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Derivation and Culture of Putative Parthenogenetic Embryonic Stem Cells in New Gelatin Substrates Modified with Galactomannan

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Abstract: Human embryonic stem cells (ESC) lines to be used for cell therapies must be created and maintained under strict conditions, excluding the use of undefined supplements. Two key steps in the creation of a new embryonic stem cell line are adherence to the substrate and derivation towards the formation of a primary colony. The bovine parthenote embryo model was used to test different matrices of gelatin nanofibers and gelatin/galactomannan films to be used for ESC derivation and culturing. Gelatin/galactomannan films were made in two concentrations of galactomannan, 0.1 and 0.3%, in an aqueous solution of gelatin and tested for gel cytotoxicity using cumulus cells (CCs). CCs showed normal cell morphology, with no sign of lysis or degeneration in any of the matrices tested. Inner cell masses of parthenote blastocysts (n=116) were placed onto the gel matrices for culture. There were three or four repeats for each matrix. Our results showed a good rate of inner cell mass (ICM) adherence on the gelatin/galactomannan films (41%-44%) and one derivative of the gel nanofiber (17% adherence to the substrate). These results encouraged us to try new gelatin formulations to increase the rates of derivation and cell proliferation under defined culture conditions to comply with good manufacturing practice directives for the potential therapeutic use of ESCs.

Keywords: gelatin, galactomannan, nanofibers, embryonic stem cells.

Introduction

Since the creation of the first human embryonic stem cell line[1] using mouse fibroblast feeder layers for derivation and colony expansion, a great deal of research has concentrated on the development of feeder-free culture conditions using single peptides or polymers matrices and culture media that do not have any undefined component on its formulation. This is an important point to be taken into account, when the embryonic stem cells are to be employed in clinical trials, as it has already been shown that pathogens may be transmitted to the cells from the culture substrate and thus to the patient.[2] In this regard, the development of protocols for derivation and culture of human embryonic stem cell (hESC) lines under defined conditions is a paramount condition for the clinical use of these cells in human therapies.

In Brazil as in several other countries, legal and ethical issues restrict the use of human gametes and embryos for research, which makes it difficult to develop new protocols that need large numbers of embryos for testing and validation. In this scenario, the cattle model is considered the best alternative due to its similarity to human developmental dynamic and easiness to generate from slaughterhouse ovaries and parthenogenetic activation. Reasonable numbers of embryos can be used to test different substrates chemically composed of pure gelatin processed into nanofibers or gelatin plus galactomannan processed into films, thus creating two different three dimensional structures for cell attachment and proliferation. Previous studies have shown the effectiveness of using collagen scaffolds for the culture and differentiation of hESC[3-7] and human somatic stem cells.[8,9] The collagen scaffolds allowed for good cell proliferation in an undifferentiated state and retention of stemcellness. However, none of these previous

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reports mentioned have performed the initial adherence and derivation steps from the embryonic inner cell mass to a primary colony of human ESCs in the collagen/gelatin matrix. Cell attachment to the substrate and initial colony growth are key steps in establishing a new embryonic stem cell line.

It is well-know from the literature that gelatin may be used scaffolds for cell culture.[10-20] Many extracellular matrix (ECM) proteins have been applied as scaffolds for tissue engineering.[10,12] It was found that collagen, the major structural protein present in ECM, is rich in arginine-glycine-aspartic acid (RGD) sequences. One of the most important properties of RGD is to promote cell adhesion and proliferation.[11] It is also known that the RGD sequence refers to particular integrin joint locations of focal adhesion, outside the cell membrane. Similar to collagen, gelatin - a partially hydrolyzed collagen - also possesses the RGD sequences of collagen, making it highly effective for cell adhesion.[21] In addition, gelatin has lower antigenicity than collagen. For this reasons, many cell assays have applied gelatin as an auxiliary component for adhesion, and its uses have been reported as for either coating plates or as scaffolds.[11,12,17,22,23] However, due to the poor thermal stability of gelatin, its use as a scaffold in tissue regeneration presents some limitations.[18,24] In order to improve gelatin properties, many attempts have been made from chemical crosslinking, by blending with other polymers, or physical treatment.[13,16,19,20] However, results were not satisfactory enough to provide a good adhesion and proliferation for different cell lines. Possibly, the reason for that is mostly related to the specific composition and topography of the gelatin scaffolds.[10]

The common carbon source used in cultures of mammalian cell lines is glucose, which is mainly metabolized through the glycolytic pathway producing lactate as by-product.[25] On the other hand, the presence of the by-product lactate disrupts the energy source of the cell. Thus, cells require an additional energy source, usually glutamine or glutamate, which generate ammonium as by-product.[26-28] As an alternative to glucose, mannose has been investigated and was found to considerably impact the levels of UDP-glucosamine and UDP-galactosamine, improving the amount of sialic acid in protein glycosylation, as well as the concentration of viable cells in the culture (25% higher than the cell culture nourished with glucose).[28-31]

In this sense, galactomannans are interesting polysaccharides chemically composed of a linear (1→4)-β-D-mannopyranosyl polymeric backbone to which single α-D-galactopyranosyl residues are attached in a random fashion.[32,33] Galactomannans are considered to provide a specific surface for cell culture.[34] The idea includes the exposure of galactose fractions to cell surfaces, while mannose fractions can act as a carbon source. For some cell types, galactose may provide an adequate growth bed, which encourages the growth of cells in culture, such as the culture of embryonic stem cell-derived hepatocytes.[35] In addition, special properties, such as low charge and hydrofilicity, make galactose based materials a suitable support for cell cultures.[34]

The ECM is not only composed of collagen fibers but many other biomolecules, among them glycoproteins and glycolipids, responsible for attaching specifically to some proteins on the cell surface. For example, proteins called lectins are present in most of human cell surfaces and exhibit various extracellular activities to mediate cell-cell and cell-matrix adhesion and migration by interacting with various glycan groups on cell surface glycoconjugates.[36] One of them, Galectin-1 (gal-1), interacts with various glycoconjugates from the extracellular matrix (*e.g.* laminin, fibronectin, β1 subunit of integrins, ganglioside GM1 and lysosomal membrane-associated proteins lamp 1 and 2); endothelial cells (*e.g.* integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, ROBO4, CD36, and CD13)[37] and on T lymphocytes (*e.g.* CD7, CD43 and CD45) where it is known to induce apoptosis.[38]

Miller and co-workers demonstrated that the commercial α-galactomannan, Davanat, derived from *Cyamopsis tetragonoloba* (commonly known as guar gum) indeed interacts with gal-1 over a relatively large surface area.[39] The interaction region is located primarily on that face of the protein opposite to its canonical lactose binding site, a property that could enhance the cell performance on galactomannan-based scaffolds.

These evidences taken together led us to investigate the performance of galactomannans in cell culture, specially its response on the culture of different cell types. The application of gelatin/galactomannan materials as substrates for the derivation of new stem cells lines - which need special environments, nourishment, and energy sources to proliferate and maintain stemcellness - is an original study, which aims to contribute to the development of a new generation of smart scaffolds for tissue engineering. Therefore, the rationale behind blending gelatin and galactomannan is the production of a new material with enhanced chemical composition to improve the compatibility between cells and substrates. The main purpose of our study was to investigate whether galactomannan modified-gelatin scaffolds are suitable for attachment and derivation of bovine inner cell mass (ICMs) to create putative primary colonies of bovine embryonic stem cells (bESC) as a model for protocols in regenerative medicine.

Experimental

***In vitro* Maturation and Parthenogentic Activation of Bovine Oocytes.** Cumulus-oocyte-complexes (COCs) were aspirated from ovarian follicles (3-6 mm diameter) and placed in groups of 20 for *in vitro* maturation (IVM) in 70 μL medium drops of maturation medium (Medium 199®, Vitrogen; Cravinhos, SP, Brazil), at 38.5 °C, in 5% CO_2 in air for 22 h. COCs with expanded cumulus cells were selected for parthenogenetic activation.

Matured COCs were exposed to M-199 supplemented with hyaluronidase (80 IU/mL) for 60 s and then gently pipetted in medium H199. Denuded oocytes were left for an additional period of 2-4 h in maturation medium, before exposure to

55 μM Ionomycin (Sigma-Aldrich) in buffered H199 medium for 5 min. Oocytes were washed three times in the same medium and transferred to CR4 medium (Vitrogen, Cravinhos, Brazil) supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) in a humidified atmosphere of 5% CO_2 in air at 39 ºC for 3.5 h and before culture in CR4 medium. Observations of embryonic development were performed at 48 h and 196 h post-activation. Activation rates were calculated as the number of 2-cell embryos on day-2 (48 h).

Inner Cell Mass Isolation. Day-9 expanded or hatching parthenogenetic blastocysts were manipulated in their culture dish with a pair of 32 G insulin needles under stereoscope. The blastocysts that have not hatched were gently removed from the zona pelucida by cutting it and pressing the embryo out. The ICM was isolated from the surrounding trophectodermal cells by holding the embryo with one needle and carefully slicing the ICM away with the other one. We were careful to remove as much as possible the trophectodermal cells, without damaging the ICM.

Preparation of Gelatin/Galactomannan Films. Aqueous solutions of gelatin (2%, w/v) (from bovine skin type B; Sigma) were prepared with the addition of galactomannan obtained by exhaustive extraction from *Delonix regia* seeds, at concentrations of 0.1% and 0.3%. Predetermined amounts of each polymer were dissolved in distilled water, separately, under stirring at 45 ºC for 60 min. Then, the solutions were mixed and kept under stirring for 24 h at room temperature. The films were obtained and chemically modified by immersion into 1-(3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC) (Sigma) solution (10 mM). The solutions were casted onto 9 cm glass plate in order to maintain the film thickness. All films were dried at 30 ºC and kept under vacuum until use.

Preparation of Gelatin Nanofibers. The gelatin solution was prepared by dissolving gelatin in trifluoroethanol (TFE) at concentration of 10% (w/v). For electrospinning, a 1 mL-syringe was filled up with the gelatin solution and delivered into a blunted medical needle spinneret (0.7 mm). The fiber mats were collected in an aluminum foil sheet. The distance between the tip of the needle and the aluminum foil was set at 12 cm. An electric potential was controlled at 15 kV. The syringe pump generates a constant flow from the needle and was set to a 0.8 mL/h flow rate. The gelatin fiber mats were obtained and chemically modified by immersion into 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich) solution (10 mM), for 1 h. After that, the fiber mats were rinsed with a sequence of ethanol:water (1:0), ethanol:water (1:1) and only water. The fiber mats were air-dried and then transferred to a desiccator prior to further use.

Morphology of the Gelatin Scaffolds. The films and the nanofibers were placed in a stub and metallized with gold before analyzes. The surface morphology images were performed using a scanning electronic microscopy (SEM) model JSM 6060, operating at an accelerate voltage of 10 keV, at the Center for Electron Microscopy of UFRGS. For the nanofibers, the fiber diameter measurements were obtained from 100 measurements from each three SEM images, using Image J software.

Derivation and Culture of Putative bESC Colonies. The isolated ICMs were placed onto the different matrices cut in 1×1 cm squares and placed at the bottom of a four-well dish (Nunc) with DMEM/F12 knockout medium (Gibco) supplemented with 20% knockout serum replacement (Gibco), glutamine (Gibco), non-essential amino acids (Gibco), penicillin-streptomicin (Sigma), β-mercaptoethanol (Gibco), and bFGF (bovine fibroblast growth factor) 10 ug/mL (BD). Adherence to the substrate was assessed as the dishes were gently swindled 24 h later. The putative primary bESC colonies were maintained in culture for 48 h in a humidified atmosphere of 5% CO_2 in air at 38.5 ºC. Each experiment was repeated three to four times.

Gel Cytotoxicity Test. To investigate the possible adverse effects of the different gel compositions we used fresh cumulus oophorus cells extracted from cumulus-oocyte-complexes (COCs) to perform a visual cytotoxicity assay.[40]

The same gel preparations were placed at the bottom of an empty well of four-well dishes (Nunc™) and covered with TCM199 medium. Cumulus oophorus cells were mechanically isolated from COCs retrieved from bovine ovaries for *in vitro* fertilization experiments. The cells were placed on the surface of the different gels and cultured at 38.5 ºC for 48 h in a 5% CO_2 atmosphere in air. After culture, cumulus cells and substrates were fixed with 4% paraformolaldehyde for 5 min at room temperature (RT). Each gel type was tested in duplicates.

Gels were stained with hematoxilin and eosin for 5 min in each solution. The visual analysis of cell viability was performed on an Olympus BX41 microscope using phase contrast for unstained preparations and optical microscopy for stained specimens. We based our analysis on observable cellular characteristics such as: cell spreading, cell lysis, formation of a confluent monolayer of well-defined cells, presence of intracytoplasmic granules and a well defined heterochromatic interphase nucleus or metaphasic figures. Cell density was not quantified. The gel samples were randomized, and the dishes containing the gels were blinded for analysis.

Statistical Analysis. The rate of ICM adherence to the three different substrates, gelatin nanofibers, gelatin/galactomannan films (concentrations 0,1 and 0,3) were evaluated by the Chi-square test using the Origin Lab program. A level of 5% of significance was used.

Results and Discussion

Morphologic Characterization of the Gelatin Scaffolds. Figure 1 shows the nanofibers morphology and the fiber diameter distribution. Generally, the nanofibrous scaffolds present a high surface area due the large number of porous, either interporosity (space between fibers) as intraporosity (the porosity

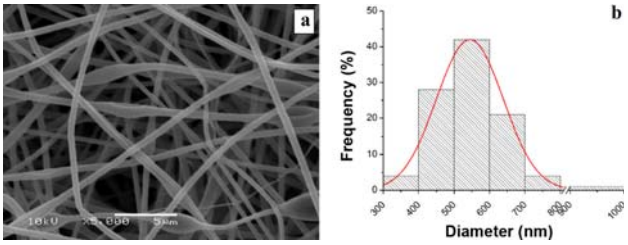

Figure 1. SEM image of gelatin nanofibers (a) and their average diameter distribution (b).

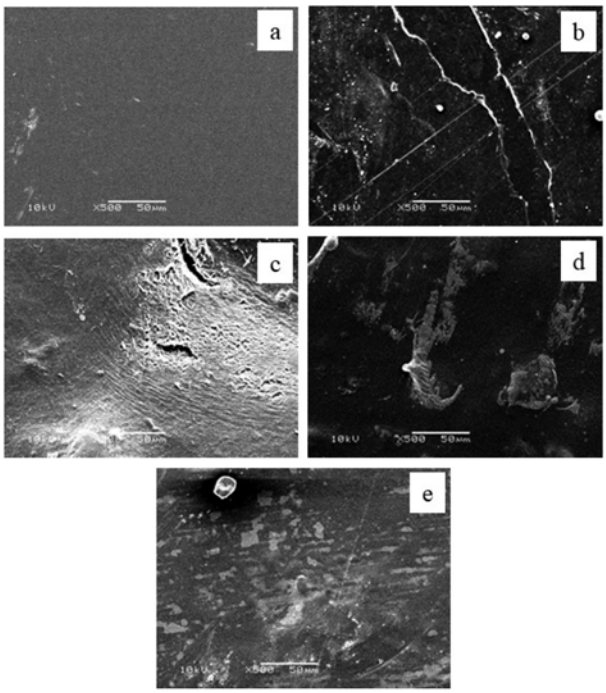

Figure 2. SEM images of film surfaces: (a) gelatin, (b) gelatin/galactomannan (0.1% w/w), (c) gelatin/galactomannan (0.3% w/w), (d) gelatin/galactomannan (0.5% w/w), and (e) galactomannan.

inherent to fibers). The fibers were mostly smooth, containing sporadic erythrocyte-like beads, presenting a homogeneous diameter distribution ranging from 300 to 800 nm and average fiber diameter of 543 nm.

As expected, the films prepared with gelatin/galactomannan showed a different surface area. As can be seen in Figure 2, the surface of all films does not present porosity. The films composed only for gelatin presented a smooth surface (Figure 2(a)), while the blends gelatin/galactomannan, as well as the films composed only of galactomannan, showed the presence of some granulates and a non-linear surface (Figure 2(b), (c), (d) and (e)).

Inner Cell Mass and Cumulus Cell Adherence and Proliferation. Results of ICM adherence and proliferation on the defined matrices with or without galactomannan are presented in Table I. Data clearly show that gels supplemented with galactomannan are better for cell adhesion, presenting

Table I. Results of Adherence and Derivation/Expansion of Embryonic Inner Cell Masses on Gel Nanofibers and Films

Substrate[a]	Number of ICMs	Adherence (Total%)[b]	Adherence Mean ± SD	Expansion
Gel	48	8 (17%)*	5,3 ± 2,3	1 (12.5%)
GG 0.1	36	16 (44%)**	4 ± 1,5	-
GG 0.3	32	13 (41%)**	4,3 ± 4	-

[a]There were three to four different repeats for each matrix. Gel=gelatin nanofibers; GG=gelatin/galactomannan films in two different concentrations of galactomannan. [b]Different superscripts within a column indicate significant differences between groups.

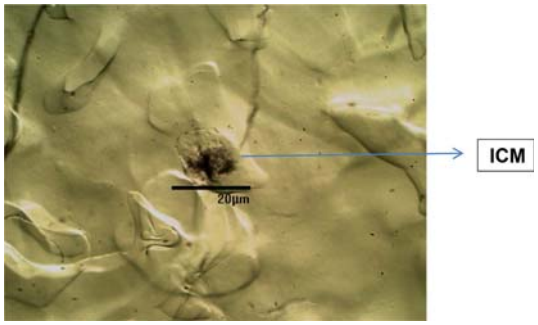

Figure 3. Colony with primary cell expansion on gelatin nanofibers matrix. ICM = inner cell mass.

reasonably good rates (41%-44%) of ICMs attached to the substrate. There was a significant difference between the gel nanofibers and the two galactomannan films regarding ICM adherence to the substrate. No statistical difference was observed between the two galactomannan concentrations regarding ICM attachment rates. However, the only inner cell mass derivation and initial proliferation was observed in a pure gelatin nanofiber substrate (Figure 3). The initial colony spread around the original ICM, but after 48 h of culture it stopped proliferating and degenerated.

Stained gels and the phase contrast observation of matrices cultured for 48 h with cumulus cells revealed two well defined cell patterns: stellate, when cells were well spread indicative of adhesion and migration in the nanofibrous substrate or round typical cumulus cells format in the film matrix (Figure 4). In both cases, normal, round heterochromatic nucleus was present in the vast majority of the cells. We also observed cell aggregates composed of round cells, which may have remained as cell clusters, when they were stripped off from the oocyte and placed onto the substrate. These cell clusters did not show any signs of cell degeneration or lysis. Also, in GG 0.1, gelatin/galactomannan (0.1% w/w), there was a clear continuous cell layer covering most of the gel area similar to the nanofibrous matrix. No clear cell lysis or pyknotic nuclei were observed, suggesting that the gel substrates employed for ICM attachment and derivation were made of non-cytotoxic components.

The present study investigated the use of polymeric matrices,

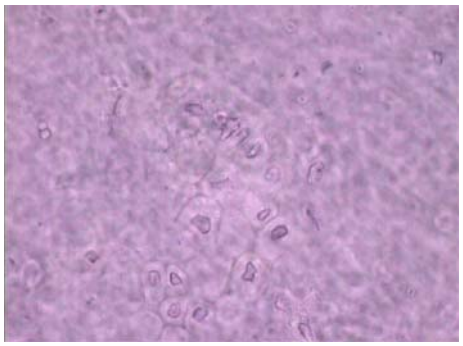

Figure 4. Cumulus cell layer among gelatin fibers after 48 h culture stained with Haematoxylin and Eosin (×400).

the gelatin nanofiber and two film formulations, by biomimicking the extra cellular matrix and cellular substrates commonly used for embryonic stem cells derivation and proliferation.[7,10-20]

Our results showed that 17% of the embryonic inner cell masses exposed to gelatin nanofibers attached to the substrate and one of them presented an initial expansion, which stopped in the first 2 days of culture. In our previous experiments using purified fibronectin as substrate,[41] we obtained 39% of adhesion of parthenogenetic ICMs on fibronectin substrate. Present results employing pure gelatin nanofibers show a significantly lower rate of adherence. On the other hand, the gelatin/galactomannan films presented similar adherence rates (41%-44%) to the former study, in both concentrations tested. In addition there was no significant difference between the two film formulations (GG 0.1 and GG 0.3).

The lack of adherence on the nanofibrous matrix may possibly be due to the lack of a cell recognition molecule, as a RDG sequence present in some extracellular matrix proteins, such as fibronectin.[42] Since its discovery in 1984,[43] RDG sequence has been given a central role in cell adhesion and proliferation. Previous reports describe the use of gelatin and synthetic polymer interface as substrate for the culture of established hESC culture.[3-7,44] These studies used established cell lines and did not perform ICM derivation on the gel matrix, a key step on the creation of a new embryonic cell line under defined conditions. However, it must be pointed out that the one initial colony observed on the gelatin nanofibrous substrate is a new and promising result that suggests that the system has the potentiality to be explored for derivation and embryonic stem cell culture under clinical grade.

Gelatin is a natural biopolymer obtained from the partial hydrolysis of native collagens and it is known that it contains many integrin binding sites for cell adhesion, migration and differentiation similar to the natural collagen. Recently, it´s use in nanofibrous scaffolds has been shown to enhance endothelial cell adhesion and proliferation.[45]

Electrospun nanofibers have a high area-to-volume ratio, which provides good substrates for cell attachment.[15] Thus, gelatin nanofibers potentially represent an ideal 3D substrate for the initial adhesion and cell proliferation, essential steps that happen on derivation of a new primary colony of embryonic stem cells. This may not be a surprising observation as it has already been shown that nanofibrous material accelerates the adhesion and growth of mesenchimal stem cells compared to smooth 2D material.[46] The present series of experiments shows that the addition of galactomannan significantly improved inner cell mass adhesion to the substrate, compared to the gelatin nanofobers alone.

It should be pointed out the fact that in the present study, using similar culture conditions, cumulus cells adhered and proliferated in gelatin films that were not successful for ICM attachment, derivation and ESC colonies formation. These results suggest that in its present form, gelatin matrices may provide the necessary cell signaling for somatic cell adhesion and proliferation, but not the required recognition sites for the pluripotent, specialized ICMs to attach and proliferate generating a new primary colony of ESCs. On the other hand, the fact that we observed one ICM that after attachment to the substrate showed signs of initial colony formation for 2 days is a new promising finding indicating that the choice of these polymers as an artificial scaffold for ESCs derivation and culture under clinical grade manufacturing protocols may be feasible, once the ideal manufacturing conditions for embryonic cells are met. Studies will now focus on new gelatin concentrations and combinations with different molecules, which may enhance cell adhesion and proliferation after adhesion.

Previous reports have already described successful derivation under defined conditions, however, under a close and more critical review, one can observe that most, if not all reported work used one or more undefined component in their ECS derivation or culture system.[7] A recent report on the use of a porous membrane to separate the feeder layer made of human endometrial cell claims successful culture and proliferation of mouse ESCs without any contact with the feeder layer.[47] The authors demonstrate that the direct contact of the ESCs with the substrate is not necessary for their proliferation and they have the benefit of the feeder secretions, claiming that this may be used as a putative system for clinical-grade human embryonic stem cells generation and therapeutic use. However, this is not a defined system, endometrial cells batches may vary so as their secretions which are totally undefined.

To possess clinical value, new materials must be non-toxic to cells surrounding or in contact with them and the culture medium and supplements shall have all its components defined to allow the exclusion of any batch variability or undesired pathogen, which may have an epigenetic effect on the hESCs in culture or under differentiation.

There are several reliable cytotoxicity tests, most of them based on time-consuming colorimetric assays and expensive equipments. We chose to perform a visual analysis of the cells placed on the different substrates based on the study of Bhatia and Yetter, where the authors reported a high degree of correlation between visual cytotoxicity ratings including cell

morphology as rounding, spreading, proliferation and quantitative cell viability measurements such as using a 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[40] The results of our visual investigation of citotoxicity revealed good cell viability in terms of morphology analysis. Cell survival and proliferation seems to have occurred in the three gels tested, which encourage us to continue using them for ICM derivation and stem cell line generation.

Conclusions

In conclusion, our present results show that laboratory engineered gelatin nanofibers are not cytotoxic as embryonic inner cell masses and somatic cumulus cells can successfully be cultured using them as matrix. Inner cell masses show a satisfactory adherence rate, when the gelatin is supplemented with galactomannan, however their expansion and colony formation seems to be inhibited under the present culture conditions. Cumulus cells did not show any apparent decrease in viability or proliferating capacity.

Further studies are underway to improve the gel fiber to mediate embryonic cell adhesion and proliferation for the creation of clinical grade embryonic stem cell lineages.

References

(1) J. A. Thomson, J. Itskovitz-Eldo, S. S. Shapiro, M. A. Waknitz, J. J. Swierqiel, V. S. Marshall, and J. M. Jones, *Science*, **282**, 1145 (1998).
(2) M. J. Martin, A. Muotri, F. Gage, and A. Varki, *Nat. Med,* **11**, 1 (2005).
(3) H. Darr, Y. Mayshar, and N. Benvenisty, *Development*, **133**, 1193 (2006).
(4) K. Gauthaman, J. R. Venugopal, F. C. Yee, G. S. Peh, S. Ramakrishna, and A. Bongso, *J. Cell. Mol. Med.*, **13**, 3475 (2009).
(5) Y. Liu, A. J. Goldberg, J. E. Dennis, G. A. Gronowicz, and L. T. Kuhn, *PLoS ONE*, **7**, e33225 (2012).
(6) D. L. Simpson, N. L. Boyd, S. Kaushal, S. L. Stice, and S. C. Dudley, Jr., *Biotechnol. Bioeng.*, **109**, 274 (2012).
(7) C. M. Crocco, N. Frantz, and A. Bos-Mikich, *JARG.* **30**, 315 (2013).
(8) E. Seyedjafari, M. Soleimani, N. Ghaemi, and M. N. Sarbolouki, *J. Mater. Sci. Mater. Med.*, **22**, 165 (2011).
(9) I. Shabani, V. Haddadi-Asi, M. Soleimani, E. Seyedjafari, F. Babaeijandaghi, and N. Ahmadbeigi, *Tissue Eng. Part A*, **17**, (2001).
(10) V. Beachley and X. Wen, *Prog. Polym. Sci.*, **35**, 868 (2000).
(11) W. T. Godbey and A. Atala, *Ann. N.Y. Acad. Sci.*, **961**, 10 (2002).
(12) R. Cancedda, B. Dozin, P. Giannoni, and R. Quarto, *Matrix Biol.* **22**, 81 (2003).
(13) W. W. Thein-Wan, V. Saikhun, C. Pholpramoo, R. D. K. Misra, and Y. Kitiyanant, *Acta Biomater.*, **5**, 3453 (2009).
(14) K. Gauthaman, J. R. Venugopal, F. C. Yee, G. S. L. Peh, S. Ramakrishna, and A. Bongso, *J. Cell. Mol. Med.*, **13**, 3475 (2009).
(15) J. H. Ko, H. Y. Yin, J. An, D. J. Chung, J.-H. Kins, S. B. Lee, and D. G. Pyun, *Macromol. Res.*, **18**, 137 (2010).
(16) Y. C. Lim, J. Johnson, Z. Fei, Y. Wu, D. F. Farson, J. J. Lannutti, H. W. Choi, and L. J. Lee, *Biotechnol. Bioeng.*, **108**, 116 (2011).
(17) S. Rungsiyanont, N. Dhanesuan, S. Swasdison, and S. Kasugai, *J. Biomater. Appl.*, **22**, 1 (2011).
(18) S.-C. Wu, W.-H. Chang, G.-C. Dong, K.-Y. Chen, Y.-S. Chen, and C.-H. Yao, *J. Bioact. Compat. Polym.*, **26**, 565 (2011).
(19) H. Wang, Y. Feng, H. Zhao, R. Xiao, J. Lu, L. Zhang, and J. Guo, *Macromol. Res.*, **20**, 347 (2012).
(20) H. Wang, Y. Feng, Z. Fang, R. Xiao, W. Yuan, and M. Khan, *Macromol. Res.*, **21**, 860 (2013).
(21) E. Rosellini, C. Cristallini, N. Barbani, G. Vozzi, and P. Giusti, *J. Biomed. Mater. Res. A*, **91**, 447 (2009).
(22) E. Yasuda, Y. Seki, T. Higuchi, F. Nakashima, T. Noda, and H. Kurosawa, *J. Biosci. Bioeng.*, **107**, 442 (2009).
(23) A. Choo and S. K. Lim, in *Embryonic Stem Cell Therapy for Osteo-Degenerative Diseases*, *Methods in Biomolecular Biology*, N. I. Z. Nieden, Ed., Springer Science, New York, 2011, Vol. 690, pp 175-182.
(24) C. Michon, G. Cuvelier, P. Relkin, and B. Launay, *Int. J. Biol. Macromol.*, **20**, 259 (1997).
(25) Y. Tsao, A. Cardoso, R. Condon, M. Voloch, P. Lio, J. Lagos, B. Kearns, and Z. Liu, *J. Biotechnol.*, **118**, 290 (2005).
(26) C. Altamirano, C. Paredes, A. Illanes, J. J. Cairo, and F. Godia, *J. Biotechnol.*, **110**, 171 (2004).
(27) C. Altamirano, A. Illanes, S. Becerra, J. J. Cairo, and F. Godia, *J. Biotechnol.*, **125**, 547 (2006).
(28) J. Berrios, C. Altamirano, N. Osses, and R. Gonzales, *Chem. Eng. Sci.*, **66**, 2431 (2011).
(29) T. Ryll, U. Valley, and R. Wagner, *Biotechnol. Bioeng.*, **44**, 184 (1994).
(30) X. Gu and D. I. C. Wang, *Biotechnol. Bioeng.*, **58**, 643 (1998).
(31) B. Follstad, U.S. Patent No.2004/00810 (2004).
(32) V. D. Shcherbukhin and O. V. Anulov, *Appl. Biochem. Microbiol.*, **35**, 229 (1999).
(33) V. D. Prajapati, G. K. Jani, N. G. Moradiya, N. P. Randeria, B. J. Nagar, N. N. Naikwadi, and B. C. Variya, *Int. J. Biol. Macromol.*, **60**, 83(2013).
(34) F. E. Noll, W. Senaratne, and Y. Wei, U.S. Patent No. 2008/0220524 A1 (2008).
(35) M. Qingyuan, A. Haque, B. Hexig, and T. Akaike, *Biomaterials*, **33**, 1414 (2012).
(36) F. T. Liu and G. A. Rabinovich, *Nat. Rev. Cancer*, **5**, 29 (2005).
(37) D. Neri and R. Bicknell, *Nat. Rev. Cancer*, **5**, 436 (2005).
(38) N. L. Perillo, C. H. Uittenbogaart, J. T. Nguyen, and L. G. Baum, *J. Exp. Med.*, **185**, 1851 (1997).
(39) M. C. Miller, A. A. Klyosov, and K. H. Mayo, *Glycobiology*, **19**, 1034 (2009).
(40) S. K. Bhatia and A. B. Yetter. *Cell Biol. Toxicol.*, **24**, 315 (2008).
(41) R. R. Ruggeri, Y. Watanabe, F. Meirelles, F. F. Bressan, N. Frantz, and A. Bos-Mikich, *J. Assist. Reprod. Genet.*, **29**, 1039 (2012).
(42) E. Ruoslahti, *Annu. Rev. Cell Dev. Biol.*, **12**, 697 (1996).
(43) M. D. Pierschbacher and E. Ruoslahti, *Nature,* **309**, 30 (1984).
(44) E. F. Irwin, R. Gupta, D. C. Dashti, and K. E. Healy, *Biomaterials*, **32**, 6912 (2011).
(45) H. Hajiali, S. Shahgasempour, M. R. Naimi-Jamal, and H. Peirovi, *Int. J. Nanomedicine*, **6**, 2133 (2011).
(46) A. Finne-Wistrand, A. C. Albertsson, O. H. Kwon, N. Kawazoe, G. Chen, I. K. Kang, H. Hasuda, J. Gong, and Y. Ito, *Macromol. Biosci.*, **8**, 951 (2008).
(47) N. Desai, J. Ludgin, J. Goldberg, and T. Falcone, *J. Assist. Reprod. Genet.*, **30**, 609 (2013).