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The influence of microenvironment and extracellular matrix molecules in driving neural stem cell fate within biomaterials

Thomas Wilems[1], **Sangamithra Vardhan**[1], **Siliang Wu**[1], and **Shelly Sakiyama-Elbert**[1,*]

[1] Department of Biomedical Engineering, University of Texas at Austin, Austin, Texas, 78712, U.S.A

Abstract

Transplantation of stem cells is a promising potential therapy for central nervous system disease and injury. The capacity for self-renewal, proliferation of progenitor cells, and multi-lineage potential underscores the need for controlling stem cell fate. Furthermore, transplantation within a hostile environment can lead to significant cell death and limited therapeutic potential. Tissue-engineered materials have been developed to both regulate stem cell fate, increase transplanted cell viability, and improve therapeutic outcomes. Traditionally, regulation of stem cell differentiation has been driven through soluble signals, such as growth factors. While these signals are important, insoluble factors from the local microenvironment or extracellular matrix (ECM) molecules also contribute to stem cell activity and fate. Understanding the microenvironment factors that influence stem cell fate, such as mechanical properties, topography, and presentation of specific ECM ligands, is necessary for designing improved biomaterials. Here we review some of the microenvironment factors that regulate stem cell fate and how they can be incorporated into biomaterials as part of potential CNS therapies.

1. Introduction

Stem cell based therapies have garnered much interest and shown significant potential when used in a multitude of tissues for injuries and diseases. These strategies typically aim to replenish lost or defective cells and rely on the successful differentiation of stem cells into mature fates. The delivery of viable stem cells is achieved through either direct injection or incorporation within a supportive biomaterial. For many applications, the cells are cultured within the biomaterial *in vitro* then the material is implanted. In other applications, the stem cells and biomaterial precursor solution are injected into the tissue then the biomaterial reacts to form the supportive matrix. The high proliferation rates, ability for self-renewal, and potential to replace lost cell types make stem cells a potentially promising cell source for CNS therapeutics. High proliferation rates allow for the rapid expansion of cells into clinically relevant numbers, however, transplantation of undifferentiated stem cells can lead

*Corresponding author: Dr. Shelly Sakiyama-Elbert, sakiyama@utexas.edu, 107 W. Dean Keeton, BME 3.110B, 1 University Station, C0800, Austin, Texas 78712, Tel: (512) 471-3604.

to tumor formation. Thus, purification to remove or limit the presence of undifferentiated stem cells must occur prior to transplantation (Johnson et al. 2010).

During development, stem cells are exposed to soluble and insoluble factors that regulate their proliferation, differentiation, and migration. In the developing CNS, stem cells give rise to more fate-restricted, tissue-specific stem and progenitor cells fated to become neurons, oligodendrocytes, and astrocytes, termed neural stem/progenitor cells (NSPCs). The main NSPCs in the maturing and adult CNS are neuroepithelial and radial glia stem cells located within the subventricular zone of the lateral ventricle and hippocampal subgranular zone (Kriegstein and Alvarez-Buylla 2009; Ma et al. 2005), while stem cells in the adult spinal cord are thought to be restricted to ependymal cells lining the ventricles of the central canal (Johansson et al. 1999; Sabelstrom et al. 2014; Weiss et al. 1996). Fate-mapping studies suggest a heterogeneity to NSPCs that display a regional diversity for areas in the brain and spinal cord segments, making identification and characterization of endogenous NSPCs difficult (Sabelstrom et al. 2014; Ihrie et al. 2011; Bonaguidi et al. 2011).

CNS trauma drives proliferation of endogenous NSPCs; however, the response is not capable of restoring all lost specialized cell types, and the proliferative cells mainly contribute to reactive gliosis, driving scar formation that limits recovery (Buffo et al. 2008; Fitch and Silver 2008; Cregg et al. 2014; Sabelstrom et al. 2014). For this reason, CNS stem cell therapies typically utilize exogenous cell transplantation over targeting endogenous stem cells. Two main types of stem cells have been utilized in CNS injuries: mesenchymal stem cells (MSCs) and NSPCs. MSCs are easily isolated from bone marrow providing for an autologous therapy, but they are potentially limited to indirect trophic and immunomodulatory support (Hofstetter et al. 2002; Parr et al. 2007; Tetzlaff et al. 2011; Urdzikova et al. 2014; Iyer et al. 2017). This review will mainly focus on NSPCs due to their proven potential to differentiate into important CNS cell types including astrocytes, oligodendrocytes, and neurons.

Direct injection of NSPCs into the injured CNS provides trophic support and replaces lost cells but is limited by poor viability of transplanted cells due to the harsh *in vivo* environment (Pearse et al. 2007). Additionally, long-distance migration of transplanted cells away from the transplant site has been documented which could lead to ectopic colony formation that could affect healthy tissue (Guzman et al. 2007; Li et al. 2003; Pearse et al. 2007; Sharp et al. 2014; Steward et al. 2014). Thus, it is necessary to protect the transplanted cells in a supportive biomaterial or artificial extracellular matrix (ECM). The ECM microenvironment encompasses ~20% of the adult brain volume and is a heterogeneous mixture of soluble and insoluble factors that each contribute to cell survival and fate (Nicholson and Sykova 1998; Novak and Kaye 2000; Hubert et al. 2009). The ability to design artificial ECM allows for inclusion of specific cues that affect NSPC differentiation and fate to improve CNS therapies.

Classically, soluble factors, such as morphogens, cytokines, and growth factors, have been used for controlling cell differentiation *in vitro*. Inclusion of these soluble factors, which are often expensive, in biomaterials requires additional design criteria including maintenance of bioactivity of the molecule, sustained release over a desired time window, and temporal

control over dosages, all of which are difficult to do *in vivo*. Designing the biomaterial to incorporate insoluble factors that control NSPC fate would not only promote cell survival but also may limit the need for soluble cues. Many of these factors reside in the ECM, therefore understanding the dynamic ECM microenvironment can aid in developing improved CNS therapeutics.

The ECM composition, including both soluble and insoluble factors termed the matrisome, is dependent upon many factors and varies during development, maturation, injury, and location. Two methodologies that can probe the matrisome on a global scale are proteomics and bioinformatics. Proteomics using mass spectrometry techniques aid in evaluating complex ECM derived from tissue samples and cell culture systems. Solubilization or enzymatic digestion of the ECM into fragments allows for MS analysis. The fragmented ECM can be further fractionated by multiple properties, such as hydrophobicity and charge, for tandem MS to provide structural information that can determine amino acid sequences and post-translational modifications within the complex ECM mixture (Byron *et al.* 2013, Naba *et al.* 2017). MS database search tools are used to identify theoretical mass spectra of known proteins to the mass spectra of ECM protein samples (Naba *et al.* 2017). In addition, bioinformatics can help identify ECM constituents through gene-centric lists with NCBI Entrez Gene database as a reference that encompasses both mouse and human genes (Naba *et al.* 2016). These gene databases can further become annotation tools for large data sets and make peptide ion identification easier.

However, the source of the ECM is important with respect to fetal compared to adult tissue sources. Numerous studies have explored the aspects of fetal ECM, such as umbilical cord ECM, to support a higher tissue remodeling response in host implantation with better modulation of macrophage phenotypes (Sicari *et al.* 2012). Human umbilical cord ECM-based hydrogels showed higher GAG composition that also supported higher differentiation of neural stem cells *in vitro* and short gelation time in a rat cortical ischemic lesion *in vivo* (Koci *et al.* 2017). The plasticity of fetal porcine brain ECM provides the developmental cues to differentiate progenitor cells into specific mature cell types and support neural circuit formation (Caprile *et al.* 2017). Furthermore, the combination of proteins in fetal ECM aided in producing dense axonal networks with higher calcium signaling and spiking activity in neural network activities (Sood *et al.* 2015). Using the bioinformatics and proteomics tools to probe the fetal ECM can aid in our understanding of the necessary developmental cues required for neural differentiation and regeneration.

To this end, research using ECM-mimetic biomaterials have been engineered to manipulate NSPCs *in vitro* and *in vivo*. Particular focus has been given to the protein composition of the native ECM; however, the physical characteristics, such as topography and stiffness are also key factors in NSPC differentiation (Figure 1). It is important to note that many studies have utilized both soluble and insoluble factors to control NSPC differentiation and that a main benefit of many ECM-derived molecules is their ability to interact with soluble factors.

This review will focus on the current understanding of key microenvironment-specific ECM factors that influence NSPC fate and their incorporation into biomaterials for CNS therapies.

2. Biomechanical and material properties that can affect NSPC fate

The biomechanical properties of CNS tissue change throughout development, during maturation, and after injury and play a key role in NSPC differentiation (Clarke et al. 2009; Fiford and Bilston 2005; Cheng et al. 2008). Additionally, the CNS is heterogeneous with the grey matter having twice the stiffness of white matter, with this trend consistent across rodents and humans (Budday et al. 2017; Christ et al. 2010; Koser et al. 2015; Moeendarbary et al. 2017). Injury to the CNS drastically alters the mechanical properties of the tissue and may be a contributor to the lack of regeneration and recovery. In contrast to most other tissue injuries that result in fibrotic scar formation (Swift et al. 2013), CNS tissue softens at the injury site. Using atomic force microscopy indentation to measure elastic modulus the rat cortex showed a decrease in elastic modulus 8–10 days post-injury (acute stage) compared to uninjured or 3 weeks post-injury (sub-acute stage) in a stab injury model. Additionally in the same publication, crush injuries to the rat spinal cord at the C5 level led to similar decreases in elastic modulus compared uninured controls (Moeendarbary et al. 2017). These results show that CNS tissue softening after injury occurs in both the brain and spinal cord, and in both sharp and blunt injuries. In addition to mechanical properties, the ECM's topography, alignment, and architecture also affect cell fate and activity. Understanding how these changes affect NSPC will provide important insights for designing biomaterials that could lead to better control over cell fate and improved therapeutic outcomes. For this section, we will provide a brief overview of the effects of ECM mechanical properties and topographic cues on NSPC fate.

2.1 Material stiffness affects NSPC fate

The material stiffness is known to affect cellular activities, such as migration, apoptosis, proliferation, and differentiation in multiple tissue types (Guilak et al. 2009). Dissociated rat NSPCs cultured on laminin-coated polyacrylamide gels with a stiffness of ~200 Pa (adult rat brain ~300 Pa) improved neuronal differentiation compared to stiffer gels (~9000 Pa), which generated more astrocytes. In the same article, similar results were found using fibrin hydrogels (Georges et al. 2006). Adult rat NSPCs were cultured on laminin-coated variable moduli interpenetrating polymer networks (vmIPNs) synthesized by polymerizing poly(ethylene glycol) within a polyacrylamide hydrogel. The stiffness or modulus of the vmIPNs could be adjusted from ~10–10,000 Pa. Softer vmIPNs (100–500 Pa) appeared to favor neurogenesis, and stiffer vmIPNs (1,000–10,000 Pa) promoted astrocyte differentiation. Interestingly, hydrogels with very soft moduli (~10 Pa, similar to the injured CNS) resulted in decreased cell spreading, proliferation, and differentiation (Saha et al. 2008).

Generally, a large range of moduli is reported for influencing cell differentiation because it is difficult to determine the exact modulus that drives cell fate decisions. NSPCs harvested from adult rat brains and cultured on methacrylamide chitosan biomaterials of variable moduli were capable of neuronal differentiation on soft substrates (<1,000 Pa) and oligodendrocyte differentiation on hard substrates (>7,000 Pa). Note that for these studies, oligodendrocyte maturation and myelination was improved on the soft substrates that favored neuronal differentiation (Leipzig and Shoichet 2009). Additionally, oligodendrocyte

progenitors (cultured on polyacrylamide gels with moduli ~1,000 Pa) resulted in increased proliferation, migration, and differentiation into oligodendrocytes. The range of biomaterial moduli that appears to favor oligodendrocyte differentiation, (myelination) and neuronal differentiation mimics the range of stiffness of the white matter (between 500–2000 Pa) (Weickenmeier et al. 2016; Budday et al. 2015; Christ et al. 2010).

2.2 Mechanotransduction via focal adhesions and topography affect NSPC fate

The ECM plays a major role in dictating a tissue's biomechanical properties and in mechanotransduction of signals to the cells through focal adhesion complexes that link their cytoskeletons to the ECM. Integrins, adaptors, and signaling proteins together form the focal adhesion protein complexes that connect the actomyosin cytoskeleton and the ECM (DuFort et al. 2011a). Focal adhesions can consist of more than 100 proteins and serve as channels for signal transduction in response to physical forces (DuFort et al. 2011b; Stukel and Willits 2016). The sequence of force transduction starts from integrin-ECM interactions and proceeds through adaptor proteins bound to actin. The forces are then transmitted from the actin filaments through the myosin head (Roca-Cusachs et al. 2012). The mechanism of mechanotransduction is not fully understood, but multiple downstream signaling pathways have been shown to be linked with mechanotransduction (Gattazzo et al. 2014). For example, the activation of focal adhesion kinase (FAK) by integrin binding regulates neural differentiation. Phosphorylation of FAK contributes to expression of the mature neuronal marker and dendritic protein, microtubule associated protein 2 (MAP2) (Teo et al. 2013). Focal adhesions also regulate cytoskeleton rearrangement and organization through the Rho/ROCK, Src family kinases, and ERK1/2 signaling pathways (McBeath et al. 2004; Roca-Cusachs et al. 2012; Mammoto et al. 2012). The inhibition of integrin β1 mediated-binding and the Rho-associated signaling pathway, a key pathway in mechanotransduction, reduced NSPC differentiation (Yang et al. 2014). Blocking the MEK-ERK signaling pathway with a small molecule inhibitor (U0126) decreased human NSPC adhesion, spreading, neurite outgrowth, and differentiation into neurons or glia (Yang et al. 2013).

In addition to the biomechanical properties, the topography of the ECM substrate also affects cell fate. The ECM topography can affect cell shape by regulating cell adhesion and cytoskeleton arrangement, which applies mechanical forces to the cell that are then transduced into a chemical response. Cells sense topographic cues including 3D and 2D environments, substrate dimensions, and geometry at nano- and micro- scales (Guilak et al. 2009). Control of surface topography can be achieved using micro-patterned substrates, however terminology describing the type of patterning is not uniform throughout the field. For this review, a graphical depiction of types of patterning is displayed in Figure 2. A groove is defined as a cut or valley in the substrate and the terrace is the distance between grooves. Note that studies have also used topographies not centered on grooves and terraces but on holes, pillars, and other 3D shapes (Moe et al. 2012). Adult rat hippocampal NSPCs cultured on a laminin-modified polystyrene micropatterned substrate (13 μm terrace width, 16 μm groove width, 4 μm groove depth) exhibited over 75% alignment in the groove direction and increased expression of βIII-tubulin (neuronal differentiation marker) compared to a planar substrate (Recknor et al. 2006). This indicates that the 3D topography of the substrate promotes neuronal differentiation of NSPCs. In another study, NSPC

differentiation on a micropatterned substrate (1.5 μm terrace width, 1.5 μm groove width, 625 nm groove depth) that had 10 nm nanopores on the surface of the grooves promoted neuronal differentiation and increased expression of focal adhesion proteins compared to a flat substrate (Yang et al. 2014).

Surface topography can also influence the mechanotransduction of physical properties to cellular activity through interactions with focal adhesions. Binding of focal adhesions induces cytoskeleton rearrangement and generates tension within the cytoskeleton, which is transduced to the cell nucleus and causes reorganization of the nucleus that alters gene expression and affects cell fate. A fibronectin-coated micropatterned substrate with smaller groove width (300 nm) enhanced focal adhesion formation of NSPCs and promoted differentiation toward neurons and astrocytes compared to a substrate with larger groove size (1500 nm). Image analysis indicated that smaller grooves may provide more contact points that facilitate NSPC focal adhesion formation, and western blot results revealed that phosphorylated FAK was upregulated in NSPCs cultured on substrates with smaller groove size (Yang et al. 2013). In addition, the MEK-ERK signaling pathway has been shown to be linked with mechanostransduction-induced NSPC differentiation (Yang et al. 2013). Vaysse *et al.* fabricated laminin-coated micropatterned substrates with 25 μm deep microchannels. Adult NSPC differentiation was evaluated on different terrace and groove widths (Béduer et al. 2012). Larger terrace and groove width (20/20 μm) promoted more neuronal differentiation of NSPCs compared to narrower substrates (5/5 μm). Individual neurons had more neurites and longer neurite length on the wider micropatterned substrate compared to the narrower ones. The direction of neurite extension was also affected by substrate topography, where neurites extended in all directions on flat substrates, while neurites cultured on aligned micropatterned substrates extended in the direction of alignment.

Electrospun fibers of variable fiber diameter and alignment also effect NSPC differentiation. When cultured on laminin-coated electrospun fibers, rat hippocampus-derived adult NSPCs showed a 40% increase in oligodendrocyte differentiation on ~300 nm fibers and a 20% increase in neuronal differentiation on 750 nm fibers compared to a laminin-coated tissue culture polystyrene surface. The NSPCs on 300 nm fibers also showed a stretched morphology that is similar to oligodendrocytes and extended in multiple directions to follow the underlying fibers (Christopherson et al. 2009). Adult NSPCs cultured on either aligned or random electrospun fibers showed greater neuronal differentiation and cell body elongation along the fiber axis of aligned substrates (Lim et al. 2010; Mahairaki et al. 2011). NSPCs induced with retinoic acid differentiated into a higher percentage of neurons on aligned fibers over random fibers or unpatterened surfaces. Interestingly, alignment led to decreased survival and attachment rates of differentiated oligodendrocytes compared to random fibers (Lim et al. 2010).

In addition to 3D/2D structure and substrate dimensions, the geometry of the substrate also affects cell fate. Moe et al. used multi-architecture arrays to introduce multiple, distinct topographies on a single substrate. Anisotropic grooves on the substrate (250 nm or 2 μm wide grooves) promoted neuronal differentiation of primary murine NSPCs, while an isotropic substrate with either holes (2 μm diameter) or pillars (1 μm diameter) promoted glial differentiation (Moe et al. 2012). Similar results were also reported by the same group

on human embryonic stem cell-derived NSPCs where the anisotropic pattern promoted neuronal differentiation, and the isotropic pattern promoted glial differentiation (Ankam et al. 2013).

Tuning both stiffness and topography of the biomaterial should enable more control over NSPC differentiation. To this end, aligned fibrillar fibrin hydrogels were fabricated with a stiffness of ~1,000 Pa and promoted increased neuronal differentiation over randomly oriented fibers (Yao et al. 2016). When transplanted into a rat spinal cord dorsal hemisection, endogenous neural crest cells migrated along the aligned fibers, and axonal extension into the transplant occurred along the fibers to form axonal “cables”. Recent work evaluated the differentiation of fetal NSPCs on aligned polycaprolactone electrospun fiber mats with large (mimicking blood vessel topography, 10 μm) or small (mimicking radial glial process topography, 800 nm) fiber diameters with or without laminin (Czeisler et al. 2016). The smaller fiber diameters improved neuronal differentiation, axonal extension, and migration compared to the large fibers. These studies suggest that both alignment and surface topography of the ECM and biomaterial play roles in NSPC differentiation and presentation of bioactive cues.

3. Effect of ECM-derived constituents on NSPC fate and their use in biomaterials

The modulus, topography, and effect on cell shape appear to be key drivers of NSPC fate; however, important ECM-derived factors can be incorporated into biomaterials to provide additional regulatory cues. Discovery of these factors is typically achieved through studying the predominant ECM proteins in the developing, mature, and injured CNS. During development, NSPCs are seen migrating and differentiating through the CNS in heterogeneous environments, which makes the constituents of their microenvironment difficult to analyze. In the mature CNS, adult stem cells reside in unique specialized niches, termed fractones due to the fractal ultrastructure that is essential to regulating these cells. Research into fractone constituents will improve our understanding of what factors are necessary to maintain the stemness of cells and potentially what factors specify cell fates. Laminin, collagen IV, and heparan sulfate proteoglycans (HSPGs) are major constituents of fractones (Mercier 2016; Mercier et al. 2002) Table 1.

3.1 Major ECM components of the stem cell niche/fractones

Laminin is a family of trimetric proteins containing α, β, and γ subunits with only certain subunits found in mammals and is a key component of basal lamina, a layer of ECM found in many tissues including the brain and blood vessels in the CNS (Nirwane and Yao 2018). Laminin-α1β1γ1, a commonly researched isoform also termed laminin-1 or laminin-111, is known to regulate axonal guidance, neuronal extension, and synaptic formation (Barros et al. 2011; Franco and Muller 2011; Nirwane and Yao 2018). Loss of laminin or laminin isoforms results in neurological impairment or death due to decreased neuronal survival, migration, and guidance (Coles et al. 2006; Franco and Muller 2011; Ichikawa-Tomikawa et al. 2012). Many laminin isoforms (α1-α5, β1-β4, and γ1-γ3) are present throughout the developing

CNS - for a detailed review of laminin expression in the CNS refer to Nirwane and Yao, 2018.

Laminin coatings are commonly used to improve cell adhesion to substrates. Studies have shown laminin substrates and laminin-rich Matrigel® produced robust neuronal differentiation of human embryonic stem cells compared to fibronectin or collagen I substrates (Ma et al. 2008). This is possibly due to the interactions of laminin with integrin α6 and β1 subunits on cell surfaces, suggesting laminin as a key factor to induce neuronal over glial differentiation.

CNS injury increases basal membrane deposition, which largely consists of laminin, around the lesion sites, thus modulating binding of growth factors. Astrocytes interact with the basal membrane deposits and contribute to scar formation by contracting or constricting the scar around the lesion area (Stichel and Muller 1998). However, after injury, expression levels of laminin within the lesion area are significantly decreased, possibly contributing to the lack of axonal extension into and through the lesion (Moeendarbary et al. 2017).

Laminin and laminin-derived peptides were tethered to substrates at varying densities to determine the optimal level for neuronal differentiation (Yang et al. 2015; Dodla and Bellamkonda 2006). Note, a significant decrease in optimal concentration was measured when switching from tissue culture plastic substrates to 3D culture within poly(ethylene glycol) hydrogels (Yang et al. 2015). 3D culture of neurospheres from murine NSPC within methylcellulose scaffolds functionalized with the peptide IKVAV, derived from the laminin α1 subunit, upregulated expression of neuronal and oligodendrocyte precursor markers (Stabenfeldt et al. 2010). Self-assembled nanofiber networks formed from amphiphilic peptides designed to present the peptide IKVAV induced differentiation of NSPCs into neurons over oligodendrocytes and astrocytes, showing the potential for laminin α1 to drive neuronal differentiation (Mammadov et al. 2014; Silva et al. 2004).

Collagen is a major constituent of ECM throughout the body but is less prevalent in the CNS, limited largely to the meninges and basement membranes. There are twenty-nine types of collagens, with the possibility of various isoforms for each type and each having a distinct function. Thus, collagens represent a large, diverse family of extracellular proteins characterized by a triple helical quaternary structure. Collagen type I, IV, and XVIII may play a role in axonal guidance during neural development with collagen IX suggested as a repellant for growing axons due to its chondroitin sulfate glycosylation (Ring et al. 1996; Hubert et al. 2009; Klapka and Muller 2006; Leclere et al. 2007; Rautavuoma et al. 2004; Schneider and Granato 2006; Xiao and Baier 2007). Collagens and collagen-derived peptide sequences largely interact with cells through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. Importantly, collagen I, IV, and XVIII have been found in fractones, the ECM microenvironment of the subventricular zone adult SC niche, where they may be involved in controlling neurogenesis (Mercier et al. 2002; Kerever et al. 2007; Mercier 2016). Incorporation of collagen I-derived peptide sequences aided neuronal differentiation from NSPCs (Cooke et al. 2010; Nakajima et al. 2007; O'Connor et al. 2001), whereas collagen IV reduced neuronal differentiation (Cooke et al. 2010). On the other hand, fibroblasts upregulate collagen I and IV following CNS injury, leading to deposition within the ECM and interaction with reactive astrocytes

that promote glial scar formation (Hara et al. 2017; Liesi and Kauppila 2002; Neo and Tang 2017).

Collagens have long been used as biomaterials to support cell transplantation in the CNS and other tissue types. Primary NSPCs from rat neuroepithelium dispersed in type I collagen containing basic fibroblast growth factor (bFGF) provided cues for neuronal differentiation with functional receptors, indicating glutamatergic and GABAergic activity (Ma et al. 2004). Furthermore, these neurons were able to show recycling of synaptic vesicles, suggesting functional synaptic connections. Complexes of NSPCs and collagen I promoted neuronal differentiation and synapse formation when transplanted into rat brains under cerebral ischemia (Yu et al. 2010).

Incorporation of cell-adhesive collagen-derived peptide sequences into biomaterials enables interaction with integrins without the need for purified collagen. Human NSPCs adhered to the GFOGER peptide sequence, derived from collagen type 1, $\alpha 1$ chain, through the interaction with β_1 integrin and differentiated into neurons and astrocytes (Que et al. 2018). Recently, collagen-based hydrogels were fabricated with mechanical properties tailored to mimic the developing spinal cord and incorporated with hyaluronic acid and laminin. These biomaterials demonstrated directed differentiation of NSPCs to oligodendrocytes *in vitro* and improved functional recovery when transplanted into a rat unilateral cervical contusion SCI model (Geissler et al. 2018; Hatami et al. 2009).

As mentioned earlier, an important benefit of many ECM-derived molecules is their interaction with growth factors. To this end, ***heparin sulfate proteoglycans*** (HSPGs) play a key role in NSPC differentiation through their association with FGF and other growth factors (Ford-Perriss et al. 2003). HSPGs are present in all tissues and consist of two main types: 1) perlecans that are deposited within the ECM, and 2) syndecans located on cell surfaces (Cui et al. 2013). Perlecan in the fractone aids in regulation of FGF-2 signaling, and perlecan deficiency blocks FGF-2 from playing a role in neurosphere formation (Kerever et al. 2007). Perlecan deficiency in telencephalons led to decreased neurogenesis in the neuroepithelium during development and lower amounts of neurons within the cortical plate and subplate of the neocortex (Giros et al. 2007). Syndecans are expressed by cells in the adult stem cell niche at times of high NSPC proliferation and migration (Ford-Perriss et al. 2003). Syndecan-1 and syndecan-4 localize in the stem cell niche in the ventricular zone of the brain during development (Ford-Perriss et al. 2003). Syndecan-3 is found on the surface of neurons during development and may promote neurite extension (Raulo et al. 2005). Therefore, understanding the role of perlecans and syndecans in the developing, mature, and injured CNS may aid in future biomaterial design.

The role of HSPGs in CNS injury is largely unknown apart from if the HSPGs have increased or decreased expression. After brain injury in adult mice, all syndecan forms were upregulated around necrotic tissue and mRNA expression levels peaked at day 7 (Iseki et al. 2002). After CNS injury, there are higher levels of sulfated glycosaminoglycan (GAG) side chains in the HSPGs, increasing the association of growth factors with the HSPGs (Properzi et al. 2008). For example, upregulation of syndecan-1 in astrocytes at the injury site may increase FGF concentrations and promote astrogliosis (Properzi et al. 2008). Incorporating

HSPGs into ECM-mimetic material design to influence NSPC fate is a promising technique when combined with growth factors. Agarose hydrogels conjugated with a laminin α1-derived peptide sequence (RLQVQLSIR, shown to bind syndecans) aided HSPG-regulated NSPC adhesion and proliferation (Yamada et al. 2012). *In vitro* testing has shown that perlecan-coated plates and perlecan addition to the media increased NSPC attachment and proliferation while suppressing astrocyte proliferation (Nakamura et al. 2015). Thus, HSPGs in the form of perlecans and syndecans can aid in regulating differentiation through the association with growth factors; however, the effect on proliferation or cell fate may be caused more by the growth factor than HSPGs.

3.2 Additional major ECM constituents

Outside of the stem cell niche, the CNS ECM consists of a heterogeneous mixture of molecules that is largely maintained by astrocytes. Astrocytes are highly dynamic, heterogeneous throughout the CNS, and undergo reactive gliosis following injury. The ability to control astrocyte phenotype enables control over the ECM microenvironment or the production of either inhibitory or regenerative ECM. For example, incorporation of decellularized ECM derived from protoplasmic (grey matter) astrocyte phenotypes into hyaluronic acid hydrogels reduced the glial scar, improved axonal extension into the injured area, and had immunomodulatory effects in a rat spinal cord injury model (Thompson et al. 2018). The protoplasmic ECM was shown to have increased levels of axonal growth-promoting proteins including collagen 4α1, perlecan, and laminins (α1, α5, β1, and γ1) (Thompson et al. 2017).

Fibronectin, a glycoprotein, is transiently expressed in the developing spinal cord and coincides with phases of increased neuronal differentiation (Krolo et al. 1998). In the adult CNS, fibronectin is found in low levels but mainly deposited in the basement membrane (Lau et al. 2013). CNS injuries commonly lead to the formation of a cystic cavity surrounded by a glial scar; however, in some instances the injury area is dominated by fibrotic scars containing high amounts of fibronectin among other constituents (Cooper et al. 2018). Fibronectin is mostly associated with adhesion and motility for many cell types, including neural SCs, but has been shown to limit morphological differentiation of oligodendrocytes and remyelination (Lau et al. 2013; Siskova et al. 2006; Stoffels et al. 2013). Fibronectin dissolved in plasma can enter the CNS via disruption of the blood-brain barrier to promote migration and proliferation of oligodendrocyte precursors (Baron et al. 2002; Stoffels et al. 2015). Thus, fibronectin can be used to recruit and proliferate oligodendrocyte precursors but must be removed to allow maturation into myelin forming mature oligodendrocytes. Collagen-based scaffolds incorporated with fibronectin did improve survival and migration of transplanted NSPCs compared to cell transplants alone, but collagen scaffolds with laminin further improved cell survival and functional recovery in mice with traumatic brain injury (Tate et al. 2009). Most studies involving fibronectin incorporate the RGD/RGDS peptide sequence into biomaterials to enhance cell adhesion with limited focus on differentiation.

In addition to the major constituents of fractones, ***tenascin***-C (TN-C) is an important glycoprotein involved in development of the CNS through regulation of cell adhesion and

signaling (Faissner and Reinhard 2015; Garcion et al. 2004; Jones and Jones 2000). NSPCs in mammalian CNS are initially FGF-2 responsive but transition to EGF responsiveness that drives the production of neuronal over glial precursors (Garcion et al. 2004). TN-C aids the transition around embryonic day 13.5–15.5 from early neurogenic FGF-2 responsive cells to late gliogenic FGF-2 and EGF-responsive NSPCs in mice (Karus et al. 2011). Knockdown of EGF receptors results in a decrease in glial precursors and more production of neurons. Studies investigating loss of tenascin showed that FGF receptor 3-expressing immature astrocytes had delayed migration and increased proliferation of NSPCs suggesting a connection between TN-C and FGF/EGF regulation. Furthermore, TN-C deficiency resulted in a shift of the patterning genes Nkx6.1 and Nkx2.2 that correlated with increased GFAP-positive mature astrocytes. Thus, TN-C may contribute to proliferation and regulation of NSPCs and glial progenitors during development of the CNS (Karus et al. 2011).

TN-C is also present in the mature and injured CNS with adult TN-C deficient mice displaying a reduction in spontaneous recovery following spinal cord injury compared to wild-type mice. Additionally, overexpression of TN-C reduced the glial scar and lesion volume, and improved axonal sprouting and functional recovery (Chen et al. 2010). Berns et al. encapsulated neurospheres-derived cells in hydrogels containing aligned nanofibers of a self-assembled TN-C mimetic peptide and showed improved neurite growth possibly due to interaction with β1 integrin receptors (Berns et al. 2016). Exploration of the use of 2D surfaces and 3D hydrogel scaffolds with TN-C peptide epitopes showed that a 3D environment produced the extensive neurite growth and high neuronal marker expression of PC12 cells (Sever et al. 2018). The neuroactive peptides from TN-C can covalently modify the nanofibers to enhance adhesion of neurons (Ahmed et al. 2006). These TN-C peptide scaffolds highlight the potential of using ECM derived constituents to direct NSPC differentiation into neuronal fates upon transplantation into the injured CNS. In contrast, after adult traumatic brain injury in mice, TN-C expression is upregulated, localized over the lesion site, and associated with GFAP-expressing reactive astrocytes (Chen et al. 2010; Laywell et al. 1992). The role of TN-C and lack of regeneration or functional recovery following injury needs to be further investigated. Another member of the tenascin family, **tenascin-R**, is expressed solely in the CNS and has been linked to NSPC proliferation, neuronal differentiation, and generation of GABAergic neurons through β_1 integrin interactions (Tsai et al. 2014; Hargus et al. 2008; Liao et al. 2008).

In development, ***chondroitin sulfate proteoglycans*** (CSPGs) modulate neural tissue morphology and function through their GAG side chains connected to a core protein. In the mature CNS, CSPGs maintain synaptic connections through the formation of perineuronal nets and are expressed by astrocytes throughout the brain and spinal cord. The molecules are also expressed by adult NSPCs and radial glia. CSPGs in the CNS are largely studied in the context of glial scar formation following injury. They are a key constituent that provides inhibitory chemical cues when deposited by reactive astrocytes around the injury site. Cleavage of the GAG side chains from the core protein of CSPGs via enzymatic treatment with chondroitinase ABC (chABC) improves axonal extension and cell migration into and through the injured area, with some studies showing improvements in functional outcomes (Hu et al. 2018; Bradbury et al. 2002; Bartus et al. 2014). The inhibitory CSPGs (aggrecan,

brevican, neurocan, and phosphacan) are well-documented for their effects on cell migration and axonal growth, with limited study of their effects on NSPC fate (Siebert et al. 2014). Dissociated mouse NSPCs were formed into neurospheres with chABC in the media and resulted in astrogliosis over neurogenesis. Furthermore, chABC treatment affected NSPC differentiation in the neurospheres through regulation of FGF-2 (decreased neuronal differentiation) and EGF (decreased glial differentiation) (Sirko et al. 2010). Chondoitin sulfate GAG side chains (CS-GAGs) used without the core protein and modified with crosslinkable methacrylate functional groups were shown to retain incorporated FGF-2 for up to one week with minimal loss. Transplantation of encapsulated NSPCs within the FGF-2 loaded crosslinked CS-GAGs matrix showed maintenance of the undifferentiated state up to 4 weeks after injury in a rat model of traumatic brain injury (Betancur et al. 2017). This is attributed to the CS-GAGs' ability to associate with the growth factor and limit diffusion of the protein out of the material. These studies suggest that NSPC differentiation is responsive to FGF-2 and EGF, which can be regulated by CS-GAGs. However, increased deposition of CSPGs by reactive astrocytes in the injured CNS causes a chemical barrier that limits axonal regeneration.

Hyaluronic acid (HA) is a major constituent of the CNS matrix. HA has domains that bind to TN-C and proteoglycans to form a HA-proteoglycan network that supports NSPCs (Ruoslahti 1996). HA is highly abundant during fetal brain development and surrounds NSPCs to aid differentiation in the spinal cord (Banerjee and Toole 1991; Meszar et al. 2008). Testing with monoclonal antibody for HA-binding protein found that HA was prevalent in the neuroectoderm during development and concentrated in the layer of neuroectoderm that contains dense neuronal processes (Banerjee and Toole 1991). Mouse embryonic NSPCs were cultured within methacrylate functionalized HA hydrogels that were crosslinked via photo-initiation. NSPCs encapsulated in soft hydrogels (compressive modulus ~3000 Pa) increased neuronal differentiation, while NSPCs encapsulated in stiffer hydrogels (compressive modulus ~5000 Pa) increased astrocyte differentiation (Seidlits et al. 2010). Encapsulation of human induced pluripotent-NSPCs as either single cell suspensions or cell aggregate spheroids within crosslinked HA hydrogels (compressive modulus of ~500 Pa) were capable of neuronal differentiation and neurite extension (Wu et al. 2017). HA has the beneficial roles of promoting axonal growth and reducing the effects of scarring from CNS injury. Hydrogels composed of high molecular-weight HA reduced the presence of immune cells and reactive astrocyte CSPG deposition at the lesion site in a rat dorsal hemisection spinal cord injury model (Khaing et al. 2011). Furthermore, HA hydrogels crosslinked through click chemistry have tunable moduli and shear thinning properties that allow for injection of a crosslinked material directly into the CNS lesion, making them an ideal biomaterial candidate for CNS therapies (Nimmo et al. 2011; Thompson et al. 2018; Owen et al. 2013; Smith et al. 2018).

4. Conclusion

This review focused largely on major constituents of the ECM microenvironment that affect NSPC differentiation. Here we reviewed both physical and chemical cues found in the ECM that drive NSPC differentiation and regulate cell fate. Physical properties, such as material stiffness and surface topography are important factors during development, maturation, and

injury. Bioengineered materials can be tailored to provide specific mechanical and topographical signals, leading to increased differentiation of NSPCs into astrocytes, oligodendrocytes, or neurons. The biomaterials can also incorporate ECM-derived constituents that directly or indirectly influence NSPC differentiation. Major ECM components located in fractones, such as laminins, collagens, and HSPGs, aid in proliferation, migration, and differentiation through integrin-mediated binding or regulation of growth factors. Additional ECM constituents found throughout the CNS, including fibronectin, tenascin, and CSPGs, were also shown to affect NSPC differentiation.

Current work focuses on using proteomics and bioinformatics to compare the CNS matrisome differences during developmental stages, in various locations throughout the CNS, and after injury. This could lead to improved biomaterial designs that incorporate multiple factors at levels known to improve tissue regeneration. For example, it has been noted that ECM derived from fetal tissue may provide improved tissue regeneration compared to adult tissue (Ren et al. 2015).

It was previously mentioned that NSPCs are difficult to characterize due to the heterogeneous mixture of cells that is found within primary isolates that have regional diversity depending on the location in the brain and spinal cord from which they are obtained. This review has assumed the NSPCs used in the reported studies have the ability to differentiate into neural cell types including astrocytes, oligodendrocytes, and neurons. However, it should be noted that the NSPCs used different experimental studies are not, nor should they be expected to be, uniform and in many cases the NSPCs will contain other cell types that may influence the results. This heterogeneity could lead to research showing a specific ECM constituent promotes glial fates, while other research with the same ECM constituent promotes neuronal fates with the only difference in the experiments being the NSPCs used for the studies. Pre-sorting NSPCs prior to use can alleviate some heterogeneity from the cultures and allow for improved reproducibility. Determining and creating a universal classification system is difficult because many factors expressed by NSPCs are also in other cell types, meaning an array of markers must be used.

It is important to note that the use of potent soluble factors *in vitro* allows for exact temporal and dosage control of multiple factors to direct differentiation into specific neural cell types. These techniques have been fundamental to refining NSPC differentiation protocols to derive unique subtypes of neural cells. For example, NSPCs exposed to caudalizing agents (retinoic acid) will drive a spinal identity, then the cells can undergo dorsoventral patterning through exposure to cocktails of soluble factors (bone morphogenic proteins, sonic hedgehog, smoothened agonists, Notch inhibitors, FGF, Wnt) that can result in the generation of specific neural cell types (dorsal/ventral interneuron populations, motor neurons, glial restricted precursors, astrocytes). This level of control is not yet possible by solely relying on ECM-derived insoluble cues; however, the importance of the microenvironment is evident. Thus, it is important to consider the types of both soluble and insoluble factors used for development of CNS therapies as they play key roles in controlling NSPC fate and repopulation of desired cell types.

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Highlights:

- Neural stem cell fate is influenced by local microenvironment factors
- Local microenvironment cues include mechanical and topographical signals
- Presentation and abundance of specific microenvironment ligands affect cell fate
- Knowing factors that affect neural stem cell fate will improve biomaterial design

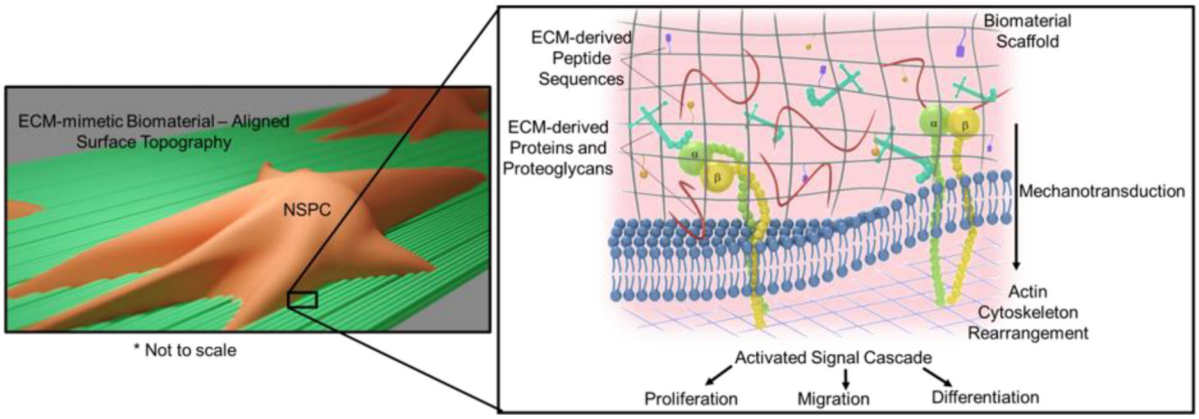

Figure 1:

Graphical representation of factors that can be incorporated into biomaterial design to control cell fate including surface topography, mechanotransduction, and ECM-derived peptides, proteins, and proteoglycans.

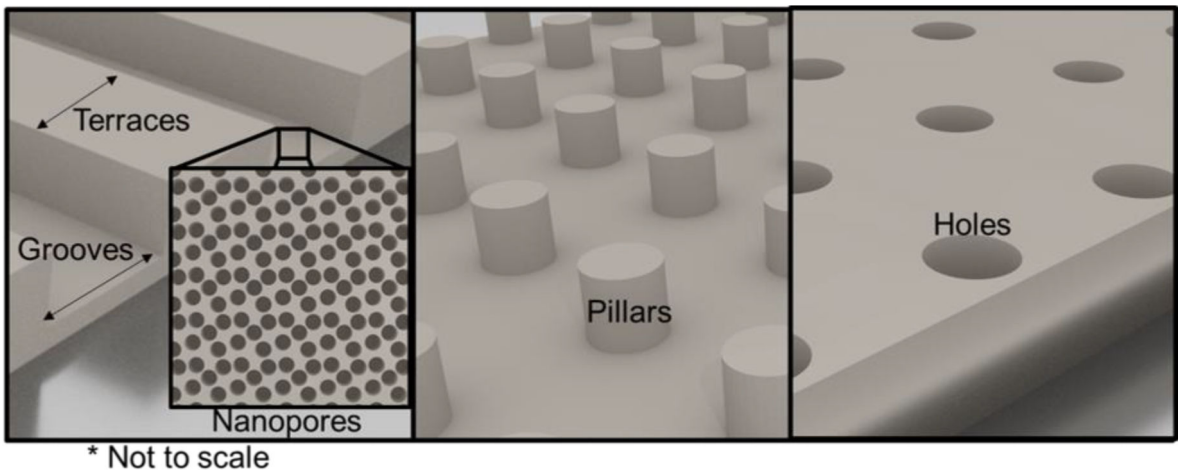

Figure 2:

Graphical representation of common micropatterned surfaces with controllable terrace width, groove width and depth, pillar height and diameter, hole depth and diameter, and inclusion of nanopores.

Table 1:

Summary of the major ECM components of the CNS.

Location	ECM Component	Role
Major ECM constituents of the stem cell niche/fractone	Laminin	Cell adhesion, migration, guidance
	Collagen	Cell adhesion, migration, guidance; structural integrity
	Heparin sulfate proteoglycans	Associate with growth factors to regulate differentiation
Additional constituents of the CNS ECM	Fibronectin	Expression coincides with phases of neuronal differentiation during development; Aids cell adhesion, migration, and survival
	Tenascin-C, Tenascin-R	TN-C: Cell adhesion; associate with growth factors to regulate differentiation TN-R: Associated with NSPC proliferation and neuronal differentiation
	Chondroitin sulfate proteoglycans	Stabilize synaptic connections. Major component of the inhibitory glial scar formed by astrocytes after injury.
	Hyaluronic acid	Cell adhesion. Aids differentiation in the developing CNS. Supportive matrix for NSPC transplantation that also decreases immune response and reactive gliosis.