Laminin-Inspired Cell-Instructive Microenvironments for Neural Stem Cells

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ABSTRACT

Laminin is a heterotrimeric glycoprotein with a key role in the formation and maintenance of the basement membrane architecture and properties, as well as on the modulation of several biological functions, including cell adhesion, migration, differentiation and matrix-mediated signaling. In the central nervous system (CNS), laminin is differentially expressed during development and homeostasis, with an impact on the modulation of cell function and fate. Within neurogenic niches, laminin is one of the most important and well described extracellular matrix (ECM) proteins. Specifically, efforts have been made to understand laminin assembly, domain architecture, and interaction of its different bioactive domains with cell surface receptors, soluble signaling molecules, and ECM proteins, to gain insight into the role of this ECM protein and its receptors on the modulation of neurogenesis, both in homeostasis and during repair. This is also expected to provide a rational basis for the design of biomaterial-based matrices mirroring the biological properties of the basement membrane of neural stem cell niches, for application in neural tissue repair and cell transplantation. This review provides a general overview of laminin structure and domain architecture, as well as the main biological functions mediated by this heterotrimeric glycoprotein. The expression and distribution of laminin in the CNS and, more specifically, its role within adult neural stem cell niches is summarized. Additionally, a detailed overview on the use of full-length laminin and laminin derived peptide/recombinant laminin fragments for the development of hydrogels for mimicking the neurogenic niche microenvironment is given. Finally, the main challenges associated with the development of laminin-inspired hydrogels and the hurdles to overcome for these to progress from bench to bedside are discussed.

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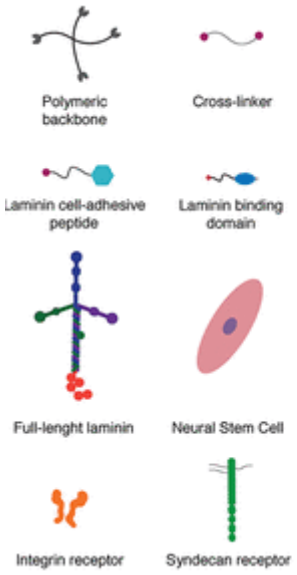

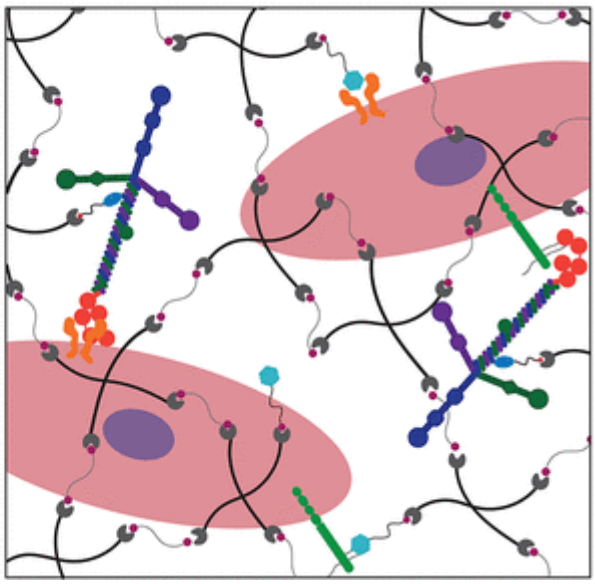

1. Laminin

1.1. Structure and Domain Architecture

Laminins are large heterotrimeric glycoproteins (400–900 kDa) composed by three polypeptide subunits, α, β, and γ, which assemble into cross-shaped molecules (Figure 1A). To date 5 α, 3 β, and 3 γ chains have been identified and associated with the formation of 16 different laminin isoforms (Figure 1B), whose expression patterns differ among different tissue types and development stages (for a more comprehensive review, see refs (1–3)). Heterotrimers containing laminin α1 (e.g., laminin-111) are mainly expressed during embryogenesis, disappearing progressively from most basement membranes (BMs) during development, while those comprising the α5 chain (e.g., laminin-511 and -521) are the most ubiquitous form in the adult organism.(4–7) Laminin isoforms 211 and 221 are mainly present in the BM of skeletal and cardiac muscles,(8–10) while laminin-411 and -421 are abundant in endothelial BMs.(11–13) Laminin-332, in turn, is specific for the basal lamina underlying epithelial cells.(14,15) In addition to the laminin isoforms identified to date, and summarized in Figure 1B, novel potential laminin chain combinations have been proposed: 212/222 (α2β1γ2/α2β2γ1);(16) 312 (α3β1γ2);(17) 333 (α3β3γ3);(18) 422 (α4β2γ2);(17) 522 (α5β2γ2),(19) though their expression in humans has not been demonstrated to date.

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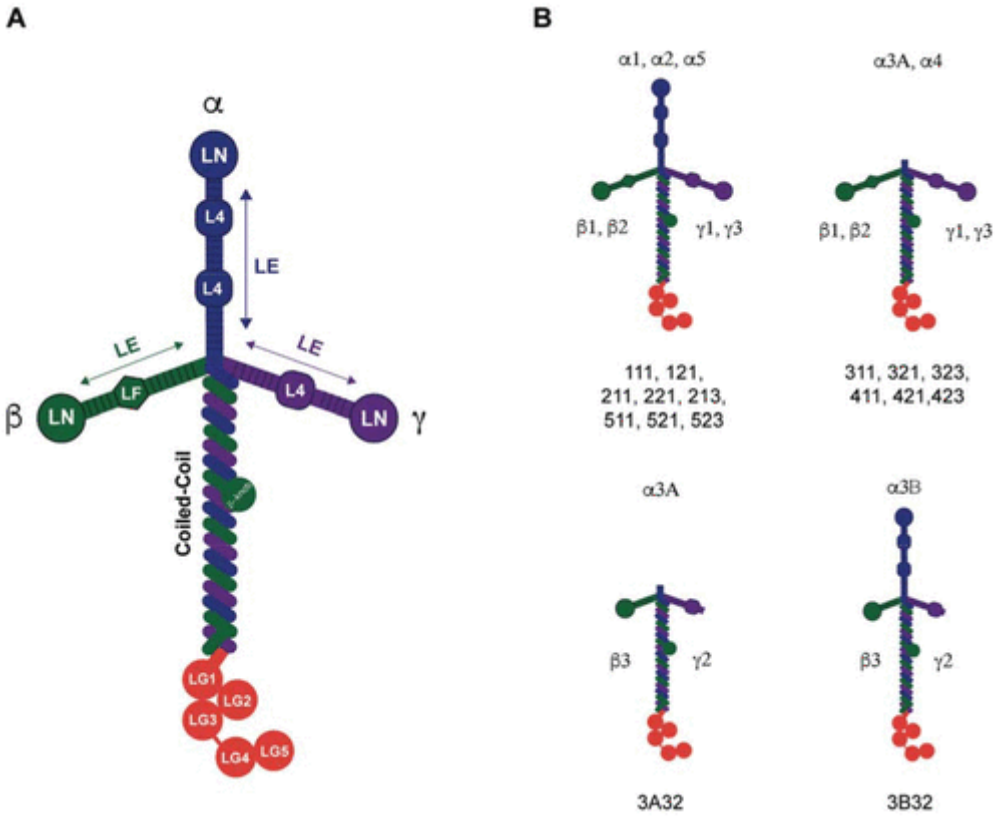

Figure 1. Laminin structure and domain architecture. (A) Representative model of laminin structure composed of three polypeptide subunits, α (blue), β (green), and γ (purple). LN, laminin N-terminal domain; LE, laminin-type epidermal growth factor like domains; L4, laminin 4; LF laminin four domains; LG laminin globular domains. (B) The 16 different laminin isoforms described to date, grouped according to their domain similarities. The laminin molecules are named according to their chain composition. Thus, laminin-511 contains α5, β1, and γ1 chains, for example. Adapted from ref (7). Copyright 2013 Landes Bioscience, with permission from Taylor & Francis Ltd. www.tandfonline.com.

Laminin isoforms are composed of three short arms and a triple α helical coiled-coil domain (long arm), formed by the combination of α, β, and γ subunits (Figure 1A). Short arms, with the exception of the α3A, α4, and γ2 chains (Figure 1B), are composed of a large globular domain at the N-terminus, the laminin N-terminal (LN) domain, and by tandem repeats of laminin-type epidermal growth factor like (LE) domains interspersed with globular domains (laminin 4 (L4) and laminin four (LF) domains), whose number vary among laminin subunits and whose function is still unknown.(1) Long arms present a highly conserved domain structure composed of α-helical domains, rich in heptad repeats $(abcdefg)_n$ of charged and nonpolar amino acids, folded into a trimeric coiled-coil structure.(20−22) β-Subunits all have a short stretch of amino acids, termed the β-knob, whose function has not yet been unveiled.(20) The long arm coiled-coil domain of the α-chain comprises a large globular domain,

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which is divided into five laminin globular (LG) domains (LG1–LG5) grouped into functional and structural distinct subdomains, LG1–3 and LG4 and LG5.(23) The coiled-coil is thought to help to orientate the LG domains so that they can be available to interact with cells via cell surface receptors, including integrins,(23–25) syndecans, and dystroglycan,(23,26–28) and bind to growth factors(29) and other extracellular matrix (ECM) proteins.(23)

1.2. Main Functions

The multiple bioactive domains of laminin are involved in the modulation of a plethora of biological functions, including ECM deposition and cell–ECM and cell–cell interactions (Figure 2A; for a more comprehensive review, see ref (30)). More specifically, the laminin short arms (N-terminus) are involved in laminin ability to polymerize,(31–33) even in the absence of other BM components, forming the molecular network that will be in contact with the cellular surface.(7) Laminin polymerization is a key process to direct BM assembly and organization and occurs by a thermally reversible mechanism dependent on the presence of calcium ions.(31–33) This process is better explained by the three-arm interaction model (Figure 2B),(32,33) which proposes that the globular N-terminal LN domains located at each end of the three laminin short arms (Figure 1B) interact with those of other laminins to form a polygonal network (Figure 2B).(31–35) The long arm of the laminin heterotrimer, in turn, is predicted not to be involved in the network formation, being free to interact with cells out of the plane of the polymer. Laminin assembly is not restricted to the self-polymerization of one specific laminin isoform with the formation of a homopolymer but can also occur by copolymerization of laminin heterotrimers from different laminin isoforms.(33) Additionally, different studies suggest that *in vivo* the process of laminin assembly is favored by laminin interaction with cell surface receptors (e.g., integrins, dystroglycan).(35–38) The importance of laminin polymerization is evidenced by studies showing that mutations in tissue-specific laminin genes that result in the impairment of laminin polymerization cause severe diseases in humans, such as congenital muscular dystrophy and epidermolysis bullosa, which are characterized by BM defects.(5) In addition, the relevance of this process is supported by reports showing that the repair of laminin polymerization can ameliorate such pathological phenotypes.(39) Moreover, laminin ability to polymerize helps to correctly orientate some of the key laminin bioactive epitopes, so they can be available to interact with cells via cell surface receptors (e.g., integrins, syndecans, and dystroglycan), as well as to bind to growth factors and other ECM proteins.

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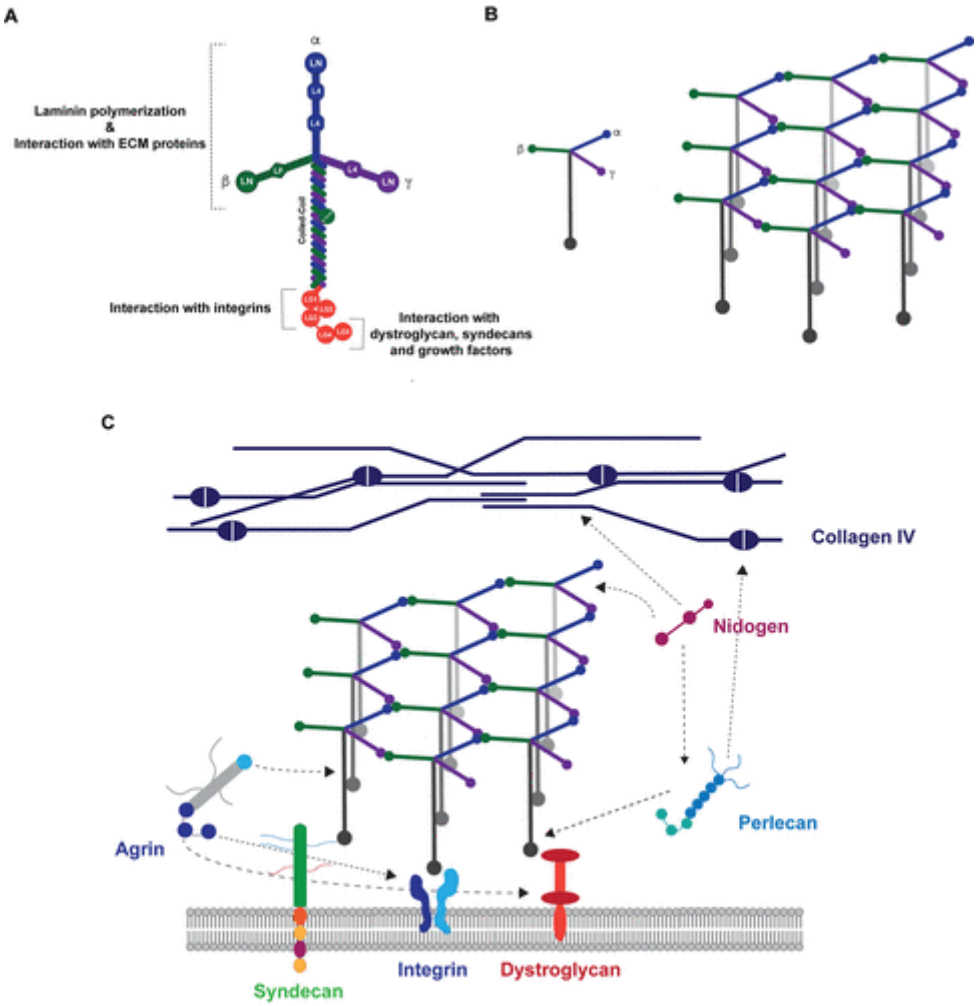

Figure 2. Main biological functions mediated by laminin (not to scale). (A) Schematic representation of the major functions of laminin. The laminin short arms (N-terminus) mediate the process of laminin polymerization and the interaction with other ECM proteins, contributing to the assembly and stability of BMs. The globular domains LG1–3 within the end of laminin long arm (C-terminus) mediate laminin interactions with cell integrin receptors, while globular domains LG4–5 mediate interactions with dystroglycan, syndecans, and growth factors, being responsible for the modulation of different cell functions (e.g., adhesion, proliferation, migration, and differentiation). Adapted from ref (7). Copyright 2013 Landes Bioscience, with permission from Taylor & Francis Ltd. www.tandfonline.com. (B) Schematic representation of laminin polymerization. N-terminal domain of α, β, and γ chains interact to form a polygonal network. Adapted from ref (49). Copyright 2012 Landes Bioscience, with permission from Taylor & Francis Ltd. www.tandfonline.com. (C) Schematic representation of supramolecular assembly of the BM. Laminin polymeric network interacts with different components of the BM, including nidogen, perlecan, agrin, and collagen IV, contributing to the stability and assembly of BM. Nidogen and perlecan mediate laminin–collagen IV binding, whereas agrin binds to laminin through its N-terminal domain. Both agrin and the laminin network are anchored to the cell surface by interactions with integrins, syndecans, and α-dystroglycan. Adapted from ref (49). Copyright 2012 Landes Bioscience, with permission from Taylor & Francis Ltd. www.tandfonline.com.

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Laminin short arms also mediate laminin interaction with other ECM proteins (e.g., nidogen, netrin 4, heparan sulfate proteoglycans (HSPGs), and collagen IV) (Figure 2C), playing a key role in the assemble and stability of the BMs.(40) The interactions mediating the incorporation of laminin polymeric structures into the supramolecular network of the BM depend on the laminin isoform, namely, on the structural domains present on laminin short arms (Figure 1B). Indeed, while an LE module within the laminin γ1 subunit(41,42) mediates a strong interaction with nidogen,(43) a sulfated glycoprotein ubiquitously found in BMs, a weaker nidogen binding has been observed in laminin γ2 and γ3 subunits.(44) Nidogen also interacts with collagen IV and perlecan, one of the major HSPGs, thus working as an intermediate for the integration of laminin into the BM. Agrin, a multidomain HSPG, was shown to mediate a high affinity interaction with a sequence of 20 conserved residues within laminin γ1 chain (dissociation constant (KD) ≅ 5 nM) through its N-terminal (NtA) domain.(45) This interaction is required for the integration of agrin into the synaptic basal lamina and other BMs.(46) Perlecan and agrin, through the direct or indirect interaction with laminin, will mediate the interaction of the laminin polymeric network with the cell surface assembled collagen IV, thus contributing to BM assembly and stabilization.(47−49)

In addition to its structural role, laminin comprises multiple bioactive domains that interact with cell surface receptors (e.g., integrins, dystroglycans, syndecans, and Lutheran) and growth factors (Figure 2B,C). These interactions are mediated by the LG domains located in the C-terminal end of laminin α chain. Whereas LG1−3 domains mediate interaction with integrin receptors and Lutheran,(23−25,50) the LG4−5 pair contains binding sites for α-dystroglycan and syndecans.(23,26−28) While the interaction with different cell surface receptors is known to be essential for the modulation of different cell functions, including cell adhesion, proliferation, migration, neurite outgrowth, and differentiation, as well as for ECM deposition,(7,10,51) interaction of laminin with growth factors (e.g., vascular endothelial growth factor; platelet-derived growth factor; fibroblast growth factor; bone morphogenic protein; neurotrophin; endothelial growth factor; CXCL chemokines) was only recently described.(29) Thus, further studies to assess its potential contribution for cell function still need to be conducted. The classical laminin-binding integrins α3β1, α6β1, α7β1, and α6β4 present different specificities depending on the laminin isoform(52) and most specifically on the laminin α chain. Laminin isoforms composed of α5 are the preferred ligands for laminin-binding integrins, whereas the ones composed of α4 chains constitute the poorest ligands.(53) Integrins binding to LG domains require that both the LG1−LG3 domains and the coiled-coil are intact. Indeed, some studies showed that modifications of the LG1−LG3 domain or the coiled-coil structure abolish cell adhesion-promoting activity.(54−56) Although integrins bind to the LG1−3 domain comprised of the α-chain,(3,6,57) β- and γ-chains are also involved in laminin−integrin interactions.(58,59) In fact, recently, the Glu residue in the C-terminis of laminin γ1 chain was found to be required for the binding of γ1-laminins to α3, α6, and α7 integrins.(60) In addition to the LG1−3 domains, integrins also interact with adhesive motifs present in the short arms of laminin α chains.(61−64) The LN domain of laminin α5 chain binds to integrin α3β1,(61) while the two RGD sequences present in the L4b domain of laminin α5 chain are recognized by β1 and αVβ3 integrins.(62) The short arm of mouse laminin α1 chain also contains an RGD sequence that mediates cell adhesion via α5β1 and α6 integrin subunit but requires proteolytic cleavage to be exposed.(63−65) Compared to integrins, α-dystroglycan displays a narrower binding spectrum, presenting high affinity only for laminin α1 and α2 chains.(3) Although the LG4−5 domains are the preferred α-dystroglycan binding domains, the LG1−3 domains of the α2 chain contain additional α-dystroglycan binding sites.(3,23,57) Syndecans are type I transmembrane HSPG cell receptors, which interact with laminin LG4−5 domains. These receptors have a key role in the modulation of all stages

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of stem cell maintenance and neurogenesis (e.g., proliferation, self-renewal, differentiation, migration, and maturation), either through independent signaling or by working alongside with other receptors, such as integrins.(66–68) In addition to α-dystroglycan and syndecans, other nonintegrin receptors have been described to interact with laminin domains different from the LG domains. These include the 110 kDa(69) and the 67 kDa cell surface receptors,(70) which bind with high affinity to the IKVAV amino acid sequence within the laminin α1 chain and the YIGSR sequence within the laminin β1 chain, respectively.

Ultimately, the laminin-associated matrix proteins, in concert with cell-surface receptors, are key for the proper assembly or deposition of laminin matrices and work in an orchestrated manner to fine-tune both matrix formation and its function.

2. Laminin in the Central Nervous System (CNS) and in Neurogenic Niches

2.1. Laminin in the CNS

Laminin is differentially expressed within the CNS, during both development and adulthood, with an impact on the modulation of cell function and fate. Despite its involvement in many aspects of CNS physiology and neuronal functions, there is still a lack of understanding of the exact role of laminin in the formation, development, and function of the neuronal networks. In this regard, several studies have been conducted to better characterize the expression and distribution of specific laminin chains (and possible laminin isoforms) in the CNS during embryonic development and in adulthood, as well as their site of origin (for a comprehensive review, see ref (71)).

Evidence from human fetal and mouse embryonic studies demonstrated that laminin-111 is expressed throughout the CNS during embryogenesis and progressively disappears during development.(4–7) Different laminin subunits (α1, α2, α3, α4, α5, β1, β2, γ1) may be found in the developing CNS, namely, in regions such as the spinal cord, ventricular (VZ) and subventricular (SVZ) zones, cortical plate, and cerebellum, but the biological significance of the presence of these chains is still not well understood.(71–73) In addition, laminins comprising the α5 chain have been shown to have a functional role in neural tube closure during mouse embryogenesis.(72,74) Of note, the expression of laminin β3 chain at the embryonic stage is still subject of great controversy.(75,76)

In the adult CNS, laminin can be found in almost all cell types, with a key role on the modulation of distinct functions.(71,77) Among the different laminin isoforms, heterotrimers comprising the α5 chain (e.g., laminin-511 and -521) have been identified as the major neuronal laminins in adulthood.(4–7)

2.2. Laminin in Neurogenic Niches

Neural stem cells (NSCs) reside within specialized structures, the neurogenic niches, where cell–cell interactions and local microenvironmental cues, including those from neighboring cells, humoral factors, and ECM, are key to regulate stem cell behavior. During development, NSCs are distributed

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along the VZ of the neural tube, while in adult brain NSCs are restricted to specific neurogenic niches, which include the subgranular zone (SGZ) of the dentate gyrus in the hippocampus or the SVZ on the lateral ventricles, which is the largest and most studied neurogenic niche.(78)

The ventricular and subventricular zone (V-SVZ) of the adult brain (Figure 3) are composed of support cells (endothelial cells and astrocytes), immature precursors, and a monolayer of ependymal cells lining the lateral ventricle.(79) Moreover, the resident stem cell lineage in the SVZ comprises the relatively quiescent NSCs (type B cells),(80) which can self-renew or generate transit-amplifying cells (type C cells). The latter can, in turn, give rise to migratory neuroblasts (type A cells) and oligodendrocyte precursors.(81,82) In the niche, NSCs are surrounded by fractones, branched structures closely associated with blood vessels rich in several ECM components, including laminin, collagen IV, nidogen, and proteoglycans.(79) These fingerlike projections of ECM can sequester neurogenic growth factors, such as fibroblast growth factor 2 from the ventricular cerebrospinal fluid and, as such, are believed to be key for the modulation of neurogenesis via ECM–growth factor interactions.(83) Moreover, fractones are suggested to have a key role on the maintenance of NSCs within the niche, mediated by cell–cell interactions. This was supported by studies showing that the in vivo blockage of the laminin α6β1 receptor expressed by SVZ NSCs favors the release of cells from the basal lamina and a subsequent increase in proliferation.(84)

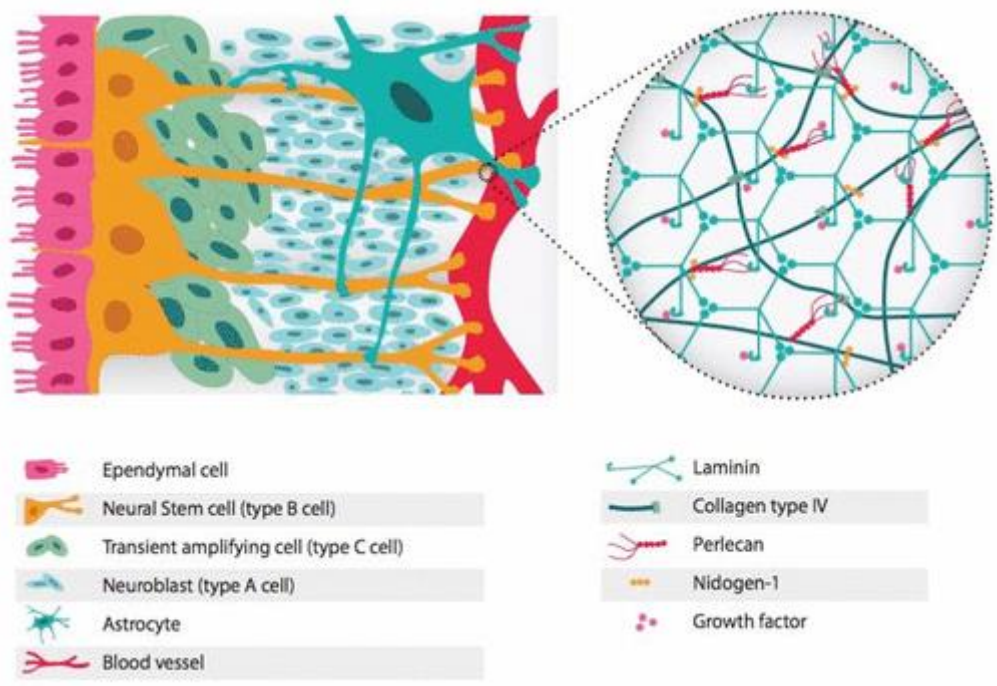

Figure 3. Representation of the adult ventricular and subventricular zones (V-SVZ). Three types of progenitor cells are found in close proximity to the ependymal cell layer and include a population of quiescent NSCs and radial glia-like cells (type B cells) that can generate transit-amplifying cells (type C cells), which, in turn, will give rise to neuroblasts (type A cells). Endothelial cells and astrocytes, which function as support cells, as well as immature precursors can also be found within the NSC niche. The detailed inset depicts the fractones, branched structures closely associated with blood

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vessels, which include laminin, collagen IV, nidogen and proteoglycans, which can sequester neurogenic growth factors. These contact with the different cell types composing the niche and are involved on the modulation of different cell functions.

Among the ECM proteins found in adult neurogenic niches, laminin is one of the most important and well described.(77,83) This is evidenced by its key role in the modulation of neuronal progenitor proliferation and survival, (85,86) neurodevelopment,(71) and hippocampal regeneration.(87) In neurogenic niches, multiple α, β, and γ laminin chains have been detected in the outer surface of blood vessels and in fractones, as well as in the ependymal cell layer(77) and around clusters of proliferating transit-amplifying cells (type C cells) and neuroblasts.(77) Notably, fractones from the adult rat, mouse, or human brain are rich in laminin β1 and γ1 chains but not in laminin α1 chain, which can be found on the BM of the blood vessels.(83) In the adult mice hippocampus BM, laminin-511 was shown to be the major neuronal laminin and important for hippocampal regeneration.(88) Within the CNS, the differential expression of laminin isoforms has been associated with different functions. For example, laminin isoforms comprising the α2 chain where shown to be required for oligodendrocyte maturation and CNS myelination in adult mice(89) under homeostasis conditions, while laminin-411 or -511 and laminin-111 or -211 produced by endothelial cells and astrocytes, respectively, participate in the formation of the blood–brain barrier.(90) Mutations in laminin β2 (mice),(91) γ1 (mice),(92) and γ3 (mice and human) (91,93) chains, in turn, were shown to disturb processes like cortical development, as well as the formation of the pial BM. Nevertheless, the mechanism by which differentially expressed laminin isoforms modulate adult neurogenesis is not yet fully understood. In this regard, some studies suggest that rather than differences in ECM composition, changes in the expression of the major laminin receptors, integrins, dystroglycan, and syndecans, seem to be responsible for the changes in ECM signaling that contribute to stem cell activation.(77,84,94) Under homeostatic conditions, NSCs have limited interaction with the laminin-rich microenvironment due to the low expression of laminin receptors and, as a result, remain relatively quiescent.(77) After stimulation and activation of NSCs (e.g., in the regenerative niche that is triggered in the aftermath of a lesion), different laminin receptors are upregulated (including α6β1 integrin and syndecan-1) potentiating its interaction with the cellular and extracellular microenvironment.(77)

The interplay between the niche components and NSCs will determine the balance between stemness and differentiation, quiescence and proliferation. In this regard, a number of experimental approaches perturbing the niches either through the genetic or cytotoxic ablation of the precursor cells or using specific culture models to evaluate the effect of specific components of the niches have been proposed to better understand how changes in the ECM receptor expression regulates NSC behavior (for a more comprehensive review, see ref (95)). However, the effect of the matrix itself remains to be elucidated. In the past few years, much attention has been devoted toward the development of three-dimensional (3D) matrices able to recapitulate the complex arrangement of cells and ECM in the nervous system and dissect the different roles exerted by the ECM and its molecular components on NSC behavior. These are expected to mimic the in vivo characteristics of NSC niches and ultimately allow the development of more efficient neuroregenerative approaches.

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3. Laminin-Inspired Hydrogels to Recreate the Microenvironment of Neurogenic Niches

As discussed in the previous section, laminin is known to have a key role in the modulation of NSC function and fate within neurogenic niches. More specifically, this ECM protein was shown to be crucial for the modulation of cell adhesion and viability(96) and neuronal outgrowth and migration.(97–99) Works published to date exploring the immobilization of full-length laminins (Table 1) or their peptide analogues (Table 2) into hydrogel matrices to recreate the NSC niche microenvironment are summarized in this section. The developed 3D platforms were applied for the in vitro study of NSC biology and to assess their interactions with the surrounding microenvironment, as well as to evaluate the potential of the developed matrices to serve as vehicles for NSC transplantation in the context of neurological disorders. In addition to mimicking the ECM composition, replicating the mechanical and structural properties of the native niche is key for the modulation of different stem cell functions, including survival, proliferation, and differentiation.(100–102) Indeed, studies using substrates with mechanical properties similar to that of native CNS (low compressive moduli, 0.1–1.0 kPa) have shown ability to support NSC function.(103–108)

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Table 1. Hydrogels Functionalized with Full-Length Laminin

3D matrix composition				biological outcome		
laminin concentration in the hydrogel[a]	polymeric backbone	laminin immobilization strategy	cell type	*in vitro*	*in vivo*	ref
1.6 ng/mg OXMC	oxidized methylcellulose (OXMC)	Schiff base reaction between laminin primary amines and carbonyl groups on OXMC	primary rat cortical neurons	enhanced cell adhesion and viability as compared to OXMC hydrogels without laminin		115
8.2 μg/mL	methylcellulose (MC)	reaction between laminin primary amines and N-hydroxysuccinimide (NHS) esters on sulfo-SANPAH ((sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate)) and coupling of the photosensitive laminin conjugate to MC by exposure to ultraviolet (UV) light	mouse neural stem cells (NSCs)	enhanced cell survival and neurite outgrowth as compared to hydrogels with physically entrapped laminin; reduced apoptosis and provided an environment that fostered differentiation	cell transplantation into a murine traumatic brain injury model favored a moderate increase in functional recovery	116, 117, 123, 124
100 μg/mL	thiolated hyaluronic acid (HA-S)	Michael-type addition of laminin free thiol groups to vinyl groups on poly(ethylene glycol) divinyl sulfone (PEGDVS) prior to the cross-linking of HA-S by PEGDVS	mouse neural stem/progenitor cells (NSPC)	supported cell chemotactic migration in response to SDF-1α, and this ability was critically dependent on both HA and laminin	favored the retention and migration of transplanted cells in response to SDF-1α in an intact mouse brain model	110, 111
100 μg/mL	HA-S + salmon fibrinogen	incorporation in the hydrogel precursor solution prior to the addition of thrombin, to cleave fibrinogen and thus induce the formation of an interpenetrating polymer network hydrogel	human NSPC and cocultures of hNSPCs and human endothelial colony-forming cell-derived endothelial cells (hECFC-ECs)	supported cell proliferation and differentiation similarly to hydrogels without laminin, but neurite outgrowth was most robust in hydrogels containing laminin; supported enhanced vascularization (vessel area percentage) as compared to fibrin hydrogels		109
0.120–0.162 μg/mL	agarose	reaction between laminin primary amines and NHS esters on sulfo-SANPAH and coupling of the laminin photosensitive conjugate to agarose by exposure to UV light	dorsal root ganglia (DRG)	neurite outgrowth was higher in anisotropic hydrogels containing gradients of photoimmobilized laminin, compared to isotropic hydrogels		125
1–100 μg/mL	collagen I	incorporation in collagen solution prior to thermally induced hydrogel gelation	DRG	addition of laminin reduced neurite growth without impacting the stiffness of the hydrogels		121
1.5 mg/mL	collagen I + HA	incorporation in hydrogel precursor solution prior to thermally induced hydrogel gelation	rat NPCs	hydrogels containing laminin supported increased NPC oligodendrocytic differentiation (or higher oligodendrocyte survival) compared to hydrogels without laminin	NPC transplantation within the three-component hydrogel promoted increased functional recovery over 6 weeks in a rat model of spinal cord injury (SCI) as compared to the media control group	122
1–100 μg/mL	acrylate (Ac)−poly(-ethylene glycol) (PEG)−NHS + PEG−diacrylate (DA)	reaction between laminin primary amines and NHS groups on Ac-PEG-NHS (4 NHS/1 laminin) and coupling of the laminin conjugate to PEG-DA by exposure to UV light	DRG	neurite outgrowth increased with laminin concentration, but no significant differences were observed between hydrogels with covalently immobilized laminin and those with physically entrapped laminin		120
50 ng/g DIBO-PEG	dibenzocyclooctyne (DIBO)−PEG	reaction between laminin primary amines and NHS groups on azide-PEG-NHS and coupling of the laminin conjugate to DIBO-PEG by copper-free azide−alkyne reaction prior to the addition of the cross-linker (four arm PEG tetrazide)	rat NSPC	hydrogels modified with laminin and a potent neurogenic differentiation factor (interferon-γ) supported high cell viability and efficiently directed cell differentiation to neurons without the need for additional supplementation		126
100 μg/mL	PEG−4maleimide (PEG-4MAL) functionalized with a thiol-containing mono-PEGylated rhNtA domain	reaction between laminin and NtA-functionalized PEG-4MAL, prior to the addition of a mixture of protease degradable and nondegradable cross-linkers	human NSCs	site-selective immobilization of laminin better preserved protein bioactivity in terms of ability to promote NSC proliferation, neuronal differentiation, and neurite extension when compared to physically entrapped laminin		119

[a]Studies published to date exploring full-length laminin immobilization all used laminin-111 purified from Engelbreth-Holm-Swarm mouse sarcoma cells.

Table 2. Hydrogels Functionalized with Laminin-Derived Peptides

laminin-derived peptide sequence	origin	polymeric backbone	cell type	biological outcome		ref
				in vitro	*in vivo*	
CCRRIKVAVWLC (cyclic IKVAV)	mouse laminin α1 chain	poly(ethylene glycol) (PEG)	human neural stem/progenitor cells (NSPCs)	enhanced cell adhesion, proliferation, migration, and neuronal differentiation compared to hydrogels functionalized with linear-IKVAV peptide		137
IKVAV	mouse laminin α1 chain	silk fibroin	human neural stem cells (NSCs)	increased cell viability, proliferation, neuronal differentiation, and maturation, compared to unmodified hydrogels		138
		PEG	mouse neural progenitor cells (NPCs)	long-term cell survival and proliferation compared to unmodified gels		139
			PC12	supported cell adhesion and viability resulting in increased outgrowth and neuronal differentiation compared with YIGSR-modified hydrogels		140
		fibrin	dorsal root ganglia (DRG)	enhanced neurite outgrowth with increased density of covalently bound peptide		141
		peptide amphiphile (PA)	mouse NPCs	supported cell viability and resulted in increased outgrowth and neuronal differentiation relative to laminin or soluble peptide	cells transplanted within PA-IKVAV hydrogels into a rat model of traumatic brain injury showed enhanced cell survival and higher tendency toward neuronal differentiation compared to those in plain PA hydrogels	143
			rat NSCs			142
			mouse NPCs	IKVAV presentation on aligned PA nanofibers enhanced neurite outgrowth; after 2 weeks of culture, the neurons displayed spontaneous electrical activity and ability to establish synaptic connections	IKVAV presentation in combination with PA nanofiber alignment promoted an oriented growth of cellular processes on cells transplanted to a rat model of SCI	144
		agarose	PC12	enhanced neurite outgrowth compared to unmodified agarose gels		145
YIGSR	mouse laminin β1 chain	PEG	mouse NPCs	promoted long-term cell survival and proliferation compared to unmodified gels		139
			PC12	promoted cell viability and retention of the neuronal phenotype following cell release from the gels		140
		fibrin	DRG	increased neurite outgrowth with increased density of covalently bound peptide		141
		agarose	DRG	enhanced neurite outgrowth		145
RGD	short arm region of laminin α5 chain and mouse laminin α1 chain	fibrin	DRG	fibrin functionalization with RGD elicited a biphasic effect on neurite outgrowth, maximum enhancement being reached at intermediate densities of covalently bound peptide		141, 146
RNIAEIIKDI	mouse laminin γ1 chain	fibrin	DRG	enhanced neurite outgrowth with increasing densities of covalently bound peptide		141
RKRLQVQLSIRT (AG73)	LG4 domain of mouse laminin α1 chain	alginate	PC12	enhanced cell attachment and neurite outgrowth compared to unmodified hydrogels		147
		collagen				148
		agarose				149
		fibrin	human NSCs	efficiently promoted neurite outgrowth		150
PPFLMLLKGSTR (G3P)	LG3 domain of laminin α3 chain	collagen	rat NSCs	enhanced cell adhesion and survival compared to unmodified collagen hydrogels		151

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3.1. Hydrogels Functionalized with Full-Length Laminin

The incorporation of full-length laminin has been widely explored for the development of biomimetic hydrogels to provide the gels with the cell adhesiveness levels of the native protein. Most of these studies (Table 1) showed that the modification of specific matrices with full-length laminin renders the microenvironment more permissive for NSC neurite extension and differentiation, while also contributing to enhanced nervous tissue regeneration (Table 1). As an example, interpenetrating networks of hyaluronic acid (HA) and fibrin containing laminin showed increased ability to support NSC neurite outgrowth when compared to hydrogels without laminin (109) (Figure 4).

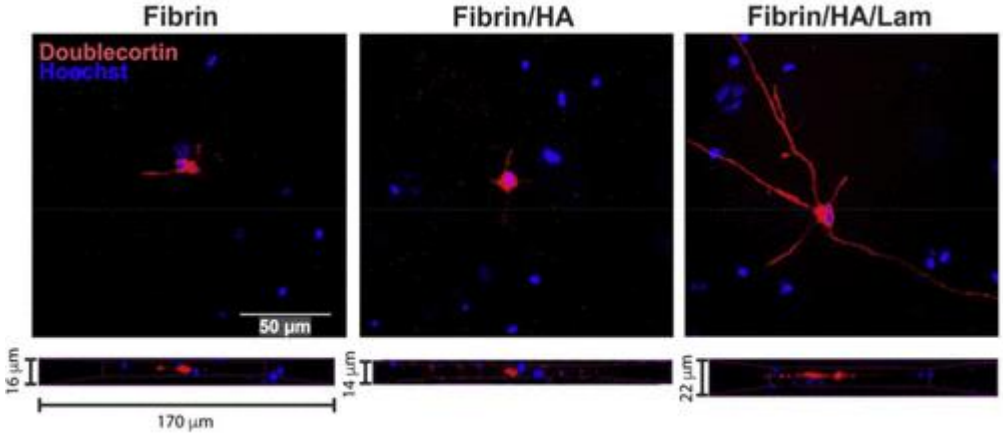

Figure 4. Human NSCs differentiate within hydrogels based on salmon fibrin, hyaluronic acid, and laminin. hNSCs cultured for 14 days formed neurons positive for the early neuronal marker doublecortin within the represented hydrogels; however, neurite outgrowth was most pronounced in hydrogels containing laminin. Adapted from ref (109). Copyright 2016 Acta Materialia, Inc., with permission from Elsevier.

The Stabenfeldt group also explored the incorporation of laminin into HA-based hydrogels to provide them with cell adhesive cues enabling NSPC adhesion and migration. Laminin immobilization significantly enhanced NSPC migration through HA-based hydrogels in response to gradients of stromal cell-derived factor 1α (SDF-1α), which is a potent chemoattractant for recruitment of endogenous NPSCs to sites of injury.(110) Moreover, HA–laminin hydrogels revealed significantly increased NPSC transplant retention and migratory response to SDF-1α in an intact mouse brain model.(111)

Since the biological features of a protein result from its multiple bioactive domains and its conformation,(112−114) the immobilization strategy and chemistry explored will have a critical impact on the biofunctionality of the engineered hydrogels.(115−119) Indeed, this is evident in some of the studies reported in Table 1, which point out that the immobilization approach significantly affects laminin function.(116,118,119) Strategies explored to date, for full-length laminin immobilization have relied either on its transient noncovalent incorporation or physical entrapment (109,120−122) or, alternatively, on its nonselective covalent immobilization (110,111,115−117,120,123−126) by taking advantage of functional groups present in multiple sites of the laminin structure, such as amines and thiols (see Figure 5 for illustrative examples based on PEG hydrogels). While physical entrapment ensures no conformational changes of the protein due to

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chemical conjugation, the absence of a stable binding may allow protein release by diffusion, especially in noncovalently cross-linked hydrogels. Covalent immobilization, in turn, although it provides a stable conjugation of the protein to a substrate, does not allow laminin exchangeability and, thus, the establishment of a dynamic biomimetic system. Moreover, and despite being widely used, these strategies lack the ability to control the conformation and orientation of bioactive molecules upon immobilization. As such, the exposure of key laminin bioactive domains, including those involved in laminin polymerization and in the modulation of NSC function and fate, can be compromised. To control the conformation of laminin upon its immobilization and the exposure of laminin bioactive domains, our group has recently explored an affinity-based approach to immobilize laminin that takes advantage of the native high affinity interaction between the N-terminal domain of agrin (NtA) and the coiled-coil domain of laminin.(118,119) Site-specific immobilization of laminin was shown to better preserve laminin ability to self-polymerize and mediate cell adhesion, when compared to nonselective covalent immobilization.(118) Moreover, synthetic hydrogels containing affinity-bound laminin better supported human NSC proliferation, neuronal differentiation, and neurite extension when compared to hydrogels with physically entrapped laminin.(119) Overall, the affinity-based approach proposed assures not only the site-specific conjugation of laminin but also the establishment of a versatile and dynamic biomimetic system, which more closely resembles what happens in a biological context.

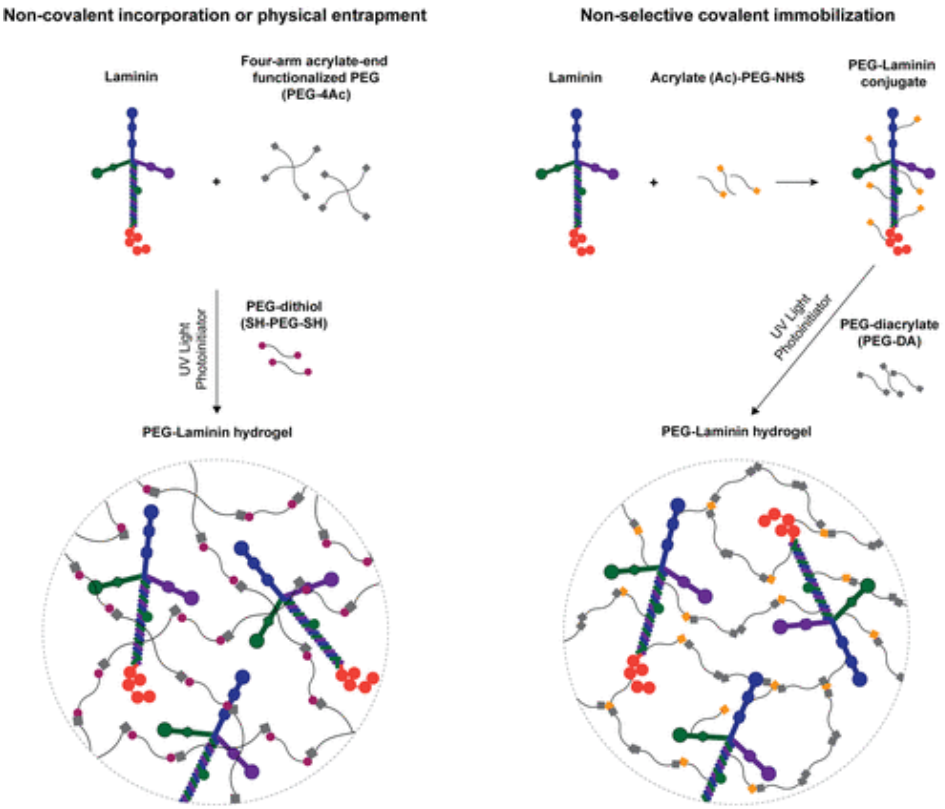

Figure 5. Strategies currently explored for laminin immobilization into 3D cell instructive microenvironments based on PEG hydrogels. Noncovalent incorporation or physical entrapment is

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used under the assumption that the entrapped protein retains the overall properties of laminin matrices in vivo. Nonselective covalent immobilization, in turn, takes advantage of functional groups (e.g., amines and thiols) present in multiple sites on the laminin structure. Both strategies lack the ability to control the orientation and conformation of laminin upon immobilization, which may compromise the exposure of key laminin bioactive epitopes.

The studies published to date, exploring full-length laminin immobilization, all used laminin-111 purified from Engelbreth-Holm-Swarm mouse sarcoma cells, as this isoform was already shown to successfully promote neuronal outgrowth and differentiation. Nevertheless, considering the key role of other laminin isoforms (e.g., laminin-521) on the in vitro modulation of neural cell behavior including neuronal adhesion, viability, and network formation, (96,127) future studies evaluating their impact on NSC behavior and function, after incorporation within 3D matrices, should be conducted.

Therapeutic strategies relying on the recruitment of endogenous progenitors to improve neural repair and functional outcome in the adult CNS have failed to contribute to cell replacement, despite the differentiation potential of endogenous NSCs.(128) In this sense, efforts have been made to develop hydrogels permissive to the infiltration of endogenous NSPCs recruited to the injury site and capable of guiding their fate to promote tissue regeneration.(129) More specifically, a laminin-functionalized HA-based hydrogel was shown to efficiently support endogenous cell infiltration and angiogenesis, while inhibiting the formation of the glial scar and promoting neurite extension in a rat model of brain lesion.

In addition to their application in the framework of nervous regeneration and tissue engineering, the incorporation of full-length laminin in 3D matrices has also been explored for application in different disease contexts, including skeletal muscle, (130,131) intervertebral disc, (132,133) and vascular regeneration.(134,135)

3.2. Hydrogels Functionalized with Laminin-Derived Peptides

Laminin-derived small adhesive sequences have been increasingly explored, in alternative to full-length laminin, to confer bioactivity to 3D matrices. These small sequences have several advantageous features, including their ability to be chemically synthesized in large scale, higher resistance to denaturation and enzymatic degradation, ability to be incorporated at higher densities for similar amounts of native protein, and lower risk of inducing immune rejection.(136) Different small peptide sequences derived from laminin, including YIGSR, IKVAV, RGD (from laminin α1 chain), RNIAEIIKDI, and RKRLQVQSIRT, are known to mediate neuronal adhesion, neurite outgrowth, and NSC migration and, as such, have been widely explored for the design of biomimetic 3D matrices for cell culture and transplantation (Table 2).

Peptides IKVAV, found in laminin α1 chain,(153) and YIGSR, found in laminin β1 chain,(70,154,155) bind to the 110 kDa(69) and 67 kDa(70) cell surface receptors, respectively, and are both recognized by the β1 integrin receptor subunit, which is highly expressed by neural stem/progenitor cells (NSPCs).(156,157) These peptides showed potential to promote neuronal attachment, migration, and neurite outgrowth (Table 2). For example, a study using self-assembling hydrogels modified with IKVAV evidenced the ability of this peptide sequence to be used as guiding cue to direct NSC

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adhesion and neuronal differentiation, as shown by the increased number of cells expressing the neuronal markers β-III tubulin and MAP2 (Figure 6A).(143) In addition, only few GFAP positive astrocytes were detected. In vivo results showed that when injected in a rat model of traumatic brain injury, RADA16-IKVAV hydrogel supported NSC survival and neuronal differentiation, as evidenced by the increased number of β-III tubulin-positive cells, as well as by the enhanced expression of mature neuronal markers such as MAP2 and NF-H, when compared to those with unmodified hydrogels (Figure 6B). In turn, the GFAP-positive astrocytes were shown to be more abundant in unmodified hydrogels (Figure 6B).(143) Overall, results in this study demonstrate that NSC transplantation within IKVAV-modified self-assembling hydrogels significantly enhanced neurogenesis while suppressing astrocytic differentiation, thus constituting a promising matrix for brain tissue regeneration.

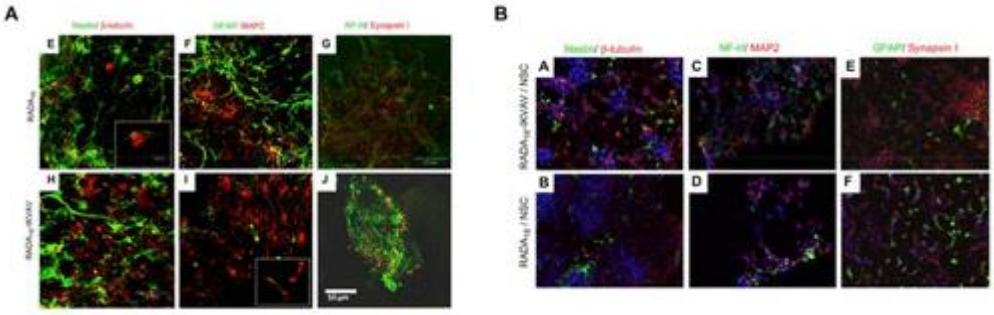

Figure 6. IKVAV-modified self-assembling hydrogel supports NSC neuronal differentiation and improvement in brain tissue regeneration. (A) Rat NSCs cultured for 14 days within unmodified (RADA16) and IKVAV-modified (RADA16-IKVAV) self-assembling hydrogels. (B) Immunohistochemical analysis of injured rat brain tissues 6 weeks post-transplantation. Cells were stained for nestin (neural progenitors; green); GFAP (astrocytes; green); β-III tubulin (premature neurons; red); MAP2 (mature neurons; red); NF-H (neural cytoskeleton; green), and synapsin I (neurotransmitters; red). Adapted from ref (143). Copyright 2012 Elsevier Ltd., with permission from Elsevier.

RNIAEIIKDI, a sequence present in mouse laminin γ1 chain,(158,159) was found to promote neurite outgrowth of DRG.(141) The RKRLQVQSIRT sequence derived from the LG4 module of mouse laminin α1 chain(160) was shown to interact with syndecan-1(161,162) and -4(163) and was used to modify different natural hydrogel matrices, including alginate, agarose, collagen, and fibrin, with promising results in terms of ability to promote neurite outgrowth of PC12 cells(147–150,164) and hNSCs.(150) The RGD sequence found in many ECM proteins, including fibronectin and laminin, was also explored for the functionalization of both natural and synthetically derived matrices, resulting in improved NSC adhesion and neurite outgrowth.(140,144,165–167) Of note, the RGD sequence explored in most of these studies was that derived from fibronectin, RGDS, and this is the reason why these studies were not included in Table 1. In fact, no study to date has explored the few RGD sequences found in laminins for the functionalization of hydrogels.(62,64)

Laminin-derived peptide fragments produced by recombinant DNA technology have also been explored for the development of 3D matrices. More specifically, a peptide derived from the LG3 domain of laminin α3 chain, G3P peptide (PPFLMLLKGSTR), which binds with strong affinity to α3β1 integrin,(168) was recombinantly modified to include a collagen binding domain. The resultant recombinant peptide was further explored for the functionalization of a collagen-based hydrogel,

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which evidenced ability to efficiently support NSC adhesion and survival.(151) A different study explored the incorporation of a recombinant heterodimer combining peptides derived from the LG3 domain of mouse laminin α1 chain and the C-terminal peptide from laminin γ1 chain (LP)(152) on collagen hydrogels. Alternatively, a recombinant laminin fragment, E8 fragment, which is a truncated protein composed of the C-terminal region of the α, β, and γ chains, was explored.(169) Unlike the recombinant peptide fragments previously described, this truncated protein retains laminin integrin-binding activity, as evidenced by the ability of laminin-511-derived E8 fragment to strongly promote the adhesion and proliferation of human pluripotent stem cells, when compared to Matrigel and the full-length laminin isoform.(170) Accordingly, envisaging the development of collagen matrices with laminin-like adhesive activity, the N-terminal ends of individual chains (α, β, and γ) in the truncated protein were modified with a collagen-binding domain (CBD), to confer collagen-binding activity to the E8 fragment. The developed matrices were shown to support the proliferation of human induced pluripotent stem cells (hiPSCs), in both 2D and 3D, though the morphology of cells grown in each condition was quite different.(169)

To better recapitulate the biological activity of laminin chains, in the past few years several studies have been exploring the additive or synergistic effect of the combined incorporation of different laminin cell adhesive peptides (e.g., IKVAV, YIGSR, RGD, AG73, RNIAEIIKDI). For example, a study exploring the modification of fibrin hydrogels with a combination, in equimolar ratios, of RGD, IKVAV, YIGSR, and RNIAEIIKDI showed that these peptides have a synergistic effect on neurite outgrowth when used to bridge a 4 mm gap in a rat dorsal root.(141) In a different study, the modification of dextran hydrogels with a mixture of equimolar concentration (1:1) of IKVAV and YIGSR, revealed better support of in vitro DRG adhesion and neurite outgrowth when compared to RGD-modified hydrogels.(171) Despite the promising results obtained to date, the use of the "typical" equimolar concentration of each peptide has not always proven to be efficient in terms of supporting cell survival and differentiation.(172) Therefore, an important factor one should consider when developing hydrogels incorporating different ECM-derived peptides, is the need to understand the effect of each individual factor, as well as the interacting factor effects for each system. With this in mind, to make the process less time-consuming, Segura and co-workers proposed the use of multifactorial experiments. Here, candidate adhesion motifs and growth factors were systematically varied in vitro to determine the individual and combinatorial effects of each factor on cell activity, enabling the optimization of a system capable of supporting hNPC (iPS-NPC) survival, proliferation, and differentiation.(172,173) The optimized hydrogel was further tested in vivo, in an animal model of stroke. Results evidenced the ability of the proposed matrix to support the survival of encapsulated hNPCs (iPS-NPCs) after transplantation into the stroke core, as well as to differentially tune transplanted cell fate through the promotion of glial, neuronal, or immature/progenitor states.(173)

4. Conclusions and Future Perspectives

In this review, we provide a detailed overview of hydrogels incorporating full-length laminin or laminin derived peptides or fragments to mirror the neurogenic niche microenvironment. Proof-of-principle of the suitability of these hydrogels to create a permissive microenvironment for NSC cell growth and differentiation in vitro, as well as for use as matrices for NSC delivery in vivo, was herein highlighted. The results obtained to date, strongly suggest that these hydrogels may serve as powerful artificial niches to modulate NSC fate and function in regenerative medicine applications.

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Despite the significant advances made in recent years using laminin-inspired hydrogels (Tables 1 and 2), a number of requisites still need to be fulfilled and a few key issues must be taken into consideration before one can see the translation of these hydrogels to a clinical setting. A main challenge one faces when developing cell-instructive hydrogels is the efficient integration of the different factors that impact stem cell fate. The latter include physical properties (e.g., mechanical and structural properties),(100−102) which should be thoroughly characterized in a number of in vitro and in vivo set ups before considering clinical translation. The degradation rate of the proposed systems is also a key aspect to consider, as the designed scaffold has to degrade at a rate matching that of cellular infiltration and ECM deposition (for a more comprehensive review, see ref (174)). Strategies to render chemically cross-linked hydrogels degradable on cell demand, such as those relying on the use of cross-linkers incorporating protease-sensitive peptides, are therefore of much interest.(175) These are expected to allow the fine-tuning of hydrogel degradation rate and its adjustment to the process of tissue remodeling. The presentation of bioactive cues (e.g., ECM proteins or peptides, growth factors, or drugs), as well as the control over their spatial and temporal exposure are also key features to take into consideration. Laminin-derived peptides or fragments (Table 2) have been widely explored for the development of laminin-inspired hydrogels, as they can provide important information regarding NSC biology, function, and fate. Nevertheless, one should not forget that in terms of biorelevance and clinical benefit, the entire laminin molecule may be necessary to provide the bioactivity levels of the native protein. Accordingly, in the past few years, several works have focused on the development of hydrogels functionalized with full-length laminin (Table 1). When developing hydrogels with tethered bioactive motifs, such as adhesive proteins and peptides, the selection of the most appropriate immobilization chemistry is crucial to ensure control over peptide or protein conformation and temporal availability. Protein conformation upon immobilization has been shown to significantly affect protein bioactivity and ultimately its ability to modulate cellular behavior;(112−114,118) therefore, immobilization strategies allowing the selective and controlled presentation of bioactive epitopes are highly desirable. These are expected to provide a higher retention of bioactivity and, as such, to more closely mirror the native ECM. In this sense, in recent years immobilization strategies have shifted toward site-specific conjugation approaches, such as bio-orthogonal chemical reactions (click chemistry), enzymatic ligation, or affinity binding, using either unnatural amino acids or engineered site-selective amino acid sequences (for a more comprehensive review, see ref (176)). To control the orientation of laminin while preserving the exposure of its multiple bioactive domains, we have recently explored an affinity-binding approach to immobilize laminin that takes advantage of the native high affinity interaction between the NtA and the coiled-coil domain of laminin.(118,119) When compared to the nonselective covalent immobilization,(118) the site-specific immobilization of laminin better preserved laminin bioactivity as evidenced by its enhanced ability to self-polymerize and mediate cell adhesion. Moreover, we showed the potential of affinity-bound laminin synthetic hydrogels, to be used as a dynamic 3D platform enabling human NSC proliferation, neuronal differentiation, and neurite extension.(119)

Laminin-111 purified from Engelbreth-Holm-Swarm mouse sarcoma cells has been, to the best of our knowledge, the isoform of choice for the development of laminin-inspired hydrogels (Table 1). Accordingly, the putative immune reaction against laminin is a key issue to be considered when envisaging the use of this ECM protein for the development of cell-instructive hydrogels. Studies conducted to date, in which laminin-inspired hydrogels were used either for endogenous NSC recruitment(129) or NSC transplantation (Table 1), report no evidence for the development of an adaptive immune response against the heterotrimeric glycoprotein. Nevertheless, more detailed studies assessing the immunogenicity of full-length laminin should be conducted. Currently,

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companies like Biolamina, Thermo Fisher Scientific, or Corning offer an extensive portfolio of human recombinant laminin cell culture substrates for a variety of applications. The developed matrices are chemically defined and animal component-free, thus surpassing the main issues regarding the immune reaction. Moreover, this also enables one to surpass another important feature, the scalability, as these companies have established efficient strategies allowing the production of clinical grade human purified laminin in large scale and with good yields.

Another important issue to take into consideration while designing laminin-inspired hydrogels is the selection of the laminin isoform (16 different laminin isoforms were identified so far(1−3)). In fact, while laminin-111 has been the isoform of choice for the development of laminin-inspired cell-instructive microenvironments (Table 1), recent evidence has shown that laminin isoforms comprising the α5 chain (e.g., laminin-511 and -521) have a key role in the in vitro modulation of neural cell behavior including neuronal adhesion, viability, and network formation.(96,127) Therefore, an issue still to be addressed is the impact of these laminin isoforms on NSC behavior and function within laminin-functionalized 3D matrices.

When envisaging the design of cell-instructive hydrogels for cell transplantation, the selection of the most suitable polymeric system for each particular application is an important issue to take into consideration (for a more comprehensive review, see ref (177)). In particular, hybrid hydrogels have been widely explored, as they combine the properties of biological (macro)molecules with the tunable and reproducible structural and mechanical features of synthetic polymers.(178,179) However, when envisaging the use of such hydrogels for cellular therapies, mimicking the complexity and functionality of the natural ECM without compromising important features for application in a clinical setting, like injectability, still remains a major challenge. In fact, in a highly organized tissue such as the CNS, hydrogels able to be precisely injected into the CNS through the use of minimally invasive surgical procedures are highly desirable, to inflict minimal injury to the remaining healthy brain/spinal cord and minimize further neuronal damage. In addition, these hydrogels are ideal for NSC transplantation into complex CNS injury sites, such as spinal cord and brain, as they are able to adapt to the defect cavity. Hydrogels provided with reversible cross-links are particularly promising for this purpose, due to the noncovalent nature of the cross-links.(180,181) Their shear thinning properties (viscous flow under shear stress) enable injection of cell-carrying hydrogels already in the gel phase, which, besides protecting cells from the mechanical forces experienced during flow, can prevent cell dissemination into off-target sites of the CNS.

High-throughput cell encapsulation platforms are also expected to contribute substantially to the development of multifunctional and dynamic hydrogels with cell adhesiveness, degradability, and mechanical properties optimized to support NSC self-renewal or, alternatively, to direct their differentiation into specific phenotypes.(182,183) These high-throughput methods are valuable tools to screen through many combinations of variables and ultimately help to evolve and test hypotheses related to cell–ECM signaling.(184) These can therefore be highly advantageous when envisaging the clinical translation of cell-laden matrices.

Overall, this review highlights the significant progress that has been made on the development and application of laminin-inspired cell-instructive microenvironments to modulate NSC fate and function in regenerative medicine applications. Nevertheless, although it is still a long way off, the advances made in the past few years in understanding the interplay between the niche components

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and NSCs are expected to contribute to the establishment of more accurate platforms with application to in vitro studies and regenerative medicine.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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