer substrate in order to study the mechanical changes made by the addition of the nanoparticles. This study was conducted using AFM analysis. The layered structure was found to be stable, with the first layer (gold nanoparticles-based collagen) sustaining the second one (laminin). This procedure was repeated 3 times.

The elastic modulus of the films was assessed using force mapping measurements in 100 different positions of the substrate. The substrate was not plastically deformed during the analysis. The measurements performed on the same area reasonably well. The force probe approach velocity applied was 600 nm s^{-1} (1 Hz). For the mechanical calculations, we applied the linear elasticity theory. In order to fit the force curves, Hertz–Sneddon theory was used [36].

The elasticity modulus (E values) for the collagen substrate was 152.0 ($\pm$63.9) MPa and, for gold-coated collagen, 879.7 ($\pm$417.4) mPa. Figure 1 presents the AFM images of a typical collagen fiber (A) and metallized collagen (MC) fiber (B) at the points selected for force measurement analysis. The representative force curves of collagen (blue) and the

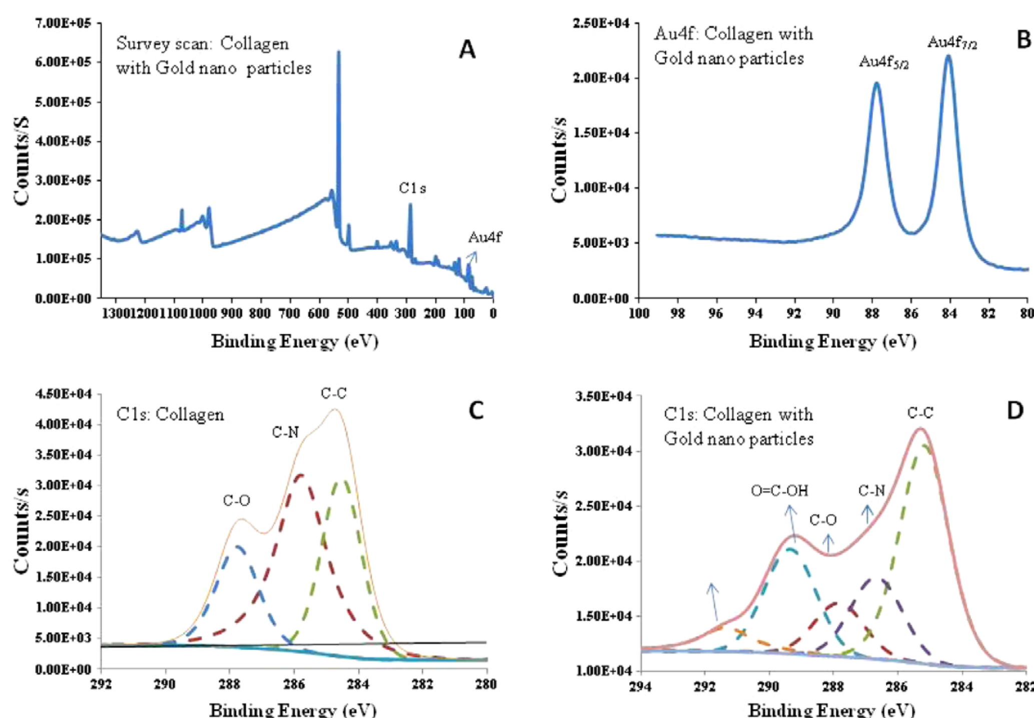

Figure 1. (A)–(D) X-ray photoelectron spectroscopy of collagen molecule-decorated gold nanoparticles: (A) survey scan of the sample, (B) detailed scan of gold, (C) detailed scan of the carbon (C 1s) peak in the sample that has only collagen without gold, (D) carbon spectra in the gold-decorated collagen sample.

metallized collagen on gold substrate (black) are presented in figure 2(C). From the statistical analysis, we concluded that the calculated elastic modulus for the metallized collagen substrate was significantly higher compared with the collagen value ($p < 0.05$). E values were calculated by averaging over all points where the force curves were collected. Furthermore, we determined the force curves for both substrates, collagen and metallized collagen. In this case, the metallized collagen material was compressed about 3 nm at 15 nN force (figure 2(C) blue) compared with pure collagen, which was compressed about 11 nm under a similar 15 nN force (figure 2(C) black).

Previous reports have shown that, with an increase in the stiffness, both the focal adhesion and cytoskeletal organization increase [37–40]. The stiffness of the substrate is important in influencing the cells' response to different substrates, but is not a bulk parameter for all types of cells. It has been recently reported that the relationship between stiffness and cell behavior is associated through the mechanical feedback of the ECM [41]. Trappmann *et al* [41] have also investigated how the mechanical properties influence cell fate. They cultured human epidermal stem cells and MSCs on polydimethylsiloxane and polyacrylamide hydrogel surfaces coated with collagen. This group found that cell differentiation was not affected by the polydimethylsiloxane stiffness and that the cells did not adhere to the polyacrylamide substrate due to the low elastic modulus (0.5 kPa). However, when collagen was cross-linked with hydrogel-nanoparticles, the cell attachment was significantly affected. The authors believed that the stiffness of the substrate is directly linked with cell behavior, but they reported that the stiffness of the substrate changes the anchoring densities of the cells to the collagen substrate. It is known that the cell attachment on the collagen substrate is mediated by integrin molecules; thus, when the ECM is loosely bound to the substrate, the signaling is affected. In addition, the surface chemistry and the topology of the substrate are believed to be two major factors in directing cell fate. The roughness of the collagen fiber can be controlled during the cross-linking with gold nanoparticles and also by altering its concentration. The roughness of the collagen substrate was found to be Rq: 39.8 nm and Ra: 31.8 nm, compared with that of the metallized collagen of Rq: 45.7 nm and Ra: 34.2 nm, respectively.

This combined study of the elastic modulus and surface topology indicates that, after the nanoparticles' surface treatment, the collagen substrate's stiffness increases dramatically. The differentiation potential increases when the roughness of the same substrate increases. Earlier studies have shown that the stiffness can control cell adhesion, viability, and proliferation [42–44]. Therefore, it is of great interest in the preparation of substrates with controlled mechanical properties and topography, as well as signaling molecules. By using different types of nanoparticles, with different shapes, sizes and concentrations, these challenges can be addressed.

3.2. Testing the efficiency of the hybrid substrates on the differentiation of MSCs into neuronal and cardiac cells

Other parameters that should be considered in the construction of a successful scaffold are the molecules used for the construction of the substrate. These molecules should act as tissue

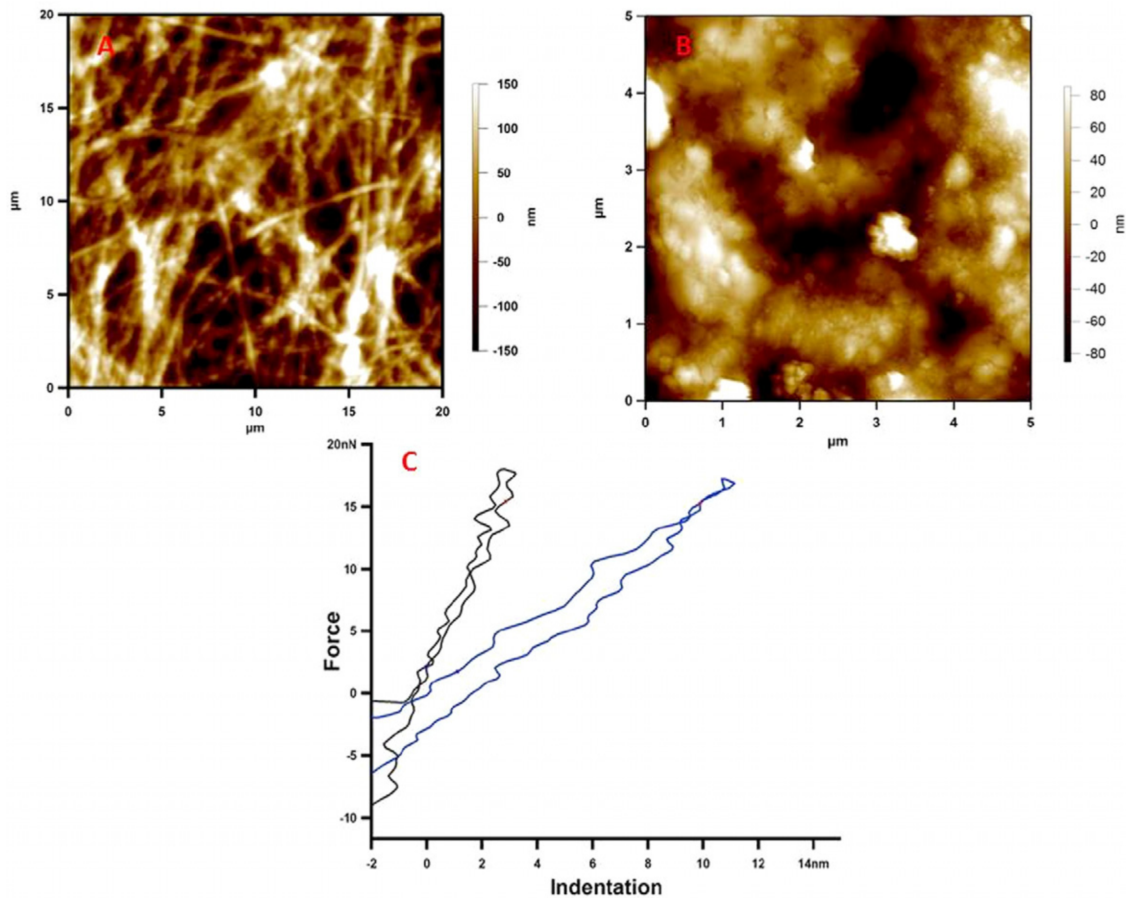

Figure 2. AFM images of a typical collagen substrate (A) and metallized collagen (B) representative force curve of collagen (blue) and metallized collagen on a gold substrate (black) (C).

substituents and should sustain and mimic tissue functions. Moreover, it is also mandatory to use multi-composite signaling biomolecules as cross-linking agents in order to further approach the bio-signaling pathways that direct cell fate. The goal is to synthesize an 'artificial extracellular matrix' with controllable topology and mechanical properties.

Furthermore, we propose to show the importance of using biomolecules, e.g., a combination of collagen and laminin, as substrate components because they are natural signaling agents that direct the cells' fate. We constructed an artificial extracellular matrix, a composite scaffold based on gold nanoparticles, collagen I, and laminin, in order to test its capacity to differentiate MSCs into neuronal and myocardial progenitor cells in the presence of differentiation media.

In order to assess the biocompatibility of Ch-MSCs with the substrates, we used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and FDA (fluorescein diacetate) viability tests. The adult mesenchymal stem cells were cultivated on the collagen + laminin substrate and a nano-ECM substrate under different conditions: in the presence of stem cell media (control) and treated with neuronal and myocardial differentiation medium for seven days. Undifferentiated chorion-derived placental MSCs cultivated on laminin + collagen (CL) and metallized collagen + laminin (MCL) resulted in increased proliferation. A statistically significant difference was observed for MCL when compared to the control (figure 3(A)). The cell proliferation and growth was statistically significant for the collagen + laminin substrate (figure 3(B)) when the cells were cultivated in the presence of neuronal differentiation media. For myocardial differentiation, the values were found to increase for cells cultivated on both substrates, with no statistically significant difference (figure 3(C)). MTT assay is a colorimetric method which measures the mitochondrial enzymes' activity and can reflect the metabolic status of cells and consequently the number of viable cells. FDA freely diffuses into cells and is rapidly esterified once it enters the cell. The viability of the cells is assessed from the hydrolysis product, fluorescein, which cannot escape from live cells. Fluorescent signals are correlated with the number and the size of individual viable cells.

In order to determine if there was a difference in the biocompatibility properties of the substrates and control samples, we performed further analysis using a two-way ANOVA Bonferroni post-test comparison of grouped data in relation to the substrates. No significant differences were observed between the analyzed data (figure 3(D)). A fluorescein diacetate (FDA) test, a fluorimetric method, was used to investigate the biocompatibility of cells cultivated on CL and MCL, in the same differentiation conditions. The results were very similar to those obtained from MTT assays in control samples

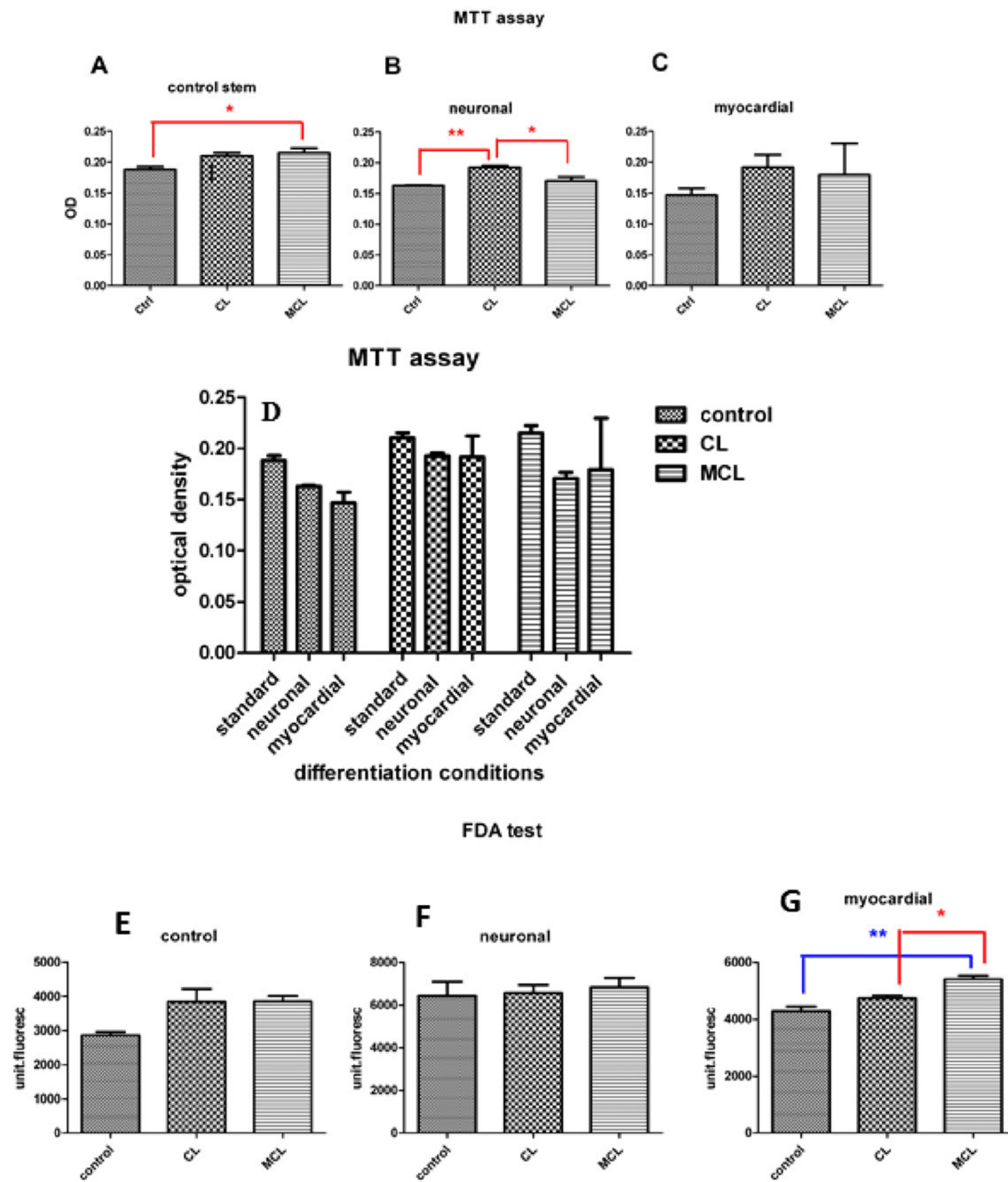

Figure 3. MTT viability assay for Ch-MSCs after seven days of cultivation such as: in the presence of stem cell media (control), neuronal differentiation medium (B) and myocardial differentiation medium (B), without substrates and on collagen + laminin and metallized collagen + laminin (MCL). Statistical significance was set at $p < 0.05$. No statistically significant differences were observed between substrates. (D) FDA fluorimetric assay of Ch-MSC cultivated in standard conditions (control without substrate), on collagen + laminin (CL) and metallized collagen + laminin (MCL) substrates. (E) stem cell medium; (F) neuronal differentiation medium; (G) myocardial differentiation medium.

(figure 3(E)). Ch-MSCs induced with neuronal differentiation medium showed no difference between control and CL or MCL substrates (figure 3(F)). Metallized collagen + laminin induced an increase in cell viability in myocardial differentiation conditions (figure 3(G)).

The viability tests were confirmed by microscopic analysis. For neuronal differentiation, Ch-MSCs were cultivated on Permanox chamber slides and Petri dishes coated with gold metallized collagen (MC) and a gold metallized collagen + laminin (MCL) substrate in the presence of growth factors (EGF, bFGF, and neuronal supplements B27 and N2 supplement) for 48 h, with a subsequent exposure for four weeks to retinoic acid, IBMX, B27, and N2 supplement. Cells cultivated without substrate were used as controls. A particular phenomenon was observed in the case of MSCs cultivated on Lab-Tek Permanox chamber slides coated with MC and MCL. The number of cells decreased rapidly after exposure to N_2 medium, and cells extended long neural-like processes and aligned, with the further appearance of some cellular fibrillar structures resembling neuronal axons (figures 4(B) and

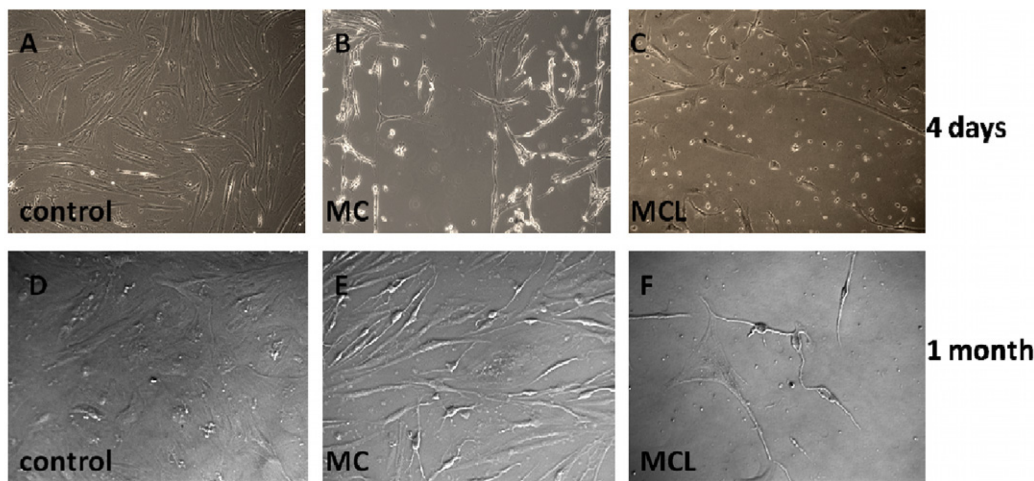

Figure 4. Contrast-phase images of Ch-MSCs cultivated with neuronal differentiation medium without substrate (control) and on MC and MCL substrates for four days and one month. Upper panel: cells cultivated on Lab-Tek Permanox chamber slides coated with MC and MCL: images were taken after four days of exposure to neuronal differentiation medium. Lower panel: cells cultivated for one month on Petri dishes coated with MC and MCL.

(C)). The differentiation process was accelerated for the MCL substrate, and the cells developed neural-like extensions and appeared to respond with the highest frequency of neural-like cells when compared with cells cultivated without substrate that responded after four weeks. The difference between MC and MCL was an increased uptake of gold nanoparticles in the case of the MCL substrate, as shown in figure 4(F).

Heaton *et al* [45] assessed possible laminin influences by comparison with collagen on neuronal adhesion and nerve fiber expression, concluding that laminin is more suitable for initiating the differentiation process. In another study, it has also been shown that laminin sustains neural expression and growth. Moreover, the progenitor expression was blocked by the antibody against integrin alpha6 or beta1 subunit [46]. Immunohystochemical staining of the samples revealed (figure 5) the expression of some neuronal differentiation markers: GFAP was strongly expressed in cells cultivated on all substrates used (metallized collagen—figure 5(B); metal absorbed collagen + laminin—figure 4(C)) when compared to the weak fluorescence of GFAP in the control sample (figure 5(A)).

Staining for neurofilaments (NF), filaments found specifically in neurons, revealed that cells differentiated on all of the substrates—laminin, MC and MCL—expressed this protein with a greater staining intensity for metallized collagen and with a neurite-like morphology for the MCL substrate. NF expression was present after four weeks of induction of neuronal differentiation, as illustrated in figures 5(D)–(F). The combination of gold nanoparticles, laminin, and collagen led to better proliferation, growth, and differentiation efficiency. The molecular configuration of the substrates, especially the presence of laminin, was identified to be of critical importance for neurite outgrowth and polarization during development and regeneration [47, 48]. Recently, Solanki *et al* [49] and García-Parra *et al* [50] confirmed that the mechanical or topographical features of micropatterned substrates (formed from ECM components, especially laminin) can guide in a controlled manner cell–cell and cell–ECM interactions and finally promote neurogenesis. Studies of the neural differentiation of embryonic stem cells have shown the advantage of laminin or laminin-rich Matrigel on neural progenitors and neurite outgrowth in a dose-dependent manner [46]. Mruthyunjaya *et al* [51] observed a similar laminin-1 effect on the neuronal phenotype of bone-marrow-derived MSCs cultivated on different ECM components. Cells plated with laminin-1 showed accelerated changes with the development of a neurite-like morphology. This team demonstrated the involvement of integrin $\alpha6\beta1$ and FAK-MEK/ERK signaling pathways of adherent cells to the laminin-1 substrate. Different nanomaterials were used for neuronal differentiation of stem cells, e.g., carbon nanotubes with diameters and lengths similar to ECM molecules (collagens and laminin). These scaffolds have a high stability and maintain their structural and mechanical properties during cell differentiation and growth [52]. Gold nanoparticles were used primarily for non-invasive imaging of cells *in vivo* or *in vitro* using the surface-enhanced Raman scattering (SERS) method. Karataş *et al* [53] and Sathuluri *et al* [54] demonstrated the mitochondrial localization of GNPs.

Cardiomyogenic differentiation of Ch-MSCs was investigated in relation to the substrate type collagen + laminin and gold metal absorbed collagen + laminin, in comparison with controls cultivated without substrate. The differentiation protocol consisted of a four-week exposure to demethylating agent 5-azacytidine (10 μM) for 24 h with one cycle of exposure/week (in total-four cycles of 5-AZA). In order to determine the induction of cardiac differentiation, immunostaining with cardiac markers was performed after four weeks of cultivating the cells in the presence of myocardial differentiation medium. We used antibodies against early cardiac specific homeobox protein Nkx 2.5, atrial natriuretic peptide cardiac hormone (ANP), and a staining protocol with phalloidin TRITC for the rearrangement of filamentous actin fibers (figure 6). In control samples, without substrate, phase contrast images highlighted the characteristic stick-like morphology of

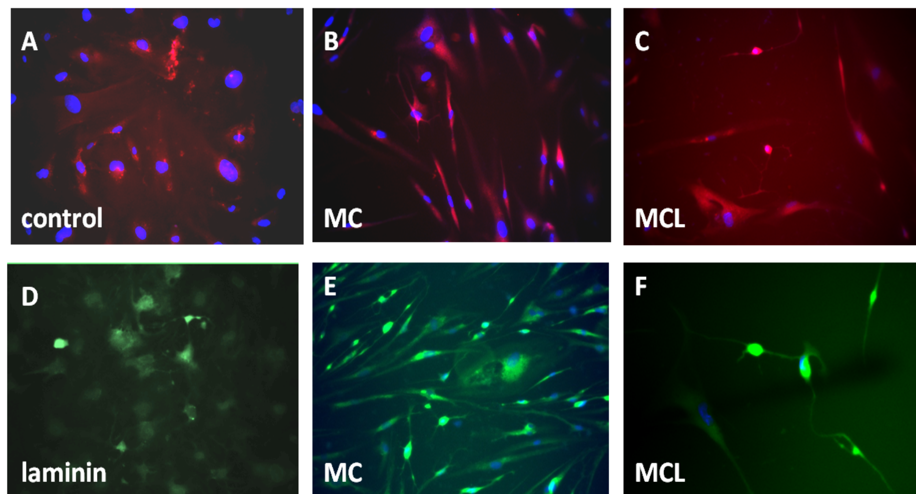

Figure 5. Immunostaining for GFAP–Texas Red (counterstaining with DAPI) after four weeks with neuronal differentiation medium: control Ch-MSC without substrate (A); Ch-MSC on the gold metallized collagen substrate (B), Ch-MSC on metal absorbed collagen + laminin (magnification 400×) (C); immunostaining for NF-FITC and the correspondent image in white light of Ch-MSC induced for neuronal differentiation on laminin (D), metallized collagen (E), and metallized collagen + laminin (F) after four weeks (400× magnification).

induced cardiac progenitors (figure 6(B)) and a weak positivity for Nkx 2.5 in immunofluorescence (figure 6(B)). MSCs cultivated on both substrates changed shape, adopting a polygonal morphology, explained by the rearrangement and assembly of myofibrils on the actin-stress fibers template coupled with focal adhesion complexes [55, 56]. Differentiation on the MCL substrate was characterized by a strong uptake of GNP in the perinuclear compartment and the rearrangement of F-actin in a characteristic manner, as observed in (figures 6(C) and (E)) by optical microscopy, with expression of intranuclear Nkx 2.5 in fluorescence microscopy (figures 6(D) and (F)).

A characteristic stick-like morphology (figure 7) with the formation of typical striated sarcomeres was attained by Ch-derived MSCs cultivated on metallized collagen + laminin after an initial step of pre-differentiation without substrate for 31 days (figure 7(B)). The control sample showed a weak rearrangement of F-actin filaments (figure 7(A)).

The morphological changes induced in Ch-MSCs with 5-AZA exposure cultivated on the metallized collagen + laminin substrate for four weeks were similar to those observed in the case of the pre-differentiated cells protocol, with the mention that in the pre-differentiation experiment the typical striated sarcomers appeared only after a shorter time of cultivation on the MCL substrate. ANP expression and the arrangement of actin fibrils suggest a more differentiated state of the cells cultivated on the MCL substrate (figures 7(C) and (D)). In this study, even though GNPs were used only as functionalization tool of collagen fibers (without being released as vehicle molecules), the complex substrate of metallized collagen + laminin was able to trigger essential signals for starting the differentiation process, in the presence of the guidance action of specific neuronal and myocardial differentiation medium.

The possibility of using stem cells in cardiovascular diseases for restoration and regeneration has been intensively studied in the past few years. Cell therapies are indicated in heart diseases because of the poor regeneration capacity of myocardial tissue. The main disadvantages of stem cell therapies are low cell retention and the lack of targeted localization, with less than 1% homing of delivered cells in the intravenous route, and 90% cell death one week after implantation [57]. Hematopoietic stem cells, for example, do not trans-differentiate into myocardial cells; instead, they were shown to become mature blood cells [58]. One of the goals of tissue engineering is to activate resident stem cells or to enhance their recruitment from stem cell niches to the site of injury. On the other hand, the contribution of extra-cardiac stem cells can be improved with genetic engineering and nanotechnology [59]. An ideal scaffold would be biodegradable, biocompatible, and possess mechanical properties similar to those of the myocardium [60]. ECM components, with their capacity for self-assembling in 3D structures, offer a favorable microenvironment as cell signal triggers, and their porosity facilitates the adhesion, colonization, and proliferation of cells. The mechanical properties of substrates have an important influence on myocardial differentiation [61]. Kim *et al* [62] have shown that ECM promotes the critical microenvironment that the cells need to proliferate and migrate. Rowlands *et al* [63] have shown that substrates with more than 9 kPa stiffness induced myocardial markers and sustained cell proliferation without using DNA demethylation agents. Moreover, Kim *et al* [64] demonstrated that nanotopography has a significant impact in guiding the role of human tissue, activating its specific functions and promoting its regeneration.

Another strategy proposed in cardiac tissue regeneration is the development of controlled delivery systems for promoting neovascularization by using, for example, encapsulated growth factors, such as VEGF (vascular endothelial growth factor) or PDGF (platelet-derived growth factor) in poly-(lactic/glycolic acid) (PLGA) microspheres [65]. Gold nanoparticles can serve

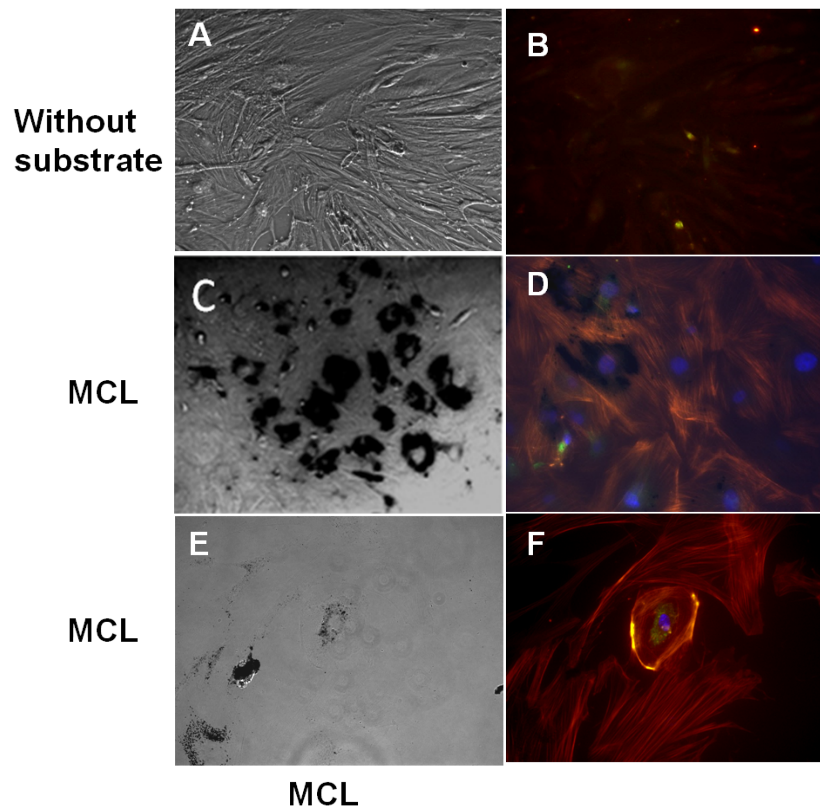

Figure 6. Immunostaining for cardiomyocytemarkers (Nkx 2.5-FITC and TRITC phalloidin; counterstained with DAPI) and, in the lower panel, corresponding phase contrast images of placental MSC exposed four weeks to 5-azacytine therapy (10 μM, one cycle of therapy/week): (A) phase contrast image of Ch-MSC induced for myocardial differentiation, control without substrate (magnification 100×), (B) Nkx 2.5-FITC expression of chorion MSC without substrate. (C) and (D) Nkx 2.5 and actin F staining of chorion MSC cultivated on a metallized collagen + laminin substrate; increased cellular intake of GNP (magnification 200×). (E) and (F) Nkx 2.5-FITC and actin F-phalloidin TRITC staining of Ch-MSC cultivated on a metallized collagen + laminin substrate (400× magnification).

as vehicles for various biomolecules or genes implicated in stem cell differentiation or for the initiation of vascularization.

The future of stem cell research and therapy will continue to provide novel avenues for diagnostics, therapeutics, and tissue regeneration.

Herein, we show that our synthesized substrate is promising for Ch-MSCs cellular differentiation and proliferation into both cardiac and neural cells. Based on the composition (collagen and laminin) and the presence of the gold structures, this substrate has the potential ability to activate specific signaling pathways by triggering the synthesis of specific growth factors or other biologically active molecules implicated in the control and fate of mesenchymal stem cells. In a short period of time, using specific differentiation media, neuronal and cardiac stem cells were obtained.

This technology may possibly represent a solution to overcome the existing limitations in the treatment of neurodegenerative diseases. However, the mechanisms that govern the interactions between various substrates and stem cells is still under investigation. It has been reported that the implanted stem cells could contribute to neuro-regeneration by stimulating the formation and production of neurotropic factors, reducing the neuro-inflammation or even by replacing the non-neuronal cells [66]. For example, in the case of Parkinson's disease, where the dopaminergic neurons are affected, using mesenchymal stem cell transplantation, the partial alleviation of symptoms has been reported [67]. Moreover, other cell therapies using neuronal stem cells isolated from olfactory mucosa [68], embryonic stem cells derived from dopaminic neurons [69], etc, have been proposed. Therefore, a variety of complex clinical approaches to address current limitations in treating various medical conditions are expected to emerge soon. Macchiarini *et al* [70] have reported the successful clinical transplantation of the stem cells from a patient suffering from failing airways, with none of the immune rejections typical of traditional organ transplantation. Another promising application of stem cell research is the use of pre-differentiated MSCs in treating cardiac diseases [71]. In this case, conventional approaches cannot currently provide successful therapies for heart failure. Similar to the case of neurodegenerative diseases, after heart failure or a myocardial infarction, the regenerative potential of the muscle is low, having no capacity to fully regenerate. For this purpose, different types of cells have been tested: hematopoietic stem cells were transplanted, but the improvement of cardiac functions was rather limited because of the onset of immunogenic problems [72, 73]. Since MSCs were reported to have HLA-DR molecules on their surface with immunosuppressive properties, they are therefore

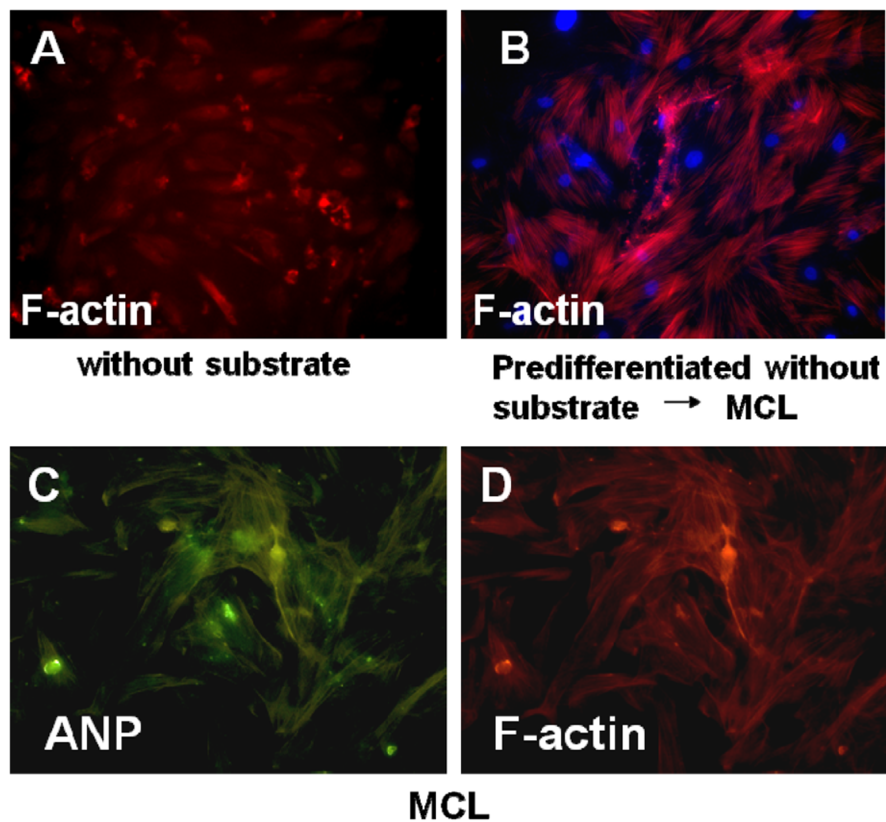

Figure 7. Immunochemical staining with TRITC—phalloidin for evidence of F-actin. (A) Chorion MSC cultivated 31 days without substrate, (B) chorion MSC pre-differentiated 31 days without substrate with five therapies of 5-AZA followed by a passage on gold metallized collagen + laminin for 19 days with one therapy of 5-AZA. White arrow indicates a cardiomyocyte with typical striated sarcomeres (magnification 200×). Immunochemical staining for ANP-FITC (C) and F-actin-TRITC phalloidin (D) expression of Ch-MSCs on metallized collagen + laminin substrates for 31 days with five therapies of 5-azacytidine (four weeks of cultivation) (magnification 200×).

highly promising compared to other types of cells. However, more studies need to be performed in order to determine the type of cell lines that need to be used for a specific condition [74]. The exploration of the pluripotent properties of MSCs has enormous potential for the treatment of various currently incurable diseases. In all these exciting medical developments, one of the real promising approaches include the use of nanostructural engineered materials with multifunctional properties and characteristics [75–81]. In the near future, clinical trials showing the effectiveness of stem cell therapy are expected to generate exciting medical developments in the treatment of various conditions and diseases that currently do not have treatments.

4. Conclusions

A complex nanosubstrate composed of gold nanoparticles, metallized collagen, and laminin was synthesized, and its ability to sustain cell differentiation was tested. The synthesized substrate, based on its complex composition, enhanced all contact stimuli inhibitory cues and signaling molecules, making it an ideal candidate for Ch-MSCs cellular differentiation and proliferation into cardiac and neural cells. First, in order to demonstrate the importance of using gold nanoparticles in the substrate composition, we tested the change in the mechanical properties of the substrate, using AFM microscopy. In this case, a higher elasticity modulus, roughness, and curve forces of the metallized substrate were observed. Thus, we concluded that the use of gold nanoparticles in the substrate composition leads to better control of their structural and mechanical characteristics. Moreover, based on its attached gold nanoparticles, this substrate has the potential to deliver growth factors or other biologically active molecules to the cells in order to control and regulate their differentiation, leading to defined populations of cells. The signaling molecules of the substrate, such as collagen and laminin, along with the mechanical changes induced by the presence of the gold nanoparticles contributed efficiently to differentiation (in comparison with the controls—no substrate comprised of a combination of collagen and laminin) into cardiac and neural cells. The synthesized substrate enhanced all contact stimuli, inhibitory curves, and signaling molecules, making it an ideal candidate for Ch-MSCs cellular differentiation and proliferation. The efficiency of the substrate was tested using MSCs cultivated in differentiation media. Cytotoxicity and immunohistochemistry studies were performed in order to show their biocompatibility and to mark the expression of specific markers upon differentiation of MSCs into neuronal and cardiac progenitors.

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