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Adapting collagen/CNT matrix in directing hESC differentiation

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Abstract

The lineage selection in human embryonic stem cell (hESC) differentiation relies on both the growth factors and small molecules in the media and the physical characteristics of the micro-environment. In this work, we utilized various materials, including the collagen-carbon nanotube (collagen/CNT) composite material, as cell culture matrices to examine the impact of matrix properties on hESC differentiation. Our AFM analysis indicated that the collagen/CNT formed rigid fibril bundles, which polarized the growth and differentiation of hESCs, resulting in more than 90% of the cells to the ectodermal lineage in Day 3 in the media commonly used for spontaneous differentiation. We also observed the differentiated cells followed the coarse alignment of the collagen/CNT matrix. The research not only revealed the responsiveness of hESCs to matrix properties, but also provided a simple yet efficient way to direct the hESC differentiation, and imposed the potential of forming neural-cell based bio-devices for further applications.

Keywords

Human embryonic stem cell; collagen; CNT; differentiation; ectodermal lineage

Introduction

Human embryonic stem cells (hESC) have the potential to remain undifferentiated in a tight colony format or differentiate into all three primary germ layers including endoderm, mesoderm and ectoderm cells that form all tissues in human body [1]. Both the scenarios occur depending on the growth factors and small molecules available in the media. Due to the immense potential of hESCs in regenerative medicine for many hard-to-treat-diseases, tremendous amount of research has been dedicated to harness the pluripotency of hESCs mainly by fine-tuning the media composition. Such methods rely heavily on our understanding of traditional mammalian developmental biology. Nevertheless, recent studies have revealed that stem cells are extremely responsive to their immediate physical environment [2,3], the extracellular matrix (ECM). ECM regulates the cell fate by the ECM molecules in concert with its physical properties [4–9], which have been frequently reported to be critical in cell lineage specification. For instance, cell shape, controlled by matrix geometry, acted as a cue in the commitment of human mesenchymal stem cells to adipocyte or osteoblast [4]; murine neural progenitor cells aligned with fibrous scaffolds, and the differentiation with long neurofilament was more preferentially shown on the nanoscale scaffolds than on the microscale scaffolds [6]; mesenchymal stem cells were shown to specify lineage and commit to neurogenic,

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myogenic and osteogenic phenotypes in response to matrix elasticity [7]. These physical effects of ECM are imposed by affecting the cytoskeletal tension, altering the cell adhesion, and deforming the cell membrane. In conjunction with stimulants added in the media, they are now deemed to be the foremost players in directing lineage specification of stem cells [10,11].

Our previous studies on the spontaneous differentiation of hESCs used a high serum concentration with DMEM media in the presence of non-essential amino acids without additional growth factors [12]. When gelatin or matrigel were used as a matrix, the medium allowed rapid spontaneous differentiation into all three lineages with no specification. According to our atomic force microscopic (AFM) analysis, such matrices are structure-less and soft. To investigate the impact of structured matrix on hESC differentiation, we chose type I collagen and its composite with carbon nanotube (collagen/CNT) as the cell culture matrices in this work. With the same medium for spontaneous differentiation, we endeavored to monitor the effects of collagen and collagen/CNT on cell lineage specification at the early stage of hESC differentiation.

Type I collagen is the most abundant collagen in human body and is a component of tendons, skin and artery walls. Upon gelation, it forms a network of interconnected fibers. A single collagen fibril is composed of three monomers that twist to form a single left handed superhelix with a well-ordered periodic structure termed the D period. Type I collagen is an ECM protein, and is known to support the growth of many neuronal cell types [13] as well as the neural differentiation of hESCs in a neural differentiation medium [5]. In this study, we attempted the use of type I collagen as a substrate to direct the differentiation of hESCs in the medium of spontaneous differentiation. In order to justify the effects of physical factors, we further manipulated the substrate properties by preparing the collagen/CNT composite material. CNTs, a one-dimensional nano-material, are frequently used in biological studies due to their high tensile strength and electrical properties [14,15]. Properly functionalized CNTs are non-toxic to cells at a low concentration in the medium or in the matrix scaffolds [15,16]. We utilized the carboxyl modified single wall CNTs to make a composite material with type I collagen, and the CNTs were found to greatly impact the collagen's stiffness and structure, resulting in the early differentiation of more than 90% of the cells into nestin-positive cells. Additionally, when we roughly aligned the collagen by dip-coating preparation, the differentiated cells lined up with the pre-aligned collagen and collagen/CNT fibril bundles. Besides the understanding of the high sensitivity of hESCs to matrix properties, the research also revealed a novel strategy of forming neural cell based bio-devices for further applications.

Materials and Methods

Cell matrices preparation

Collagen type I from rat tails was purchased from Sigma (St. Louis, MO). According to the manufacturer's protocol, 1.1 mg/ml of collagen was prepared under sterile conditions in 10× PBS buffer (from Fisher, Fair Lawn, NJ) containing 1 N NaOH to achieve the pH of 7.4. Plastic slides were dip-coated with the collagen solution along one direction (to promote the alignment of fibrous scaffolds). The slides were then incubated at 37 °C for 1.5–2 hrs for gelation before being using for cell culture.

High purity single-walled carbon nanotubes (SWCNTs) were purchased from HELIX Material Solution (Richardson, TX). Raw SWCNTs were functionalized with carboxyl groups by sonicating SWCNTs in an aqueous solution containing 8 M of H₂SO₄ and 8 M of HNO₃ at 70°C for 3 hours [17–19]. After centrifugation at 5000 RPM for 10 minutes, the supernatant was extracted, and the CNTs were collected on a filter membrane. The CNTs were further washed by DI-H₂O until the pH was around 6–7, then dried in an oven at 60 °C for 3 hours to obtain the powder. The oxidation of SWCNTs was confirmed by FTIR (Thermo Nicolet,

NEXUS 470) with peaks at 3400 cm^{-1} and 1700 cm^{-1} , characteristic for the carboxyl groups [20]. The higher dispersion of functionalized CNTs in water also distinguished them from the raw CNTs.

A low dose of CNT is less toxic to cells [15,16]. In the preparation of the collagen/CNT composite material, we added the oxidized SWCNTs to a 1.1 mg/ml collagen solution at a final CNT concentration of 0.005 mg/ml. This was followed by the same gelation procedure as for pure collagen, except a longer gelation time (2.5–3 hrs).

hESC culture

H9 cells from WiCell (Madison, WI) were grown according to standard Wicell protocol. In brief, cells were routinely cultured on feeder cells derived from mitotically inactivated mouse embryo fibroblast (MEFS). Cells were maintained in a DMEM/F12 (Mediatech, Herndon, VA) medium, containing 20% knockout serum replacement, 1% nonessential amino acid, 1 mM l-glutamine, 100 μM beta-mecaptoethanol, and 4 ng/ml bFGF (Invitrogen, Carlsbad, CA). Confluent hESCs were split using 0.5% trypsin EDTA and plated on the prepared matrices at a ratio of 1:6. The cells were grown in a DMEM (high glucose) medium, containing 10% fetal bovine serum and 1% non essential amino acids, commonly used for spontaneous differentiation of hESCs.

Immunofluorescence

Day 3 cells grown on the collagen, collagen/CNT and gelatin matrices were fixed and permeabilized with pre-cold methanol for 5 minutes and rinsed with PBS. The cells were then incubated with the primary antibody against nestin (purchased from Millipore, Phillipsburg, NJ) with 1% BSA in PBST for one hour at room temperature followed by the incubation with alexa fluor 594 fluorescent conjugated secondary antibody for one hour at room temperature. The nuclei were stained with DAPI for 5 minutes. A Nikon TE-U 2000 fluorescence microscope was used in the study.

AFM measurements

Collagen, collagen/CNT or gelatin coated substrates were thoroughly rinsed with DI- H_2O before the surfaces were gently dried with nitrogen while retaining the moisture on the surfaces. The AFM imaging was carried out in air-tapping mode using a multimode Nanoscope IIIa AFM (Veeco Metrology, Santa Barbara, CA), equipped with a J-scanner. Single crystal silicon tips at a resonance frequency of 300–350 kHz were used in the studies.

The substrate stiffness measurements were conducted by using Si_3N_4 tips operated in fluid contact mode in DI- H_2O . AFM nano-indentation method was applied to obtain the Young's Modulus using the formula [21–23]

$$E = K\pi(1 - \nu^2) \Delta d / [2 \tan \alpha (\Delta z - \Delta d)^2] \quad \text{Eq. 1}$$

where E is the young's modulus, k is the spring constant of the AFM tip, ν is the poisson's ratio (commonly takes 0.5 for biological samples), $\alpha (= 35^\circ)$ is the half angle of the tip, Δd is the relative cantilever deflection and Δz is the relative sample displacement. The spring constant of the tips was $0.056 \pm 0.002\text{ N/m}$, as calibrated by using reference cantilevers with known spring constants [22,24]. From a force spectrum, which illustrates the tip deflection-displacement relationship during the tip approaching and retraction from a substrate, Δd and Δz can be experimentally determined and thus Young's modulus can be evaluated. The average

E value of each matrix type was estimated based on at least 25 separate measurements at five different areas and on different samples with different tips.

Results

hESC differentiation on various matrices

Undifferentiated hESCs grow in compact colonies, with well-defined round-shaped boundaries as shown in Figure 1a. Three days after they were re-seeded on the substrates without the MEFS feeder layer or the conditioned media, the cells on gelatin showed random cell morphologies (Fig. 1b), deviating from the small spherical shape of undifferentiated hESCs. However, the cells barely expressed the markers of *sox17* (endoderm), *brachury* (mesoderm) and *nestin* (ectoderm) for early stage differentiation. With collagen or collagen/CNT as the matrices, more than 90% of the cells elongated and well spread with long filaments (Fig. 1e,h).

The cells and their filamental extensions preferentially aligned along one direction due to the dip-coating preparation of collagen or collagen/CNT cell culture matrices, which will be analyzed later. The same alignment was also obeyed by most nuclei, evident in Fig. 1g and j.

The phenotypic feature of the cells implies some degree of ectodermal lineage specification. When the differentiated cells at Day 3 were stained by the antibody against *nestin*, an early neural progenitor marker [25], to our surprise, cells on the collagen/CNT matrix (Fig. 1i) demonstrated significantly higher level of *nestin* expression than those on the pure collagen matrix (Fig. 1f), regardless of the similarity in cell shape in the two cases. This was reproducibly observed with no exceptions. Note that Fig. 1e–j illustrate the regions with relatively high cell densities. The same cell morphology and the marker expression characteristics were shown at regions of low cell densities. Over a longer term (Day 6) of culture in the same medium, we observed increased *nestin* expression level on pure collagen while the expression level on collagen/CNT remained higher. Meanwhile, a low level of *nestin* expression showed up on cells cultured on gelatin. Taken together, while hESCs spontaneously differentiate into various cell lineages in the absence of the MEFS feeder and the conditioned media, the collagen and collagen/CNT matrices specify the differentiation towards *nestin*-expression cells, which are likely neural progenitor cells. The collagen/CNT matrix stimulates the hESC differentiation to the ectodermal lineage at a much faster pace, making the composite material a superior matrix for producing neural progenitors.

Matrix surface characterization

To unveil the difference in physical property among the gelatin, collagen and collagen/CNT matrices, we carried out AFM characterization. As illustrated in Fig. 2, the gelatin surface is structure-less, while one-dimensional fibrous nano-scaffolds are dominant on the collagen and the collagen/CNT surfaces. Notably, thinner, better spread, flexible and tangled collagen fibrils (5–15 nm in diameter) are frequently seen on the surface of pure collagen, in contrast to the thicker, rigid fibril bundles (35–55 nm in diameter) with less surface coverage in collagen/CNT samples. At high resolution (see insets of Fig. 2b and c), the D-periods of collagen are clearly visible, and were measured at 68 ± 1 nm and 70 ± 1 nm, respectively, on the pure collagen and the collagen/CNT samples. The difference is small yet conclusive according to the statistical analysis (based on more than 30 measurements for each matrix type).

Besides the structural modification, the addition of CNT to collagen altered the matrix stiffness. The stiffness of the three matrices was compared via the elasticity measurement by the nano-indentation method. The Young's Moduli of gelatin, collagen and collagen/CNT were calculated using Eq. 1, and were 0.07 MPa, 0.59 MPa, 1.88 MPa and, respectively, in contrast to ~700 MPa measured on blank tissue culture dishes (see Table 1). Gelatin provides a much

softer surface for cell culture. Apparently, CNT confers additional stiffness to the collagen fibrils. Note that CNTs were not visualized on the collagen/CNT samples, though CNTs can be well resolved on samples from pure CNT preparation. Since both CNT and collagen are one-dimensional materials, and the –COOH functionalized CNTs can strongly interact with the amine and acid groups of collagen via the interactions such as hydrogen bond, electrostatic interaction and the hydrophilic interactions, we infer that the CNTs may be “woven” into the collagen fibers, forming an integrated structure at the molecular level. The increased D-period in collagen/CNT supports such an assumption. Further study of the mechanism of collagen-CNT interaction is undertaken in the lab.

Cell – matrix interaction

Collagen fibril bundles were not visualized in Fig. 1 on the methanol fixed samples. Most likely, the fixation distorted the collagen structures. To understand the cell-matrix interaction, we performed live cell imaging. Figure 3 shows the phase contrast images of live hESCs at Day 3 of differentiation. A region of low cell density was chosen to highlight the relative locations of the cells and the collagen fibers. Consistent with the result of AFM study, we observed pure collagen formed thin, flexible and tangled fibril bundles and were well spread across the substrate. In the collagen/CNT sample, the fibril bundles were thicker with less coverage on the substrate. Coarse collagen alignment is visible on both samples, with a better alignment in collagen/CNT due to its thick and rigid fibril bundles. Consequently, cells on collagen/CNT mostly grew long bipolar filaments along the side of the well-oriented thick fibril bundles; whereas cells on pure collagen grew filaments frequently branching and extending along variable directions. Occasionally, cells were found at the collagen-free regions (e.g., the cell in the yellow circle at the lower portion of Fig. 3b). These cells exhibit random geometry, and differ from those grown at the collagen-rich areas. They barely stain nestin according to the immunofluorescence. Taken together, we have shown that the growth and the development of individual hESCs are remarkably responsive to the structure and the stiffness of matrices, indicating a strong cell-matrix interaction.

Discussions

hESCs can spontaneously differentiate to cells in endoderm, mesoderm and ectoderm lineages. When bio-factors remained the same in the media, our results have shown that the microarchitecture of a cell matrix plays a critical role in controlling the overall growth and differentiation pattern of hESCs. A structure-less and soft gelatin matrix results in the hESC differentiation to cells in all three lineages. A collagen or a collagen/CNT matrix, characterized by one-dimensional fibril structures, causes preferential development of hESCs to elongated cells with long filaments. Both the cell nuclei and the extensions showed coarse alignment when the collagen was pre-aligned (upon dip-coating). The observation is consistent with those by others [4,6], suggesting that the microstructure of a cell matrix can provide the guidance for cell shape development by exerting anisotropic local stresses to the cells to influence the positions in which cells assemble their focal adhesions and the orientation in which they spread. The cells are also sensitive to the difference between collagen and collagen/CNT. The addition of CNTs not only modified the nanostructure of collagen (increase of D-period), but also enhanced the collagen-collagen interaction, leading to the elimination of flexible, thin, tangled fibrils and the development of rigid, long, straight and thick fibril bundles (Fig. 2). Cells were responsive to such matrix properties by growing extremely long, bipolar filaments closely adhered to the collagen/CNT fibril bundles (Fig. 3b).

It is not a surprise that a large majority of hESCs grown on the collagen and the collagen/CNT matrices developed into elongated cells with long filaments, resembling ectodermal cells. Strikingly, the cells on a collagen/CNT matrix expressed nestin at a very high level at as early

as Day 3 in the medium for spontaneous differentiation, suggesting a remarkable polarization in the early differentiation choices toward the ectodermal lineage. To our knowledge, this is the first report of differentiating hESCs to neural progenitor cells in three days in a simple yet efficient way while bypassing the formation of embryoid bodies. Pure collagen also specified the ectodermal lineage at a later stage, evident by the increased level of nestin expression at Day 6. The difference in the rate of ectodermal lineage commitment of hESCs on the collagen and the collagen/CNT matrices is ascribed to their distinctions in both the microstructure and the stiffness. In particular, cells are very sensitive to ECM stiffness, which determines the level of tensional forces in the cell cytoskeleton. These forces, mediated by the transmembrane adhesion proteins, e.g., integrins and cadherins, can substantially affect the expression of the signaling proteins in stem cells [6–8]. Collagen type I is an ECM ligand for integrins. The cytoplasmic tail of these integrins is bound by integrin linked kinase (ILK) [26], a focal adhesion protein that resides next to actins. It was reported that ILK activation is implicated in accumulation of cytoplasmic beta-catenin, thus positively affects the wnt-beta catenin signaling which is essential for neuronal differentiation [27]. We speculate that the increased tensional forces offered by the stiffer collagen/CNT fibrils modulate the stresses at the actin structure, which are then transduced into the wnt-beta catenin pathway via ILK, promoting the neuronal differentiation.

This research explored the use of a collagen/CNT composite material as an *in vitro* cell culture matrix to direct the hESCs' early differentiation to neural progenitor cells at a high yield. In our future work, incorporation of biochemical studies will be pursued to unveil the signal pathway of the matrix regulated differentiation events. A detailed time-dependent study is expected to provide the insight into the differentiation routes of hESCs on various cell culture matrices. The development of methods for quantitative spatial control of collagen/CNT alignment will render the patterned hESC differentiation to ectodermal, and further neural cells, which naturally form well confined nano/micro bio-devices for further applications.

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Figure 1.

Cell morphology and nestin expression of hESCs after cultured in the medium of spontaneous differentiation for three days on various matrices. The cells were fixed by methanol for 5 minutes. (a) Phase-contrast image of a colony of undifferentiated cells; (b–d) Phase-contrast (b) and immunofluorescent images of differentiated cells on gelatin, showing the staining with nestin (c) and the co-staining with nestin and nuclei (d); (e–g) Phase-contrast (e) and immunofluorescent images of differentiated cells on collagen, showing the staining with nestin (f) and the co-staining with nestin and nuclei (g); (h–j) Phase-contrast (h) and immunofluorescent images of differentiated cells on collagen/CNT, showing the staining with nestin (i) and the co-staining with nestin and nuclei (j). The yellow arrows in g and j indicate the coarse alignments of the cells. The amplifications are 4× for (a) and 20× for (b)–(j)

Figure 2. AFM characterization (amplitude images) of the surface structures of gelatin (a), collagen (b) and collagen/CNT (c) matrices prepared under the cell culture conditions. Image size: $8.5 \times 8.5 \mu\text{m}^2$, inset $2 \times 2 \mu\text{m}^2$.

Figure 3. Phase-contrast images of live hESCs cultured on collagen (a) and collagen/CNT (b) for three days in the medium of spontaneous differentiation. The yellow circles refer to cell bodies; the red arrows indicate the variable directions of filament development in (a) and the bipolar filament development in (b). In (a), collagen fibrils are shown as dark, fine, curving structures covering most of the area; several thin, flexible and tangled collagen fibrils are highlighted in cyan in the blue circle. In (b), several rigid, thick, straight collagen/CNT fibrils are highlighted in cyan. The amplification is 10 $\times$.

Table 1

Physical Properties of cell culture matrices characterized by AFM

	Collagen	Collagen-CNT	Gelatin	Subs.
Young's Modulus (MPa)	0.59 ± 0.05	1.88 ± 0.55	0.07 ± 0.01	$700 \pm 1\text{Mpa}$
D-Period (nm)	68 ± 1	70 ± 1		