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Mimicking Stem Cell Niches to Increase Stem Cell Expansion

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Summary

Niches regulate lineage-specific stem cell self-renewal vs. differentiation *in vivo* and are comprised of supportive cells and extracellular matrix components arranged in a 3-dimensional topography of controlled stiffness in the presence of oxygen and growth factor gradients. Mimicking stem cell niches in a defined manner will facilitate production of the large numbers of stem cells needed to realize the promise of regenerative medicine and gene therapy. Progress has been made in mimicking components of the niche. Immobilizing cell-associated Notch ligands increased the self-renewal of hematopoietic (blood) stem cells. Culture on a fibrous scaffold that mimics basement membrane texture increased the expansion of hematopoietic and embryonic stem cells. Finally, researchers have created intricate patterns of cell-binding domains and complex oxygen gradients.

Introduction

Stem cells have widespread potential for regenerative medicine and the treatment of genetic disorders and cancer [1]. Although stem cell therapies are moving steadily towards the clinic, it has proven very difficult to increase tissue-specific stem cell numbers *ex vivo* without loss of stem cell potential. This contrasts with extensive self-renewal *in vivo* for the lifetime of an individual. It is now generally accepted that adult stem cells reside in specialized niches that coordinate self-renewal vs. differentiation [2–4]. This has led to the hypothesis that mimicking the stem cell niche will facilitate stem cell self-renewal and controlled differentiation *ex vivo*.

The various tissue-specific stem cell niches share many similar features. Heterologous (hub or stromal) cells provide critical cell-cell contacts and paracrine signaling, and a number of signaling molecules have been highly conserved from invertebrates to humans [2,3]. The extracellular matrix (ECM) retains stem cells in the niche and also serves to initiate signal transduction events – either alone or in synergy with cytokines. Further adding to the complexity, glycosaminoglycans (GAGs) locally concentrate and present soluble growth factors. The niche established by supportive cells and the ECM likely regulates stem cell fate via complementary mechanisms including the presentation of immobilized signaling molecules in a defined manner, the modulation of matrix stiffness, and the creation of cytokine gradients (Figure 1). The physiochemical environment, including oxygen tension (pO₂) and pH, also contributes to the regulation of stem cell replication and differentiation. In contrast to tissue-specific stem cells, embryonic stem cells (ESCs) are present only transiently in the

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developing embryo and therefore do not have a stable niche *in vivo*. ESCs also differ from tissue-specific stem cells in that they can be readily expanded in culture over extensive time periods. However, the culture systems that have been successfully used for ESC expansion suggest that ESC self-renewal vs. differentiation is regulated in a similar manner via interactions with other cells, ECM components, soluble factors and the physicochemical environment [5].

The complexity of the stem cell niche is challenging to reproduce. However, a number of microscale technologies have been developed for tissue engineering [6] and a wide range of natural and synthetic materials have been successfully used for stem cell culture [7,8]. In this review we focus on recent efforts to mimic cell-cell and cell-matrix interactions representative of the stem cell niche – with an emphasis on systems that presented ligands in a defined manner and that modulated stem cell self-renewal or differentiation (Table 1).

Cell-cell interactions

Direct interactions between stem cells and supporting cells modulate stem cell retention and regulation. The essential role of cadherins in stem cell retention is illustrated by the observation that differentiation-defective germline stem cells displaced normal stem cells from their niche in the *Drosophila* ovary by upregulating E-cadherin expression [9]. Nagaoka *et al.* immobilized mouse E-cadherin as a fusion protein with the Fc domain of mouse IgG1 (E-cad-Fc) and showed that undifferentiated embryonic carcinoma cell lines, which form compact colonies on collagen or fibronectin, scatter on E-cad-Fc and display a spindle-shaped morphology with E-cadherin-rich protrusions [*10]. In contrast, mammary and kidney epithelial cell lines and primary hepatocytes formed tight colonies on E-cad-Fc, which suggests that the mechanisms of E-cadherin-mediated adhesion and migration vary with the state of cell differentiation [*10].

Juxtacrine activation of Notch signaling via cell-presented ligands has been implicated in many different stem cell niches [3,4]. Two recent studies suggest that inclusion of immobilized Notch ligand selectively increases the self-renewal of hematopoietic stem cells (HSCs) in cultures with cytokine cocktails optimized for primitive cell expansion. Suzuki *et al.* showed that coating plates with a fusion protein of Delta1 and the Fc domain of human IgG1 (Delta1-Fc) did not increase the overall expansion of human cord blood (CB) CD133⁺ cells or the production of CD133⁺CD34⁺CD38[−] cells during 3-week cultures, but approximately doubled the production of multi-potent progenitor cells (CFU-Mix) [*11]. Culture on Delta1-Fc doubled the number of cells able to reconstitute multi-lineage human hematopoiesis in immunodeficient mice, and bone marrow cells collected from primary recipients supported engraftment of human cells in two of three secondary transplant recipients [*11]. In a similar manner, Jagged1 immobilized on Sepharose beads had little effect on the *ex vivo* expansion of lineage negative (Lin[−]) mouse bone marrow cells during two weeks in culture, but greatly increased the production of male Lin[−] cells able to generate hematopoietic cell chimerism in secondary female transplant recipients [12]. In the presence of epithelial differentiation-inducing growth factors, immobilized rat Jagged1-Fc (via adsorbed protein G or bound anti-Fc antibody), but not soluble Jagged1, greatly enhanced Notch/CBF-1 signaling and differentiation of rat primary esophageal epithelial stem cells [13].

Immobilized growth factors

Many cytokines, including stem cell factor (SCF) and leukemia inhibitory factor (LIF), are presented in a membrane-bound form by supportive cells in the stem cell niche. Further, a large number of growth factors may be sequestered by ECM components including proteoglycans. Diffusible growth factors can also be immobilized in an active conformation. Covalent linkage of LIF to polyester fiber mesh or surfaces coated with poly(octadecene-*alt*-maleic anhydride) was shown to support expansion of murine ESCs with retention of ESC markers [14,15].

Covalent binding of fibroblast growth factor (FGF)-2 to polyamide nanofibrillar surfaces inhibited the rapid degradation of FGF-2 in solution and supported expansion and colony formation of human ESCs [16]. Strong noncovalent interactions can also be used to immobilize cytokines in an active form, as illustrated by the attachment of biotinylated SCF to biotinylated polyethylene glycol (PEG) chains via a NeutrAvidin linker [17] and attachment of a fusion protein of vascular endothelial growth factor (VEGF)₁₂₁ and the collagen-binding domain of fibronectin to surfaces coated with gelatin or collagen [18]. Iwata and colleagues developed a multi-domain chimeric protein that contains epidermal growth factor (EGF), ciliary neurotrophic factor (CNTF), a globular capping domain that blocks CNTF activity (linked via a thrombin cleavage sequence), and a decahistidine linker that binds to surface-immobilized Ni(II) ions [19]. Rat neural stem cells (NSCs) cultured on surfaces containing the full chimeric protein expanded in number and maintained 85% undifferentiated cells. However, ca. 70% of the cells differentiated into astrocytes on surfaces treated with thrombin to expose the CNTF domain [19].

Cell-ECM interactions

Fibronectin effects on stem cells have been extensively studied. Covalently linking fibronectin to aminated polyethylene terephthalate (PET) film supported 10-fold greater expansion of CB CD34⁺ cells than tissue culture polystyrene (TCPS) or unmodified PET [20]. Fibronectin has multiple cell-binding domains that may be differentially displayed when fibronectin is adsorbed. Fibronectin fragments and peptide mimics, such as those containing RGD (ligand for $\alpha 5\beta 1$ and other integrins) and LDV ($\alpha 4\beta 1$ integrin), have been used to study the effects of individual domains. CB CD34⁺ cells cultured in serum-free medium (SFM) for 10 days on LDV linked to a PET film contained twice as many cells able to initiate long-term hematopoiesis *in vitro* (LTC-ICs) as those cultured on PET controls and supported low levels of engraftment in 3 of 4 lethally irradiated immunodeficient mice [21]. Surfaces modified with RGD or control peptides did not support engraftment in mice, but produced only 20% fewer LTC-ICs than those with LDV [21].

Several laminin cell-binding domain peptide mimics have been evaluated in stem cell cultures. Human adipose stem cells adhered and spread more extensively on poly(caprolactone) (PCL)-based scaffolds covalently linked to IKVAV, as compared to the laminin-derived peptides RGD or YIGSR [22]. Rat adult hippocampal progenitor (AHP) cells cultured for 8 days on IKVAV peptides covalently linked to a supported phospholipid bilayer showed similar expansion and retention of undifferentiated state as AHP cells cultured on polyornithine/laminin adsorbed to SiO₂ [23]. However, cells on IKVAV-presenting surfaces formed a network of interconnecting colonies, while cells were evenly distributed on laminin [23]. Derda *et al.* evaluated a wide variety of laminin peptides presented in self-assembled monolayers on gold for their ability to support human ESC adhesion and proliferation [24]. The IKVAV and RGD peptides showed variable results. However, four different peptides from the laminin γ chain and one peptide from the β chain supported ESC expansion and expression of the primitive markers Oct4, alkaline phosphatase, and SSEA4 to a similar extent as Matrigel in 6-day cultures [24].

Covalently linked GAGs support stem and progenitor cells. Partially thiolated hyaluronic acid (HA) conjugated to polydopamine-functionalized polystyrene supported adhesion and proliferation of the megakaryoblastic M07e cell line [25]. PCL and polylactide surfaces conjugated with heparin supported high-affinity adsorption of BMP-2, as well as adhesion and proliferation of the C3H10T1/2 murine mesenchymal stem cell (MSC) line [26]. PEG hydrogels functionalized with heparin sustained human MSC viability and induced osteogenic differentiation [27].

Presentation of multiple ligands enhances surface functionality and more closely mimics the niche. Immobilization of fibronectin peptide mimics RGD and LDV additively increased M07e cell adhesion [17], while presenting both HA and tropocollagen I synergistically increased adhesion of CD133⁺ cells from mobilized peripheral blood [28]. A multi-domain peptide containing two IKVAV sequences, a heparin-binding sequence from laminin, and a hydrophobic sequence for stable adsorption to polystyrene supported greater adhesion of C2C12 pluripotent murine myoblasts than IKVAV alone and also supported the BMP-2-induced osteogenic differentiation of MC3T3-E1 murine osteoprogenitors [29]. Sol-gel-derived thin films incorporating RGD from fibronectin and YIGSR from laminin – with or without NID – supported the retinoic acid-induced differentiation of P19 embryonic carcinoma stem cells to neurons and astrocytes [30]. Cultures on RGD/YIGSR/(NID) produced more astrocytes and fewer neurons, but longer neurite extensions, than those on collagen [30]. Nakajima *et al.* used microlithography and derivatized alkanethiols on gold to evaluate combinations of covalently linked ECM components and growth factors on fetal rat NSC differentiation [31]. Although the growth factor effects predominated (e.g., EGF-induced maintenance of NSCs and NGF-induced neural differentiation), the ECM also played a role, as illustrated by much less extensive NT-3-induced neural and CNTF-induced astroglial differentiation on laminin vs. fibronectin [31]. Maruyama *et al.* immobilized extracellular domains from membrane-bound signaling molecules and evaluated their effects on the outgrowth of thalamocortical axons [32]. Axon growth was promoted or inhibited depending on the combination, concentrations, and spatial distribution of the signaling molecules and the presence of proteins from the upper (laminin) or deep (N-cadherin) layers of the developing cortex [32].

Matrix stiffness

Organs and tissues have been adapted for their function and vary in stiffness due to differences in ECM composition, crosslink density, and mineralization. Cell membrane mechanical properties also differ with cell type and differentiation stage. For example, human MSC membranes are twice as stiff as those of osteoblasts, but MSCs extend much longer tethers than osteoblasts [33]. While substrate compliance is known to influence mature cell function, stem cell responses have not been studied extensively. Hsiong *et al.* showed that the stiffness of alginate hydrogels functionalized with RGD had little effect on the growth of the clonally derived D1 murine MSC line, but that more mature MC3T3-E1 cells and D1 cells induced to differentiate down the osteogenic pathway exhibited greater growth rates on alginate disks with greater stiffness [34]. Heterogeneous human MSCs also showed higher growth rates on stiffer gels [34]. Engler *et al.* used collagen-coated polyacrylamide gels with elastic moduli similar to brain (1 kPa), muscle (10 kPa), and collagen (100 kPa) to show that substrate stiffness alters human MSC differentiation even in the absence of soluble inducers [35]. The MSCs exhibited a neuron-like phenotype on soft gels, myoblast-like morphology on substrates of intermediate stiffness, and an osteoblast-like phenotype on the stiffest gels. Soluble inducing factors synergized with matrix stiffness to increase lineage-specific gene expression [35].

Topography

Cells *in vivo* are exposed to diverse topographies including fibrous ECM and rough mineralized bone. Murine ESCs cultured on electrospun polyamide nanofibers that mimic basement membrane texture yielded 2-fold greater cell expansion than on coverslips, while retaining Nanog expression and differentiation potential [36]. Binding fibronectin to PET fiber scaffolds increased CB CD34⁺ cell expansion 5-fold compared to that on PET films and produced cells that reconstituted hematopoiesis in immunodeficient mice [20]. CB CD34⁺ cells cultured in SFM for 10 days on polyethersulfone (PES) nanofibers functionalized with C₂- or C₄-tethered amino groups supported similar total cell expansion as did cultures on TCPS, but the PES

nanofibers yielded 4-fold greater CD34⁺ cell expansion and 50% greater LTC-IC production [**37]. After transplantation into lethally irradiated immunodeficient mice, cells cultured on the nanofibers resulted in 5-fold greater human cell content than fresh cells or cells cultured on TCPS [**37]. Diblock co-polymers of polystyrene and poly-2-vinylpyridine or poly-4-vinylpyridine formed dot-like (6 nm) or worm-like (3 nm) surface nanotopography, respectively, via controlled microphase separation [38]. The worm-like surfaces supported greater human MSC proliferation, more elongated cells, and thicker ECM deposits [38]. Charest *et al.* used hot embossing to create patterns in polycarbonate surfaces coated with fibronectin; surfaces with parallel 5–75 μm grooves resulted in preferential alignment of C2C12 and primary myoblasts, but did not affect cell expansion or differentiation compared to smooth surfaces or those arrayed with 5–75 μm holes [39]. When nanogrooves were superimposed perpendicular to the microgrooves, MC3T3-E1 cell bodies aligned with the microgrooves, but many cell processes aligned with the nanogrooves [40].

Oxygen gradients

Stem cell niches are often located in regions of low oxygen tension and low pO₂ typically decreases the rate of stem cell differentiation and enhances stem cell proliferative potential (reviewed in [41]). Murine fetal cortical neural progenitor cells expanded in culture at 2–5% O₂, but declined at 20% O₂ [42]. Oligodendrocyte progenitors, but not committed neuronal progenitors, exhibited more extensive apoptosis at 20% O₂ [42]. It has recently been proposed that oxidative stress suppresses N-cadherin-mediated HSC adhesion to osteoblasts and induces HSCs to exit the niche [43]. CD34⁺ cells in CB are normally exposed to higher pO₂ values than those in bone marrow. Exposure of CB CD34⁺ cells to 2% O₂ significantly decreased their survival compared to that at 20% O₂, but cell death was substantially decreased by engagement of the $\alpha 4\beta 1$ integrin [44].

It is difficult to control pO₂ at low levels. Park *et al.* have developed a microfabrication-based approach to pattern a wide variety of oxidative microgradients by using titanium/platinum microelectrodes arranged along intricate geometries to generate O₂ via electrolysis [*45]. Rat C2C12 myoblasts, which undergo apoptosis and differentiation under high pO₂, were used to confirm the predicted gradients [*45].

Patterning cells and ligands

The stem cell niche exhibits a distribution of different cell types and ligands. Lee *et al.* used photolithography and inkjet printing to control homogeneous and heterogeneous cell-cell interactions; the number of hepatic (HepG2) cells in a cluster was controlled by varying the size of collagen islands from 30 (1 cell) to 100 μm (ca. 20 cells) and the intervening space was filled with 3T3 fibroblasts [46]. Patterning has also been used to induce several differentiation fates in a single culture. Phillipi *et al.* created islands of immobilized BMP-2 on a fibrin matrix and showed that muscle-derived stem cells cultured in a myogenic medium differentiated into osteogenic cells within the BMP-2 islands, while those in the surrounding area formed multinucleated myotubes [47].

Receptors are not uniformly distributed on stem cells. It has been shown that lipid rafts help to regulate HSC cycling and that blocking receptor clustering interferes with cytokine signaling [48]. Therefore, it will be important to modulate ligand patterns within a single-cell scale. Doh and Irvine immobilized biotinylated anti-CD3 antibody and ICAM-1 to polymer thin films in patterns that resemble the immunological synapse [*49]. When randomly migrating T-cells encountered a patterned synapse, they changed from polarized to rounded morphology and became activated [*49]. Presentation on fluid lipid surfaces allows for cell-directed reorganization of ligands. Thid *et al.* covalently linked IKVAV peptide to palmitoyl-oleyl-phosphocholine (POPC) and presented this within a fluid supported POPC bilayer [50]. AHP

cell attachment to IKVAV-POPC was sigmoidal with a threshold of 3 pmol/cm² and maximal binding at 8 pmol/cm², and was essentially the same as attachment to IKVAV covalently linked to tethered PEG chains [50]. Nam *et al.* showed that MCF-10a mammary epithelial cells cultured on a fluid supported lipid bilayer reorganized lipid-linked EGF into large clusters over a period of several hours [51].

Toward the future

While progress has been made to increase the frequency of adult stem cell renewal, we are still far from reaching the goal of selective and sustained expansion of tissue-specific stem cells. Many different factors must be presented in the correct arrangement and orientation within a well-defined 3-dimensional construct of defined stiffness and topography in the presence of pO₂ and growth factor gradients in order to mimic the complexity of the stem cell niche. While it will probably not be necessary to mimic all aspects of the niche to greatly increase stem cell self-renewal, it will almost certainly be necessary to simultaneously mimic multiple components of the niche. The advances discussed above provide a foundation for evaluating a wide range of combinations of different immobilized factors on materials with varying compliance and texture. A recent discovery has enabled the covalent modification of virtually any material using the biocompatible adhesive coating polydopamine [25]. This coating is prepared from aqueous solution and is amenable for patterning and conjugation with molecules containing free amines or thiol groups. Another recent study has demonstrated the patterning of fluid lipid domains within a background of covalently linked fibronectin [52]. This will allow for the simultaneous presentation of immobilized and mobile ligands. The approaches and technologies described in this review provide ideas and tools for multi-parameter design of improved stem cell culture systems.

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Figure 1. Schematic diagram illustrating several factors to consider when mimicking stem cell niches

Variations in the co-localization, clustering, surface concentration, and gradients of two immobilized ligands are represented by red spheres and blue pyramids. Matrix stiffness and topography are depicted by the rippled membrane surface. Image drawn by Mark Seniw.

Table 1
Mimicking stem cell niches to alter the regulation of stem cell expansion and controlled differentiation

Niche Properties	System Details	Species	Cell Type	Observations	References
Ligand Presentation	Immobilized Dectal-Fc	Human	HSPC	↑ Maintenance of undifferentiated cells, NOG engraftment	[*11]
	Jagged1 immobilized on Sepharose beads	Mouse	HSPC	↑ Notch signaling, male BDF1 engraftment in female BDF1 mice, secondary engraftment	[12]
	Multifunctional chimeric proteins immobilized by Ni chelating with decabistidine tag	Rat	NSC	Expansion and maintenance of undifferentiated cells; thrombin-treatment exposes secondary region and induces astrocyte differentiation	[19]
	Immobilized IKVAV peptide in lipid bilayer surfaces	Rat	AHP	Expansion and maintenance of undifferentiated cells; formed networks of 3D colonies	[23]
	Array of 500 μm × 500 μm LN derived peptides	Human	ESC	Peptides identified for undifferentiated cell proliferation	[**24]
	Heparin on PEG hydrogel	Human	MSC	Osteogenic differentiation	[27]
	Ormosil-immobilized adhesion peptides	Rat	ESC	Neuronal differentiation, ↑ neurite length	[*30]
	Immobilized LN, FN, or Col w/EGF, NGF, NT-3, or CNTF	Rat	NSC	Identified combinations for maintenance of undifferentiated cells or neuronal vs. astroglial differentiation	[**31]
	BMP-2 islands on fibrin gel	Mouse	MDSC	Cells on BMP-2 islands became osteogenic, while surrounding cells formed myotubes	[47]
Matrix Stiffness	RGD-modified alginate gels	Human	MSC	Stiff gels ↑ proliferation of hMSCs and committed progenitor cell lines	[34]
	Collagen-coated polyacrylamide gels	Human	MSC	Directed differentiation into neuron, myoblast, or osteoblast lineages based on gel elastic modulus	[**35]
Topography	FN-coated PET nanofiber mesh	Human	HSPC	↑ Expansion and maintenance of undifferentiated cells, NOD/SCID engraftment	[20]
	Polyamide nanofiber mesh	Mouse	ESC	↑ Expansion and maintenance of undifferentiated cells	[36]
	Aminated PES nanofiber mesh	Human	HSPC	↑ Expansion and maintenance of undifferentiated cells, NOD/SCID engraftment	[**37]

HSPC: Hematopoietic stem and progenitor cell; NSC: Neural stem cell; MSC: Mesenchymal progenitor cell; ESC: Embryonic stem cell; MDSC: Muscle-derived stem cell; NOD/SCID: Non-obese diabetic severe combined immuno-deficient mice; NOG: NOD/SCID mice intercrossed with IL-2 receptor γ chain-knockout; BDF1: (C57Bl/6 × DBA/2)F1; PEG: Polyethylene glycol; Ormosil: Organically modified silica; LN: Laminin; FN: Fibronectin; EGF: Epidermal growth factor; NGF: Nerve growth factor; NT-3: Neurotrophin-3; CNTF: Ciliary neurotrophic factor; BMP-2: Bone morphogenetic protein-2; IKVAV: Laminin-derived peptide; RGD: Fibronectin-derived peptide; PET: Polyethylene terephthalate; PES: Polyethersulfone