Stem cells in tissue engineering – dynamic cultivation requirement

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SUMMARY
Stem cells have shown great potential for in vitro tissue engineering, regenerative medicine, cell therapy and pharmaceutical applications. All these applications, especially in clinical trials, will require guided production of high-quality cells. Traditional culture techniques and applications have been performed for the majority of primary and established cell lines and standardized for various analyses. Still, these culture conditions are unable to mimic dynamic and specialized three-dimensional microenvironment of the stem cells’ niche from in vivo conditions. In an attempt to provide biomimetic microenvironments for stem cells in vitro growth, three-dimensional culture techniques have been developed. In our study advantages of newly developed porous scaffolds as the most promising in vitro imitation of niche that provides physical support, enables cell growth, regeneration and neovascularization, while they are replaced in time with newly created tissue was explained. Furthermore, dynamic cultivation techniques have been described, as new way of cell culturing that will be the main subject of our future research. In that manner, by developing an optimal dynamic culturing method, high-quality new cells and tissues would be possible to obtain, for any future clinical application.

Keywords: stem cells; culture technique; scaffolds; ALBO-OS; bioreactor

INTRODUCTION
Stem cells (SCs) have the ability to self-renew and differentiate into mature types of cells that develop all organs and tissues in human body. There are two major categories of SCs defined by their origin and potency - embryonic stem cells, and adult, mesenchymal stem cells [1]. Embryonic stem cells (ESCs) are pluripotent, capable of unlimited self-renewal and differentiation into any type of cell in the body. Mesenchymal stem cells (MSCs) are isolated from adult sources such as bone marrow, adipose, and dental tissue. MSCs are multipotent cells that can differentiate into a limited number of cell types. Also, self-renewal and differentiation potential is dependent on the tissue they are isolated from, and age of a donor. The advantages are accessibility and less ethical concerns for their usage [2, 3]. SCs have shown great potential for in vitro tissue engineering, regenerative medicine, cell therapy and pharmaceutical applications. All these applications, especially in clinical trials, will require a guided production of high-quality cells [4].

TRADITIONAL CULTURE TECHNIQUES
SCs are propagated as a monolayer in two-dimensional (2-D) plastic culture plates. 2-D culture techniques and applications have been practiced for the majority of primary and established cell lines and standardized for various analyses, from isolation and characterization of cells, to the studies of disease development and drug testing [5]. To grow cells on plastic culture dishes, ESCs have to be seeded on precoated surface to aid in attachment. 2-D expansion of ESCs has been improved by applying defined and xenogenic-free culture media and attachment substrates. However, uniform expansion of ESCs is still difficult to achieve as 2-D culture methods for propagation of ESCs are challenging, expensive and require high level of expertise [5, 6]. Differentiation of ESCs has been achieved by using specific induction media into ectodermal, mesodermal, and endodermal lineages. An important advantage of monolayer culture is controlled differentiation of human ESCs that allows uniform treatment for differentiation of cells. However, differentiation in monolayer culture often results in mixed populations of differentiated cells [4, 5, 7].

Unlike embryonal, MSCs have natural ability to adhere to plastic and glass surfaces. Xenogenic substrates are not necessitated for attachment, although they are usually cultured in the media containing animal serum. Use of animal-derived media can potentially transmit pathogens and limit reproducibility between cultures. Recently, xenogenic-free media has become available for cultivation of MSCs [8]. Expansion of MSCs in monolayer has its limitations. Monolayer culture needs routine passaging to maintain self-renewal and potency of cells, which is
highly inefficient for large-scale expansion of cells. Maintenance of uniform distribution, growth, and harvesting processes is needed and consequently, heterogeneity is minimized and cell yield is high. Phenotypic changes occur in MSCs while culturing in monolayers, and cells’ fate and differentiation potential are altered after numerous passages. Despite the limitations, 2-D culture has been used for differentiation of MSCs into many specialized cells, including chondrocytes, osteocytes, adipocytes, cardiomyocytes, smooth muscle cells, and hepatocytes, by using cell-specific differentiation media. Assessment of differentiation stages is commonly done by specific transcriptional gene expression and extracellular matrix (ECM) depositions. The main downside of monolayer differentiation is a lack of providing functionally competent cells. They often differentiate into precursor-like cells, suggesting the possibility to re-differentiate during extended culturing. Prior to clinical application, modification of differentiation protocols should be made. Overall, 2-D culture conditions are unable to mimic dynamic and specialized three-dimensional (3-D) microenvironment of the SC niche from in vivo conditions.

THREE-DIMENSIONAL CULTURE TECHNIQUES

In an attempt to provide biomimetic microenvironments for stem cells in vitro growth, 3-D culture techniques have been developed. These methods have a common goal to mimic the ECM composition and stiffness of SC niche in vitro. Challenge lies in protocol optimization depending on cell type and the aim of analysis. Therefore, the uniform expansion of SCs without loss of genetic stability or differentiation potential has to remain regardless of applied technique.

Static three-dimensional culture

1. Spheroids

Formation of spheroids that consist of cell aggregates and allow cell interactions in the absence of additional substrates is one of the simplest 3-D culturing method. A wide range of adherent cell types have the ability to form spheroids by spontaneous cell aggregation when they are seeded in low-adhesion culture plates in suspension culture, in a form of a hanging drop, or in rotating culture. MSCs are successfully maintained and expanded by spheroid method where they exhibited increased clonal growth and multipotency, and activation of pluripotency genes. However, as in monolayer cultivation, long-term culture of MSCs in spheroids spontaneously led to differentiation. In comparison with monolayer culture, MSCs grown in 3-D spheroids have shown increased chondrogenic and osteogenic differentiation in vitro. In particular, chondrogenic differentiation of MSCs has been shown as more effective in high-density cell culture methods utilizing pellet culture or spheroids, in comparison to 2-D culture. Still, this method is not applicable for cell expansion due to the inability to control aggregate size, leading to agglomeration, apoptosis, and inhibition of cell proliferation. Depending on tissue that cells are isolated from, spheroids could consist of heterogeneous population of cells, with different proliferative capacity. Even more important drawback is limited diffusion of oxygen and nutrients in the center of the spheroid, which gradually leads to a hypoxic environment, and at the end, formation of necrotic center. Due to these drawbacks, spheroids have been more successfully employed in study of 3-D cell structures, cell differentiation and cancer biology rather than homogenous cell proliferation and production of high-quality uniform cell cultures.

2. Scaffolds

Various natural and synthetic biocompatible and biodegradable materials have been used to mimic the biochemical and biophysical properties of SC niches that stimulates cell proliferation and/or differentiation. Natural biomaterials (agarose, fibronectin, hyaluronic acid, chitosan) in vitro often transduce biological signals to cells. As a result, biomaterials can aid in cell maintenance and differentiation. However, problems such as variability and the potential of xenogenic media components to cause disease limit their use. Synthetic polymers (polyethylene glycol, poly-l-lysine, poly-lactic acid, poly-glycolic acid, and poly-dl-lactic acid-co-glycolic acid) are the group of artificially made scaffolds with different mechanical properties (pore size, elasticity, adhesion, tensile strength). Biodegradable, porous scaffolds represent the most promising in vitro imitation of SC niche that provides physical support, enables cell growth, regeneration and neovascularization, while they were replaced in time with new bone. Innovative scaffold construction that consists of ceramic part mimicking bone structure, and thin polymer layer above that contribute its’ better mechanical properties and biocompatibility, showed to be very promising biomaterial for bone tissue engineering. Scaffolds are commonly used as carriers that mimic ECM, promote expansion, migration, and differentiation of SC. For SC culturing, scaffolds can be prefabricated, afterward, cells are seeded onto the scaffold, and allowed to migrate and proliferate. Scaffolds can incorporate growth factors and cytokines, and provide mechanical stimulation for SC differentiation. On the other side, there are scaffolds with self-assemble encapsulating cells that are incorporated in biomaterial at the time of its fabrication. Due to these advantages, prefabricated scaffolds are commonly used for seeding SCs prior to their differentiation and usage in tissue engineering. We recently reported successful application of composite scaffold, combination of calcium hydroxyapatite and poly (lactic-co-glycolic) acid, named ALBO-OS, as a bone substitute for bone tissue engineering. Scaffold with very high porosity and nanotopology showed to be very suitable for cell adhesion and proliferation, providing larger surface area, allowed better adhesion and provided more area for differentiation of MSCs. Furthermore, it has been shown the formation of new mineralized matrix, osteoconductive and certain
osteoinductive effect (Figure 3). Compressive strength of ALBO-OS showed to be a good mechanical support during the whole period of its transformation into new bone [21, 24, 25].

It has been shown that scaffold mechanical properties stimulate differentiation of MSCs into various lineages. Softer substrates induced MSC differentiation into neural and beta islet cells, chondrocytes and adipocytes. On the other hand, increase in substrate stiffness supported MSC differentiation into myoblasts and osteoblasts. Compressive forces mimicking joint action via mechanotransductive scaffold increased chondrogenic gene expression in MSCs [27, 28]. Since the composition and mechanical properties of biomaterials guide MSCs differentiation into specific cell lineages, it is important to be optimised and easily producible. Cell-cell and cell-ECM interactions in static 3-D culture during maintenance and expansion of...
MSCs can provide insight on basic cell biology processes and mechanisms. The main challenge of 3-D static cultivation remains extensive cell expansion in individual scaffold constructs. By increasing in size of scaffold, cell growth in the center could be compromised in a similar way as in spheroids. Flow of oxygen, nutrients, and waste in whole scaffold space might be limited [4]. Methods that could provide 3-D cell growth and expansion without nutritional drawbacks are the subject of current investigations.

**Dynamic three-dimensional culture**

Bioreactors are used to enable, monitor, and control biological processes. In tissue engineering, they enable culturing high densities of cells, allow cell growth and proliferation, and minimise variability observed in traditional culturing. The new way of cell culturing requires defined and well-regulated conditions regarding temperature, oxygen and nutrients concentration, pH, as well as metabolites removal. Various types of 3-D culture techniques provide effective cell proliferation and differentiation [29]. There are differences in techniques used to develop bioreactor systems that affect their application.

Several bioreactor designs have been developed during recent years that can be used for expansion and guided differentiation from a single cell to tissue culture. Spinner flasks are one of the simplest and most convenient bioreactor systems. They operate by using local hydrodynamic forces that create shear stress on cells. In the center of the system is cell/scaffold assemble, while magnetic bar provides a constant flow of medium around scaffold. The system is placed in an incubator with controlled conditions. The degree of shear stress is defined by stirring speed. The downsides represent possibility of dense layer formation of cells on surface that could compromise nutrition of cells in the center, and unequal shear stress, with the highest level at the bottom of the flask [30]. Rotating bioreactors were designed for improved control of shear stress by horizontal rotation and simulation of microgravity. In this way limitations regarding the equal flow of nutrients and waste products are reduced [31]. The awareness of limitations of diffusion systems based on rotation led to development of perfusion bioreactors. Flow perfusion systems allow constant exchange of nutrients and waste, providing better control of culture parameters. They enable transport of nutrients and oxygen through the entire scaffold, while media must be changed at regular intervals [32]. Lastly, mechanical force bioreactors have been developed to mimic tissue physiology, by using direct mechanical strain such as compressive and tensile forces. Mechanical stimulation could be in the form of bending, stretching, contraction and compression. It has been shown that direct mechanical stimulation induced proliferation, osteogenic differentiation, and formation of ECM. A drawback could be diffusional limitations in larger constructs [33].

Future prospective research should include culturing of MSCs on porous scaffolds as promising tool for cells expansion and differentiation, in dynamic constructions, as flow perfusion systems, that would provide homogenous culturing conditions. In that manner, high-quality new cells and tissues would be possible to obtain regardless of scaffold size, which represents qualitative foundation for any clinical research.

**CONCLUSION**

Biomedical applications of stem cells require production of high number of uniform cells that nowadays exceed millions of cells produced by traditional culturing techniques. Recent studies have shown that transplantation of higher cell concentration had better outcome. Billions to trillions of cells will be required in clinical trials involving cell therapy. Advancements in the developing xenogenic-free media, culturing techniques and devices are of great importance. Large scale of cell culturing demands collaboration between biomedical researchers and engineers, to develop an optimal dynamic culturing method that will provide therapeutic use of stem cells.

**REFERENCES**

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282(5391):1145–7. [DOI: 10.1126/science.282.5391.1145] [PMID: 9804556]
2. Chen KC, Mallon BS, McKay RD, Robey PG. Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. Cell Stem Cell. 2014; 14(1):13–26. [DOI: 10.1016/j.stem.2013.12.005] [PMID: 24388173]
3. Ćetenović B, Čolović B, Vasilijić S, Pašalić S, Jokanović V, Marković D. In Vitro Biocompatibility of Nanostructured Endodontic Materials Using SCAP Cells. Balk J Dent Med. 2017; 21(3):167–70. [DOI: 10.1515/bjdm-2017-0029]
4. McKee C, Chaudhry GR. Advances and challenges in stem cell culture. Colloids Surf B Interfaces. 2017; 159:62–77. [DOI: 10.1016/j.colsurfb.2017.07.051] [PMID: 28780462]
5. Burdick JA, Vunjak-Novakovic G. Engineered microenvironments for controlled stem cell differentiation. Tissue Eng Part A. 2009; 15(2):205–19. [DOI: 10.1089/teng.2008.0131] [PMID: 18694293]
6. Gušak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell. 2009; 5(1):17–26. [DOI: 10.1016/j.stem.2009.06.016] [PMID: 19570510]
7. Pineda ET, Nerem RM, Ahsan T. Differentiation patterns of embryonic stem cells in two-versus three-dimensional culture. Cells Tissues Organs. 2013; 197(5):399–410. [DOI: 10.1159/000346166] [PMID: 23406658]
8. Santos FD, Andrade PZ, Abecasis MM, Gimble JM, Chase LG, Campbell AM, et al. Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions. Tissue Eng Part C Methods. 2011; 17(12):1201–10. [DOI: 10.1089/ten.tec.2011.0255] [PMID: 21895491]
9. Hanley PJ, Mei Z, da Graca Cabreira-Hansen M, Klis M, Li W, Zhao Y, et al. Manufacturing mesenchymal stromal cells for phase I clinical trials. Cytotherapy. 2013; 15(4):416–22. [DOI: 10.1016/j.jcyt.2012.09.007] [PMID: 23489051]
10. Friedenstein AJ, Chalákhyan RK, Latsnik NV, Panasyuk AF, Keilis-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation. 1974; 17(4):331–40. [PMID: 4150881]
11. Erickson GR, Gimble JM, Franklin DW, Rice HE, Awad H, Gušak F. Chondrogenic potential of adipose tissue-derived stromal
Matične ćelije u tkivnom inženjerstvu – potreba za dinamičnom kultivacijom

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UVOD

Matične ćelije (MĆ) imaju sposobnost samoobnavljanja i usmerravanja u pravcu zrelih tipova ćelija koje grade sva tkiva i organje ljudskog organizma. Postoje dve osnovne grupe MĆ definisane po poreklu i potencnosti – embrionalne matične ćelije i zrele, mezenhimalne matične ćelije [1]. Embionalne matične ćelije (EMĆ) jesu pluripotentne, sa sposobnošću neograničenog samoobnavljanja i usmerravanja u pravcu svih tipova ćelija prisutnih u organizmu. Mezenhimalne matične ćelije (MMĆ) izolovane su iz zrelih tipova tkiva, kao što su koštana srž, mala, mezodermalno i endodermalnoj lozi. Važna prednost MMĆ [8]. Pri gajenju MMĆ u 2-D uslovima javljaju se određena prilike načina gajenja ćelija koje omogućuje podjednaku izloženost ćelije medijumu. Ipak, ovakvo usmerravanje često za rezultat ima mešovitu populaciju zrelih ćelija i tkiva visokog kvaliteta za svaki klinički priliku.

Ključne reči: matične ćelije; tehnike gajenja; nosači; ALBO-Os; bioreaktor

TRADICIONALNE TEHNIKE GAJENJA ĆELIJA

MČ se uzgaju u vidu jednog sloja ćelija, u dve dimenzije (2-D), na plastičnim podlogama ploča i boca namenjenih uzgajanju ćelija. Gajenje ćelija u 2-D uslovima primenjeno je na većini primarnih ćelijalnih kultura i ćelijalnih linija, i standardizovano za različite analize, od izolacije i karakterizacije ćelija do istraživanja razvoja oboljenja i testiranja lekova [2, 3]. Da bi se uzgajale na plastičnim podlogama, MĆ se moraju zaseći na prethodno pripremljene površine kako bi se olakšalo vezivanje ćelija za podlogu. Gajenje EMĆ u 2-D uslovima poboljšano je primenom određenih hraniljivih medijuma bez prisustva proteina životinjskog porekla i dodataka za poboljšanje vezivanja.
One često diferentuju u prekursorne čelije, te postoji mogućnost da tokom vremena dediferentuju [4]. Pre kliničke primene neophodno je izvršiti izmene u protokolima za usmeravanje čelija. Uopšteno, 2-D uslovi gajenja čelija nisu u mogućnosti da imitiraju dinamičnu i specijalizovanu trodimenzionalnu (3-D) mikrosredinu niše u kojoj rastu MČ u in vivo uslovima.

TRODIMENZIONALNE TEHNIKE GAJENJA ĆELIJA

U pokušaju stvaranja mikrosredine u kojoj se imitiraju biološki uslovi rasta MČ u in vitro uslovima, razvijene su 3-D tehnike gajenja čelija. Zajednički cilj ovih metoda je da se smiješavaju sferne i specijalizovane za usmeravanje i kontrolu čelijama varijabilnosti u nizima i mogućnosti za usmeravanje čelijske niše koje imitiraju trodimenzionalne uslove u kojima je omogućen autorregulativni pristup mehanici fizičke stimulacije [11].

Statična trodimenzionalna kultura

1. Sfere

Sfere su jedan od osnovnih 3-D metoda gajenja čelija i predstavljaju akumulaciju čelija u kojima je omogućena međučelijska interakcija u osnovnom jednom slojku. Čelije u sfere se mogu zasejati u homogenim populacijama i stvaraju visokokvalitetne i uniformne čelijske komponente. Zbog ovih prednosti sfere se često primenjuju za imitaciju trodimenzionalne biokompatibilnosti [12].

2. Nosači

Različiti prirodni i veštački biomaterijali i biorazgradivi materijali se primenjuju za imitaciju biokompatibilnosti [12]. Strogo definisana potencijalna efikasnost trodimenzionalnih metoda gajenja čelija za usmeravanje, koncentraciju i proizvodnju određenih biokomponenti i biokomponenta čelija. Ovaj metod je najbolje definisana za njegovu primenu u statičkim 3-D uslovima [13].

Za razliku od drugih metoda, sfere su jedna od osnovnih 3-D metoda gajenja čelija i predstavljaju akumulaciju čelija u homogenim populacijama i stvaraju visokokvalitetne i uniformne čelijske komponente. Zbog ovih prednosti sfere se često primenjuju za imitaciju trodimenzionalne biokompatibilnosti [12].

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Stomatološki glasnik Srbije. 2018;65(1):37-44
Dinamična trodimenzionalna kultura

Bioreaktori omogućuju odvijanje, nadzor i kontrolu bioloških procesa. U tkivnom inženjerstvu omogućavaju uzgajanje velike gustine ćelija, rast i umnožavanje ćelija i umanjuju raznolikost prisutnu u statičnim uslovima uzgajanja ćelija. Novi način uzgajanja ćelija traži definisane i dobro kontrolisane uslove temperature, koncentracije kiseonika i hranljivih materija, pH sredine, kao i uklanjanje produkata metabolizma. Različite vrste 3-D tehnika uzgajanja ćelija obezbeđuju dobro umnožavanje i usmeravanje MČ ka zrelih ćelijama [29]. Različite tehnike koje su primenjene u razvoju sistema bioreaktora određuju njihovu namenu.

U proteklim nekoliko godina razvijeno je više tipova bioreaktora namenjenih umnožavanju i vođenju diferencijacije od pojedinačne ćelije do uzgajanja tkiva. Rotirajuće boce za gajenje ćelija su najjednostavniji i najpraktičniji sistem bioreaktora. Lokalne hidrodinamske sile predstavljaju osnovu sistema i na ćelije deluju silama smicanja. U središtu sistema se nalazi nosač sa ćelijama, dok magnetna mešalica omogućuje stalni protok medijuma oko nosača. Sistem je smešten u inkubatoru sa kontrolisanim uslovima sredine. Vrednost sila smicanja definisana je brzinom mešanja medijuma. Nedostaci ove metode sadržani su u mogućnosti stvaranja gustog sloja ćelija na površini, koje mogu ugroziti ishranu ćelija u dubljim strukturama nosača, kao i stvaranja nejednakih sila smicanja, uz najveće vrednosti na dnu boce u kojoj se ćelije gaje [30]. Potencijalni nedostatak može predstavljati ograničenje u protoku medijuma kod većih nosača [33].

Buduća prospektivna istraživanja bi trebalo da uključe gajenje MMČ na poroznim nosačima kao najpogodnijim strukturama za umnožavanje i usmeravanje ćelija u dinamičnim sistemima, kao što je bioreaktor sa perfuzijom, kako bi se omogućili homogeni uslovi gajenja. U tom smislu, biće omogućen razvoj visokokvalitetnih novih ćelija i tkiva nezavisno od veličine nosača, što predstavlja kvalitetnu osnovu za sva buduća klinička istraživanja.

ZAKLJUČAK

Primena matičnih ćelija u biomedicini zahteva gajenje velikog broja uniformnih ćelija, koji premašuje milione ćelija koje je danas moguće gajiti tradicionalnim tehnikama. Nedavne studije su pokazale da transplantacija velikog broja ćelija daje bolji ishod lečenja. Kliničke studije koje će uključiti ćelijsku terapiju će zahtevati milijarde i triljone novih ćelija. Napredak u primeni medijuma bez proteina životinjskog porekla, tehnikama uzgajanja i opremi su od velike važnosti. Gajenje velikog broja ćelija zahteva saradnju istraživača iz oblasti biomedicine i inženjerstva, kako bi se razvio optimalni dinamični metod gajenja ćelija koji će omogućiti sprovođenje ćelijske terapije.