Review

Combining protein-based biomaterials with stem cells for spinal cord injury repair

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Abstract

Introduction

Protein-based biomaterials can be combined with stem cells to develop therapies that aid in the regeneration of injured spinal cords. These approaches use various protein scaffolds to support the survival and differentiation of implanted stem cell-derived populations into functional neurons and glial cells with the aim of overcoming the inhibitory scarring that restricts cell regrowth after spinal cord injury. The following review evaluates two key protein-based biomaterials–fibrin and collagen–in combination with both pluripotent and multipotent stem cells as strategies for spinal cord injury repair. Recent studies on the protein network of decellularised extracellular matrix are also highlighted as an exciting area for future work.

Conclusion

The combination of protein-based biomaterials with stem cells shows significant promise as a strategy for spinal cord injury repair.

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Introduction

Biomaterials are materials used with biological systems and can be derived from natural sources or synthetically produced. Various combinations of biomaterials with stem cells can be used to replace lost or damaged tissue following spinal cord injury (SCI). Proteins fulfil many structural and functional roles to support cells *in vivo*, making them excellent candidates for tissue engineering applications. In addition, they are natural biomaterials, possessing the desirable properties of biocompatibility and biodegradability. Two key examples of protein-based biomaterials, fibrin and collagen, are discussed in this review.

Pluripotent stem cells (PSCs) are characterised by immortality–the ability to continuously self-renew–and pluripotency–the ability to differentiate into all somatic cell types. PSCs include both embryonic stem cells (ESCs) and induced PSCs (iPSCs). ESCs originate from the inner cell mass of an early stage embryo and were first derived from mice by Evans and Kaufman[1] in 1981 and from humans by Thomson et al.[2] in 1998. Nearly a decade later in 2006 and 2007, Takahashi et al.[3,4] generated the first iPSCs showing that murine and human somatic cells could be reprogrammed to behave like ESCs by introducing four defined transcription factors via viral transduction. These factors, Oct3/4, Sox2, c-Myc and Klf4, were termed the Yamanaka factors. The development of iPSCs has tremendous implications for regenerative medicine due to the possibility of generating patient-specific cell therapies and the ability to generate PSC lines without the use of embryos.

Multipotent stem cells can give rise to multiple mature phenotypes and exist within specialised niches in many adult tissues. Temple[5] first described neural stem cells (NSCs) isolated from the rat forebrain and characterised them by their ability to develop into the primary cells of the central nervous system (CNS). In 1992, Reynolds and Weiss[6] successfully demonstrated that isolated cells from adult mouse striatum could be induced to differentiate into neurons and astrocytes using epidermal growth factor. Unlike PSCs, NSCs possess a fixed capacity only to differentiate into the cells of the nervous system.

Both pluripotent and multipotent stem cells can generate the necessary quantities of cells required for transplantation due to their ability to continuously divide. These cells can then be differentiated into desired phenotypes for therapeutic applications. For SCI treatment, stem cells are differentiated into neural cells to overcome the inhibitory glial scarring which seals off the injury site and replaces the functional cells lost during injury. Stem cell-derived neural progenitor cells (NPCs) transplanted in a non-inhibitory environment survive and differentiate into neurons and oligodendrocytes leading to regeneration[7] while the environment of an injured spinal cord inhibits NPC survival and promotes differentiation into astrocytes contributing to glial scarring[8]. Therefore, many stem cell-based therapies seek

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to promote the generation of neurons and oligodendrocytes while reducing the differentiation of astrocytes. Another therapeutic approach utilises the protective function of astrocytes to improve the conditions after SCI[9]. This review considers the differentiation of pluripotent and multipotent stem cells into various neural cell types in combination with fibrin and collagen biomaterials for SCI treatment applications.

Fibrin

Unlike other extracellular matrix (ECM) proteins associated with the mature tissue structure, fibrin acts as a temporary scaffolding component formed during the wound-healing process. *In vivo*, it is the end product of the coagulation cascade initiated in response to injury, ultimately forming the fibrous mesh of a blood clot. Fibrin circulates in the bloodstream as a zymogen in the form of fibrinogen. It is polymerised by the enzyme thrombin and then cross-linked into a protein mesh by factor XIII in the presence of calcium chloride. Several commercial fibrin hydrogel products replicate this process and such hydrogels have been translated into surgical sealants used for clinical applications[10]. The properties of fibrin hydrogels can be controlled by two main variables, which include differing the concentrations of fibrinogen and thrombin, leading to greater matrix stiffness when increased[11], as well as the use of protease inhibitors such as aprotinin to prevent the degradation of fibrin by cell-secreted proteases.

Fibrin hydrogels

The Sakiyama-Elbert lab[12-16] developed fibrin scaffolds for repair of SCI. Willerth et al.[12] focused on optimising fibrin-seeding conditions for the neural differentiation of embryoid bodies (EBs)–aggregates of cells containing neural progenitors–derived from mouse ESCs. In their *in vitro* studies, the optimal concentrations

of fibrin (10 mg/mL or 12.8 mg/mL), thrombin (2 NIH U/mL) and aprotinin (5 µg/mL) were determined. These optimised scaffolds supported the differentiation of ESC-derived neural progenitors into neurons and astrocytes, with intact EBs demonstrating more robust growth and survival in three-dimensional (3-D) culture compared with dissociated EBs. A later study by Kolehmainen and Willerth[17] demonstrated that 3-D fibrin scaffolds are also an effective platform to support the neural differentiation of EBs derived from mouse iPSCs.

Building on their earlier work, Willerth et al.[13] showed that murine ESC-derived NPCs responded to soluble growth factors when seeded inside fibrin matrices for 14 days. In this study, neurotrophin-3 (NT-3) and sonic hedgehog (Shh) were found to increase the yield of neurons and oligodendrocytes, whereas platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were shown to increase cell viability compared with untreated cells. Fibrin scaffolds incorporating an affinity-based drug delivery system were then used to deliver these neurotrophic factors in a controlled manner over time[14]. Simultaneous controlled release of NT-3 and PDGF successfully promoted the proportion of murine ESC-derived EBs that differentiated into NPCs, neurons and oligodendrocytes while reducing the proportion of astrocytes compared with untreated cells.

This work was translated for *in vivo* studies by Johnson et al.[15,16] by transplanting mouse ESC-derived NPCs encapsulated in fibrin into a rat SCI model. The fibrin scaffolds protected the cells from the inhibitory environment presented by the injury site, as indicated by increased cell survival compared with transplanted cells without fibrin. Fibrin scaffolds containing NT-3 and PDGF increased proliferation of the transplanted cells and differentiation into neurons both

in the presence and in the absence of an affinity-based heparin-binding drug delivery system (Figure 1). After 8 weeks, the addition of growth factors without a delivery system encouraged proliferation and aided in functional recovery, whereas over-proliferation of the non-NPC population was observed with the controlled release of the growth factors leading to a loss of behavioural function. The ESC-derived EBs produced using a retinoic acid (RA) induction protocol[18] were shown to yield approximately 70% NPCs and the over-proliferation observed correlated with the proliferation of the 30% non-NPC cell population, suggesting the importance of transplanting a pure NPC population.

Lu et al.[19] investigated the ability of NSC-derived neurons to regenerate axons *in vivo* after neural transection. Both rat and human foetal spinal cord-derived NSCs were embedded into growth factor-containing fibrin matrices and grafted into rat SCI lesion sites 2 weeks post-transection. Grafted cells differentiated into neurons with a large number of long axons and formed synapses with host cells. Host oligodendrocytes also myelinated the transplanted cells. Functional recovery was enhanced in groups receiving NSCs grown in growth factor-containing fibrin matrices 3 weeks post-grafting compared with the non-treated control group. Furthermore, these human ESC-derived neural progenitors combined with fibrin and growth factors expressed neural markers *in vivo*, demonstrating that ESC-derived cells could also differentiate into neurons and extend axons at the inhibitory injury site.

Fibrin hydrogel composites

Fibrin scaffolds can also be combined with other biomolecules to enhance its properties. For example, Sarig-Nadir and Seliktar[20] modified fibrin hydrogels with polyethylene glycol (PEG), creating a scaffold with a
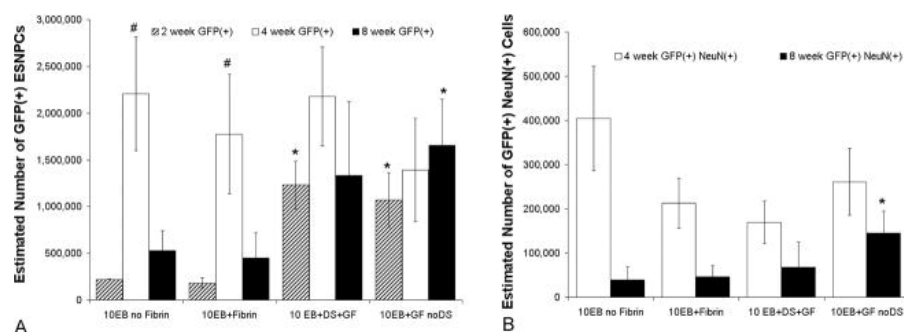
Figure 1: Stereological counts of GFP expressing ESNPCs 2, 4 and 8 weeks after transplantation and GFP-expressing ESNPCs that also expressed the neuronal marker NeuN 4 and 8 weeks after transplantation. (a) Count of GFP-expressing ESNPCs was performed to analyse survival and proliferation of the transplants from each experimental group. There was significant increase in the estimated number of GFP-positive ESNPCs between the 2 and 4-week time points in the 10EB no Fibrin and 10EB + Fibrin. No significant difference in the estimated number of GFP-positive ESNPCs was observed between experimental groups at the 4-week time point. Eight weeks following transplantation, the ESNPCs transplanted in fibrin scaffolds containing neurotrpohin-3 (NT-30 and platelet-derived growth factor-AA (10EB + GF no DS, $n = 8$) had a significantly greater number of ESNPCs (eError bars represent SEM, $^*P < 0.05$ vs. same group at 2 weeks, $*P < 0.05$ vs. 10EB no Fibrin and 10EB + Fibrin at same time point). (b) Count of GFP-expressing ESNPCs that also expressed NeuN (marker for mature neurons) was performed to analyse neuronal differentiation. After 4 weeks, no significant difference in the estimated number of NeuN-positive ESNPCs was found between experimental groups. The ESNPCs transplanted in fibrin scaffolds containing NT-3 and PDGF-AA with no HBDS (10EB + GF no DS) had a significantly higher count of NeuN positive ESNPCs when compared with ESNPCs transplanted alone (10EB no Fibrin) (error bars represent SEM, $* P < 0.05$ vs. 10EB no Fibrin at same time point). ESNPCs, embryonic stem cell-derived neural progenitor cells; GFP, green fluorescent protein; HBDS, heparin-binding delivery system; NeuN, neuronal marker; NT, neurotrpohin; PDGF, platelet-derived growth factor.
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greater tunable mesh size and permeability. Composition of the PEGylated fibrin hydrogels was controlled using different protein concentrations and PEG:fibrinogen ratios. They showed that dorsal root ganglion (DRG) cell neurites preferentially invaded the PEGylated fibrin compared with unmodified fibrin over a 4-day period, as indicated by morphological assessment.

In a different approach, Lee et al.[21] developed a biological printing technique for constructing cell–hydrogel composites with growth factor-releasing fibrin. In this study,

fibrin hydrogels containing vascular endothelial growth factor (VEGF), reported to have *in vitro* effects on NSC migration and proliferation, were printed between layers of collagen. Murine NSC cells were then printed into the collagen surrounding the fibrin. Cells within the printed tissue demonstrated high viability, growth factor-induced morphology characterised by elongation and extension of neurite processes, and larger migration range compared with the controls without VEGF-containing fibrin after 4 days of *in vitro* culture. The studies demonstrate the

versatility of fibrin scaffolds when combined with other materials.

Collagen
Collagen describes the family of proteins that make up the main component of the ECM in many species, including humans. The specific properties of collagen depend on the type and the tissue from which it is derived. Collagen-I is the most abundant form of the dozens of types identified. In general, collagen is composed of distinct polypeptide chains that self-assemble under physiological conditions into triple helices that are covalently cross-linked into fibril structures. Collagen is used to fabricate hydrogels as well as other micro- or nano-scale substrates, including microspheres and freeze-dried porous scaffolds[22]. The mechanical properties and degradation characteristics are typically modified by varying the protein concentration, but can also be tuned in hydrogels by varying the solution's pH level.

Collagen hydrogels
Collagen scaffolds support neural differentiation of murine ESCs both *in vitro* and *in vivo*. Kothapalli and Kamm[23] found that hydrogels from collagen reconstituted at a higher pH (7.4–9) favoured neural differentiation of murine ESC-derived NPCs treated with soluble RA and Shh compared with those at lower pH (5.5), which favoured astrocyte lineages *in vitro*, as shown in Figure 2. In a xeno-transplantation study, Hatami et al.[24] showed that human ESC-derived NPCs seeded in collagen scaffolds could improve motor and sensory function after 5-weeks post-injury in a rat SCI model compared with collagen scaffolds without cells present.

Ge et al.[25] showed that expansion and differentiation of rat NSCs *in vitro* can be accomplished in 3-D collagen scaffolds. After 42 days, quantitative assessment of fluorescently labelled cultures indicated high viability as

well as over 40% expression of the early neuronal marker β-III-tubulin (TUJ1). Nakaji-Hirabayashi et al.[26] demonstrated that 3-D collagen hydrogels incorporating an integrin-binding peptide supported higher viability levels of foetal rat-derived NSCs after 7 days of *in vitro* culture compared with collagen hydrogels alone.

Collagen scaffolds can also be modified to serve as drug delivery systems for neurotrophic factors. In two separate studies, Yang et al.[27,28] showed a decrease in the required brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor using controlled release from the collagen hydrogel when compared with soluble factor delivery. These modified drug-releasing scaffolds also improved cell adhesion, survival, proliferation and neural differentiation. Huang et al.[29] found that collagen hydrogels incorporating BDNF had a consistent release profile after an initial burst release with nearly 100% delivery by day 10. When used *in vitro*, they found that the BDNF-collagen hydrogels resulted in higher neuronal differentiation of rat NSCs after 7 days compared with collagen hydrogels alone. Furthermore, BDNF-releasing collagen resulted in a heterogeneous population of differentiated cells with more neurons than astrocytes while collagen alone resulted in an increased number of astrocytes compared with neurons.

Micro- and nanoscale collagen scaffolds

Yao et al.[30] successfully fabricated collagen-based microspheres to serve as carriers of oligodendrocyte progenitor cells (OPCs) *in vitro*. Collagen microspheres were produced using a water-in-oil technique and cross-linked with 1-ethyl-3-(3dimethylaminopropryl) carbodiimide. OPCs from 1-day post-natal rats were seeded onto collagen microspheres and cultured in OPC medium for 3 days; OPCs developed
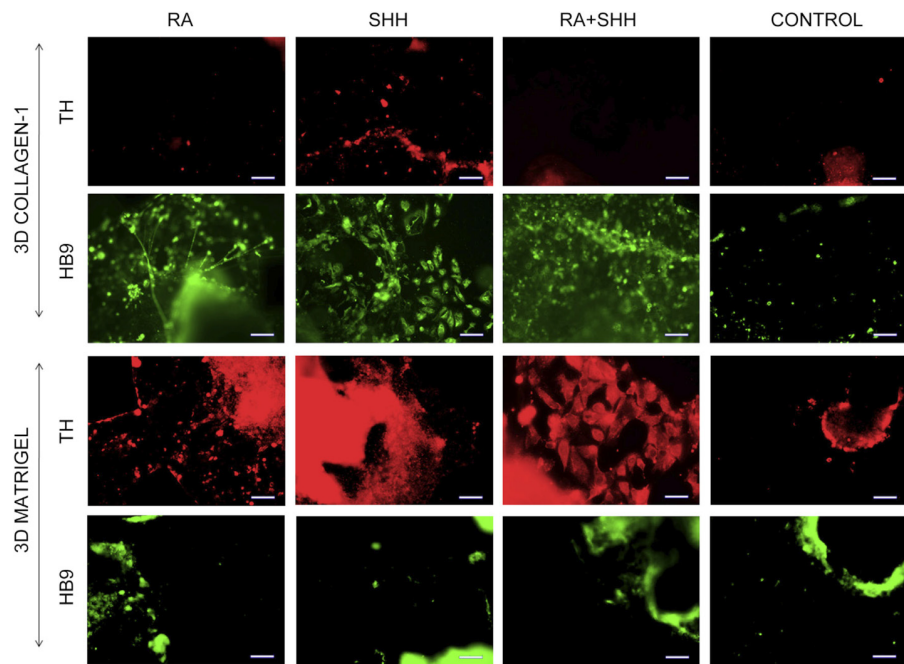

Figure 2: Representative immunofluorescence images of dopaminergic (TH-stained) and motor neurons (HB9-stained) formed within collagen-1 or matrigel 3D scaffolds, in the presence of 1 µM RA or 300 nM Shh or both. Control cultures in the respective cases did not receive any signalling molecules. Scale bar: 100 µm. RA, retinoic acid; Shh, sonic hedgehog; TH, tyrosine hydroxylase.
Source: Figure and caption reprinted from Biomaterials, Kothapalli and Kamm[23] with permission from Elsevier.

short processes and remained attached to the microspheres. Collagen microspheres pre-seeded with OPCs were then co-cultured *in vitro* with DRGs in order to investigate the ability of OPCs to contribute to neural myelination. After 8 days of co-culture, multiple processes from the microspheres wrapped around DRG neurites and contributed to myelin sheath formation, as shown in Figure 3. These findings show the potential of collagen microspheres as carriers of neural stem cells to treat SCI.

In a different approach, Li et al.[31] fabricated highly porous collagen scaffolds using a freeze-drying technique that resulted in over 98% porosity with pore sizes ranging from 60 to 200 µm. When seeded with rat NPCs, these scaffolds supported cell growth in the lesion site of a rat SCI model. Furthermore, the scaffold could be modified by functionalising collagen

with epidermal growth factor receptor (EGFR) and exposing it to 0.5 mg/mL myelin. After 12-weeks post-implantation, this collagen-EGFR-myelin scaffold resulted in greater differentiation of NPCs to neurons with increased staining of microtubule-associated protein 2 and neurofilament. Astrocyte differentiation was also suppressed with less glial fibrillary acidic protein –positive staining compared with NPCs seeded in collagen alone.

Collagen has also been used to immobilise the surface of synthetic polymeric electrospun nanofibres. Li et al.[32] show that nanofibres coated with collagen significantly improve the viability and attachment of NSCs derived from prenatal rats compared with unmodified nanofibres after 4 days of *in vitro* culture. All these studies show the range of ways that collagen can be processed to support stem cell culture.
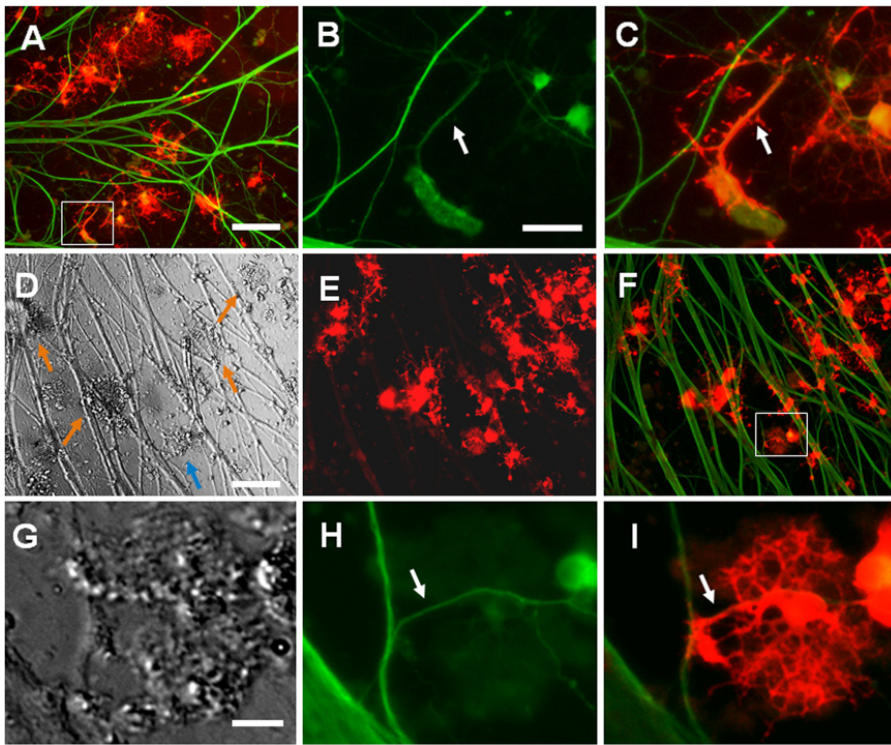
Figure 3: (a) Co-culture of DRGs with OPCs in cell culture plate. Scale bar: 200 μm. (b) and (c) Magnified images shows the OLs wrap DRG neurites. Scale bar: 50 μm. (d–f) Co-culture of DRGs and OPCs carried out by collagen microspheres. Scale bar: 200 μm. (d) Bright field image shows that collagen microspheres attach to the cell culture plate. The arrows point at the collagen microspheres. (e) and (f) OPCs on collagen microspheres differentiated into OLs. The processes of OLs wrapped the neurites of DRGs. (g–i) Magnified images for the microsphere indicated by blue arrow in (d) show the irregular shape of the collagen microsphere and the neurites of DRGs were wrapped by OLs. Scale bar: 100 μm.DRGs, dorsal root ganglions; OLs, oligodendrocytes; OPCs, oligodendrocyte progenitor cells.

Source: Figure and caption reprinted from Yao et al[30]. under the Creative Commons Attribution License http://creativecommons.org/licenses/by/4.0.

Discussion

The authors have referenced some of their own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

Both fibrin and collagen in combination with stem cells have demonstrated potential for the treatment of SCI. The advantages of these protein-based scaffolds include compatibility with all stem cell types, commercial availability and fabrication into various scaffold architectures. Furthermore, they can be functionalised with various molecules and include drug delivery systems for the controlled release of neurotrophic factors. However, one of the main disadvantages of fibrin and collagen is the relative lack of control over scaffold mechanical properties. Although modification is possible by varying protein concentration, the level of control does not approach the degree and precision of tuning possible with synthetic biomaterials. As the scaffold degradation rates for fibrin and collagen often outpace cell growth,

it is challenging to characterise their long-term performance *in vivo*.

At present, the use of iPSC-derived NPCs combined with protein-based biomaterials remains underrepresented compared with the more established and commonly used ESCs and NSCs. As technologies for the generation of iPSCs continue to be refined, it will be increasingly important to include iPSC-derived NPCs in the development of SCI therapies. In addition, due to the current challenges associated with producing pure populations of NPCs from stem cells, development of efficient and reproducible neural differentiation protocols for the generation of specific neural cell subtypes in conjunction with these scaffolds continues to be an important area for investigation.

A promising extension of the work on protein-based biomaterial scaffolds involves the use of decellularised ECM. In this approach, a harvested tissue is processed to remove cells while leaving the ECM intact. The resulting material contains native proteins and polysaccharides as well as other bound growth factors. Crapo et al.[33] used this technique to prepare hydrogel scaffolds from decellularised ECM derived from tissues of the porcine CNS. These CNS–ECM scaffolds were found to retain laminin and myelin as well as VEGF, and bFGF; the optic nerve-derived ECM also contained nerve growth factor. An *in vitro* assay using PC12 cells (a neural cancer cell line) demonstrated that these scaffolds supported cell proliferation, migration and differentiation after 24–48 h. Furthermore, the level of cell function depended on the source of ECM, suggesting the presence of tissue-specific properties. This concept was further investigated in a later study by the same group[34] which showed that murine neuroblastoma cells seeded on ECM hydrogel scaffolds exhibit high viability after 7 days *in vitro* with the most neuronal differentiation observed on spinal cord-derived ECM

and the largest neurite extensions on non-CNS-derived ECM. Zhang et al.[35] have used chemically extracted acellular muscle as a graft for the repair of SCI, taking advantage of the parallel cylindrical structure of muscle basal lamina to provide an environment similar to endoneural tubes. Muscle-based ECM scaffolds also make it possible to allograft the tissue from one site of the host to the site of SCI, eliminating immunogenic reactions. In a rat SCI model, integration with the host tissue was observed with sprouting axons growing into the scaffold after 4 weeks. More neurons were observed in the grafted site for the group with ECM-based scaffolds compared with the injury-only group. Important future work will include the combination of decellularised ECM scaffolds with pluripotent and multipotent stem cells to further enhance their therapeutic potential.

Conclusion

A key advantage of protein-based biomaterials is their ability to support cell growth and differentiation through the presentation of important chemical and mechanical cues similar to those found in the complex 3-D protein–polysaccharide ECM network. Furthermore, decellularised ECM can serve as an excellent protein-based biomaterial that retains the desirable biomolecular composition and key structural features that may enhance cell-specific differentiation. The combination of protein-based biomaterials with stem cells shows significant promise as a strategy for SCI repair.

Abbreviations list

BDNF, brain-derived factor; bFGF, basic fibroblast growth factor; CNS, central nervous system; DRG, dorsal root ganglion; EBs, embryoid bodies; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; NSCs, neural stem cells; NPCs, neural progenitor cells; NT-3, neurotrophin-3; OPCs, oligodendrocyte progenitor cells; PDGF, platelet derived growth factor; PEG, polyethylene glycol; RA, retinoic acid; Shh, sonic hedgehog; SCI, spinal cord injury; VEGF, vascular endothelial growth factor; 3-D, three-dimensional.

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