THE USE OF BIOMATERIALS WITH STEM AND PRECURSOR CELLS IN DISEASES OF THE CENTRAL NERVOUS SYSTEM; A STEP TO CLINICAL TRIALS

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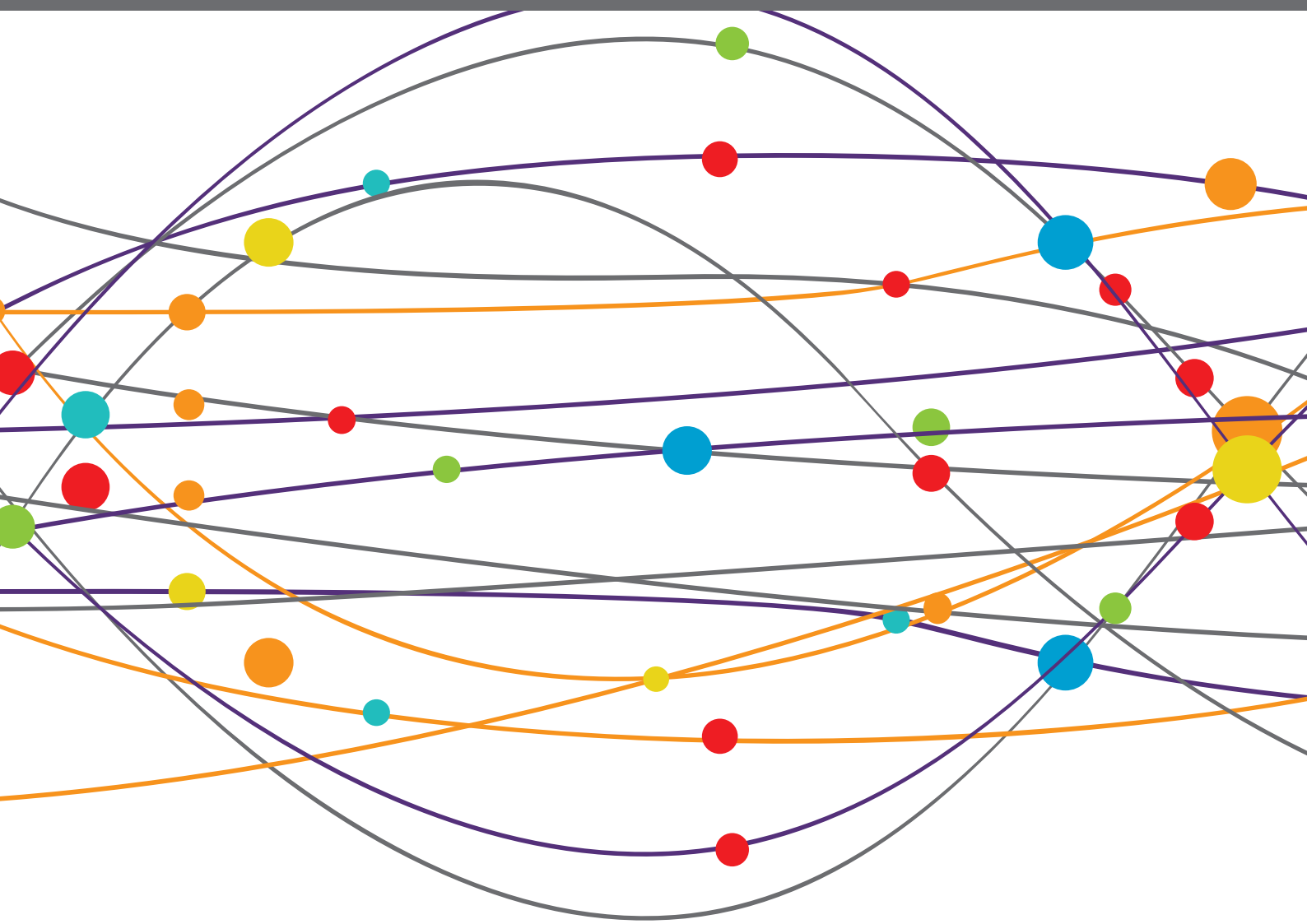

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THE USE OF BIOMATERIALS WITH STEM AND PRECURSOR CELLS IN DISEASES OF THE CENTRAL NERVOUS SYSTEM; A STEP TO CLINICAL TRIALS

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Table of Contents

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EDITORIAL
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Editorial: The Use of Biomaterials With Stem and Precursor Cells in Diseases of the Central Nervous System; A Step to Clinical Trials

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Editorial on the Research Topic

The Use of Biomaterials With Stem and Precursor Cells in Diseases of the Central Nervous System; A Step to Clinical Trials

With a participation of 12 articles, the Research Topic "The use of Biomaterials with Stem and Precursor Cells in Diseases of the Central Nervous System; A Step to Clinical Trials," has had an impact that demonstrates the relevance and interest of translational research in scientific readers.

The link between the basic experimental scenario and the decision making process in the clinic is a highly demanding condition for public health and it strengthens the collaboration links between basic and applied research. With basic topics related to unique and combined molecular strategies and their diverse potential applications, this topic forms a mosaic of possibilities of technological development and therapeutic strategies in the short term.

Spinal cord injury, bioactive implants, scaffolds for tissue regeneration and proposals for chronic degenerative diseases such as Alzheimer's, cancer, and stroke, are evaluated with high scientific and ethical rigor. Their results promise new routes in the therapeutic possibilities from molecular and genetic concepts. These reports explore the handling of precursor cells and biomaterials, both as scaffolds and as vehicles of different bioactive factors with regenerative potential.

The evaluation of the state of the art in the use of biomaterials and stem and precursor cells is a crucial point in order to establish the goals and objectives in addressing new scientific and clinical challenges. At this point, Ojeda-Hernandez et al., include a detailed and extensive review on the biomedical applications of chitosan and its derivatives in the Central Nerve System (CNS). Several research groups have described the use of chitosan as a versatile biomaterial in the development of therapeutic strategies. The work presented by Lara-Velazquez et al., describes multiple studies highlighting the advantages and challenges of chitosan-based gene and drug delivery systems (nanocapsules, nanospheres, solid-gel formulations, nanoemulsions, microshperes, and micelles) for the treatment of brain tumors. The work presented by Ruiz-García, Alvarado-Estrada, Krishnan et al., addresses potential and newest developments in the area of bioengineering and cell therapy. It is an exquisite work, exploring state of the art approaches in the therapy of glioma. In recent years, the use of mesenchymal stem cells have taken a leading role in the development of therapeutic strategies

in various pathologies of the nervous system. Hmadcha et al., describes the controversial role of the Mesenchymal Stem Cells (MSCs) in contributing to cancer pathogenesis mainly because the differentiation into the cancer associated fibroblast and also in cancer suppressing because the immunomodulation effect. MSC-based anti-cancer agent delivery systems were also described as a new potential application of MSCs.

Another pathology where the use of biomaterials preloaded with stem cells has been proposed to promote neural restoration is stroke. Esteban-Garcia et al., describe in their review the use of biomaterials for neurorestauration after ischemic stroke. One of the most common strategies to target this problem is biomaterials combined with cellular therapy. The authors address the limitations and consequences that originate after stroke, the endogenous repair mechanisms and the critical keys that can contribute to a successful therapy using biomaterials and stem cells.

A clear example of a topic for both basic and translational research is the review presented by Purvis et al., the authors describe the neurogenic potential of the adult brain and the capacity of neurogenic niches to serve as a potential source of cells that facilitate neuronal replacement injured or damaged regions of the brain. They show different strategies that are being studied in order to promote neuronal replacement from endogenous neural stem cells; cover different aspects from pharmacological approaches using neurotophic factors-loaded biomaterials. The work continues with a description of tissue engineered cellular scaffolds that allow neuroblast toward migration from neurogenic niches damaged brain areas. They propose the use of this particular technology for the development of therapies to treat several types of neurological disorders as well as for the treatment of traumatic brain injuries.

A new approach to the use of biomaterials is the design and *in vitro* three-dimensional (3D) modeling of neurodegenerative diseases such as Alzheimer's, Hernández-Sapiéns et al., in their research look for a novel Alzheimer disease study model. Similarly, Gazarian et al., for the first time, report the expression of tau protein and mRNA in Dental Pulp Stem Cells (DPSCs) in an *in vitro* model, this model is effective to study the mechanisms that promote tau aggregation and neurofibrillary tangles (NFT's) formation. Both reports manage to generate a valid and useful *in vitro* model useful in the area of personalized medicine. Along the same lines and with a similar methodological approach, Ruiz-Garcia, Alvarado-Estrada, Schiapparelli et al., give a comprehensive view of the multiple aspects of the glioma microenvironment which are fundamental for supporting tumor growth and phenotype, further describing how researchers have taken into account all these information for setting up 3D models able to recapitulate all these tumor aspects.

A further step in the advancement of preclinical trials is the development of experimental preclinical models that simulate pathologies such as spinal cord injury. Buzoianu-Anguiano et al., in their study, used predegenerate nerve scaffolding and bone marrow cell transplantation for spinal cord injury in a chronic complete transection model in rats. They describe beneficial effects with respect to axonal regeneration in the combined treatment groups, and attribute to this observation the slight motor functional improvements observed. In a similar model, Rodríguez-Barrera et al., investigate the impact of the combined strategy to improve morphological and functional recovery in rats with chronic spinal cord injury, addressing the use of stem cells, immunization strategies with neural peptides and scar removal.

Finally, an example of the convergence of basic research and its transfer to the clinic is the work presented by Ramos-Zúñiga et al., which addresses in the description of a clinical case of the surgical treatment to seal the skull floor. In this article, he reported the first case with the use of a novel bilaminar chitosan scaffold that can potentially be used in seal skull floor repair, primarily targeting the bony part of this structure.

We are confident that the path traced by this topic in biomedical research, can generate more projects and interest in the scientific community, from the synergy of the different domains of science, for the benefit of the population in the short term.

AUTHOR CONTRIBUTIONS

RR-Z, HG-C, UG-P, and JM-G: manuscript drafting and critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Therapeutic Potential of Mesenchymal Stem Cells for Cancer Therapy

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Mesenchymal stem cells (MSCs) are among the most frequently used cell type for regenerative medicine. A large number of studies have shown the beneficial effects of MSC-based therapies to treat different pathologies, including neurological disorders, cardiac ischemia, diabetes, and bone and cartilage diseases. However, the therapeutic potential of MSCs in cancer is still controversial. While some studies indicate that MSCs may contribute to cancer pathogenesis, emerging data reported the suppressive effects of MSCs on cancer cells. Because of this reality, a sustained effort to understand when MSCs promote or suppress tumor development is needed before planning a MSC-based therapy for cancer. Herein, we provide an overview on the therapeutic application of MSCs for regenerative medicine and the processes that orchestrates tissue repair, with a special emphasis placed on cancer, including central nervous system tumors. Furthermore, we will discuss the current evidence regarding the double-edged sword of MSCs in oncological treatment and the latest advances in MSC-based anti-cancer agent delivery systems.

Keywords: mesenchymal stem cells, cancer, cell therapy, therapeutic agents, anti-tumor activity

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INTRODUCTION

Mesenchymal stem cells (MSCs), also referred to as mesenchymal stromal cells, are adult stem cells capable of self-renewal and multilineage differentiation (Jiang et al., 2002). They were originally found in the bone marrow (Friedenstein et al., 1970), but they were later identified in other tissues including adipose tissue, muscle, peripheral blood, hair follicles, teeth, placenta and umbilical cord (da Silva Meirelles et al., 2006). Although MSCs may exhibit different characteristics depending on their tissue of origin, they must meet the three minimal criteria defined by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). First, MSCs must show plastic-adherence when grown *in vitro*. Second, MSCs must express the surface antigens CD73, CD90, and CD105 while lacking expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. Third, MSCs must differentiate into mesodermal cell types (i.e., adipocytes, chondrocytes, and osteoblasts) when cultured under specific conditions. In addition to mesodermal linage, MSCs are capable of differentiating into cells of non-mesodermal origin (i.e., ectodermal and endodermal lineages),

such as neuronal cells, cardiomyocytes, hepatocytes or epithelial cells (Lee et al., 2004; Paunescu et al., 2007; Quevedo et al., 2009; Gervois et al., 2015). This plasticity of MSCs confers benefits in tissue regeneration.

Mesenchymal stem cells have become as the top used stem cell type for clinical application due to numerous advantages (Connick et al., 2012; Gotherstrom et al., 2014; Karantalis et al., 2014; Rushkevich et al., 2015; Thakkar et al., 2015; Vega et al., 2015; Fernandez et al., 2018) (**Figure 1**). In addition to different source and multilineage differentiation potential, MSCs also possess the capacity to migrate to injured sites in response to environmental signals and promote tissue regeneration mediated by the release of paracrine factors with pleiotropic effects. Through interaction with the host niche, MSCs are able to inhibit the immune system, promote cell survival, or induce angiogenesis among others pleiotropic activities (Salgado et al., 2010). Of these, the immunosuppressive role of MSCs is particularly interesting for clinical use since it confers resistance to rejection by the host immune system after transplantation. Furthermore, MSCs can be obtained from easily accessible sources by minimally invasive methods (e.g., peripheral blood, adipose tissue) and can be rapidly expanded in large-scale for clinical use (Escacena et al., 2015). This allows to produce a patient-specific medicinal product (i.e., autologous medicinal product) within a therapeutic time window. In addition, the possibility of obtaining MSCs from adult tissue circumvent the ethical issues associated with the use of embryonic source (Lo and Parham, 2009; Ramos-Zuriga et al., 2012). All these advantages of MSCs make this cell type a powerful tool for clinical application in regenerative medicine.

Although MSCs have shown tremendous therapeutic potential in various diseases, their application for cancer treatment remains controversial. While some studies indicate that MSCs may contribute to cancer pathogenesis, emerging data support the beneficial effects of MSCs for oncological treatment. In this review, we provide an overview on the therapeutic application of MSCs for regenerative medicine and discuss the double-edged sword of MSCs for cancer.

THERAPEUTIC POTENTIAL OF MSCs

Over the past decades, a large number of studies have emerged using MSC-based therapies in preclinical studies to treat many different pathologies, including neurological disorders, cardiac ischemia, diabetes and bone and cartilage diseases (Si et al., 2012; van Velthoven et al., 2013; Jones et al., 2015; Liu et al., 2016; Ozeki et al., 2016; Capilla-Gonzalez et al., 2018; Chau et al., 2018; Rehorova et al., 2019; Soria et al., 2019). The therapeutic potential

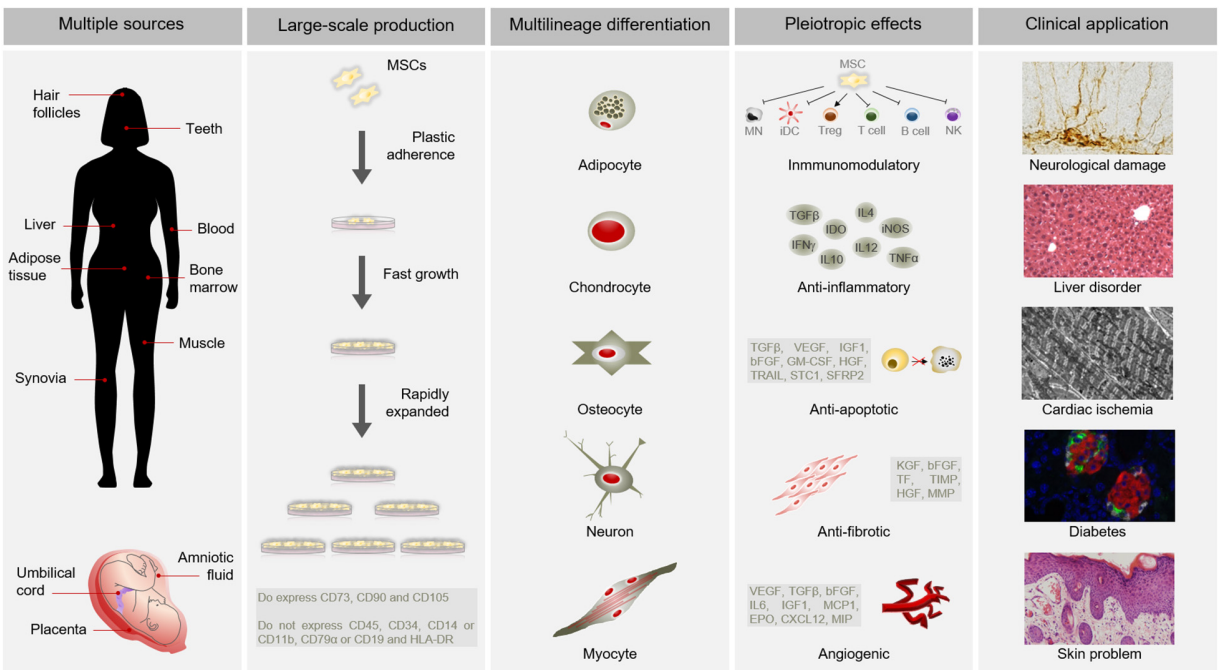

FIGURE 1 | Advantages of MSCs for clinical use. MSCs possess multiple advantages for clinical application. Among other benefits, MSCs can be isolated from several sources, are large-scale produced, differentiate into a variety of cell types and have pleiotropic effects. All these advantages make MSCs suitable for clinical application in different pathological conditions, such as neurological damages, liver disorders, cardiac ischemia, diabetes or skin problems. Abbreviations: HLA-DR, major histocompatibility complex class II DR; MN, monocyte; iDC, immature dendritic Cell; Treg, Regulatory T cell; NK, natural killer cell; TGFβ, transforming growth factor; INFγ, interferon γ; IDO, indoleamine 2,3-dioxygenase; IL10, interleukin 10; IL4, interleukin 4; IL12, interleukin 12; iNOS, inducible nitric oxide synthase; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor; IGF1, insulin like growth factor 1; bFGF, basic fibroblast growth factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HGF, hepatocyte growth factor; TRAIL, TNF-related apoptosis-inducing ligand; STC1, stanniocalcin 1; SFRP2, secreted frizzled related protein 2; KGF, keratinocyte growth factor; TF, tissue factor; TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinases; IL6, interleukin 6; MCP1, monocyte chemoatractant protein 1; EPO, erythropoietin; CXCL12, C-X-C motif chemokine 12; MIP, macrophage inflammatory protein.

of MSCs is firstly mediated by their inherent ability to migrate toward damaged tissues. Then, engrafted cells secrete bioactive mediators, such as growth factors, cytokines and extracellular vesicles that exert immunosuppressive, anti-apoptotic, anti-fibrotic, angiogenic, and anti-inflammatory effects (Salgado et al., 2010). For instance, a study using a neonatal stroke rat model showed that intranasal delivery of bone marrow MSCs reduces infarct size, gray-white matter loss, and motor deficits (van Velthoven et al., 2013). These beneficial effects were in part explained by an increased cell proliferation in the ischemic hemisphere of transplanted rats. In a mouse model of Friedreich's ataxia, intrathecal injections of bone marrow MSCs improved motor function and delayed neurodegeneration by releasing the neurotrophic factors Neurotrophin-3, Neurotrophin-4, and brain-derived neurotrophic factor, which are implicated in neuronal survival (Jones et al., 2015). Human MSCs derived from umbilical cord showed benefits by improving ventricular function in a porcine model of myocardial ischemia (Liu et al., 2016). In this study, the authors described that MSC-treated pigs exhibit increased angiogenesis, reduced apoptosis and decreased fibrosis in the ischemic heart. Bone marrow-derived MSCs have also shown benefits in improving insulin sensitivity associated with an increased GLUT4 expression in type 2 diabetic rats (Si et al., 2012). More recently, the intranasal application of human adipose-derived MSCs was found to prevent neurocognitive impairments induced by cranial radiation in mice (Soria et al., 2019). The neuroprotective role of intranasally delivered MSCs was mediated by limiting pro-inflammatory processes, restricting oxidative damage accumulation, and reducing neuronal loss after radiation. Another study reported beneficial effects of umbilical cord-derived MSC extracts for atopic dermatitis in a murine model by reducing the T cell response (Song et al., 2019). These reports uncover two main properties of MSCs that determine their therapeutic potential; the capacity to migrate toward the lesion site and the ability to repair damaged tissues.

Migration Toward Damaged Tissues

The success of an advanced therapy medicinal product initially depends on its ability to reach target tissues. MSCs possess inherent tropism toward damaged sites that is controlled by a large number of factors and mechanisms, including chemoattractant signals. For instance, the C-X-C motif chemokine ligand 12 (CXCL12) is a frequent triggering factor at the site of injury. It has been demonstrated that a subpopulation of MSCs expresses the C-X-C chemokine receptor type 4 (CXCR4) that binds to its ligand, the CXCL12, to mediate cell migration (Wynn et al., 2004; Ma et al., 2015). Aside from CXCR4, MSCs express other chemokine receptors, such as CCR1, CCR2, CCR4, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, and CX3CR1 (Sordi et al., 2005; Von Luttichau et al., 2005; Honczarenko et al., 2006; Ringe et al., 2007). These receptors are essential to respond to triggering factors at the site of injury. In addition, MSCs also express cell adhesion molecules, including CD49d, CD44, CD54, CD102, and CD106 (De Ugarte et al., 2003). These chemokines and cell adhesion molecules orchestrate the mobilization of MSCs to sites of injury, in a similar manner to white blood cells do (Kolaczkowska and Kubes, 2013). MSC mobilization is a multistep process that encompasses the attachment of free circulating MSCs in the blood stream to transmigrate between endothelial cells with the ultimate goal of migrate and engraft to the target tissue (**Figure 2**).

Tissue Repair Ability

Once recruited in the injured site, MSCs contribute to tissue repair and regeneration through activation of several mechanisms. A growing body of research has demonstrated that MSCs display pleiotropic effects, which give them an enormous therapeutic potential (**Figure 1**). In response to injury signals, MSCs secrete a variety of mediators of tissue repair, including anti-apoptotic, anti-inflammatory, immunomodulatory, anti-fibrotic and angiogenic agents (Caplan and Dennis, 2006; Meirelles Lda et al., 2009; Maltman et al., 2011; Escacena et al., 2015). Among pleiotropic effects, anti-inflammatory and immunomodulatory properties are mainly responsible for the therapeutic benefits of MSCs. As sensors of inflammation, MSCs release soluble factors, such as transforming growth factor β (TGFβ), Indoleamine 2,3-dioxygenase (IDO), Tumor Necrosis Factor α (TNFα), Interleukin 10, and Interferon gamma (INFγ), which interfere with the immune system and modify the inflammatory landscape (Prockop and Oh, 2012). Pivotal studies showed that MSCs inhibit the proliferation of T and B cells (Di Nicola et al., 2002; Corcione et al., 2006; Song et al., 2019), suppress the activation of natural killer cells (Sotiropoulou et al., 2006), and prevent generation and maturation of monocyte-derived dendritic cells (English et al., 2008; Spaggiari et al., 2009). Furthermore, MSCs are able to promote the generation of regulatory T cells (Maccario et al., 2005), which exert immunosuppressive effects. Although soluble factors play a key role in the immunosuppressive activity of MSCs, cell-to-cell contact also influences immune responses (Ren et al., 2010;

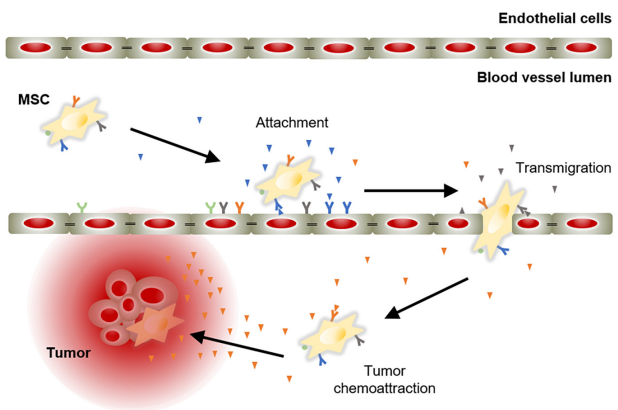

FIGURE 2 | Model of chemoattractant-induced MSC migration toward tumor lesion. Mobilization of MSCs initiates with their incorporation into the circulation. Then, MSCs migrate via the blood stream to areas of injury in response to chemoattractant cues. Ligand-receptor bindings allow MSCs to attach to endothelial cells lining the blood vessels. Subsequently, MSCs activate and initiate the process to cross the endothelium to move toward the target tissue, guided by a chemotactic gradient.

Li Y. et al., 2019). For instance, direct contact between MSCs and proinflammatory macrophages has been shown to induce immune tolerance through induction of tumor necrosis factor-stimulated gene-6 (TSG-6) production (Li Y. et al., 2019). MSC-mediated modulations of the immune response set in motion essential inflammatory processes that significantly promote tissue repair and regeneration by driving healing, scarring and fibrosis (Julier et al., 2017).

Another typical property of MSCs that is involved in their therapeutic effects is the multilineage differentiation potential. In addition to mesodermal linage, MSCs can differentiate into cells of ectodermal and endodermal origin, such as neuronal cells, cardiomyocytes, hepatocytes or epithelial cells (Lee et al., 2004; Paunescu et al., 2007; Quevedo et al., 2009; Gervois et al., 2015). This ability to differentiate into cell types of non-mesodermal origin has been questioned by researchers claiming that differentiated cells from MSCs are able to dedifferentiate and transdifferentiate into cells of another developmental lineage (Song and Tuan, 2004). Notwithstanding, the versatile differentiation potential of MSCs allows the replacement of damaged or dead cells from different tissues. However, several studies indicate that, after administration, MSCs transiently engraft at the injury site for a short period of time and then disappear. The latter suggest that MSCs must activate mechanisms in the host niche which contribute to tissue repair. For instance, the cross-talk between MSCs and the damaged tissue microenvironment results in the secretion of specific agents involved in proliferation and differentiation of local precursor cells. In this context, a study suggested that systemic administration of MSCs improves radiation-induced intestinal epithelium injury in mice, by increasing the activation of the Wnt/β-catenin signaling pathway that drives proliferation and maintenance of intestinal stem cells (Gong et al., 2016). In a mouse models of Alzheimer's disease, intravenous administration of MSCs stimulated proliferation and differentiation of hippocampal neuronal progenitor cells into mature neurons by increasing the Wnt signaling pathway (Oh et al., 2015).

DIVERGENT ROLES OF MSCs IN CANCER TREATMENT

The therapeutic benefits of MSCs have prompt their use in cell-based strategies to treat different diseases, including cancer. Similar to damaged tissues, tumors exert chemoattractant effects on MSCs that influence their recruitment to tumor sites (**Figure 2**). The CXCL12/CXCR4 axis is one of the most frequently studied signaling pathways in the mobilization of MSCs to tumor microenvironment (Gao et al., 2009; Xu et al., 2009; Lourenco et al., 2015; Wobus et al., 2015; Kalimuthu et al., 2017). However, the ability of MSCs to migrate toward cancerous tissue is also controlled by other agents, including diffusible cytokines such as IL8, growth factors such as TGFβ1 or platelet derived growth factor (PDGF), and extracellular matrix molecules such as matrix metalloproteinase 2 (MMP-2) (Nakamizo et al., 2005; Birnbaum et al., 2007; Bhoopathi et al., 2011). Once the tumor niche is reached, MSCs interact with cancer cells via direct and indirect mechanisms that affect tumor development (**Figure 3**). The paracrine function of MSCs is one of the main mechanisms involved in cancer regulation and is mediated by multiple factors, including growth factors and cytokines. These paracrine factors affect cellular processes involving tumor cell cycle (i.e., cell proliferation), cell survival, angiogenesis, and immunosuppression/immunomodulation, allowing MSCs to regulate cancer. The paracrine agents can be directly secreted into the extracellular space or packaged into extracellular vesicles to be spread in the tumor milieus (Rani et al., 2015). The interaction of MSCs with tumor cell cycle is the most commonly accepted process by which MSCs exert their therapeutic effects (Fathi et al., 2019). By inhibiting proliferation-related signaling pathways, such as the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), MSCs can induce cell cycle arrest and reduce cancer growth (Lu et al., 2019). In addition, MSCs can undergo differentiation into other cell types, such as cancer-associated fibroblasts (CAFs), to directly contribute to cancer progression (Jotzu et al., 2011; Barcellos-de-Souza et al., 2016; Aoto et al., 2018) (**Figure 3**).

Accumulating evidences indicate that the cross-talk between MSCs and tumor cells results in both pro-tumor and anti-tumor effects, raising safety concerns for clinical application in oncology (Barkholt et al., 2013) (**Figure 3**). The discrepancies in the ability of MSCs to promote or suppress tumor development may be attributable to differences in experimental tumor models, MSC tissue source, dose or timing of the MSC treatment, cell delivery method, control group chosen, and other experimental conditions (Bortolotti et al., 2015; Bajetto et al., 2017). In this regard, a study demonstrated that direct (cell-to-cell contact) or indirect (released soluble factors) interaction between umbilical cord MSCs and glioblastoma stem cells produces divergent effects on cell growth, invasion and migration (Bajetto et al., 2017). Additionally, the application of MSCs for cancer patients is a more complex situation in which other factors have to be taken into consideration. For instance, the pathological conditions of each patient may induce cellular and molecular changes in MSCs that interfere with their therapeutic effects (Capilla-Gonzalez et al., 2018; Perez et al., 2018; Rivera et al., 2019). We must, therefore, be cautious in the conclusions we draw from a single study regarding the therapeutic effects of MSCs in cancer.

Pro-tumor Activity

The pleiotropic effects of MSCs that promote tissue repair and regeneration may also confer pro-tumor functions to these cells. For instance, metastatic human breast carcinoma cells were found to induce the secretion of the chemokine (C-C motif) ligand 5 (CCL5) from MSCs, which enhanced tumor invasion (Karnoub et al., 2007). Seminal reports demonstrated that MSCs are also able to inhibit apoptosis in tumor cells by secreting pro-survival factors such as VEGF and basic fibroblast growth factor (bFGF) (Konig et al., 1997; Dias et al., 2002).

Numerous studies converged on the finding that MSCs contribute to cancer pathogenesis by releasing inflammatory factors that promote immunosuppressive effects. For example, an

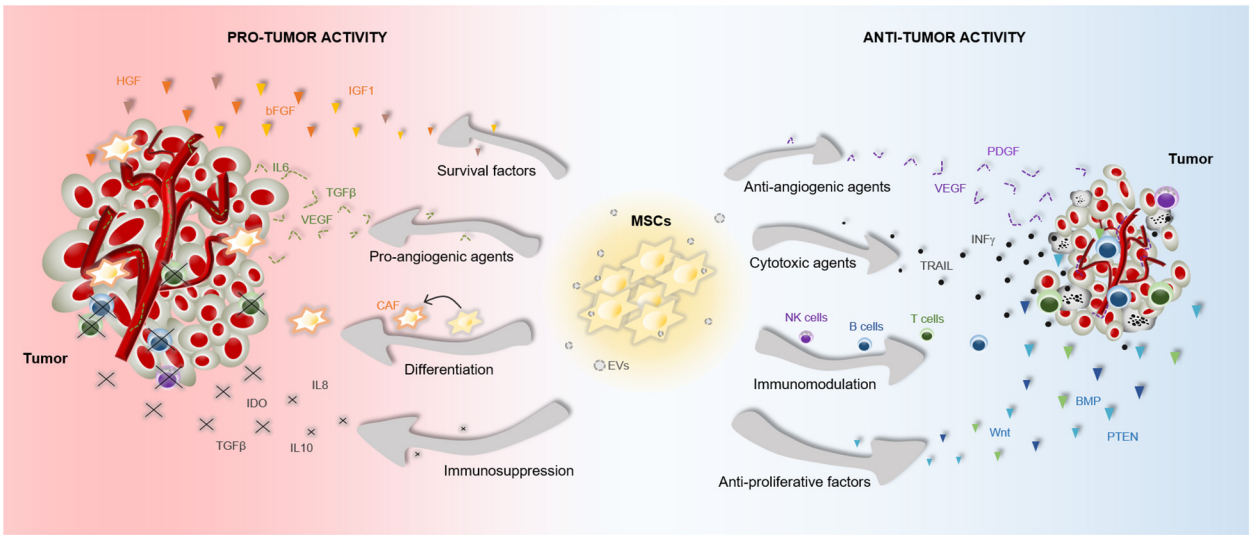

FIGURE 3 | Pro- and anti-tumor effects of MSCs. The particular properties that make MSCs excellent therapeutic agents, can also influence tumor progression. MSCs are able to release multiple agents with pro- and anti-tumor effects, which affect survival, proliferation and angiogenesis among other cell functions. These paracrine agents can be directly secreted into the tumor milieus or secreted via EVs. Furthermore, MSCs can differentiate into CAFs to support tumor progression. Abbreviations: bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; CAF, cancer-associated fibroblasts; HGF, hepatocyte growth factor; EVs, extracellular vesicles; IGF1, insulin like growth factor 1; IL6, interleukin 6; IL8, interleukin 8; IL10, interleukin 10; INFγ, interferon gamma; IDO, indoleamine 2,3-dioxygenase; NK, natural killer; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase; PDGF, platelet derived growth factor; TRAIL, TNF-related apoptosis-inducing ligand; TGFβ, transforming growth factor; VEGF, vascular endothelial growth factor.

in vitro study showed that MSCs isolated from gastric tumors mediate cancer progression through secretion of Interleukin 8 (IL8) (Li et al., 2015), a pro-inflammatory chemokine that favors the recruitment of leukocytes. It is known that recruited leukocytes, such as macrophages and neutrophils, facilitate cancer initiation and progression (Guo et al., 2017; Powell et al., 2018). Similarly, MSCs are able to secrete TGFβ that promotes macrophages infiltration at the tumor site and facilitates tumor escape from immune surveillance (Kim et al., 2006; Byrne et al., 2008).

Compelling evidences indicate that MSCs can also support tumor angiogenesis, an essential process in cancer progression that supplies tumors with oxygen and nutrients. For instance, MSCs recruited in breast and prostate tumors were found to increase the expression of angiogenic factors, including TGFβ, VEGF and Interleukin 6, which contribute to tumor growth and vascularization (Zhang et al., 2013). Similarly, a correlation between increased expression of TGFβ1 and higher microvessel density was observed in hepatocellular carcinomas of mice receiving intravenous injections of human MSCs (Li et al., 2016). This study further supported that MSCs may enhance tumor angiogenesis via TGFβ.

Furthermore, MSCs can also respond to soluble factors secreted from cancer cells and differentiate into CAFs, a cell type within the tumor microenvironment capable of promoting tumorigenesis (Mishra et al., 2008). In particular, TGFβ secreted from cancer cells plays a key role in the differentiation of MSCs into CAFs (Jotzu et al., 2011; Barcellos-de-Souza et al., 2016; Aoto et al., 2018). It is known that the transition of MSCs into CAFs contributes to tumor progression in part by their active secretome, which includes immune-modulating agents (CXCL12, Granulocyte Macrophage Colony-Stimulating Factor), pro-angiogenic factors (VEGF, TGFβ, PDGF), pro-survival factors (Hepatocyte Growth Factor, Insulin like Growth Factor 1, Interleukin 6), and extracellular matrix modulators (MMP, Tissue Inhibitor of Metalloproteinases) among others (Kalluri, 2016). Cell engulfment has also been identified as an interaction process between MSCs and cancer cells that enhances tumor aggressiveness. A recent report demonstrated that breast cancer cell engulfment of MSCs leads to changes in the transcriptome profile of tumor cells, mainly associated with oncogenic pathways (Chen et al., 2019). This MSC engulfment enhances epithelial-to-mesenchymal transition, stemness, invasion, and metastasis of breast cancer (Chen et al., 2019).

Anti-tumor Activity

Although compelling evidences show a pro-tumorigenic role of MSCs, these cells also have potent tumor suppressive effects that have been exploited as cancer therapeutics. Previous studies have demonstrated that MSCs release cytotoxic agents, such as TNF-Related Apoptosis-Inducing Ligand (TRAIL) that selectively induces apoptosis in different types of cancer (Wiley et al., 1995; Hao et al., 2001; Takeda et al., 2001; Akimoto et al., 2013). Recently, a report indicated that bone marrow MSCs promote apoptosis and suppress growth of glioma U251 cells through downregulation of the PI3K/AKT signaling pathway (Lu et al., 2019). Similarly, intravenously transplanted MSCs were found to suppress tumor growth by blocking AKT activation in a Kaposi sarcoma mouse model (Khakoo et al., 2006). In mammary carcinomas, umbilical cord MSCs attenuated cell

growth and triggered apoptosis through inhibiting ERK1/2 and AKT activation (Ganta et al., 2009). The Wnt signaling pathway has also been involved in the ability of MSCs to inhibit tumor cell proliferation (Qiao et al., 2008a,b). A mechanistic study of the inhibitory effect of MSCs on breast cancer cells demonstrated that the protein Dickkopf-1 (Dkk-1) released from MSCs blocks tumor growth via depression of Wnt signaling (Qiao et al., 2008a).

In contrast to investigations describing the pro-angiogenic effect of MSCs (Zhang et al., 2013; Li et al., 2016), the anti-tumor activity of MSCs via inhibition of tumor angiogenesis has also been documented. A study reported that bone marrow MSCs restrict vascular growth in ΔGli36 glioma xenograft through downregulation of the PDGF/PDGFR axis (Ho et al., 2013). In particular, the expression of PDGF-BB protein was significantly reduced in tumor lysates when treated with MSCs, which correlated with reduced levels of activated PDGFR-β and the active isoform of its downstream target AKT (Ho et al., 2013). In a melanoma mouse model, transplanted MSCs inhibited angiogenesis in a concentration-dependent manner, leading to a reduced tumor growth (Otsu et al., 2009). Confirmatory *in vitro* studies suggested that the anti-angiogenic effect was due to MSC-induced capillary degeneration (Otsu et al., 2009).

Furthermore, MSCs have elicited anti-tumor immune responses through released inflammatory mediators, such as the multifunctional cytokine TGFβ. Similar to several signaling molecules, TGFβ plays a dual role in cancer development (Bierie and Moses, 2006). Besides the aforementioned pro-tumor functions, TGFβ signaling exhibits suppressive effects in cancer (Dong et al., 2007; Guasch et al., 2007). In fact, while the expression of the type III TGFβ receptor (TβRIII) decreases during breast cancer progression, restoring TβRIII expression suppresses tumorigenicity (Dong et al., 2007).

MSCs AS CARRIERS OF ANTI-CANCER PAYLOADS

Over the past decade, research efforts have focused on investigating the potential of stem cells as Trojan horses to selectively deliver anti-cancer payloads to tumor cells. In this context, MSCs have attracted much attention as therapeutic carriers due to their inherent capacity to migrate to tumor sites. Genetic engineering is one of the most common strategies used to produce MSCs delivering tumor-suppressing agents into cancer cells. Typically, MSCs have been genetically modified with viral particles to express cytokines, such as Interferon β (INFβ) (Studeny et al., 2002; Shen et al., 2016). It has been reported that human umbilical cord MSCs transduced with adenoviral vectors expressing IFNβ effectively inhibit the growth of breast cancer cells through induction of apoptosis (Shen et al., 2016). Interleukins are another group of cytokines used as tumor-suppressing agents (Chen et al., 2008; Liu et al., 2018). A recent study using lentiviral transductions showed that human umbilical cord MSCs expressing interleukin-18 inhibit the proliferation and metastasis of breast cancer in mice (Liu et al., 2018). Genetically engineered MSCs with TRAIL have also shown strong anti-tumor activity in different types of cancer (Ciavarella et al., 2012; Fakiruddin et al., 2014; Guo et al., 2016; Jiang et al., 2016). In a fascinating study, X. Jiang and colleagues developed a non-viral method using nanoparticles to produce human MSCs engineered to express the suicide protein TRAIL for targeting and eradicating intracranial gliomas in mice (Jiang et al., 2016). When transplanted in a mouse model of orthotopic patient-derived glioblastoma xenografts, TRAIL-expressing MSCs inhibited tumor growth, induced apoptosis, reduced the occurrence of microsatellites, and extended animal survival.

Beside cytokines, several other proteins have been used as tumor-suppressing agents in MSC engineering for cancer therapy. For instance, Bone Morphogenetic Protein 4 (BMP4)-expressing MSCs were found to efficiently suppress tumor growth and prolong survival of glioma-bearing mice (Li et al., 2014; Mangraviti et al., 2016). Similarly, MSCs modified to express the tumor-suppressor gene Phosphatidylinositol 3,4,5-Trisphosphate 3-Phosphatase (PTEN) induced cytotoxicity of glioma cells (Guo et al., 2016).

MicroRNAs (miRs) have gained special interest in cancer therapy because of their ability to modulate post-transcriptional gene expression. It is known that MSCs express a variety of miRs that can be packaged into extracellular vesicles, and delivered to neighboring cells to exert therapeutic effects (Collino et al., 2014). Taking advantage of this property, MSCs have been engineered to carry specific miRs with anti-cancer properties (Lee et al., 2013; Lang et al., 2018; Sharif et al., 2018; Li X. et al., 2019). For instance, lentiviral vectors were used to engineer MSCs to produce extracellular vesicles carrying high levels of miR-124a, which had an effective anti-tumor action in multiple patient-derived glioma stem cell lines (Lang et al., 2018).

The loading of MSCs with oncolytic viruses has being used as an effective anti-tumor therapy. MSCs infected with the oncolytic adenovirus ICOVIR5 provided therapeutic benefit for the treatment of lung carcinoma in mice, through inhibition of tumor growth and promotion of T cell recruitment to the tumors (Rincon et al., 2017). Similarly, MSCs carrying the oncolytic adenovirus CRAd5/F11 inhibited tumor progression in a subcutaneous murine xenograft model of colorectal cancer (Guo et al., 2019). Different variants of the oncolytic herpes simplex virus has also been used to arm MSCs that effectively track metastatic tumor lesions and prolong survival of mice with brain metastatic melanomas (Du et al., 2017).

Another cellular Trojan horse that has been used for cancer treatment is MSCs loaded with anti-cancer drugs. For instance, an *in vitro* study determined that the conditioned media from gingival papilla-derived MSCs primed with Paclitaxel, Doxorubicin or Gemcitabine inhibit squamous carcinoma growth (Cocce et al., 2017). Paclitaxel-loaded MSCs also exhibited anti-tumor effects in glioma-bearing rats (Pacioni et al., 2015). Latest investigations have focused on the development of strategies to improve the payload and delivery capacity of MSCs. In this sense, nanoparticles are a promising approach to increase the anti-tumor efficacy of MSCs loaded with anti-cancer drugs (Layek et al., 2018; Wang et al., 2018; Moku et al., 2019). Drug-encapsulated nanoparticles offer multiple therapeutic benefits by

providing preferential accumulation at the target site, preventing burst release and reducing side effects.

LIMITATIONS OF MSC-BASED THERAPIES FOR CANCER: A CHALLENGE FOR BIOMATERIALS

The use of engineered MSCs has emerged as a new therapeutic paradigm to treat cancer. However, efficient engraftment and survival of delivered MSCs remains a potential obstacle that limits their therapeutic application. Biomaterials are used in cell therapy as scaffolds that improve the retention of transplanted cells in specific sites to treat different pathologies. This combined use of biomaterials and stem cells allows from the treatment of restricted defects to the repair and replacement of entire organs (Zeng et al., 2015; Wang et al., 2017; Diomede et al., 2018). In the cancer research area, a recent study described a method for the delivery of therapeutic MSCs on biomaterials to treat postoperative brain cancer (Sheets et al., 2018). This approach bases on the implantation of biodegradable fibrin scaffolds seeded with MSCs into the resection cavity to eradicate residual tumor cells in patients receiving surgical removal, with the ultimate goal of increasing cancer-free survival. Another study used cryogel-housed MSCs that were engineered to release anti-CD33-anti-CD3 bispecific antibody for effective immunotherapy in acute myeloid leukemia (Aliperta et al., 2017). In addition to scaffolds, biomaterials are used to encapsulate cells, protecting them from the host while allowing the diffusion of nutrients and therapeutic agents. Microcapsules designed with alginate, cellulose and agarose have shown benefits in cell-based anti-cancer therapies (Pelegrin et al., 1998; Sakai et al., 2005; Schwenter et al., 2011; Johansson et al., 2013). The group of Simone P. Niclou demonstrated that the interstitial delivery of alginate-encapsulated cells expressing the soluble form of the leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) inhibited tumor growth in orthotopic patient-derived glioblastoma xenografts mouse model (Johansson et al., 2013).

Biomaterials can be designed to have their own anti-cancer activities. Among biomaterials used in oncology, Gliadel represents one of the major success in the development of interstitial therapies for brain cancer (Brem and Gabikian, 2001). Gliadel is a biodegradable medicinal implant made of polifeprosan that is inserted into the resection cavity and slowly releases the anti-cancer agent carmustine over 2–3 weeks. The use of Gliadel in patients receiving surgical removal of brain tumors is associated with moderated survival benefits (Bregy et al., 2013). Latest investigations have discovered a thermo-responsive biodegradable paste that allows delivering of multiple anti-cancer agents with improved results in glioma patients survival (Smith et al., 2019). Once optimized the composition and design of biomaterials, it may be possible to use them in combination with stem cells to release anti-cancer agents in a cooperative manner. Therefore, the application of biomaterial in MSC-based therapies is a potential approach for the treatment of cancer that merits further investigation.

CLINICAL APPLICATION OF MSCs FOR CANCER THERAPY

The last decade has witnessed a rapid development of cell-based therapies for oncological application, being MSCs at the forefront of this new tendency. Aside from their anti-cancer effects, MSCs are of special relevance for personalized cell-based therapies because they can be easily obtained with minimally invasive procedures and rapidly large-scale expanded (Escacena et al., 2015). To date, 25 clinical trials are registered on ClinicalTrials.gov aimed to use MSCs in various cancer conditions. Among these studies, 14 trials are using MSCs as therapeutic agent to directly treat cancer (**Table 1**). Most of these trials are ongoing phase 1 or 2 studies that are evaluating the safety and efficacy of MSC application in cancer patients. Of special note is a completed phase I/II clinical trial from 2013 that investigated the use of bone marrow-derived autologous MSCs infected with the oncolytic adenovirus ICOVIR5 (CELYVIR) to treat metastatic and refractory solid tumors in children and adults (NCT01844661). This exploratory study evaluated the adverse effects after intravenous infusions of CELYVIR (time frame: 48 h after each infusion) and the clinical outcome (time frame: up to 2 months after the last infusion). The authors concluded that multidoses of CELYVIR have an excellent safety profile and beneficial anti-tumor effects (Garcia-Castro et al., 2010; Melen et al., 2016). Interestingly, they documented a complete remission in one pediatric case 3 years after CELYVIR treatment (Garcia-Castro et al., 2010).

Furthermore, there are nine registered clinical trials that evaluate the application of MSCs to treat a variety of side effects of cancer treatments, such as cardiomyopathy due to anthracyclines (NCT02509156), xerostomia due to radiotherapy (NCT03874572), cisplatin-induced acute renal failure (NCT01275612), erectile dysfunction after prostatectomy (NCT01089387) or radiation-induced hemorrhagic cystitis (NCT02814864). Therefore, MSC-based therapies stand as a good therapeutic option not only to directly target cancer, but also to minimize the side effects of cancer treatments. Consequently, there are two types of participants that would be potential candidates in a clinical trial of MSC-based therapy for cancer; (1) patients with cancer that are receiving or not receiving treatment, and (2) cancer survivors that experience side effects of oncological treatments. Inclusion criteria for these individuals should be either to be diagnosed with cancer, to suffer side effects of cancer treatments or both. Despite advances are being achieved, the lack of published results involving clinical studies hinders the development of further advances in the therapeutic application of MSCs.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Mesenchymal stem cells are widely used in the treatment of various diseases due to their ability to home to damaged tissues, their ability to differentiate into various cell types and their pleiotropic effects. However, the therapeutic use of MSCs for

TABLE 1 | Clinical studies using MSC-based therapies for cancer treatment.

NCT Number	Purpose	Condition	Therapeutic agent	Phase	Start date	Status	Locations
NCT03896568	To determine the maximal tolerated and toxicity of allogeneic bone marrow-derived MSCs loaded with the oncolytic adenovirus DNX-2401 (BM-MSCs-DNX2401)	Glioma	BM-MSCs-DNX2401	I	2019	Recruiting	United States
NCT03608631	To determine the maximal tolerated and toxicity of MSC-derived exosomes loaded with KrasG12D siRNA (iExosomes)	Pancreatic cancer	iExosomes	I	2019	Not yet recruiting	United States
NCT03298763	To evaluate the safety and anti-tumor activity of MSCs genetically modified to express TRAIL (MSC-TRAIL)	Adenocarcinoma of lung	MSC-TRAIL	I, II	2019	Recruiting	United Kingdom
NCT03184935	To determine the safety and efficacy of human umbilical cord-derived MSCs (UC-MSC)	Myelodysplastic syndromes	UC-MSC	I, II	2017	Unknown	China
NCT02530047	To find the highest tolerable dose of bone marrow-derived MSCs expressing INFb (BM-MSC-INFβ) that can be given To patients with ovarian cancer and to test their safety	Ovarian cancer	BM-MSC-INFβ	I	2016	Active, not recruiting	United States
NCT02181478	To evaluate feasibility and safety of combining intra-osseous umbilical cord blood hematopoietic stem cells (UC-HSC) and MSC	Hematologic malignancies	MSCs UC-HSC	I	2015	Recruiting	United States
NCT02068794	To study the side effects and best dose of adipose tissue-derived MSCs infected with oncolytic measles virus encoding thyroidal sodium iodide symporter (AdMSC-MV-NIS)	Ovarian cancer	AdMSC-MV-NIS	I, II	2014	Recruiting	United States
NCT02079324	To determine maximum tolerable dose, safety and efficacy of intratumoral injected GX-051	Head and neck cancer	GX-051	I	2014	Unknown	Korea
NCT02270307	To evaluate the effectiveness of the use of MSCs and cyclophosphamide	Hematological malignancies	MSCs and cyclophosphamide	II, III	2014	Unknown	Russian Federation
NCT01983709	To evaluate home of bone marrow-derived MSCs (BM-MSCs) to sites of prostate cancer after systemic administration	Prostate cancer	BM-MSCs	I	2013	Terminated	United States
NCT01844661	To evaluate the safety of bone marrow-derived autologous MSCs infected with ICOVIR5 (CELYVIR) in children and adults with metastatic and refractory solid tumors	Solid tumors metastases	CELYVIR	I, II	2013	Completed	Spain
NCT01129739	To evaluate the safety and efficacy of MSCs derived from human umbilical cord/placenta (UC/PL-MSC) at a dose of 1.0E + 6 MSC/kg	Myelodysplastic syndromes	UC/PL-MSC	II	2010	Unknown	China
NCT01092026	To determine the feasibility of umbilical cord blood hematopoietic stem cell (UCB-HSC) transplantation with co-infusion of third party MSCs	Hematological malignancies	UCB-HSC with MSCs	I, II	2010	Unknown	Belgium
NCT01045382	To evaluate the capacity of MSCs to improve 1-year overall survival of patients transplanted with HLA-mismatched allogeneic hematopoietic cells	Hematological malignancies	MSCs	II	2010	Recruiting	Belgium

cancer has been hampered by contradictory results describing both anti- and pro-tumor effects in preclinical studies. Despite this reality, latest MSC-based therapies bring new hope to cancer patients by offering highly effective anti-cancer treatments in a personalized manner. Among MSC-based therapies, the use of MSCs as Trojan horses to deliver therapeutic factors represents an important step forward to a more efficient cancer treatment. The next challenge is to better understand the interaction between MSCs and cancer cells to improve the clinical safety of MSC-based therapeutic approaches. In this context, the use of MSC-derived extracellular vesicles as a cell-free therapy has emerged as a promising option that circumvent the safety concerns associated with the use of live cells. Further research will shed light on the challenges facing cell-free therapy for cancer. We are definitely moving closer to generate a safe and effective medicinal product for cancer that will improve survival and quality of life of patients suffering this devastating disease.

AUTHOR CONTRIBUTIONS

VC-G conceived the manuscript. All authors contributed to the manuscript revision and approved the submitted version.

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CASE REPORT
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Bilaminar Chitosan Scaffold for Sellar Floor Repair in Transsphenoidal Surgery

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Background: Endoscopic endonasal transsphenoidal surgery (EETS) is a standard technique used to approach sellar tumors. It is relatively safe, minimally invasive and carries a low risk of complications. However, one of the common complications reported with this technique is CSF leakage which causes morbidity, an increase in recovery time and hospital costs. This complication usually occurs from violation of the diaphragma sellae and a defect in the structures of the sellar floor or incomplete repair. In this article we report the first case with the use of a novel bilaminar chitosan scaffold which can be potentially used in the repair of the sellar floor, primarily aiming to the bony part of this structure.

Case Presentation: After a personalized design employing a tissue engineering strategy, we reconstructed the sellar floor in a 65-year-old woman who had undergone EETS for a pituitary adenoma with progressive bilateral visual loss. To repair the bony defect of the sellar floor, we used a novel bilaminar chitosan scaffold. The patient had an unremarkable postoperative course with no evidence of CSF leak. The polymer was well tolerated without toxicity, infection or complications. After 2 years of follow up the patient remains neurologically intact, and in good endocrinological status.

Conclusion: This is the first report of the use of this biomaterial and its biocompatibility in a clinical setting for the repair of the sellar floor during EETS. Our experience with chitosan bilaminar scaffold and in several preclinical studies in the literature have demonstrated good biocompatibility and effective bioengineered bone regeneration due to its excellent osteoconductive properties, this study pretends to be one landmark for further clinical research and larger case series with the use of this personalized tissue engineering materials in order to see they real efficacy to increase the surgeon armamentarium.

Keywords: biomaterial, endoscopic, sellar floor reconstruction, chitosan, CSF leak, transsphenoidal

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BACKGROUND

Transsphenoidal surgery has become a great tool for minimally invasive endoscopic resection of tumors in the anterior skull base, especially in the sellar region, there are, however complications largely reported from this approach, such as postoperative cerebrospinal fluid (CSF) rhinorrhea between others (Roca et al., 2018).

The frequency of postoperative CSF leaks depends on many factors as the technique used for reconstruction of the sellar floor and the skull base bony defect, and has been observed in as many as 5 to 75% of cases (Gardner et al., 2008a,b; Stippler et al., 2009; Greenfield et al., 2010). CSF leaks can lead to complications such as infection and pneumoencephalus, which may lead to further comorbidities, longer recovery times and increased hospital costs (Conger et al., 2018; Roca et al., 2018).

Different techniques and materials have been reported in the literature for reconstruction of these skull base defects including the use of fat grafts, autologous muscle, fascia lata, vascularized mucosal flaps, non-vascularized autografts, and different synthetic materials (Conger et al., 2018; Roca et al., 2018). They include bio-absorbable implants as collagen sponges (Kelly et al., 2001), dura mater substitute (Sandoval-Sánchez et al., 2012; Al-Asousi et al., 2017), fibrin glue, Polyethylene glycol (PEG) hydrogel dural sealant (Cosgrove et al., 2007; Burkett et al., 2011), polydioxanone plates and non-absorbable implants as porous polyethylene plates and titanium mesh (Al-Asousi et al., 2017).

Currently synthetic materials have become a great option to decrease and avoid donor-site morbidity and other complications related with the harvesting of autologous tissue (Al-Asousi et al., 2017).

The use of chitosan [poly-(β-1/4)-2-amino-2-deoxy-D-glucopyranose] has been described in several preclinical studies and tested in tissue bioengineering of bone (Zhao et al., 2002; Kim et al., 2004; Yoshida et al., 2004; Teng et al., 2008; Yuan et al., 2008; Liu et al., 2009; Xianmiao et al., 2009; Li et al., 2010; Mota et al., 2012; Pu et al., 2012; Zhang et al., 2012; Azevedo et al., 2014; Fan et al., 2014; Rodriguez-Vázquez et al., 2015; Vega-Ruiz et al., 2017) neural tissue (Simoes et al., 2011; Meyer et al., 2016; Ghasemi Hamidabadi et al., 2017; Nawrotek et al., 2017; Zhao et al., 2017) and soft tissue (Gobin et al., 2006; Paulo et al., 2009; Tchemtchoua et al., 2011; Udpa et al., 2013; Zou et al., 2017). Chitosan is a copolymer derived from the alkaline deacetylation of chitin and made of N-acetyl-D-glucosamine and D-glucosamine bonds and β bonds in which glucosamine is the main repeating unit in its structure (Rodriguez-Vázquez et al., 2015).

Observations made in studies of biosynthetic replacement of bone, the potential for reconstructive surgery was suggested due to its biocompatibility, as well as osteinductive and osteoconductive features (Zhao et al., 2002; Kim et al., 2004; Yoshida et al., 2004; Teng et al., 2008; Yuan et al., 2008; Liu et al., 2009; Xianmiao et al., 2009; Li et al., 2010; Mota et al., 2012; Pu et al., 2012; Zhang et al., 2012; Azevedo et al., 2014; Fan et al., 2014; Rodriguez-Vázquez et al., 2015; Vega-Ruiz et al., 2017). However, as of now there is a lack of clinical data demonstrating its usefulness and safety in the clinical setting (Vega-Ruiz et al., 2017).

Due to challenges frequently encountered in the repair of the sellar floor specifically the bony part, we intended to address this problem with a chitosan scaffold. This material has shown to provide useful characteristics facilitating bone regeneration (Zhao et al., 2002; Kim et al., 2004; Yoshida et al., 2004; Teng et al., 2008; Yuan et al., 2008; Liu et al., 2009; Xianmiao et al., 2009; Li et al., 2010; Mota et al., 2012; Pu et al., 2012; Zhang et al., 2012; Azevedo et al., 2014; Fan et al., 2014; Rodriguez-Vázquez et al., 2015; Vega-Ruiz et al., 2017), and scaffolding properties characterized by infiltration of fibroblasts, with subsequent deposits of organized collagen fibers, without evidence of infection or abnormal healing (Sandoval-Sánchez et al., 2012).

Our case describes the use of a novel bilaminar chitosan scaffold used for the repair of the sellar floor after endoscopic endonasal transsphenoidal surgery for a pituitary macroadenoma. Our use of such a bioactive membrane to repair the bony defect could potentially be useful for a strong and durable closure of the sellar floor, but further studies are needed to see its real efficacy, this case pretends to study the biocompatibility and performance of this material in a single patient and be a landmark for larger series.

BILAMINAR CHITOSAN SCAFFOLD

The bilaminar implant constitutes two types of different spatial structures: one of the membranes presents a flat-smooth structure, the other membrane has a tridimensional-porous structure. Each of the physical-chemical properties given to the membranes, was in anticipation of the biological effect needed in the effector tissue (**Figures 1A,B**).

The two types of membranes, synthetized for the construction of a bilaminar implant, were made with biomedical grade chitosan of medium molecular weight. It is characterized by 75–85% of deacetylation, and is available as a powder from Sigma Aldrich®, United States.

In the case of the membrane showing a flat-smooth structure, it was synthetized from a 2% chitosan solution made in diluted acetic acid acting as a solvent (Sigma Aldrich®, United States); In order to achieve a suitable solute, the mix was first set on a magnetic stirrer for 1 h. Then, the solution was brought into a sonicator at 28°C for 2 h to dissolve any air bubbles that had formed by stirring.

The other membrane (tridimensional-porous structure) was synthetized from a chitosan solution of 4% dissolved in diluted acetic acid (Sigma Aldrich®, United States);

The mix was set on a magnetic stirrer for 4 h, and thereafter the solution was brought to a sonicator at 28°C for 2 h,- again until all air bubbles formed by the stirrer were completely eliminated.

Once the solutions had been made, for the synthesis of the two membranes (the flat-smooth and the tridimensional-porous) both were set in comparable quantities (of ml/cm^2) in a Petri dish. In the case of the flat-smooth membrane, it was generated by drying under 98% of humidity loss and for the other (tridimensional-porous), a procedure of phase separating was thermically induced.

When both membranes were ready, they were assembled to create a sandwich structure. Consequently, the ensemble was put in a Petri dish and the cover lid was set up inverted. This construct was set up for drying for 24 h at room temperature and then it was precipitated in a solution of sodium hydroxide 1N, following the same protocol for each membrane separately before the construct was usable. Then we froze the mold at −196°C and

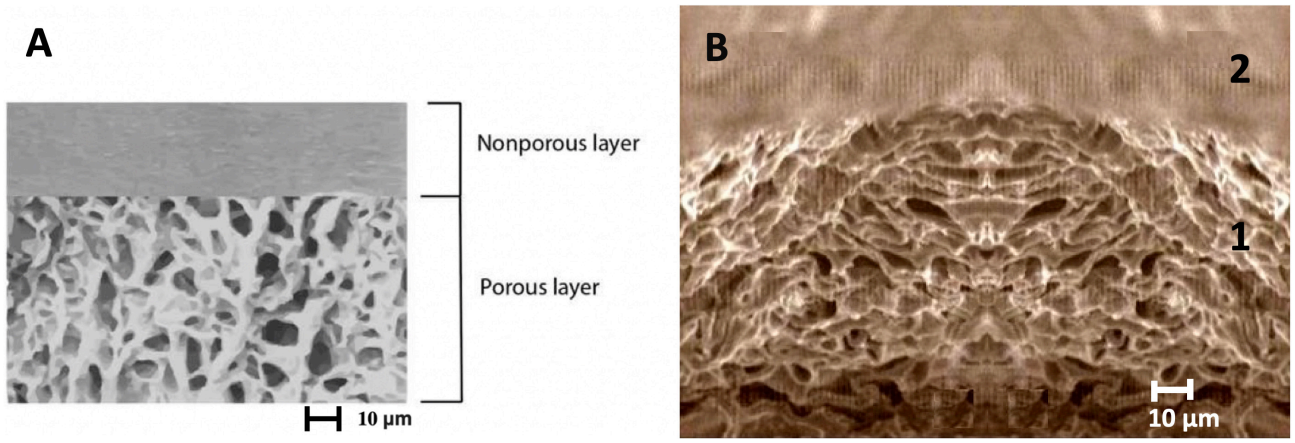

FIGURE 1 | (A) Digital design that describe the biophysical structural characteristics of the chitosan scaffold in bilayer (Non-porous and porous) and the average pore diameter of 10 µm. **(B)** Electronic photomicrograph with evidence of the microstructure of the scaffold device substitute of dura mater conformed by two layers of chitosan. (1) Porous layer of 300 µm (2) Non-porous layer 100 µm. Observe the interface of the bi-layer that is structurally similar to the histological conformation of the dura mater, and has a homogeneous and regular pattern in the diameter of its pores, in contrast with the non-porous portion that has a smooth surface. This confers the capacity of an watertight behavior as a membrane on the one hand, in addition to forming a structural network for the development of fibroblasts of the receptor tissue to favor the orderly and structured development of the collagen in the regenerative process.

then lyophilized it in a freeze dryer for 5 h (FTS Systems Inc., New York, NY, United States). The management of the implant with NaOH and the washing with distilled water result in a pH near to a neutral value.

The sterilization of the implant was performed with the use of ethylene oxide gas. We performed control tests to verify that the biomaterial structure was intact after the sterilization process. The reproducibility of the design and formulation of the chitosan scaffold has been systematized in the patent number: MX 358993 B in reference to the surgical procedure, synthesis and sterilization of the biomaterial. Nowadays is a standard procedure that combine the minimal invasive surgery and skull base surgery, in order to treat pituitary adenomas.

CASE PRESENTATION

A 65 years old right-handed woman, came to neurosurgery for consultation due to progressive bilateral visual loss in her temporal fields. This had occurred over 10 months, and 2 weeks prior to her admission she reported sudden loss of consciousness, prompting her admission to the hospital. On examination, she was alert and oriented x 3, she had a normal cranial nerve examination except for decrease visual acuity (20/200 in her left eye, 20/80 in her right eye), bitemporal hemianopia and mild atrophy of the optic disk in the left eye. Gait, motor and sensory examination was normal.

Laboratory studies showed a LH at 0.22 IU/L (reference value in Postmenopausal females 15.0–62.0 mIU/mL)and prolactin at 53 ng/mL (reference value in non-pregnant females 2–29 ng/mL)0.7. A contrast enhanced brain MRI was obtained (**Figures 2A,B**) and revealed a sellar lesion which was hypointense in T1 but hyperintense in T2 sequences with enhancement of the periphery. The lesion extended into the sphenoid sinus and parasellar space without encasement of the carotids and into the suprasellar cistern abutting the optic chiasm. The patient underwent endoscopic endonasal transsphenoidal surgery for resection of the sellar lesion (**Figures 3A,B**). Intraoperatively, the lesion appeared reddish in color and it was of soft consistency. Moderately bleeding was encountered during resection and a sample was taken for pathology. At the end of the tumor removal, the scaffold was implanted to close the bone defect in the sphenoid sinus (**Figures 4A,B**). Due to the fact that the graft could be molded into shape, it was easily set and allowed to cover the entire size of the defect. A standard fat graft was then placed in the sphenoid sinus covering the outer membrane of the chitosan graft. Finally, fibrin sealant was used, and a nasal packing was inserted in both nostrils.

The patient had an unremarkable postoperative period and after a few days the patient was discharged without evidence of CSF leak or complications. After one month the patient showed complete recovery of her visual acuity and visual fields. At follow up, the patient underwent a postoperative brain MRI (**Figures 5A,B**) illustrating gross total resection and good closure of the sellar floor. There were no signs of rejection or inflammation in the area where the chitosan scaffold was implanted.

DISCUSSION

One of the main challenges of transsphenoidal surgery is a potential CSF leak and a durable reconstruction of the sellar defect. The CSF leaks can derive from the surgical procedure or from primary erosion of the sellar floor which can be encountered in various tumors that affect this region and it remains a matter of debate of how to seal these off in the best

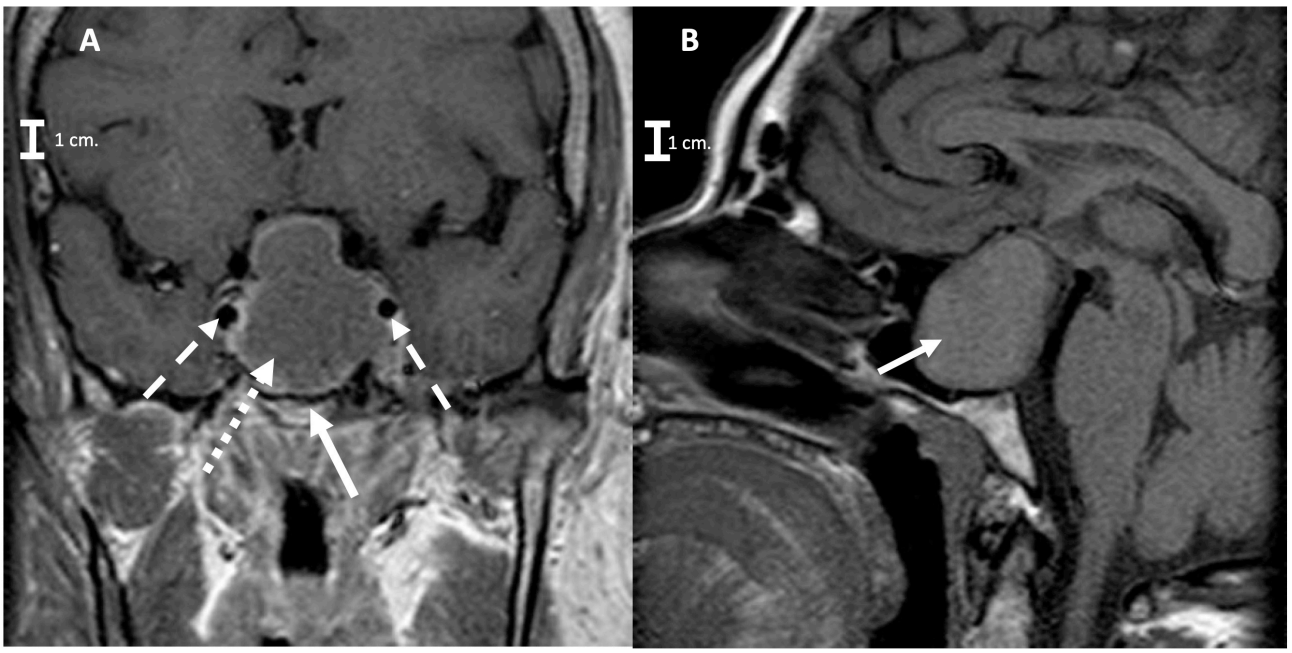

FIGURE 2 | (A) Preoperative coronal Brain MRI T1 signals respectively, showing the pituitary macroadenoma (arrow with short dashed line) with suprasellar extension and selllar floor erosion (arrow with continues line), without encasement of the carotids (arrows with long dashed line pointing both carotids). **(B)** Sagittal brain MRI T1 signals respectively (arrow with continues line is pointing to the Macroadenoma).

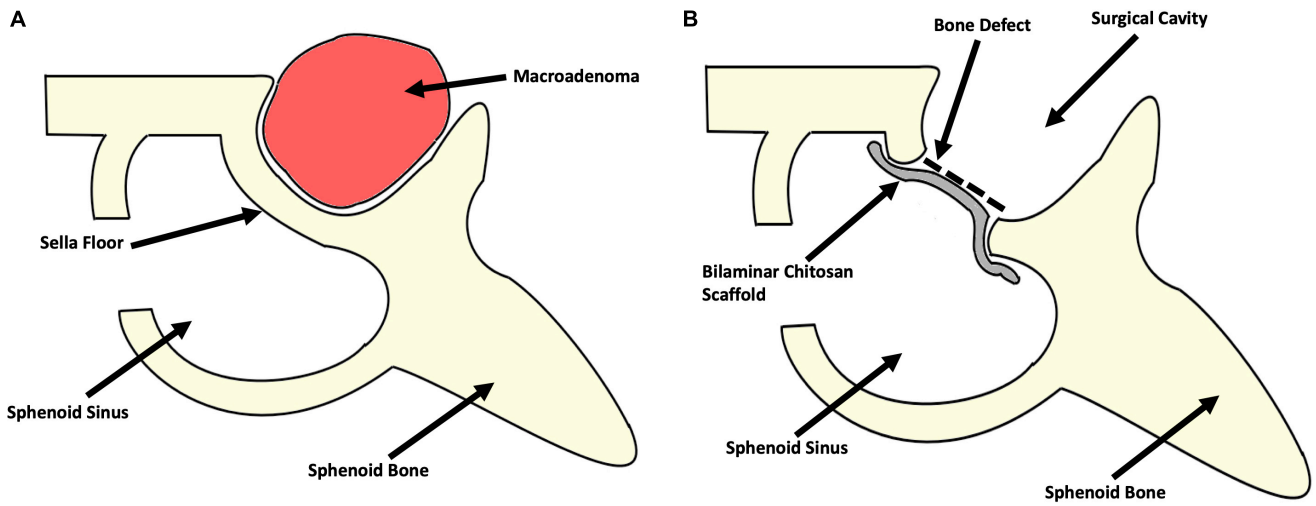

FIGURE 3 | Schematic representation of the surgical procedure. **(A)** Preoperative representation of the surgical corridor and the anatomical location of the macroadenoma. **(B)** Postoperative representation of the surgical cavity after gross total resection of the macroadenoma and the reconstruction of the sellar floor bony defect with the bilaminar chitosan scaffold (due to the easy molding of this scaffold, it was possible to cover the entire shape of the bony defect).

way (Gardner et al., 2008a,b; Stippler et al., 2009; Greenfield et al., 2010; Conger et al., 2018).

In this article we present the first biocompatibility results of using a novel bilaminar chitosan scaffold construct for repair of the sellar floor primarily aiming to the bony defect, in endonasal endoscopic transsphenoidal surgery.

We consider chitosan a very suitable material, as the potential for bone regeneration and its physical-chemical characteristics have been already established and reported in the literature. However, there is a lack of data for its use in the clinical setting and the description of its biocompatibility in human tissues is also poor.

The inclusion of this patient with a macroadenoma to this clinical trial was performed, due to the larger surgical corridor needed for the resection of the voluminous mass in comparison with smaller lesions, the requirement for a larger bony defect

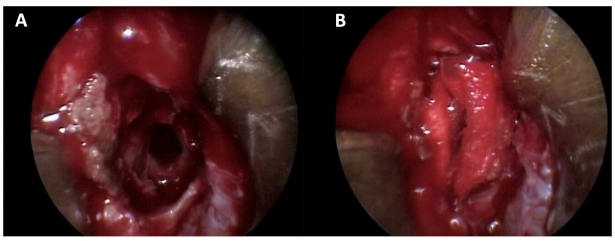

FIGURE 4 | Transoperative images of the implanting of the bilaminar chitosan scaffold in the sellar floor defect, **(A)** image of the defect before the setting of the scaffold, **(B)** image of the sellar defect after the setting of the scaffold.

in the sellar floor in order to extend the access of the resection, increase the complexity of the reconstruction of this structure and also rise the concern for a postoperative CSF leak. Also, this single patient was selected for an evaluation in terms of biocompatibility, toxicity, and the long-time biological behavior of the biomaterial before continuing to a larger series of patients. At this time, we are planning for a bigger clinical trial with the inclusion of more patients.

For that reason, we intended to show in this study that a bilaminar chitosan scaffold is a very appealing option for the bony part of sellar floor reconstruction as its use carries the advantage of chitosan being osteconductive. A bioengineered scaffolding with good mechanical properties could potentially allow a customized repair of the bone defects after surgery.

The use of chitosan was selected over other materials, due to its biodegradability, low cost, versatile molding and non-toxic properties reported in the literature, in applications such skin regeneration, tissue engineering and regenerative medicine (Sandoval-Sánchez et al., 2012; Rodriguez-Vázquez et al., 2015; Vega-Ruiz et al., 2017). The main concerns for complications with the use of this scaffoldings is the low risk for local inflammatory reactions and rejection of the implant.

The patient was discharged with oral pain medication, antibiotics, endocrinological follow up and instructed postoperatively to return to our hospital facilities at any symptom concerning of CSF leak, infection, local or systemic inflammatory response and was also followed up with a postoperative MRI and laboratory studies that included but are not limited to cell blood counts (CBC), acute phase reactants and blood chemistries. This case has been followed up for more than 3 years, without postoperative symptoms or complications, abnormalities in the laboratory or in imaging studies.

In our report we describe a good malleability of the scaffold during its intraoperative use. Advantageous practical feature are its moldable nature, the fact that it can be easily cut and adapted to the patient′s anatomical needs, allowing the surgeon to be very flexible during the surgical procedure.

The appropriate tolerance and biocompatibility between the implant and the recipient tissue were evaluated, and there was not evidence of local or systemic inflammatory reaction, toxicity, infection, or severe scar reaction or fibrosis. (Acute phase reactants and CBC were within the normal range.

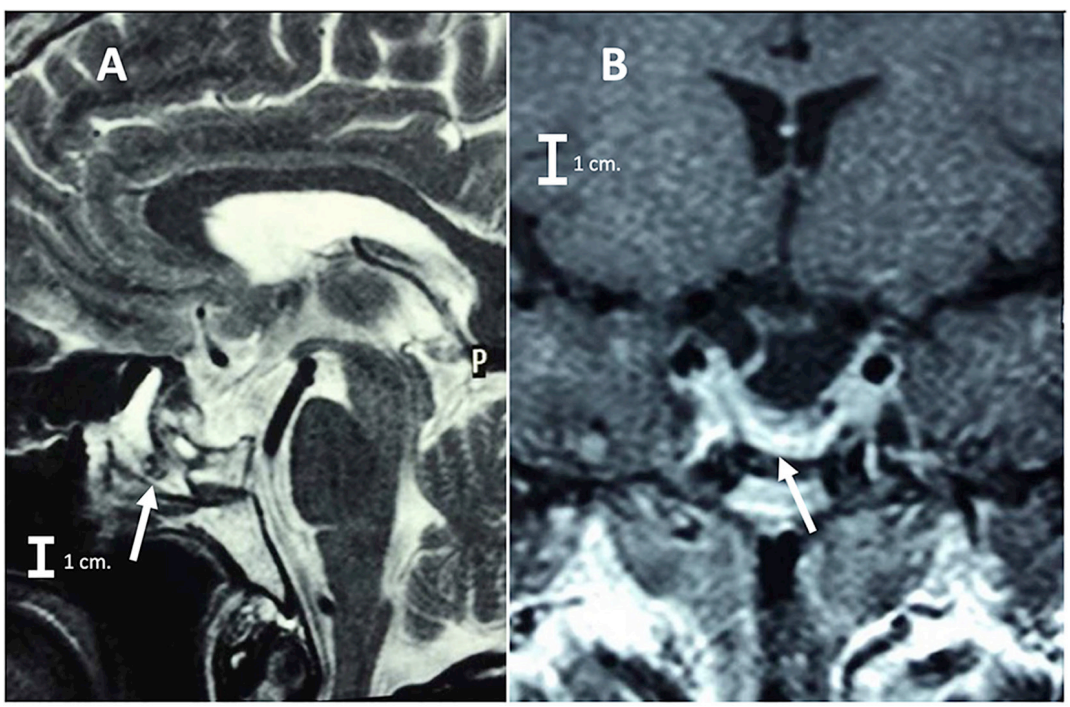

FIGURE 5 | (A) Postoperative coronal brain MRI T1 signal respectively, showing the gross total resection of the Macroadenoma and the reconstruction of the sellar floor (arrow) with the bilaminar chitosan scaffold, **(B)** sagittal brain MRI T2 signal (arrow showing also the reconstruction of the sellar floor with the bilaminar chitosan scaffold).

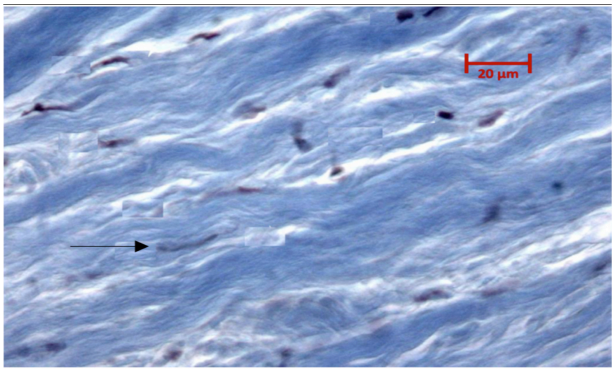

FIGURE 6 | Photomicrograph with Masson trichrome stain 180 days after the colocation of scaffold in the preclinical experimental process in which an organized structure of fibroblast and collagen growth (arrow) is demonstrated without evidence of fibrosis or acute or chronic inflammatory reaction in the scaffold from which the dura mater has regenerated.

At the local level the postoperative MRI also showed a good reconstruction and sealing of the sellar floor without signs of rejection or inflammatory response per imaging). Accomplishing the surgical objective of reconstruction of the sellar floor.

In the preclinical study, from our research group (Sandoval-Sánchez et al., 2012). The experimental model was performed in two phases: An *in vitro* phase were the bilayer chitosan scaffold was evaluated for pore size, thickness, water absorption capacity, tensile strength, strain, and toughness. In the second *in vivo* phase 27 durectomized New Zealand rabbits were randomly assigned into three duraplasty groups with autologous dura, collagen matrix or bilayer chitosan scaffold. Histology response to regeneration was evaluated through hematoxylin and eosin stain and Masson trichrome stain after the colocation of the implant (**Figure 6**; Sandoval-Sánchez et al., 2012).

We concluded that the biomaterial was biocompatible as in the *in vivo* phase there were no cases with CSF fistula, fever, or infection. In the histologic evaluation after 180 days we found an organized structure of fibroblast and collagen growth without evidence of fibrosis or acute or chronic inflammatory reaction in the scaffold from which the dura mater has regenerated. The scaffold did not compress neural tissue and conserved a watertight dural closure, these findings make the bilayer chitosan scaffold a suitable and biocompatible option for reconstruction of the sella floor.

With this study we wish to create a landmark for further testing of this biomaterial as we want to advance tissue engineering in neurosurgical procedures.

Further research is also needed to establish the biocompatibility in larger series, and to assess any potential side effects of chitosan in the clinical setting and to prove its usefulness and advantage compared with other materials already used in the clinic (Macías-Reyes et al., 2012; Sandoval-Sánchez et al., 2012; Rodriguez-Vázquez et al., 2015; Vega-Ruiz et al., 2017).

To this end it is necessary to conduct a comprehensive study of a series of such patients and to also evaluate the long-term efficacy of this therapeutic option, as well as its price tag, when compared with other materials.

CONCLUSION

A novel chitosan scaffold was created and designed by bioengineering methods for tissue repair with the concept of reconstruction of the bony part of the sellar floor in mind.

In this single case report to elucidate the biocompatibility and safety from an observation with more than 3 years of follow up, we show that the chitosan scaffold demonstrated good biocompatibility, without any observed toxicity or inflammatory reaction which was feared as a host response against the implanted biopolymer.

We wanted to verify the suitability of the scaffold in a single case with a long prospective follow up before continuing to larger series and to stablish a landmark for further clinical studies. We theorize that this bilaminar scaffold could act not only as a mechanical barrier but also as a bioactive implant which is suitable for surgical repair of bony defects due to its permissive nature in bone regeneration as reported in several preclinical studies, further research is needed to demonstrate the real efficacy of this bioengineering scaffolds in the clinical settings.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Bioethics Committee Approval: Register O63-2014. Center of Health. University of Guadalajara. All the procedures performed in this report were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed Consent for Participation: Informed consent was obtained from all individuals participants included in the study. Informed Consent for Publication: Informed consent was obtained from the participant for the publication of this case report and any potentially-identifying information/images. International Clinical Trial Register: NCT03280849.

AUTHOR CONTRIBUTIONS

RR-Z and FL-G contributed to the design and implementation of the research, to the analysis of the results, data acquisition and to the writing of the manuscript, revision for intellectual content,

gave final approval of the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. IS-D contributed to the analysis of the results, data acquisition, and to the writing of the manuscript.

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Single vs. Combined Therapeutic Approaches in Rats With Chronic Spinal Cord Injury

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The regenerative capability of the central nervous system is limited after traumatic spinal cord injury (SCI) due to intrinsic and extrinsic factors that inhibit spinal cord regeneration, resulting in deficient functional recovery. It has been shown that strategies, such as pre-degenerated peripheral nerve (PPN) grafts or the use of bone marrow stromal cells (BMSCs) or exogenous molecules, such as chondroitinase ABC (ChABC) promote axonal growth and remyelination, resulting in an improvement in locomotor function. These treatments have been primarily assessed in acute injury models. The aim of the present study is to evaluate the ability of several single and combined treatments in order to modify the course of chronic complete SCI in rats. A complete cord transection was performed at the T9 level. One month later, animals were divided into five groups: original injury only (control group), and original injury plus spinal cord re-transection to create a gap to accommodate BMSCs, PPN, PPN + BMSCs, and PPN + BMSCs + ChABC. In comparison with control and single-treatment groups (PPN and BMSCs), combined treatment groups (PPN + BMSCs and PPN + BMSCs + ChABC) showed significative axonal regrowth, as revealed by an increase in GAP-43 and MAP-1B expression in axonal fibers, which correlated with an improvement in locomotor function. In conclusion, the combined therapies tested here improve locomotor function by enhancing axonal regeneration in rats with chronic SCI. Further studies are warranted to refine this promising line of research for clinical purposes.

Keywords: axonal regeneration, bone marrow stromal cell transplant, pre-degeneration peripheral nerve transplant, BBB modified score, kinematic analysis, functional recovery

INTRODUCTION

Traumatic Spinal Cord Injury (SCI) results in partial or complete alterations of motor, sensation, and autonomic functions. Despite the advances in biomedical research for acute SCI, there is no effective treatment for axonal regeneration and functional restoration at chronic stages.

After SCI, efficient cord regeneration is mainly limited by a local inhibitory environment due in part to a biochemical barrier formed by molecules like myelin-associated proteins (1, 2) as

well as proteins constitutive of the extracellular matrix, markedly chondroitin sulfates (3, 4). In addition, a physical barrier (fibrogial scar) formed by microglia, oligodendrocytes, meningeal cells, and reactive astrocytes also inhibits axonal regrowth (5, 6).

Some strategies for promoting axonal regeneration and functional restoration in spinal cord injury models are relevant to the present study, especially those based on bone marrow stromal cell (BMSC) transplantation (7), fresh and pre-degenerated peripheral nerve (PPN) transplantation (8), and chondroitinase ABC (9, 10), all of which promote partial neuroprotective and regenerative effects after early SCI.

If used independently, the benefits of the above-mentioned strategies seem insufficient to be translated to patients, but several experimental studies have shown that combined therapies may produce a greater benefit (11–14). In fact, recently, we reported on the additive morphofunctional effect of combining PPN and BMSCs in a model of spinal cord transection (15). This justifies continuous testing for this kind of strategy.

To continue along this promising research line, the current study is designed to test the hypothesis that the combination of ChABC with PPN and BMSC transplant has a synergic effect on axonal regrowth and functional recovery in a model of complete chronic spinal cord transection. Our objective is to evaluate the reparative ability of several single and combined treatments targeting some barriers for spinal cord regeneration in rats with sequelae of severe and delayed SCI.

METHODS

Experimental Design

The Ethics Committee in Research of the Hospital de Especialidades, Centro Médico Siglo XXI, Instituto Mexicano del Seguro Social approved this study. Experiments were performed according to the official Mexican norm NOM-062. A total of 114 syngeneic Fisher 344 rats, 8–10 weeks old, and 200–220 g body weight were used. For SCI and transplants, 58 female rats were randomly divided into five groups. Twenty-eight male rats were used as sciatic nerve donors, and 28 male rats were used for BMSC procurement. Male donors were used for PPN transplants; as the nerve itself is larger than female nerves, we only used a single nerve to fill in the gap. After all surgical procedures, animals received oral enorfloxacin 10 mg/kg and ibuprofen 15 mg/kg once a day for 8 days.

Surgical Procedures and Transplant Preparation

Spinal Cord Injury

For cord injury, rats were anesthetized with a mixture of ketamine (70 mg/kg) and xylazine (10 mg/kg) given IM. A laminectomy was performed at T9, and subsequently, the dorsal portion of the dural sac was longitudinally sectioned and the spinal cord was completely transected in the transverse plane with microsurgery scissors. Complete transection was verified using a microsurgery hook to check for the absence of residual tissue. Finally, the skin wound was sutured, and the animal was returned to its home cage.

Peripheral Nerve Preparation for Transplantation

Twenty-one days before transplant, donor rats were anesthetized and subjected to complete transversal section of the sciatic nerve at the cephalic portion of the thigh; the free end of the caudal stump of the sectioned nerve was sutured with 7-0 nylon to prevent spontaneous nerve regeneration. On transplant day, rats were anesthetized to remove an ∼2 cm long segment of the sciatic nerve distal to the section. The nerve was then placed in ice-cold isotonic saline solution until transplantation.

Isolation and Culture of Bone Marrow Stromal Cells

BMSCs were obtained from the femurs and tibias aseptically harvested from donor rats euthanized with an overdose of sodic pentobarbital. Bone marrow was obtained by rinsing out each harvested bone with growing medium (Dulbecco's Modified Eagle D-MEM® GIBCO) using a 3-ml syringe; the released marrow was then centrifuged for 7 min at 1,300 rpm. The BMSCs were separated by centrifugation at 2,000 rpm at 24°C for 45 min with Ficol gradient (SIGMA) (3 ml). The cells obtained were seeded in a 75-cm^2 culture flask with 10 ml of D-MEM, 20% bovine serum (FSB, GIBCO), 2 ml L-glutamine (GIBCO), 2 ml antibiotic-antimycotic (GIBCO), and 2 ml of non-essential amino acids (GIBCO). Cells were then placed in a water-jacketed incubator at 37°C with 5% CO_2 until a monolayer of fibroblasts was formed. Afterward, a layer of BMSC on top of the fibroblasts was seeded, and cultures were kept for four passes until they became mature.

Transplantation

Four weeks after spinal cord transection, rats were randomly assigned to one of the five experimental groups. In Group 1 (control, $n = 14$), the surgical wound was reopened, and the dural sac was exposed without removing the scar. In Group 2 ($n = 14$), the dural sac was reopened in order to carefully remove the scar, leaving an ∼6-mm gap. Then, two injections of 5 µl each of DMEM medium (GIBCO) containing 3×10^4 BMSCs were injected parasagittally on each stump of the spinal cord with a 2 mm depth at the edge of both the rostral and caudal stumps. In Group 3 ($n = 14$), the spinal cord scar was removed as described for Group 2, and then, three to five PPN segments, each 6 mm long, were longitudinally implanted in the spinal cord gap. The implants were affixed with fibrin glue (BAXTER). In Group 4 ($n = 14$), the treatments described in Groups 2 and 3 were combined. Finally, in Group 5, the treatment described in Group 2 was used, with two BMSC injections administered in each stump, but adding 6 µl of ChABC (2 units/ml Seikagaku 100332; Associates of Cape Cod) to each injection (injecting a total volume of 11 µl) and with PPN implanted in the cord gap as described in Group 3.

Assessment of Locomotor Performance

Open Field Test

Hind limb locomotion was assessed by the modified BBB score for complete transaction: 22 points in four levels were evaluated, focusing on rhythmicity, movement alternation with and without body weight, and plantar support (16). Animals were evaluated 24 h after injury and weekly for the following 12 weeks. Observers

were blind to experimental groups, and the rats were not placed on a treadmill for the open field test.

Kinematic Analysis

Kinematic registration of gait was performed after 3 months of treatment. First, hindlimbs were marked with a non-toxic marker (Sharpie®) on the iliac crest, hip, knee, ankle, and fifth metatarsal phalangeal joints. Next, each animal was introduced separately into an acrylic tunnel (60 × 5 × 5 cm) marked every 5 cm. The animal was then recorded with a commercial digital video camera. Four consecutive steps (the first step was not considered in order to exclude the initial phase of the gait) were analyzed. With the help of computer software (Total Video Converter), digital photographs were obtained from each recording frame (30 frames/s). The Cartesian coordinates of each joint were determined from each photograph by using ImageJ software (Version 1.30, NIH). The registered coordinates were converted from pixels to centimeters based on the reference marks (5 cm) placed in the tunnel.

The values in centimeters were introduced into a software platform designed by CINVESTAV-IPN (17), which associates joints defined by Cartesian coordinates, drawing all the resulting lines of the movement executed by the animal's limbs during gait and the movement sequence of the extremities during walking.

Procedures for Morphological Assessments

Immunofluorescence

Twelve weeks after treatment, animals were euthanized with sodic pentobarbital IP 40 mg/kg and, immediately after, were intracardially perfused with 0.9% NaCl followed by 4% paraformaldehyde. A 2-cm-long segment of cord centered at the site of injury (**Supplementary Figure 1**) was removed. Tissues were placed in PBS with 30% sucrose for 24 h. Next, 20-μm-thick serial sagittal sections were obtained with a LEICA cryostat. Sections were washed in PBS and were then blocked with normal bovine serum (Vector Lab) (1:200 in PBS) for 30 min. They were then incubated in anti-microtubule-associated protein 1B (MAP1-B, C-20, goat polyclonal IgG, Catalog no. sc-8971; Santa Cruz Biotech), anti-growth associated protein 43 (GAP-43, H-100, rabbit polyclonal IgG, Catalog no. sc-1779), anti-glial fibrillary acidic protein (GFAP, H-50, rabbit polyclonal IgG, Catalog no. sc-65343), and anti-NGFR p75 (mouse monoclonal IgG, Catalog no. sc-71692) for 48 h at 4°C. Samples were washed with PBS and incubated with the secondary antibody (Alexa 488 Anti-rabbit or Anti-goat, Molecular Probes Invitrogen, Catalog no. A 11008) for 2 h; finally, they were again washed with PBS, contrasted with Hoechst (dilution of 5 μl/5 ml PBS; Molecular Probes, Life Technologies) for 3 min, and covered with Vectashield (Vector Lab) and a cover glass. Images were acquired using a Nikon Ti Eclipse inverted confocal microscope equipped with an A1 imaging system, controlled with the proprietary software NIS Elements v.4.5.0. Imaging was performed using either a 20× (Dry, NA 0.8) or 60× (Oil immersion, NA 1.4) objective lens. Dyes were excited in sequential mode using the built-in laser lines 403 nm (Hoechst), 488 nm (Alexa 488), and 563 nm (Alexa 546). The corresponding fluorescence was read in the following ranges: 425–475 nm (Hoechst 33342), 500–550 nm (Alexa fluor 488), and 570–620 nm (Alexa fluor 546) using the manufacturer-provided filter sets. The images were acquired and analyzed using NIS Elements V.4.50. From each section, six images were acquired, comprising the epicenter and the zones rostral and caudal to the site of injury. Using FIJI-ImageJ software, the intensity of green-channel pixels, corresponding to the Alexa 488 per area, was quantified. The calibration of the intensity by area was conducted via a spatial scale derived by using the intensity values contained within the Images bitmap (image given by the Software) to establish the basal intensity values of each image. The density is the same as the intensity per area, which was determined in relation to the control groups and expressed as pixels/mm^2; the counting area was adjusted to 1 mm^2.

Tracers to Assess Axonal Regrowth

Twelve weeks after treatment, one anterograde neurotracer in the brain and one in the spinal cord were injected into two animals from each group. Anesthetized animals underwent stereotactic surgery by drilling the skull according to the following coordinates: (−1.2 mm) anteroposterior, lateral right (−2 mm), and lateral left (+2 mm) with respect to Bregma. The tracer was injected through a micro-injector (Kd Scientific 310-plus), connecting a Hamilton syringe adapted with a microdialysis tube. The tip of the syringe was placed on the brain surface and introduced 2 mm in the drilled canals. Two microliters of mini-ruby dextran tetramethylrhodamine (Molecular Probes Life Technologies, Catalog no. D3312) were injected, at a rate of 0.5 ml/min on each side, and the skin was sutured. Afterward, the spinal cord was exposed through a laminectomy performed at T12 to inject mini-emerald dextran fluorescein trace (Molecular Probes Life Technologies, D7178). Using a stereotactic approach to the spine, a microinjection was performed in the spinal cord, close to the central artery, 1 mm from the surface; a total volume of 1 μl of the solution was delivered at a rate of 0.01 ml/min. The skin was sutured, and the animal was returned to its home cage. After 3 weeks, animals were intracardially perfused with 0.9% NaCl followed by 4% paraformaldehyde.

Twenty-micron longitudinal floating sections were obtained in a cryostat. After collection, sections were contrasted with Hoechst (dilution of 5/5 ml of PBS; Molecular Probes) and mounted with Vectashield. The tissue was analyzed with a confocal microscope to identify the stained axons with neurotracers.

Statistical Analysis

The one-way ANOVA on ranks (Kruskall-Wallis tests) and Mann-Whitney *U* tests were conducted when the data distribution required non-parametric testing. Locomotor function (BBB) data were analyzed using the repeated measures ANOVA test. Kinematics data were analyzed using a two-way ANOVA test followed by Tukey's test. Statistical analyses were performed using Graph Pad Prism V6.0. A significance level of $p < 0.05$ was set for all analyses.

RESULTS

Morphological Outcome

Phenotypification of BMSCs

Cells were characterized by the immunophenotyping technique (flow cytometry), showing that 66.5% of the population was positive to Cd90 2.85% was double-positive for Cd105 and Cd13, and 37.4% was positive for Cd13 demonstrating that the population used for transplants were adult mesenchymal stem cells (**Supplementary Figure 2A**). In the spinal cord tissue, all groups treated with BMSCs, regardless of whether they had received single or combined treatment, showed Cd90- and Cd105-positive cells in the spinal cord, grouped into colonies (**Supplementary Figures 2B,C**). In addition, these cells showed immunoreactivity for GFAP, MAP-1B, and P75 (**Figures 1A–C**) and displayed a glial star-shaped morphology with long ramifications (**Figure 1D**). These cells were absent in rats that did not receive BMSCs.

Axonal Regeneration

The qualitative exploration (analysis and observations) of GAP-43 expression revealed that the apparent amount of immunoreactive fibers in the control group (**Supplementary Figures 3A,F**) was less than that observed in groups with single treatment (**Supplementary Figures 3B,C,G,H**). In addition, in the control groups, such fibers appear disorganized. Qualitatively comparing combined (**Supplementary Figures 3D,E,I,J**) against single treatment groups, it seems that the immunoreactive fibers are more abundant, of larger caliber, and better organized after combined treatments (**Figures 2A–D**).

Quantitative analysis for GAP-43 confirms the above-mentioned observations. At the epicenter (**Figure 2E**) and in areas rostral and caudal to it (**Supplementary Figures 4A,B**), the fluorescein density of all treated groups was significantly higher than in the control group ($p < 0.001$). Density was also significantly higher for the combined treatment groups when compared to those receiving single treatments ($p < 0.05$).

For MAP-1B expression, the same qualitative and quantitative trends were observed: the greatest quantity of positive immunoreactive fibers and the best organization of these was observed in the combined-treatment groups, followed by the single-treatment groups, and finally in the control group (**Figures 3A–D** and **Supplementary Figure 5**). Quantitative

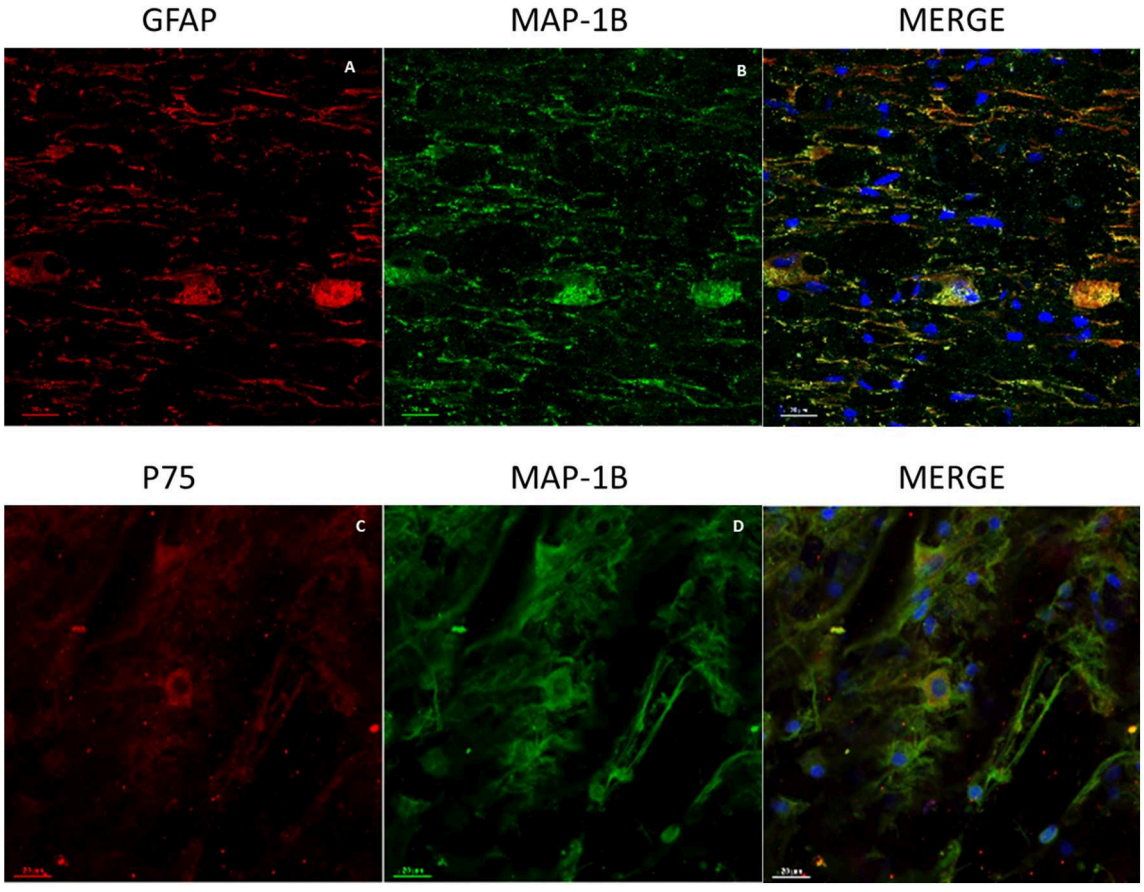

FIGURE 1 | Representative images of GFAP, MAP-1B, and P75 expression in BMSCs at the spinal cord. **(A)** Immunoreactive cells for GFAP (red). **(B)** Immunoreactive cells for MAP-1B (green). **(C)** Immunoreactive cells for P75 with star-like morphology (red). **(D)** Immunoreactive cells for MAP-1B with long extensions (green). Nuclei contrasted with Hoechst (blue). Scale bars **(A–D)** = 50 μm.

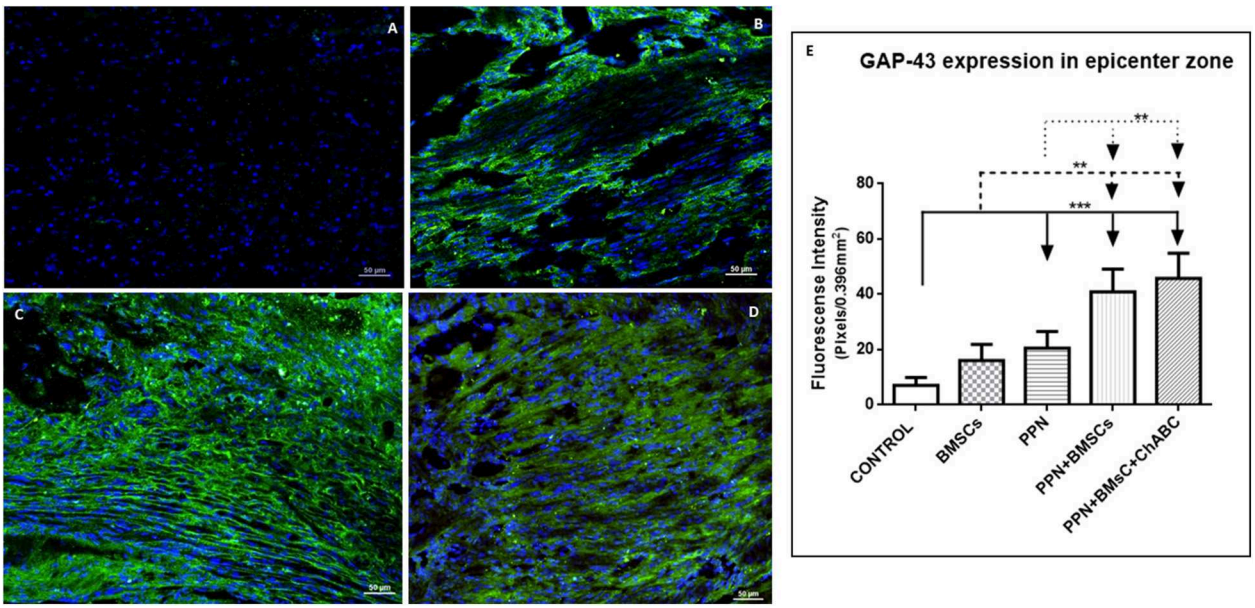

FIGURE 2 | Expression of GAP-43 at the epicenter. **(A–D)** Show representative images of immunoreactive fibers for GAP-43 (green) of groups control **(A)**, PPN **(B)**, PPN + BMSCs **(C)**, and PPN + BMSCs + ChABC **(D)**. Note that fibers in the PPN group seem disordered, scarcer, and thinner compared to combined groups. Scale bar **(A–C)** = 50 μm. Plots in **(E)** show quantification of fluorescein density at the epicenter zone. Data are expressed as the mean ± S.D. ($n = 6$). Statistical analysis: Kruskal Wallis followed by *U*-Mann Witney. ***$p < 0.0001$; **$p < 0.05$.

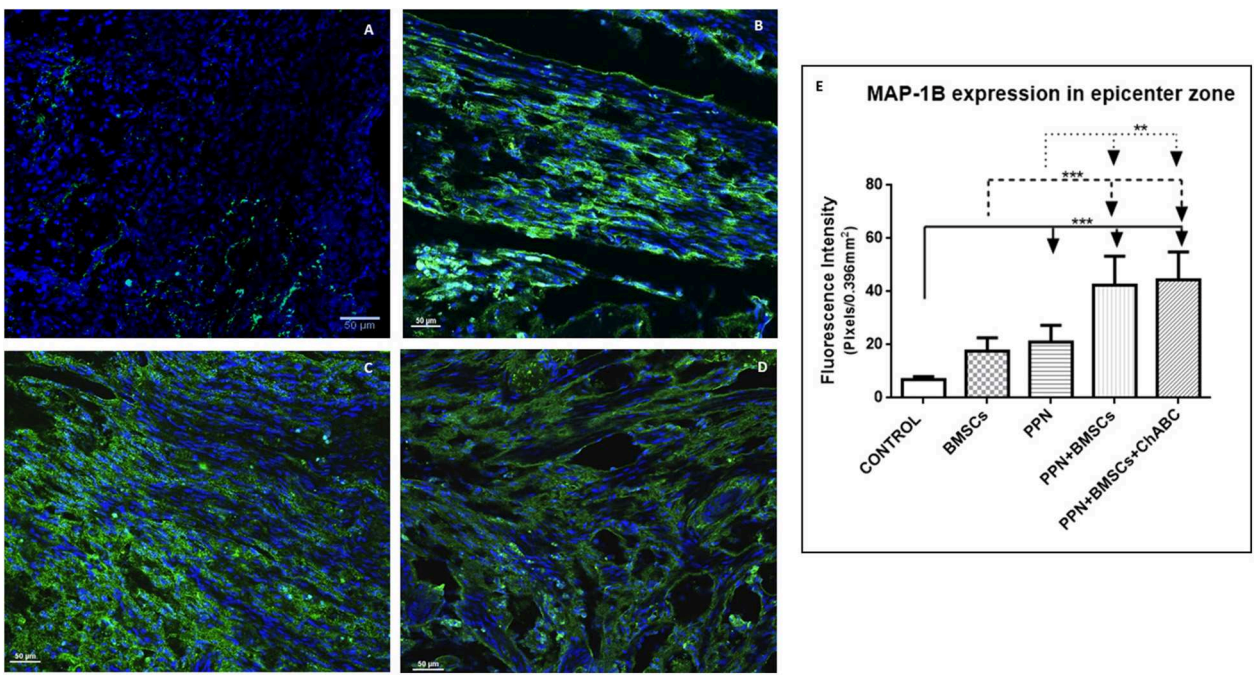

FIGURE 3 | Expression of MAP-1B at the epicenter. **(A–D)** Show representative images of immunoreactive fibers for MAP-1B (green) of groups control **(A)**, PPN **(B)**, PPN + BMSCs **(C)**, and PPN + BMSCs + ChABC **(D)**. Note that fibers in the PPN group seem disordered, scarcer, and thinner compared to combined groups. Scale bar **(A–C)** = 50 μm. Plots in **(E)** show quantification of fluorescein density at the epicenter zone. Data are expressed as the mean ± S.D. ($n = 6$). Statistical analysis: Kruskal Wallis followed by *U*-Mann Witney. ***$p < 0.0001$; **$p < 0.05$.

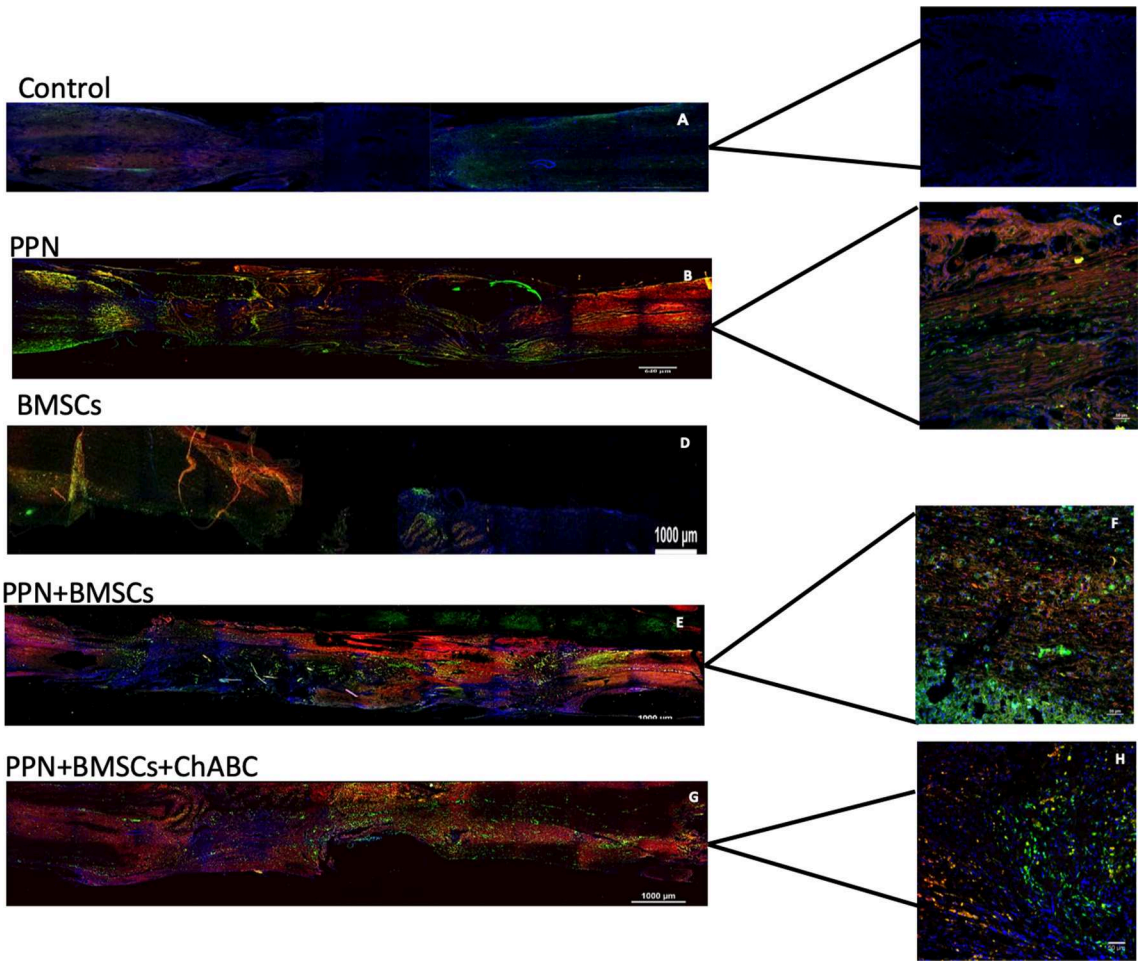

FIGURE 4 | Representative images of axonal regeneration. In **(A,B,D,G)**, descending fibers (red, mini-ruby dextran, tetramethylrhodamine) and ascending fibers (green, emerald fluoro dextran fluorescein) are shown in the rostral zone (left), epicenter (central zone), and caudal zone (right) of the spinal cord. In **(C,F,H)** positive fibers from each tracer are observed in the transplant zone (central zone). Nuclei dyed in blue with Hoechst. Scale for **A,B,D,F** = 1,000 µm and for **C,E,G** = 50 µm.

analysis revealed that the fluorescein density of all treated groups was significantly higher than in the control group ($p < 0.001$) and was significantly higher in combined treatments than in single treatments ($p < 0.05$) (**Figure 3E** and **Supplementary Figures 4C,D**).

Neurotracers in specimens of rats containing PPN as a graft, both with single and combined treatment, showed, at the epicenter as well as rostral and caudal to it, fibers marked with ruby red (Fluoro mini-ruby dextran, tetramethylrhodamine) belonging to the motor area and green emerald fibers (Fluoro emerald dextran fluorescein) coming from the lumbar area. Positive fibers for both tracers appear better organized, thicker, and increased in combined treatment compared to single treatment (**Figures 4**, **5**).

Histological Appearance of Host-PPN Apposition

At the end of the experiment, groups with pre-degenerate peripheral nerve transplantation showed a good apposition between nerve graft and spared spinal cord, with the presence of micro-cysts in both rostral and caudal interface zones; the opposite features were seen in the control group (**Supplementary Figure 6**).

Locomotor Function Outcome

The modified BBB scale showed that all treated groups scored significantly higher than the control group ($p < 0.0001$; **Figure 6A**). Combined treatment groups showed a significantly higher score than the single treatment group ($p < 0.001$). Remarkably, from the 8th week onwards, combined treatment groups showed frequent rhythmic movements in both hindlimbs.

The kinematic analysis revealed a significant increase in the amplitude of hindlimb movements in groups treated with PPN grafts alone and with combined treatments in comparison to the control and BMSC groups ($p < 0.0001$) (**Figure 6B**). The increase in the amplitude of movements was observed in the gait pendulum movement (stride) in combined groups, which was greater than in the single treatment

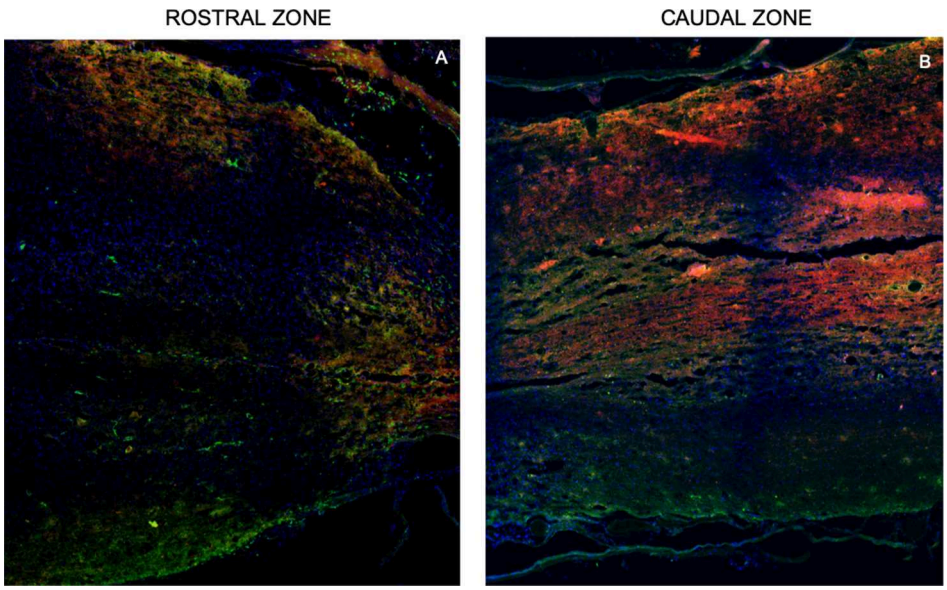

FIGURE 5 | Representative tracing images of the spinal cord. Rostral zone **(A)** and caudal zone **(B)** of the spinal cord show ascending fibers (red, mini-ruby dextran, tetramethylrhodamine) and descending fibers (green, emerald fluoro dextran fluorescein). These inmunofluorescence are representative of the staining of the neurotracers. Nuclei dyed in blue with Hoechst. Scale = 1,000 μm.

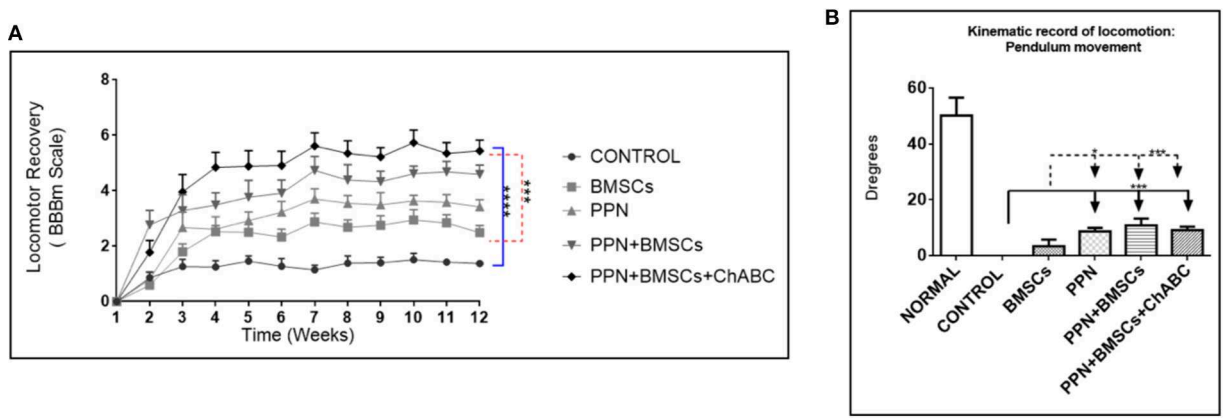

FIGURE 6 | Effect of therapeutic approaches on locomotor function. **(A)** Shows the modified BBB scores obtained during 12 weeks after treatment. Statistical analysis: repeated-measures ANOVA followed by Tukey test. ****$p < 0.0001$; ***$p < 0.001$. **(B)** Shows kinematic recording of pendular movement. Statistical analysis: two-way ANOVA followed by Tukey test. ***$p < 0.0001$; *$p < 0.05$. Data are expressed as the mean ± S.E.M. ($n = 12$).

and control groups (**Supplementary Figure 7**). This increase in the amplitude of movements was shown in both hips and knees (**Figures 7A,B**).

DISCUSSION

This study was designed to explore the potential of combined therapeutic approaches for promoting neural regeneration after complete chronic spinal cord transection, as assessed by analysis of the expression of molecules associated with axonal growth, neural tracing, and locomotor performance.

Rationale for Experimental Design

Choice of Therapeutic Procedures

The choice of therapeutic procedures tested here was based on previous reports claiming their ability to support spinal cord regeneration, each through different mechanisms.

BMSCs support axonal regrowth in both acute (18, 19) and chronic (20) cord transection. BMSCs are capable of building a niche inside the host from which diverse growth factors and molecules for axonal guidance and extension are secreted, promoting an enabling environment for axonal regrowth (21–23). In addition, BMSCs promote oligodendrogenesis (24).

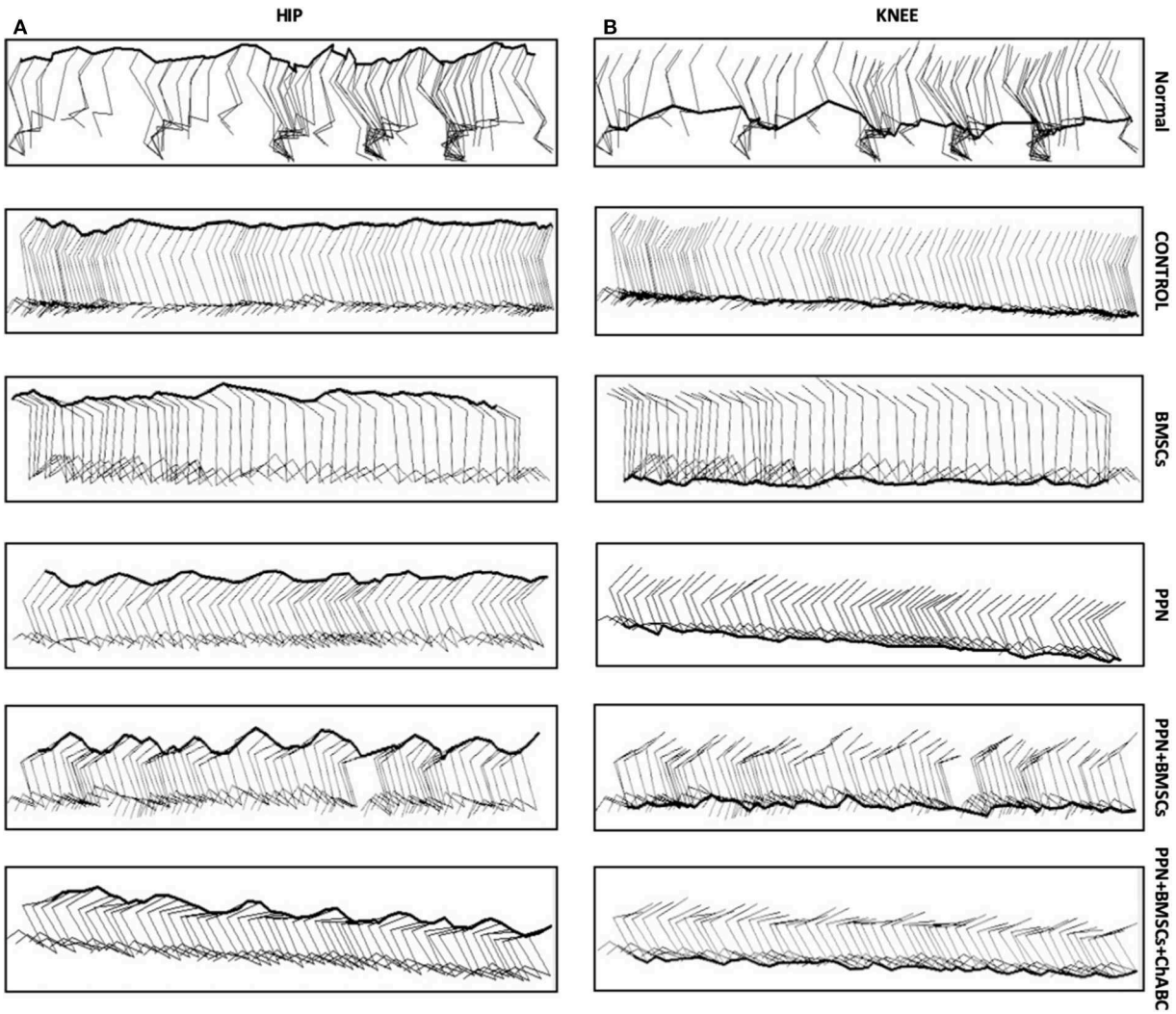

FIGURE 7 | Representative sagittal stick diagrams of kinematic gait recording. **(A)** Hip; **(B)** knee. Diagrams in intact rats show the movement of the hips and knees is in waveforms. In the control groups, diagrams show no movements; only flat lines can be seen. In the group treated with BMSC, waves are inconsistent and have small amplitude. With PPN treatment, there is an increase in the formation of waves in both joints. Noticeably, in the combined treatment (PPN + BMSCs and PPN + BMSCs + ChABC) groups, the waves show greater amplitude and are constant in both joints. The images of Normal, Control, PPN, PPN + BMSCs, and PPN + BMSCs + ChABC represent the right leg, and only the group for BMSCs represents the left leg.

The tubular structure and cells (mainly Schwann cells and macrophages) present in implants of PPN work as scaffolding and promote a permissive microenvironment for axonal regrowth in both acute and chronic spinal cord transection (25–27). Regenerated axons are able to cross both rostral and caudal graft-host interfaces (8, 15, 28, 29).

ChABC digests the glycosaminoglycan chains of chondroitin sulfate and restores plasticity to the adult spinal cord and brain, making axonal regrowth possible (9, 30). The power of this enzyme to digest the chondroitin sulfate proteoglycans that are associated with a glial scar has been exploited to facilitate the extent of axons between grafts and neighboring spinal cord (31).

Injury Model and Assessments

In order to study axonal regeneration in SCI, one must work with a partial or complete cord transection. Because of the enormous variability observed in the clinical setting, the experimental model used here appears to be a suitable alternative for studying the influence of combined therapeutic approaches, as it maintains uniformity in both the severity of injury and the time elapsed before starting treatment.

The most appropriate histological methods for evaluating axonal regeneration involve the use of neurotracers, as well as the expression of molecules directly or indirectly associated with both guiding and assembling the regenerating axons, such as GAP-43 and MAP-1B (32–34).

Although the standard BBB scale is by far the most commonly used test for motor assessment in experimental rodent SCI, here we choose a modified version of BBB design for complete transection because of its greater sensitivity to detecting movements originating distally to the site of injury (16, 35). In addition, the kinematic analysis of movement provides a valuable complementary assessment, as it is objective and accurate (36).

Injury Chronicity

Performing a therapeutic intervention in chronic stages of SCI, as in this study, represents a greater challenge for successfully promoting cord regeneration compared to earlier therapeutic interventions.

A lot of experimental strategies for cord repair at acute stages have been tested, including cell and tissue transplants, exogenous substances capable of neutralizing neurite outgrowth inhibitors like myelin-associated proteins and physical barriers, growth factors, biomaterials, and drugs (37, 38). Many of these treatments promote axonal regrowth and improve locomotor function, with different extents of efficiency. Similar treatment strategies have been tested in a limited number of studies in subjects with injury at chronic stages, usually with poorer results (9, 20, 27).

Single vs. Combined Treatments

It was shown here that by using combined treatments, axonal regeneration, as well as locomotor function recovery, increases in comparison to single treatments. These results confirm our hypothesis and underline the suitability of simultaneously targeting different barriers for spinal cord regeneration to obtain better results.

Our results are consistent with previous studies reporting that, despite the positive structural and functional results obtained with single treatments, their combination has an additive effect, improving individual positive effects (39, 40).

Some successful combinations that promoted greater axonal regeneration than single treatments include treatments combining PPN + FGFα (41), PPN + ChABC (31), PPN + ChABC and PPN + BDNF (29), BMSCs + granulocyte-colony stimulating factor (42), ChABC + NT3 (13), ChABC + rehabilitation (9), and PPN + BMSC (15).

In most studies where the effect of combined treatment for SCI has been evaluated, two procedures have been used. In the few studies where three procedures were combined, the result was better in comparison to single or dual interventions (13, 43), which supports the concept that certain combinations may have an additional additive effect, as observed in this study.

Morpho-Functional Outcome

As expected, the highest axonal regeneration corresponds with the greatest functional recovery, as observed in animals that received combined treatments.

Our observation from cytometry that PPN bridges placed in the site of injury both increase the number and support the axial orientation of regenerated axons emerging from both stumps agrees with previous studies reporting similar results from using cord hemisections treated in the acute stage, with PPN combined with other interventions (29, 44). Functionally, treatment using only PPN promotes mild locomotor function recovery in both acute (45, 46) and chronic stages (15). Similarly, the mild motor recovery observed here after single treatment with BMSCs in the chronic stage of injury agrees with the functional outcome after transplanting only BMSCs in the acute phase of injury (11, 42, 47).

As we have shown here by combining PPN + BMSCs and PPN + BMSCs + ChABC, others have previously reported that the augmentation of locomotor function obtained through single treatments is potentiated by combined treatments. An example of such benefit with treatments given early after injury are the combinations of PPN + FGFα (44), PPN + ChABC (45), and ChABC + NT-3 (13). Examples of studies where the benefit was obtained after administering treatment in chronic stages of the lesion are the combinations of PPN + BMSCs (15), BMSCs + Chitosan (11), BMSCs + G-CSF (42), and cADMSCs + ChABC (48).

Although we did not determine the precise mechanisms behind the axonal regeneration and functional recovery observed with combined treatments, it is reasonable to speculate that functional reinnervation may be established after the PPN promotes long-distance axonal regeneration, while, in the interphase areas, BMSCs release molecules capable of enhancing axonal outgrowth and providing guidance for synapse formation on appropriate targets (49). Finally, the administration of ChABC did not produce the expected beneficial additive effect as part of a combined treatment, suggesting that the administration strategy was insufficient.

Limitations and Future Prospects

While we were able to demonstrate an initial impression of the potential for our approach to regenerate the spinal cord in the late stage of injury, a limitation of the present study is the small sample size, which only allows preliminary conclusions to be drawn. Nonetheless, we believe that our results provide a basis for future studies aimed at improving this encouraging therapeutic approach and fully developing its applicability.

To gain an initial perspective on the effects of the approach tested here, histology and locomotor evaluations were performed. However, more in-depth mechanistic studies are warranted for a better understanding of how combined treatments might modify key areas within the microenvironment of the injury, including myelination and other molecular events of these therapies.

Conclusion

Using a clinically relevant model of chronic SCI in rats, we have investigated the morpho-functional effects of single and combined therapeutic procedures targeting different barriers for spinal cord regeneration.

In the long run, PPN and BMSCs given as single treatments discretely enhance axonal regeneration and locomotor function, while, by combining treatments (PPN + BMSCs and PPN + BMSCs + ChABC), a significant enhancement of axonal regeneration and a modest recovery of locomotion was observed. These findings suggest that functional spinal cord regeneration can be effectively induced in the late stages of injury.

The encouraging results reported here merit further pre-clinical studies designed to delve into this promising line of research. In addition, discernment of the precise mechanisms underlying these benefits deserves further attention.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee in Research of the Hospital de Especialidades, Centro Médico Siglo XXI, Instituto Mexicano del Seguro Social.

AUTHOR CONTRIBUTIONS

VB-A: conception and design, provision of study material, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. JR-O, EG-V, SS-T, and IJ-E: collection and/or assembly of data and data analysis and interpretation. SO-S: conception and design, provision of study material, data analysis and interpretation, and final approval of the manuscript. AV-G: collection and/or assembly of data. GG-S: data analysis and interpretation and manuscript writing. JM-C and IM: manuscript writing. FF-V: collection and/or assembly of data. IG: conception and design, provision of study material, financial support, administrative support, and final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00136/full#supplementary-material

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Use of a Combination Strategy to Improve Morphological and Functional Recovery in Rats With Chronic Spinal Cord Injury

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Immunization with neural derived peptides (INDP), as well as scar removal (SR) and the use of matrices with bone marrow-mesenchymal stem cells (MSCs), have been studied separately and proven to induce a functional and morphological improvement after spinal cord injury (SCI). Herein, we evaluated the therapeutic effects of INDP combined with SR and a fibrin glue matrix (FGM) with MSCs (FGM-MSCs), on motor recovery, axonal regeneration-associated molecules and cytokine expression, axonal regeneration (catecholaminergic and serotonergic fibers), and the induction of neurogenesis after a chronic SCI. For this purpose, female adult *Sprague-Dawley* rats were subjected to SCI, 60 days after lesion, rats were randomly distributed in four groups: (1) Rats immunized with complete Freund's adjuvant + PBS (vehicle; PBS-I); (2) Rats with SR+ FGM-MSCs; (3) Rats with SR+ INDP + FGM-MSCs; (4) Rats only with INDP. Afterwards, we evaluated motor recovery using the BBB locomotor test. Sixty days after the therapy, protein expression of TNFα, IL-4, IL-10, BDNF, and GAP-43 were evaluated using ELISA assay. The number of catecholaminergic and serotonergic fibers were also determined. Neurogenesis was evaluated through immunofluorescence. The results show that treatment with INDP alone significantly increased motor recovery, anti-inflammatory cytokines, regeneration-associated molecules, axonal regeneration, and neurogenesis when compared to the rest of the groups. Our findings suggest that the combination therapy (SR + INDP + FGM-MSCs) modifies the non-permissive microenvironment post SCI, but it is not capable of inducing an appropriate axonal regeneration or neurogenesis when compared to the treatment with INDP alone.

Keywords: SCI, Immunomodulation, MSC, Fibrin tissue adhesive, A91 peptide

INTRODUCTION

Spinal cord injury (SCI) leads to a series of anatomical and physiological changes resulting in permanent or temporary changes in spinal cord (SC) functionality (1, 2). Cell necrosis, glial reaction and especially inflammation induces the appearance of cavities, cysts and glial scars which interrupt the descending and ascending axonal tracts causing paraplegia or quadriplegia (3).

Infiltration of inflammatory cells has been correlated with the amount of tissue damaged after injury. However, it has been shown that the presence of inflammatory cells is essential for neuroprotection (4, 5) and regeneration (6, 7) of the SC, where its action depends on the effects exerted by autorreactive Central Nervous System (CNS) T-cells which are part of an autoimmune response developed after injury (4).

Protective autoimmunity (PA) is a new concept, which refers to the modulation of self-reactive mechanisms to promote neuroprotection and neural restoration (3, 4). PA is a physiological mechanism induced after SCI that could be boosted by immunizing with neural-derived peptides (INDP), a therapeutic strategy that requires the use of peptides such as A91 to induce PA. A91 is a peptide derived from the immunogenic sequence (87–99) of the myelin basic protein (MBP), the most immunogenic protein in the CNS. Activating T-cells by means of $A91_{87-99}$ peptide, induces a Th2 response, which stimulates microglia to its differentiation to an M2 phenotype, resulting in a micro-environment with lower production of free radicals, improved motor recovery, among other neuroprotective mechanisms (5, 8, 9) as well as axonal regeneration (7, 10). This therapeutic strategy has rendered encouraging results both, in acute and chronic SCI, being the chronic one the most difficult stage of injury to carry out a therapeutic approach.

In the chronic phase of SCI there is scar tissue avoiding a correct reconnection of axons by forming a physical and chemical barrier made of sulfated proteoglucans and CD44 glycoprotein, thus inhibiting the formation of growth cones and axonal prolongation (11, 12). Additionally, chronic SC injury is considered a period of low activity with a progressive decline. Therefore, in order to achieve axonal regeneration a possible alternative could be scar removal (SR), with the goal of re-establishing electrical conduction and consequently the synapses, in addition to restoring the conditions of an acute injury, such as activation of PA as wells as cytokine release and neurotrophic factors (7, 13). On the other hand, biomaterials have been used such as scaffolds to facilitate regeneration of nerve fibers (12). Fibrin glue matrix (FGM) is a fibrinogen and thrombin derivative that has been used as a cavity repair adhesive material (14). This biomaterial has successfully been used in combination with bone marrow -mesenchymal stem cells (MSCs) to promote the secretion of neurotrophic factors, giving the opportunity for a neurogenic effect and axonal regeneration (10, 15). FGM is a biocompatible element with MSCs (16). As PA has proven to exert beneficial actions after a chronic SCI, we now intended to improve this effect by combining INDP therapy with other therapeutic strategies that also have shown beneficial effects and that could complement the actions provided by INDP. The present study evaluates the effect of combining INDP plus surgical removal and inhibition of glial scar, together with a FGM impregnated with MSCs. We expect that the renewed microenvironment induced by SR and, its inhibition, will allow INDP to promote neuroprotection (after SR) and induce the production of neurotrophic factors and thus axonal regeneration and neurogenesis (7, 11, 17). In the same way, MSCs could modulate SR (18), reduce proinflammatory cytokines (19) and collaborate to induce axonal regeneration (20). Finally, FGM will provide the matrix for MSCs and the scaffold for axonal growing (21). It is plausible to expect that, the combination of these strategies, could integrate different beneficial elements that may result in a better neurological recovery, especially in the chronic stage of injury (a hostile phase for regeneration).

A recent study in our research group proved that the combined therapy of SR + INDP + FGM-MSCs in a model of complete transection improved motor and electrophysiological recovery with an increase in genes associated with regeneration and an increment in the percentage of serotonergic fibers (5-HT) (10).

In this study, we evaluated the effect of this combination therapy on motor recovery, protein expression of cytokines and molecules associated with regeneration, axonal regeneration and neurogenesis in a model of chronic contusion of SCI.

Abbreviations: CICSA, Centro de Investigación en Ciencias de la Salud; CMN Siglo XXI, Centro Médico Nacional Siglo XXI; INDP, Immunization with neural derived peptides; SR, Scar removal; MSCs, Mesenchymal stem cells; SCI, Spinal cord injury; FGM, Fibrin glue matrix; FGM-MSCs, Fibrin glue matrix with MSCs; CONACyT, National Council of Science and Technology of Mexico; SC, Spinal cord; CNS, Central Nervous System; PA, Protective autoimmunity; MBP, Myelin basic protein; 5-HT, Serotonin; BDNF, Brain derived neurotrophic factor; GAP-43, Growth associated protein-43; IL-4, Interleukin 4; IL-10, Interleukin 10; TNFα, Tumor necrosis factor alpha; INFγ, Interferon gamma; TGFβ, Transforming growth factor beta; IGF-1, Insulin-like growth factor 1; TH, Tyrosine hydroxylase; NOM 062-ZOO-1999, Mexican Official Norm on Principles of Laboratory Animal Care; DMEM, Dulbecco's Modified Eagle; FBS, Fetal bovine serum; DPY, α,α′-dipyridyl; PBS, Phosphate buffered saline; CFA, Complete Freund's adjuvant; BBB, Basso, Beattie & Bresnahan; ELISA, Enzyme-linked immunosorbent assay; BrdU, 5-bromo-2′-deoxyuridine; Dcx, Doublecortin; NPCs, Neural progenitor cells; PBT, PBS-Triton; DAPI, 4′,6-diamidino-2-phenylindole; SD, Standard deviation; BBB test, Bonferroni's *post hoc* test; ERK, Extracellular Signal-regulated Kinase; STAT 3, Signal transducer and activator of transcription 3; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma extra-large; CD4+, Cluster of differentiation 4; AKT, Protein kinase B; MAPK, Mitogen-activated protein kinase; INFγ, Interferon gamma; TGFβ, Transforming growth factor beta; ANOVA, Analysis of variance.

MATERIALS AND METHODS

Experimental Design

Sample size for this experiment was calculated using an alpha of 0.05 and beta of 0.20. Experiments were performed 60 days after SCI, with subsequent analyses carried out over the two following months. The experiment consisted of 48 rats with chronic SCI randomly distributed into four groups (GraphPad QuickCalcs: http://www.graphpad.com/quickcalcs/): (1) rats immunized with complete Freuds adjuvant + PBS (control; PBS-I) (n = 12); (2) rats only with INDP (n = 12); (3); rats with SR + FGM-MSCs (n = 12) and (4) rats with SR + INDP + FGM-MSCs (n = 12).

Locomotor function was evaluated 60 days after SCI and thereafter weekly throughout 2 months. At the end of each experiment (120 days after SCI), rats were euthanized, and the SC was then analyzed. We determined the neurogenic effect of the therapy in all studied groups at the injured site of the SC, as well as the expression of proteins for brain-derived neurotrophic factor (BDNF), growth associated protein-43 (GAP-43), interleukin-4 (IL-4), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNFα). We determined the number

of 5-HT+ and TH+ fibers in the caudal stump of the four groups. Finally, neurogenesis by Immunofluorescence was also assessed.

Ethics Statement

All procedures were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals, and the Mexican Official Norm on Principles of Laboratory Animal Care (NOM 062-ZOO-1999). In addition, the Animal Bioethics and Welfare Committee approved all animal procedures (ID: 178544; CSNBTBIBAJ 090812960). All experiments were designed and reported according to the ARRIVE guidelines.

In order to perform euthanasia, animals were previously anesthetized by an intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg).

Spinal Cord Injury

Adult female Sprague–Dawley rats (13–14 weeks old) weighing between 230 and 250 g were subjected to a moderate SC contusion. Animals were anesthetized by an intramuscular injection of a mixture of ketamine (77.5 mg/kg) (Probiomed, Mexico City, Mexico) and xylazine (12.5 mg/kg) (Fort Dodge Laboratories, Fort Dodge, Iowa, USA). One hour after induction of anesthesia, their skin was opened in layers and a laminectomy was performed at T9 vertebral level of the SC. Subsequently, a 10 g rod was dropped onto the SC from a height of 25 mm using the NYU impactor (NYU, New York, USA). Functional recovery of all groups was assessed using the BBB locomotor scale.

Postoperative Care

After SCI, animals were housed with food and water ad libitum, and received manual bladder voiding, three times a day for 2 weeks. Sterile bedding and filtered water were replaced daily. To avoid infection, Enrofloxacin (Marvel, Mexico City, Mexico) was diluted into their drinking water at an approximate dose of 64 mg/kg/day for 1 week. Animals were carefully monitored for signs of infection, dehydration, or auto mutilation with appropriate veterinary assistance as needed.

Antigen ($A91_{87-99}$ Peptide)

$A91_{87-99}$ peptide was derived from the encephalitogenic amino acid sequence 87–99 of the MBP. A non-encephalitogenic analog was obtained by replacing the lysine residue with alanine at position 91. The modified peptide was purchased from Invitrogen Life Technologies (San Diego, CA, USA). Reverse-Phase HPLC confirmed the purity of the $A91_{87-99}$ peptide (>95%).

Isolation and Phenotyping of MSCs

MSCs were obtained from female Sprague Dawley healthy rats. Animals were euthanized with an overdose of pentobarbital sodium. Bone marrow was obtained from both femurs and tibias using a 200 μL micropipette and deposited in a 15 mL conical tube with culture medium [Dulbecco's Modified Eagle [DMEM], from GIBCO]. The sample was centrifuged at 1500 rpm for 7 min. Cells were then separated using a Ficoll (Sigma-Aldrich) (3 mL) gradient centrifuged at 2,000 rpm at 24°C for 30 min. The total number of nucleated cells obtained was quantified and 9×10^6 cells were seeded into a 75 cm^2 culture flask (in 5 mL of DMEM with 20% fetal bovine serum (FBS), from GIBCO), 1 mL of L-Glutamine (GIBCO), 5 mL of HEPES (Sigma-Aldrich), and 1 mL of Penicillin-Streptomycin (GIBCO). Cells were then placed in a water-jacketed incubator at 37°C with 5% CO2 until they formed a fibroblast monolayer. Finally, MSCs were reseeded onto the fibroblast layer and maintained for 2 weeks until transplantation.

Phenotyping

MSCs expanded in culture from passage 4 were phenotypically characterized by flow cytometry. The cells were centrifuged at 1,500 rpm for 7 min and they were blocked for 30 min with normal serum, then the cells were centrifuged at 1,500 rpm for 7 min to wash. Afterwards the cells were incubated with primary antibodies Anti rat- CD13 Santa Cruz Biotechnology Inc; CD90.1 eflour 450 anti-rat (Biolegend); CD70 PE anti-rat (Biolegend); PE/Cy7 anti-mouse CD105 Biolegend; Anti rat-CD117 Millipore; CD34 (ICO 115) FITC (Santa Cruz Biotechnology Inc), all at a dilution of 1:1000 in darkness for 30 min at 4°C. They were washed twice with FACS Buffer before being centrifuged again at 1,500 rpm for 5 min finally being quantified and analyzed with flow cytometry using the Cell Quest-Pro (BD Bioscience) program.

Cell phenotype proportion: 99% of the cells were mature MSCs; 80.45% were positive for CD13 (marker for subpopulation of MSCs); 51% were double positive for CD105+CD90+ (marker for subpopulation of MSCs); 28.1% were positive for CD70 (marker for subpopulation of MSCs); 11.49% were positive for CD 117 (marker for subpopulation of MSCs) and 1.70% of cellular population was positive for CD34 (specific control marker for hematopoietic stem cells) (10). Therefore, the majority of cells transplanted were adult MSCs.

Intervention in the Chronic SCI (Combination Therapy)

Sixty days after SCI, animals were anesthetized again as previously described. Then after, using a surgical microscope, a longitudinal incision was performed and fibrosis was removed. A second longitudinal incision was carried out, the meninges were referenced to the bordering muscles and the necrotic tissue was eliminated. The scar from each stump was removed by performing a single incision with a double-bladed scalpel leaving a space of 2 mm in length. Surgery for this procedure is a reproducible technique. The surgeon was always blinded to the group of animals. This method is helpful to successfully remove the glial scar and it does not generate any additional neurological deficit. Importantly, it produces a mild lesion that allows a renewed production of growth factors and the consequent induction of a favorable microenvironment for neural regeneration. Once the scar was removed, its renewal was halted by adding α,α′- dipyridyl (DPY), an iron chelator that inhibits a key enzyme for collagen biosynthesis in the acute phase of SCI. Therefore, this iron chelator inhibits SR and promotes an extensive long-distance regeneration of injured axons. DPY was injected directly –six times– into each stump of the SC by using a Hamilton syringe. Each injection deposited 2 μL volume of DPY (16 nmol) diluted in PBS. Right after,

a mixture of MSCs were pre-labeled with the membrane dye PKH26, (Sigma-Aldrich) then 2.5×10^6 MSCs in 5 μL and FGM (10 μL, with a final concentration of fibrinogen of 5 mg/mL) was grafted using a Hamilton syringe. Finally, the meninges were sutured with a 9-0 suture and the aponeurotic plane and skin were separately sutured with a nylon thread. Rats were then immunized subcutaneously at the base of the tail with 200 μg of $A91_{87-99}$ peptide dissolved in phosphate buffered saline (PBS), emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg/ml Mycobacterium tuberculosis (Sigma-Aldrich, St. Louis, MO, USA). In order to boost the protective and regenerative action of INDP, immunization with $A91_{87-99}$ peptide was accompanied with CFA, an adjuvant that potentiates the immune response but does not influence the protection or restoration exerted by INDP (7, 10).

Assessment of Motor Recovery

Locomotor recovery was assessed using the Basso, Beattie & Bresnahan (BBB) open-field locomotor scale method. Animals were evaluated 60 days after SCI and thereafter weekly throughout 8 weeks. Three separate blinded observers evaluated all animals and the average of the three scores was used.

Enzyme-Linked Immunosorbent Assay (ELISA)

Two months after therapeutic intervention, animals were euthanized with an overdose of pentobarbital sodium (80 mg/kg) and then, SC samples were rapidly excised. Reagents, samples, and standards were prepared according to the instructions provided by the manufacturer: IL-4 ELISA Kit (Cell Applications, San Diego, CA, USA), IL-10 ELISA kit (RayBiotech, Norcross, GA, USA), BDNF ELISA Kit (Ray Biotech, Norcross, GA, USA), neuromodulin (GAP-43) ELISA kit (CUSABIO, Houston, TX, USA) and TNFα ELISA kit (Origene, Rockville, MD, USA). Briefly, 100 μl standard or 30 μg total protein sample were added to each well and incubated for 2 h at 37°C. The liquid of each well was removed and not washed. Afterwards, 100 μl of Biotin-antibody (1x) was added to each well and incubated for 1 h at 37°C, followed by aspiration and washing for 3 times. One hundred μl HRP-avidin (1x) were added to each well and incubated for 1 h at 37°C. Subsequently, it was aspirated and washed 5 times and, 90 μl of TMB substrate were added to each well, incubated and protected from light for 15–30 min at 37°C. Finally, 50 μl Stop solution were added to each well and read at 450 nm within 5 min.

Immunohistochemistry for 5-HT+ and TH+ Fibers

Two months after the therapeutic intervention, animals were euthanized with an overdose of pentobarbital sodium (80 mg/kg) and an intracardiac perfusion with 4% paraformaldehyde was performed. The affected portions of the SC were fixed overnight and then transferred to 30% sucrose for cryoprotection. Samples were embedded in Tissue-Tek (Miles Elkhart, IN, USA), and longitudinal frozen sections (40 μm thick) were performed. Immunohistochemical staining was carried out in order to count the amount of TH+ and 5-TH+ fibers. Tissues were incubated in 0.03% hydrogen peroxide to quench endogenous peroxidase activity. Subsequently, the tissue was incubated overnight with the following primary antibodies: monoclonal goat antibody against TH (1:2000; Chemicon), or polyclonal rabbit antibody against 5-HT (1:2000; Sigma- Aldrich). Following rinsing with PBS, samples were incubated for at least 2 h with donkey IgG anti-goat IgG (1:500; Chemicon) and Sheep IgG anti rabbit IgG (1:500; Abcam) secondary biotinylated antibodies. To visualize positive fibers, samples were incubated 5 min with Vector DAB kit (Vector laboratories, CA, USA). Then, samples were evaluated and analyzed by a blinded observer that counted individual fibers using a 20X objective (Olympus DP72, Japan). The number of regenerating axons 1 mm caudal to the lesion was assessed.

Immunofluorescence for Evaluating Neurogenesis

Neurogenesis was evaluated by immunofluorescence using a double stain with anti-5-bromo-2'-deoxyuridine (BrdU) and doublecortin (Dcx) antibodies. BrdU is a synthetic nucleotide analog of thymidine incorporated during the S phase of the cell cycle, whereas Dcx is a marker for neural progenitor cells (NPCs). Therefore, BrdU+/Dcx+ cells are a result of neurogenesis. For this assay, the rats received one injection of BrdU (Abcam, Cambridge, UK; 50 mg/kg) intraperitoneally every 12 h for 5 days. The SC samples were then removed (1.0 cm caudal/rostral from the injury site) perfused and fixed with 4% paraformaldehyde. Tissues were cut transversally with the cryostat into sequential serial sections (at 0, 2, 4, and 6 mm caudal and rostral from the epicenter). Slices were 40 μm thick and a total of 48 sections per animal were counted and placed on slides using the free float method. Slides were washed twice for 10 min with PBS-Triton (PBT) and incubated with ImmunoRetriever (Bio SB, Santa Bárbara, CA, USA) for 60 min at 65°C. Afterwards, slides were washed three times for 5 min with PBS and incubated for 30 min with 1N HCl at 37°C. When completed, they were incubated for 10 min with sodium borate 0.1 M and washed three times with PBT. Unspecific binding sites were blocked with standard blocking solution with fetal bovine serum for 30 min. The primary antibodies against BrdU (Roche Diagnostics, Indianapolis, USA) (mouse IgG, 1:250) and Dcx (Santa Cruz Biotechnology, Dallas, TX, USA) (goat IgG, 1:250) were allowed to incubate for 20 h overnight. The next day, the slides were washed three times for 10 min with PBT and incubated with secondary antibodies (Invitrogen, Carlsbad, CA, USA) (BrdU: donkey IgG; Dcx: rabbit IgG; all at 1:500) for 2 h. The excess antibodies were removed by washing with PBT. Slides were counterstained with DAPI. All areas were quantified as total number of cells in all SC samples by a blinded evaluator using cell counting software ImageJ 1.52a. The total number of BrdU+/Dcx+ cells was obtained by averaging the total number of cells from 3 slides (3) and confocal images were acquired using a Zeiss LSM 800 microscope.

Statistical Analysis

Data is displayed as mean ± standard deviation (SD), and statistical significance was established when $p \leq 0.05$. GraphPad

Prism 8.0 (GraphPad Software, Inc. La Jolla, CA, USA) was employed in statistical analysis. Data from the assessment of functional recovery was analyzed using an ANOVA for repeated measures with Bonferroni's *post hoc* test (BBB test). Protein expression, the percentage of 5-HT+ and TH+ fibers and neurogenesis were analyzed by One-way ANOVA followed by Tukey–Kramer *post hoc* test.

RESULTS

INDP Alone Induces the Best Locomotor Recovery After Chronic SCI

In order to test the effect of the different therapeutic strategies on neurological recovery, the motor performance was evaluated comparing the four groups. **Figure 1** shows that 60 days after injury the locomotor performance of all groups was very similar before the therapeutic intervention (6.45 ± 0.84, mean ± SD; p = 0.1020, One-way ANOVA followed by Tukey–Kramer *post hoc* test). Sixty days after the respective therapy, all treated groups presented an improvement in motor recovery when compared to the one observed in the PBS-I group. Rats submitted to SR + FGM-MSCs and the ones treated with only INDP showed the highest motor recovery compared to the rest of the groups (8.15 ± 1.56 and 9.0 ± 2.10 respectively; $p < 0.05$, ANOVA for repeated measures with Bonferroni's *post hoc* test). Rats treated with the combination strategy presented a lower motor recovery (7.55 ± 1.21) as compared to those treated with SR+FGM-MSCs or INDP alone; but the improvement was still significantly higher when compared to PBS-I rats (6.550 ± 0.49).

INDP but Not the Combination Therapy Generates a Permissive Microenvironment Where Anti-inflammatory Cytokines and Regeneration-Associated Molecules Are Increased

It has been reported that INDP induces a permissive microenvironment for neural restoration in the chronic stages of injury (4, 5). Based on these findings, we explored the induction of this permissive microenvironment by analyzing the production of one pro-inflammatory (TNFα) and two anti-inflammatory cytokines (IL-4; IL-10). Additionally, the production of specific regeneration-associated proteins such as BDNF and GAP-43 were also assessed.

Figure 2A shows that INDP alone and SR + INDP + FGM-MSCs groups elicited a significant reduction of TNFα (53.91 ± 3.35 and 53.54 ± 0.66, respectively; mean ± SD) when compared to the rest of the groups (SR + FGM-MSCs: 76.02 ± 12.82 and PBS-I: 77.96 ± 1.33). When evaluating anti-inflammatory cytokines, a significant increase in IL-10 protein levels was observed in both INDP alone and SR + FGM-MSCs groups (**Figure 2B**; 4.84 ± 0.67 and 5.28 ± 0.99, $p < 0.05$, One-way ANOVA followed by Tukey–Kramer test) when compared to the SR + INDP + FGM-MSCs and PBS-I groups (4.22 ± 0.02

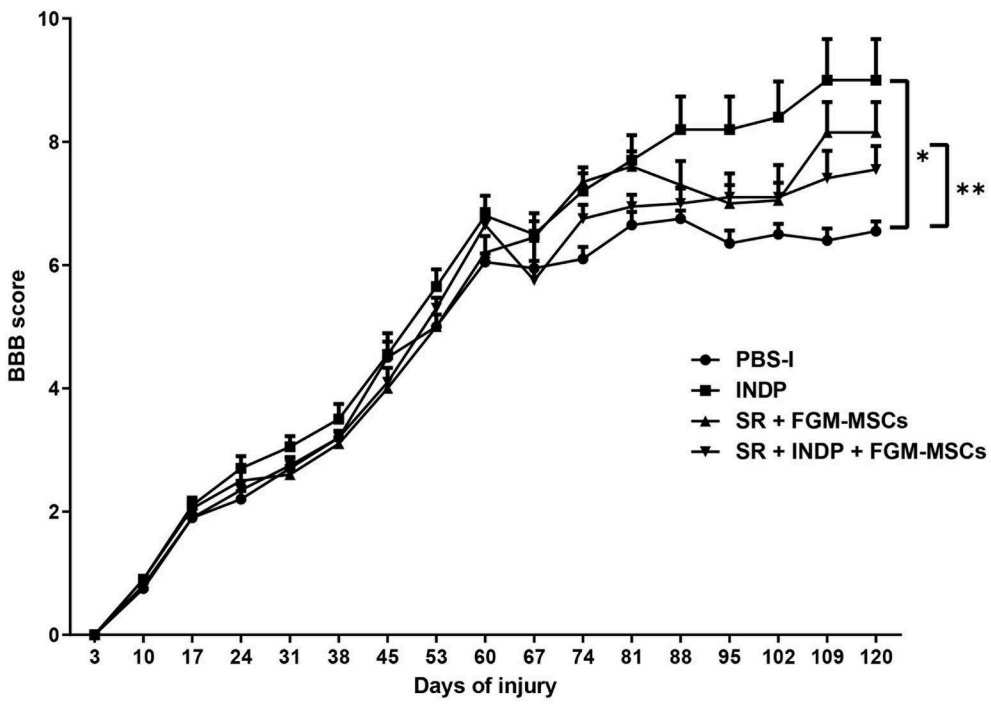

FIGURE 1 | Motor recovery after therapeutic intervention. After treatment, a significantly better motor recovery was observed in SR+FGM-MSCs and INDP alone groups. Rats treated with INDP alone showed the highest motor recovery among all groups. *$p = 0.0220$, **$p = 0.045$, ANOVA for repeated measures with Bonferroni's *post hoc* test. Each point represents the mean ± SD of 12 rats.

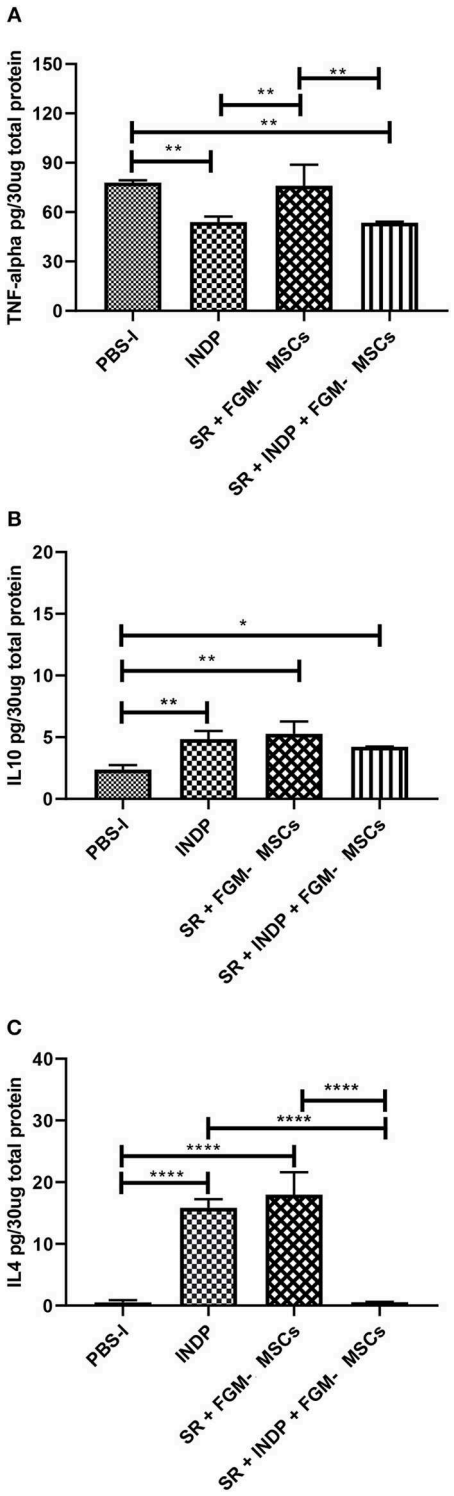

FIGURE 2 | Cytokines concentration in the site of injury. TNFα **(A)** protein concentration was significantly reduced only in the INDP and SR + INDP + FGM-MSCs groups. Both IL-4 **(B)** and IL-10 **(C)** showed significantly increased levels of protein concentration in both INDP and SR + FGM-MSCs groups. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$. One-way ANOVA followed by Tukey–Kramer *post hoc* analysis. Bars represent the mean ± SD of 4 rats. This is one representative graph of three experiments.

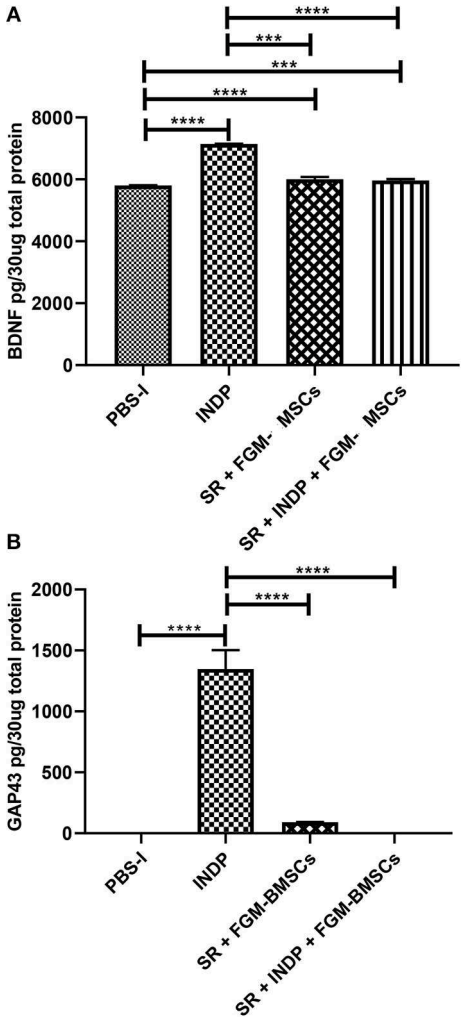

FIGURE 3 | Regeneration-associated proteins concentration in the site of injury. BDNF **(A)** and GAP-43 **(B)** protein concentration was significantly increased only in the INDP group. $^{***}p < 0.001$; $^{****}p < 0.0001$. One-way ANOVA followed by Tukey–Kramer *post hoc* analysis. Bars represent the mean ± SD of 4 rats. This is one representative graph of three experiments.

and 2.37 ± 0.36, respectively). Similarly, IL-4 protein levels significantly increased in the groups treated with INDP alone and SR + FGM-MSCs (15.85 ± 1.39 and 17.99 ± 3.63, respectively), when compared to the rest of the groups (**Figure 2C**; SR + INDP + FGM-MSCs: 0.56 ± 0.05 and PBS-I: 0.51 ± 0.36, $p < 0.05$, One-way ANOVA followed by Tukey–Kramer test).

In regard to BDNF and GAP-43, which indicate regeneration at the site of injury, a significant increase in the protein concentration of these molecules was observed in the group treated with INDP alone (**Figures 3A,B**; BDNF:7144 ± 5.312; GAP-43:1347 ± 155.5). The rest of the groups presented lower values of BDNF (SR + INDP + FGM-MSCs: 5961 ± 46.57; SR + FGM-MSCs: 6006 ± 68.53; PBS-I: 5804 ± 3.87) and GAP-43 (SR + INDP + FGM-MSCs: 0.0001 ± 0.009; SR + FGM-MSCs: 91.52 ± 1.22; PBS-I: 0.13 ± 0.27).

INDP Alone but Not the Combination Strategy Promotes Axonal Regeneration

To determine whether the microenvironment created by the different therapeutic strategies had any positive effects on axonal regeneration, we assessed the percentage of axons observed at the caudal stump of the SCI after therapeutic intervention. The percentage was obtained from the total number of fibers observed in sham-operated rats.

Figure 4 shows the percentage of TH+ (**Figure 4A**) and 5-HT+ (**Figure 4B**) fibers in all groups. The group treated with INDP alone showed a significant increase in both TH+ and 5-HT+ fibers when compared to the rest of the groups (TH+ 33.17 ± 6.58; 5-HT: 50.67 ± 3.26; $p < 0.05$, One-way ANOVA followed by Tukey–Kramer test). Interestingly, groups treated with SR + FGM-MSCs or SR + INDP + FGM-MSCs presented no significant differences in the percentages of TH+ (SR + INDP+ FGM-MSCs: 12.33 ± 6.53; SR+FMG-MSCs: 19.67 ± 5.95) and 5-HT+ fibers (SR + INDP + FGM-MSCs: 26.17 ± 9.78; SR + FMG-MSCs: 23.50 ± 10.80) when compared to the PBS-I group (TH: 14.50 ± 6.18; 5-HT: 31.33 ± 3.93; $p > 0.05$).

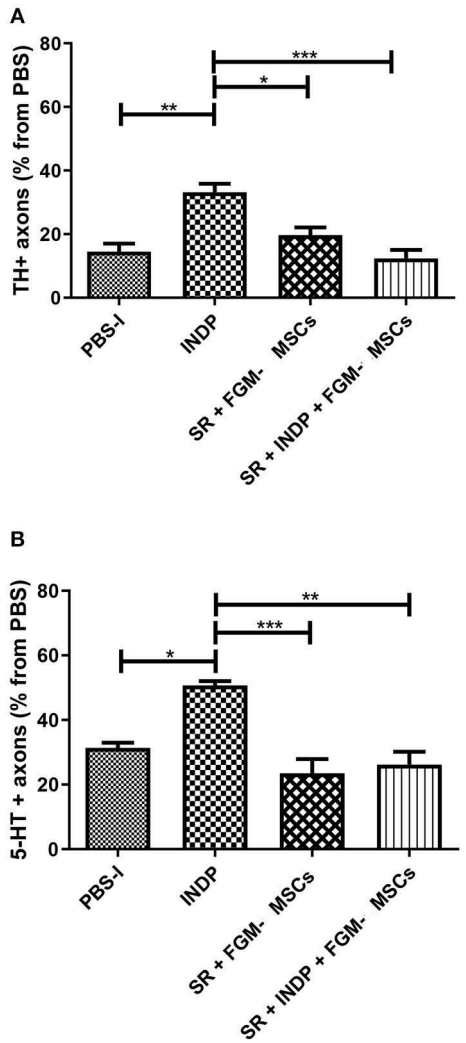

FIGURE 4 | Percentage of axons observed at the caudal stump of SCI rats after therapeutic intervention. The percentage was obtained from the total number of axons found at the same level in sham-operated rats. INDP treatment induced a significant increase of both TH+ **(A)** and 5-HT+ **(B)** fibers (*$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$, One-way ANOVA followed by Tukey–Kramer *post hoc* analysis). Animals treated either with SR + FGM-MSCs or SR + INDP + FGM-MSCs presented no significant differences in the percentage of TH+ or 5-HT+ fibers when compared to PBS-I rats. Bars represent the mean ± SD of 4 rats. This is one representative graph of three experiments.

Neurogenesis Is Increased by INDP but Not by the Combination Therapy in Rats With Chronic SCI

In order to assess the number of NPCs at the injury site, we labeled BrdU+/DCX+ cells (neuroblasts) at the epicenter, rostral, and caudal stumps of the SC. Neither the rats treated with SR + FGM-MSCs nor SR + INDP + FGM-MSCs showed significant differences in the total number of neuroblasts when compared to the PBS-I group. However, the rats treated with INDP alone presented a significant increase in the total number of neuroblasts at 2, 4, and 6 mm caudal to the epicenter of the injury (**Figures 5**, **6**); $p < 0.05$; One-way ANOVA followed by Tukey's test).

DISCUSSION

Previous studies in SCI models have shown that INDP is a promising therapeutic strategy to promote neuroprotection and neural restauration. These beneficial effects have been observed when INDP is administered in both acute and chronic phases of SCI (5, 10). Therefore, our purpose is to improve its protective and restorative benefits by combining INDP whit other successful therapeutic strategies. Previous investigations have shown that SR (22), the use of inhibitors of scar formation (17, 18, 22) or FGM-MSCs separately, promote a significant tissue restoration and motor recovery after SCI. Based on these results, combining INDP with these alternative strategies would be promising.

Chronic phase of SCI is a period of stability and low activity at the site of injury (11). In order to achieve a successful treatment, we proposed a surgical SR to break the latent chronic phase as this procedure reactivates the inflammation response (7) mimicking the initial injury process (19). This microenvironment, allows INDP to stimulate protective autoimmunity and thus, activate its neuroprotective (lipid peroxidation, inflammation and apoptosis inhibition) and restorative (IGF-1, BDNF, and GAP-43 induction) actions by modulating the gene expression of pro and anti-inflammatory cytokines as well as growth factors (7). Additionally, SR diminishes the existing physical barrier of collagen fibers and reactive glial cells. On the other hand, MSCs possess high immunomodulatory properties and they are capable of restoring injured CNS due to the anti-inflammatory molecules and trophic factors they produce to promote angiogenesis, remyelination, axonal regrowth, neural cell death inhibition, amongst others (20).

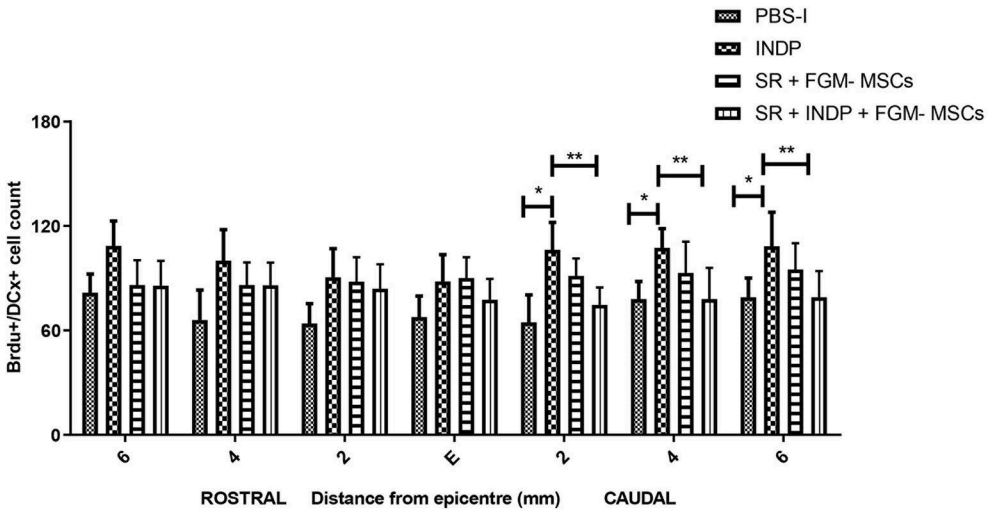

FIGURE 5 | Number of BrdU+/DCX+ cells at caudal stumps of the SC. Rats treated with SR + FGM-MSCs or SR + INDP + FGM-MSCs showed no significant differences in the total number of BrdU/DCX labeled cells (neuroblasts) compared to the PBS-I group. Animals treated with INDP alone presented a significant increase in the total number of neuroblasts at the caudal stump of the SC. Bars represent the mean ± SD of 4 rats. This is one representative graph of three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; one-way ANOVA followed by Tukey's test.

We predict that the conditions created by each of these therapies in the injury site and the synergy that they could generate together, should enhance the restoration conditions causing a better functional regeneration and thus, a better neurological recovery in the groups receiving the combined therapy. This therapeutic strategy has rendered optimistic results in SCI models involving acute contusion (5) or chronic complete transection (10). The objective of this study was to explore now the effects this combination could exert after a chronic SC contusion.

Our results show that INDP alone, SR + FGM-MSCs and the combination therapy (SR + INDP + FGM-MSCs) all increase motor recovery, but to different extents. Interestingly, the effect is reduced when the combination therapy is applied compared to the other treatment options. With the purpose of understanding this effect, the production of pro-inflammatory (TNFα) and anti-inflammatory cytokines (IL-4 and Il-10) were analyzed. We observed that there is a clear reduction of TNFα when INDP alone and the combination therapy were used, which prove how these therapies could modulate the inflammatory response, a phenomenon strongly involved in tissue destruction and restoration inhibition. On the other hand, we also found in both cases an increase in IL-10, an anti-inflammatory molecule that could help even more in the restoration process. Nevertheless, combination strategy did not promote the production of regeneration associated molecules neither improved the formation of regenerating fibers or neurogenesis. The lack of GAP-43 and BDNF—molecules favoring neural restoration- in the combination group could be -at least in part- the cause of the failure observed in these animals. It is difficult to understand why in this case, the microenvironment was not enriched with these molecules. SR for instance, has shown to improve the gene expression of BDNF and GAP-43 when combined with INDP. In the same way, this combination (SR + INDP) increased TH+ and 5-HT+ fibers and improved motor recovery (7). On the other hand, there is no evidence on any modulatory effect that could be exerted by FGM on the production of GAP-43 or BDNF. Therefore, the only option causing the lack of these molecules could be the counteraction induced by the modulating mechanisms exerted by MSCs. It is known that MSCs might inhibit the expansion of T cells not allowing the necessary response to promote the beneficial effects (21, 23). This is an issue that should be deeply studied in future investigations.

On the other hand, INDP alone was capable of providing a permissive microenvironment after a chronic SCI. This finding supports our previous reports and strengthens the idea that INDP is a promising therapy for acute and chronic SCI as now we show that INDP also promotes neurogenesis.

In the present study, INDP increased IL-10 concentrations. Regarding this, previous reports have stated that INDP alone produces an increase of this cytokine in chronic SCI (7) and stroke rat models (6). IL-10 is an anti-inflammatory cytokine that works together with CD4+ cells in glial maintenance (24) and as an apoptotic cascade blocker by increasing the levels of certain anti-apoptotic proteins such as Bcl-2 and Bcl-x leading to the decrease of Caspase-3 in motor neurons. This release of IL-10 in SCI models induces the recovery of motor function, decreases pain and tissue damage (25). In a recent study, IL-10 also proved to have NPCs homeostasis in the neurogenic niches by modulating the proliferative pathways of ERK and STAT 3 (26). Regarding IL-4, increased levels of this cytokine were found only in the group that received INDP alone and in the group treated with SR + FGM-MSCs. The fact that the combined therapy reduced IL-4 production is really interesting and could be, at least in part, the cause of its reduced morphological

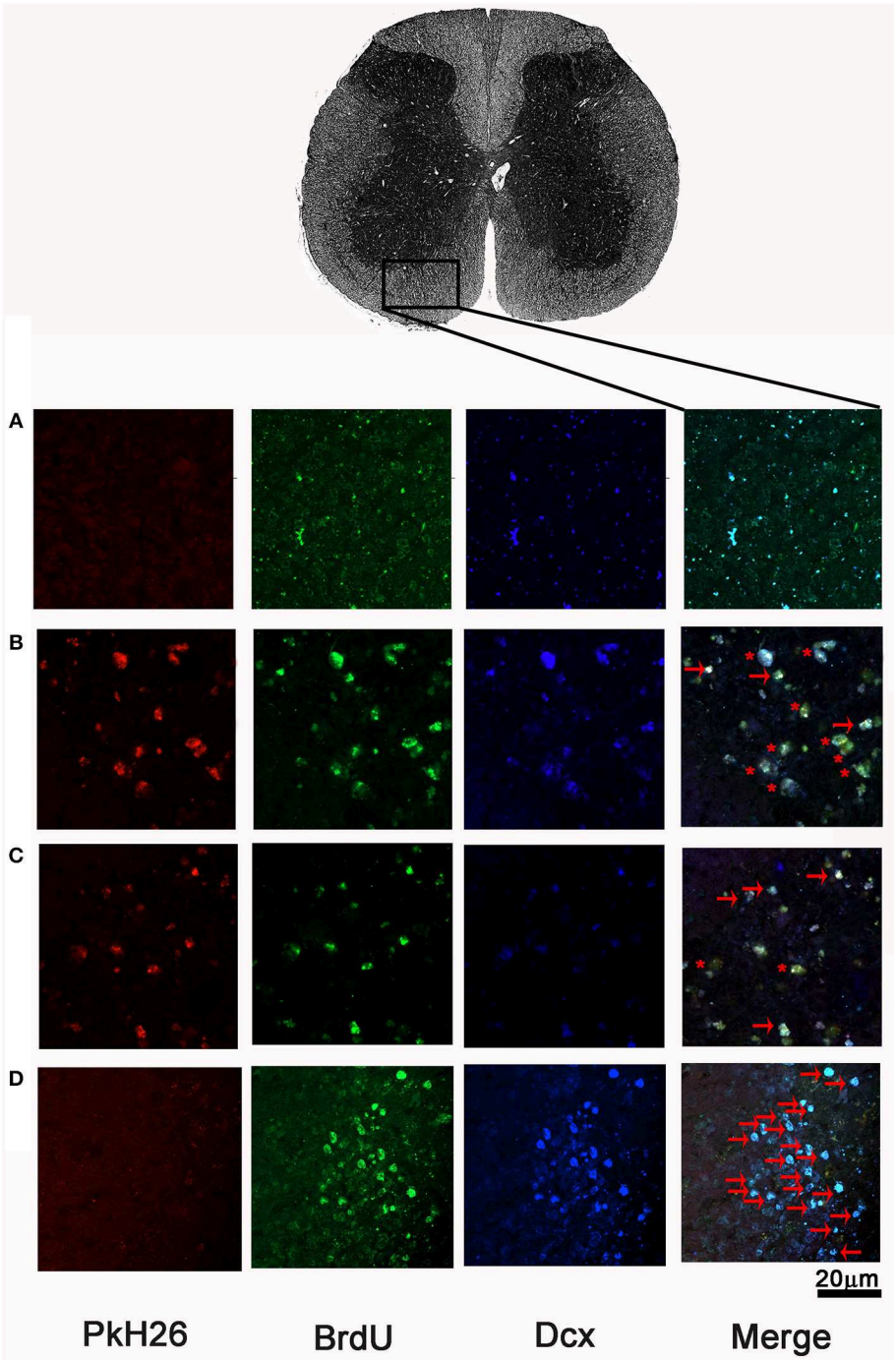

FIGURE 6 | Representative microphotographs of BrdU+/DCX+ cells at ventral horns of SCI rats after therapeutic intervention. In the first section, pkH26 positive cells (red), BrdU+ cells (green), Dcx+ cells (blue). Double-label (BrdU+/Dcx+; cyan), triple-labeling (BrdU+/Dcx+/pkH26+; yellow) show merged final section. **(A)** PBS-I, **(B)** SR + FGM-MSCs, **(C)** SR + INDP + FGM-MSCs, and **(D)** INDP. An asterisk (*) indicates neuroblasts with triple labeling. Arrows depict BrdU+/DCX+ cells. A higher number of neuroblasts was observed in the group with INDP. This is one representative photograph of three experiments. Scale bar 20 μm.

and functional effects. IL-4 can exert neuroprotective effects by regulating the acute and chronic response of the macrophages and promotes growth, phagocytic activity, and proliferation of microglial cells (27). IL-4 reduces also the production of nitric oxide and inflammatory cytokines such as, TNFα and INFγ (28–30). Another beneficial effect of IL-4 is neural restoration, this is induced by increasing the branching and maturation of oligodendrocytes through the interaction with the microglia (28).

In addition, a study by Walsh et al. proves how this cytokine induces axonal growth in *ex vivo* models. Neurons incubated with IL-4 increased their axonal elongation as well as restoring injured neurons through the activation of neuronal receptors of IL-4 and so amplifying neurotrophin signaling via AKT and MAPK (31). Our current study indicates that INDP induces a favorable microenvironment of IL-4, thus suggesting its favorable actions at the injury site. This finding partially explains the motor recovery observed in this group, which could be due to the production of these cytokine, inducing maybe the production of neurotrophins such as BDNF (7, 32).

BDNF has an important function in neural tissue repair and CNS plasticity, especially in neurogenesis, axonal growth, myelination and sympathetic plasticity (33, 34). In fact, BDNF grants immediate actions together with direct effects on synaptic transmission (35). BDNF is also associated with GAP-43 induction, which is a common mediator of the regenerative effect of BDNF (36, 37). Notoriously, GAP-43 plays an essential role in the neurotrophic functions of BDNF (36) in cervical axotomy models where it was proven that the injection with BDNF stimulates expression of GAP-43, consequently inducing axogenesis and neural repair (38). These findings can explain the increase levels of GAP-43 observed in the group treated with INDP. GAP-43 is also strongly related in the transduction signaling for axonal growth and axons direction guidance (39). Several studies mention the possible role that GAP-43 has in the regulation of neurotransmitter release (36, 40). In summary, GAP-43 helps as a useful marker for neural regeneration and has an important role in neurites formation, regeneration and neuroplasticity (37).

All of these results, support the idea that BDNF together with GAP-43 contribute to neural restoration. However, to demonstrate whether the permissive anti-inflammatory microenvironment created by the combined therapy offered in our work had positive effects on axon regeneration, we evaluated the percentage of 5-HT and TH immunoreactivity fibers at the caudal portion where the lesion occurred prior to treatment. Axons which are positive to 5-HT and TH along the SC derive from regions near the brain stem and are distributed throughout the whole SC, these axon fibers descend from neurons located in the Raphe nucleus (41) and Locus Coeruleus (42), respectively. After SCI, the distal portions of the axons are isolated from the neural nuclei, causing degeneration due to the lack of neurotrophic factors. For this reason, both 5-HT and TH markers are useful for assessing the effects that these treatments have on axonal regeneration. These fibers modulate in some way locomotor network (43–45).

The results of our study show that the microenvironment created in rats treated with INDP alone is associated with a significant increase in the number of 5-HT+ and TH+ fibers located in the caudal segment of the SC and so improving locomotor activity. Differently, in the group receiving the combination therapy there was no difference in the number of these fibers. It is important to mention that the minimal motor recovery observed could be due to other tracts such as corticospinal tracts, which are not evaluated in this study. It is known that an injury inflicted by contusion originates a stronger reaction and, thereby, a greater tissue destruction compared to an injury inflicted by transection (46). This could provide more opportunities in axonal regeneration for the latter and less for the first one. In addition, the resulting different microenvironment could likely affect the capacity to induce axonal regeneration-specially for certain tracts- and probably supporting the regenerative process of other different fibers (47). This could partially explain the contrasting results observed - in axonal regeneration- between the present study and those previously reported in rats with SC transection (10). On the other hand, the lack of regeneration of 5-HT+ and TH+ fibers also contrasts with previous findings reported in the acute phase of injury after SC contusion. The acute phase of injury is characterized by an intense inflammatory response and a great release of neural constituents (48). These factors can better activate the protective and restorative effects of the combination therapy to induce the regeneration of the tracts mentioned above. In contrast, the chronic phase is a period of generalized stability in which many of the elements that were activated as protective means or promoters of the restoration observed during the acute phase of the injury are missing (49, 50). Although in our model the SR inflicted a slight injury, it was not enough to support the beneficial effects of the combination therapy and, thereby, the regeneration of the evaluated tracts.

We also evaluated neurogenesis and observed that only the group treated with INDP alone shows a significant increase in the number of early formed neurons. The results found in this study suggest that the combination strategy did not act in synergy with INDP and more than improving, it inhibits its beneficial effects after a chronic SC contusion. This unexpected result, is the opposite of what has been observed in models of chronic SC transection where a more favorable result was obtained (10). These contrasting results demonstrate again that the microenvironment generated after a SC transection or contusion are different and therapeutic strategies should be individualized.

Some limitations of the present study, in the failure of the combination therapy, might be the ideal timing. Previous studies in our group have shown that INDP requires T cells activation and their interaction with other immune cells (51). In addition, when anti-inflammatory or immunosuppressive treatments, such as Methylprednisolone or Cyclosporin A are administrated at the same time with A91 peptide, INDP effect is not observed. The combination therapy used in this investigation uses simultaneously INDP and MSCs. Therefore, MSCs could interfere with INDP action due to their anti-inflammatory effects, which would show a similar result as the ones with methylprednisolone or Cyclosporin A. This is a topic that should be addressed in further studies. Future investigations must be carried out and focused in the assessment of whether the INDP could have a better effect when applied a few days before the MSCs transplant instead of being used simultaneously. Additionally, some complementary evaluations as electrophysiological studies should be included in order to improve the functional analysis.

CONCLUSION

The findings observed in this study, suggest that the combination therapy (SR + INDP + FGM-MSCs) modifies the non-permissive microenvironment post spinal cord injury, but it is not capable of inducing an appropriate axonal regeneration or neurogenesis when compared to the treatment with INDP alone. Therefore, indicating that the best therapy in a chronic SC contusion rat model is purely with INDP.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Mexican Official Norm on Principles of Laboratory Animal Care (NOM 062-ZOO-1999). In addition, the Animal Bioethics and Welfare Committee approved all animal procedures (ID: 178544; CSNBTBIBAJ 090812960).

AUTHOR CONTRIBUTIONS

RR-B contributed to the concept, design of experiments, as well, substantially contributed to the acquisition, analysis, interpretation of data, and drafting of the manuscript. AF-R contributed to acquisition and interpretation of data, surgical procedures and postoperative care of the experimental animals. VB-A contributed to the acquisition, analysis, interpretation of data. EG contributed to the surgical procedures. KS-Z was contributed to surgical procedures and postoperative care of the experimental animals. DI-A was involved to the acquisition and contributed in the drafting of the manuscript and postoperative care of the experimental animals. MG-L was involved to the acquisition and postoperative care of the experimental animals. JJ-VW was substantially involved in the drafting of the manuscript and postoperative care of the experimental animals. AI contributed to the conception and design of this project, general supervision of the research group and gave final approval of this manuscript. All authors read and approved the final manuscript.

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REVIEW
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Tissue Engineering and Biomaterial Strategies to Elicit Endogenous Neuronal Replacement in the Brain

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Neurogenesis in the postnatal mammalian brain is known to occur in the dentate gyrus of the hippocampus and the subventricular zone. These neurogenic niches serve as endogenous sources of neural precursor cells that could potentially replace neurons that have been lost or damaged throughout the brain. As an example, manipulation of the subventricular zone to augment neurogenesis has become a popular strategy for attempting to replace neurons that have been lost due to acute brain injury or neurodegenerative disease. In this review article, we describe current experimental strategies to enhance the regenerative potential of endogenous neural precursor cell sources by enhancing cell proliferation in neurogenic regions and/or redirecting migration, including pharmacological, biomaterial, and tissue engineering strategies. In particular, we discuss a novel replacement strategy based on exogenously biofabricated "living scaffolds" that could enhance and redirect endogenous neuroblast migration from the subventricular zone to specified regions throughout the brain. This approach utilizes the first implantable, biomimetic tissue-engineered rostral migratory stream, thereby leveraging the brain's natural mechanism for sustained neuronal replacement by replicating the structure and function of the native rostral migratory stream. Across all these strategies, we discuss several challenges that need to be overcome to successfully harness endogenous neural precursor cells to promote nervous system repair and functional restoration. With further development, the diverse and innovative tissue engineering and biomaterial strategies explored in this review have the potential to facilitate functional neuronal replacement to mitigate neurological and psychiatric symptoms caused by injury, developmental disorders, or neurodegenerative disease.

Keywords: neural precursor cells, neuroblasts, adult neurogenesis, subventricular zone, tissue engineering, biomaterials, neural regeneration

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INTRODUCTION

Neurogenesis in the mammalian brain is a continuous process that occurs in multiple stages throughout the developing brain (1, 2) and persists into adulthood (3, 4). Interest in adult neurogenesis in the human brain has gained significant momentum over the past several years. Research in the field of neurogenesis has included basic investigations of the neurogenic potential of

the brain over time as well as how this capacity can be harnessed as a neuronal replacement strategy throughout one's lifetime following brain injury and/or neurodegenerative disease. Previous observations that endogenous neurogenesis can become altered in disease states (5–8) have led to investigations of how biomaterials and tissue engineering can be utilized to augment neurogenesis in the adult brain (9–13). While these emerging strategies may have the potential to promote neuronal replacement, there are still several challenges to overcome before these technologies can be successfully utilized in application to injury and disease.

There is controversial evidence regarding whether adult neurogenesis continues in the human subventricular zone (SVZ) surrounding the lateral ventricles and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) throughout life (3, 14–17). Of particular interest in this review is the possibility that neural precursor cells (NPCs) exist in some quantity in the SVZ throughout the human lifespan. While there is considerable debate regarding the quantity of NPCs that exists within this neurogenic niche and the existence and functionality of the rostral migratory stream (RMS) as the human brain matures and ages (reviewed in section Controversy Over Human SVZ and RMS below), there is evidence from several different groups indicating that the adult human SVZ contains some quantity of NPCs (18–22).

The current evidence indicating regionally-restricted locations of NPCs within the adult brain (3, 23) and the propensity of adult SVZ NPCs to mature into an interneuron phenotype (24) limits their potential to contribute to self-repair. The various brain injuries and neurodegenerative diseases that can cause the loss of diverse neuronal types across distinct brain regions urgently calls for the development of reliable methods for diverse neuronal replacement. The advent of advanced regenerative medicine therapies has led to the production of various biomaterial-, cell-, and/or microtissue-based methods that could be utilized in an attempt to promote or enhance neuronal replacement across various brain regions.

There are currently three general strategies to replace lost neuronal populations throughout the brain: exogenous stem cell transplants (25–29), direct cell reprogramming (30–35), and redirection of endogenous NPCs (5, 36). Here, we specifically review pharmacological, biomaterial, and tissue engineering strategies that have been developed in an attempt to harness the neurogenic potential of the SVZ to serve as an endogenous, reliable NPC source to replace lost or damaged neurons throughout life. While some strategies rely on the application of extrinsic factors to modulate neurogenesis within the SVZ, others manipulated the intrinsic properties of the SVZ to redirect endogenous NPCs. Pharmacological strategies include the use of neurotrophic factors and signaling peptides to augment NPC proliferation. More recent research has also led to the fabrication of various types of acellular scaffolds and hydrogels designed to facilitate or divert immature neuroblast migration from the SVZ (37). In addition, we review an exogenously biofabricated tissue-engineered RMS developed by our laboratory that replicates the structure and function of the endogenous glial tube (38–40). This novel replacement strategy seeks to redirect endogenous neuroblast migration from the SVZ through an engineered "living scaffold" and to diverse, specific locations throughout the brain. This microtissue construct, unique from acellular biomaterial scaffolds in that it is comprised of living cells, is the first tissue-engineered neuronal replacement strategy that attempts to replicate and expand upon one of the brain's intrinsic mechanisms for neuronal replacement.

The strategies discussed in this review have the potential to enhance neuronal replacement in response to a wide range of brain disorders, including acquired brain trauma, developmental disorders, and neurodegenerative diseases. As such, the current article presents the technical details and findings to date for a range of regenerative therapies designed to facilitate endogenous neuronal replacement. Importantly, we discuss several challenges that need to be overcome in order to better utilize endogenous stem cells for repair purposes and discuss the diseases and disorders across the human lifespan for which these emerging technologies may be particularly applicable. Before we can reliably utilize these biomaterials and tissue engineering strategies as safe, effective clinical therapies to enhance neuronal regeneration, continued advancements must be made to ensure neuronal survival, reliable maturation and differentiation into appropriate cell types in target regions, proper integration, and network formation with resident neurons, and development of reliable ways to measure and modulate these factors. In particular, we recognize that the ability to influence cell fate determination is currently a major limitation of all of the strategies discussed herein. We attempt to answer what we can regarding the potential of these strategies with the understanding that success will be determined by future research indicating their ability to influence cell determination. Moreover, we consider the particular challenges in employing these new technologies as treatments to diseases and disorders that are not fully understood mechanistically. These challenges are critical to contemplate as we move toward utilization of these technologies in clinical trials. Overall, biomaterial and tissue engineering strategies that can reliably direct and enhance neurogenesis in a sustained fashion and to diverse brain regions may have the potential to successfully promote neuronal replacement in quantities large enough to effectively enhance functional recovery following a variety of injury, degeneration, and disease states that arise throughout human life.

NEUROGENESIS IN THE ADULT BRAIN

Neurogenic niches remain in some form within the mammalian brain post-development (3, 14, 41, 42). Here, we review the location of these niches and discuss the unresolved controversy over the existence and function of the SVZ and RMS in the adult human brain. We also review observations of altered NPC production and neuroblast migration in the mammalian brain following diverse types of brain injury.

Endogenous Stem Cell Sources in the Postnatal Brain

There is ongoing discussion in the field of neuroscience regarding the exact definitions of neural stem and progenitor cells. Differentiation of stem cells exists along a continuum and

multiple terms are currently used to define these different types of immature cells. In this review, we use the term neural precursor cells (NPCs), which is a broad term that encompasses both neural stem and progenitor cells. We recognize that by using this umbrella term we may lose some of the nuances of the particular cell types discussed in the literature reviewed herein. However, we choose to use this term for the sake of clarity and conciseness. With regards to the SVZ, we use the term NPCs when referring to immature cells from the time that they are activated type B1 cells in the SVZ to the time that they become type A cells (neuroblasts). We use the term neuroblast to refer to immature neurons that are migrating. We recognize that there is further detail and nuance surrounding these terms that will not be discussed in this review.

The idea that neurogenesis in mammals is restricted to embryonic development was first challenged by Joseph Altman in the 1960's when he demonstrated radioactive thymidine incorporation in cerebral neurons of adult rats (43, 44). Subsequent electron microscopy analysis provided evidence that that these newborn cells in the brain are indeed neurons (45) and demonstrated that they are capable of integrating into functional neuronal circuits (46). *In vitro* research then demonstrated that cells isolated from the adult mouse brain are capable of dividing and differentiating into astrocytes and neurons (47, 48), suggesting the existence of NPCs in the postnatal brain. As exploration of adult neurogenesis progressed, research began to focus on factors that control this NPC proliferation and neuroblast migration, including neurotransmitters (49, 50), receptors (51), signaling RNAs (52), neurotrophic factors (53, 54), and cell intrinsic signaling molecules (55, 56).

The discovery of NPCs in the SGZ of the DG and the SVZ surrounding the lateral ventricles has emerged as a reproducible finding in the brains of most mammals (3, 23, 57–59). New NPCs generated in the SGZ mature into excitatory cells in the DG and form synapses with target cells in the hilus and area CA3 (60, 61) (**Figures 1A,E**). Simultaneously, neuroblasts formed in the SVZ migrate through the RMS to the olfactory bulb (OB), where they mature into inhibitory granule and periglomerular neurons and form functional synaptic connections (24) (**Figures 1A–D**). These newly formed neurons generated from both neurogenic niches serve a wide variety of functional purposes in the mammalian brain, including cognitive functions such as odor memory, perceptual learning, and mate recognition (63–65). Additionally, there is evidence showing the presence of neurogenesis in other brain regions such as the neocortex, corpus callosum, striatum, amygdala, septum, piriform cortex, olfactory tubercle, thalamus, hypothalamus, and substantia nigra of adult mammals, including rats, mice, hamsters, rabbits, and macaques (62, 66, 67) (**Figure 1F**). However, adult neurogenesis within the human brain remains controversial. Divergent evidence recently emerged regarding the existence of adult neurogenesis in the human hippocampus (15–17). While some studies suggest that hippocampal neurogenesis continues throughout human life (68–70), competing evidence argues that hippocampal neurogenesis ceases during childhood (71, 72). There is currently insufficient evidence to conclude whether hippocampal neurogenesis continues throughout adulthood in humans (15). Additionally, there is lack of consensus regarding various aspects of adult neurogenesis within the human SVZ (3, 14), which will be discussed in detail in section Controversy Over Human SVZ and RMS.

Of particular interest to this review is neurogenesis arising from the SVZ. There has now been consensus for several decades that NPCs continue to exist in the SVZ throughout the life of fish, reptiles, and several different types of mammals (73–79). The rodent SVZ has been the most extensively studied and is the most well-characterized. The neurobiology of the rodent SVZ and the factors regulating SVZ neurogenesis have been recently reviewed by the Alvarez-Buylla group and will not be discussed here (78, 80). However, it is critical to consider the factors that control endogenous proliferation, differentiation, and migration of SVZ-derived NPCs, as well as how these factors change with aging (81), in order to understand whether endogenous SVZ neurogenesis may have the potential to be upregulated. Upregulation of SVZ-derived NPCs may be required in order to promote the success of the biomaterial and tissue-engineered technologies discussed in section Current Strategies to Augment and Redirect Endogenous Neurogenesis. Here, there is substantial evidence regarding the cell cycle and lineage tracing of SVZ-derived NPCs, as well as factors that play an important role in regulating SVZ-derived NPC proliferation in the adult rodent brain (78, 80, 82–84). Research suggests that the majority of rodent hippocampal NPCs divide asymmetrically and that there may be a limited number of proliferative cycles emerging from the rodent SGZ (85–88). In contrast, recent research has demonstrated that the majority of SVZ-derived NPCs divide symmetrically and that ∼20% of these B1 cells symmetrically self-renew, allowing neurogenesis to continue throughout adulthood (82). However, it remains unknown whether there is a limited number of times that B1 cells can symmetrically self-renew. This concept should be more thoroughly investigated as it will shed light on whether there is a limited number of proliferative cycles of SVZ-derived NPCs, a factor that will be important to consider for the success of the biomaterial and tissue-engineered technologies reviewed in section Current Strategies to Augment and Redirect Endogenous Neurogenesis.

The structure of the SVZ and mechanisms of adult neurogenesis arising from this region are highly heterogeneous among species, even between mammalian species (89, 90). In addition to differences in structure and organization of the SVZ and RMS, NPC maturation, survival, migration, and circuit integration of cells arising from the SVZ also differ among species (89). While certain mammalian species such as rodents seem to have detectable and continual SVZ neurogenesis throughout life, production of neuroblasts within the adult human SVZ may be more sparse (19, 67). Due to the challenges associated with performing neurogenesis research in the human brain, the majority of previous research on adult neurogenesis has been conducted in model organisms such as rodents and birds. However, it is essential to keep in mind that evolutionary and ecological adaptations among species contribute to differences in the mechanisms of adult neurogenesis (90, 91). Evidence elucidating mechanisms of adult neurogenesis that is gathered from basic research conducted in model organisms cannot

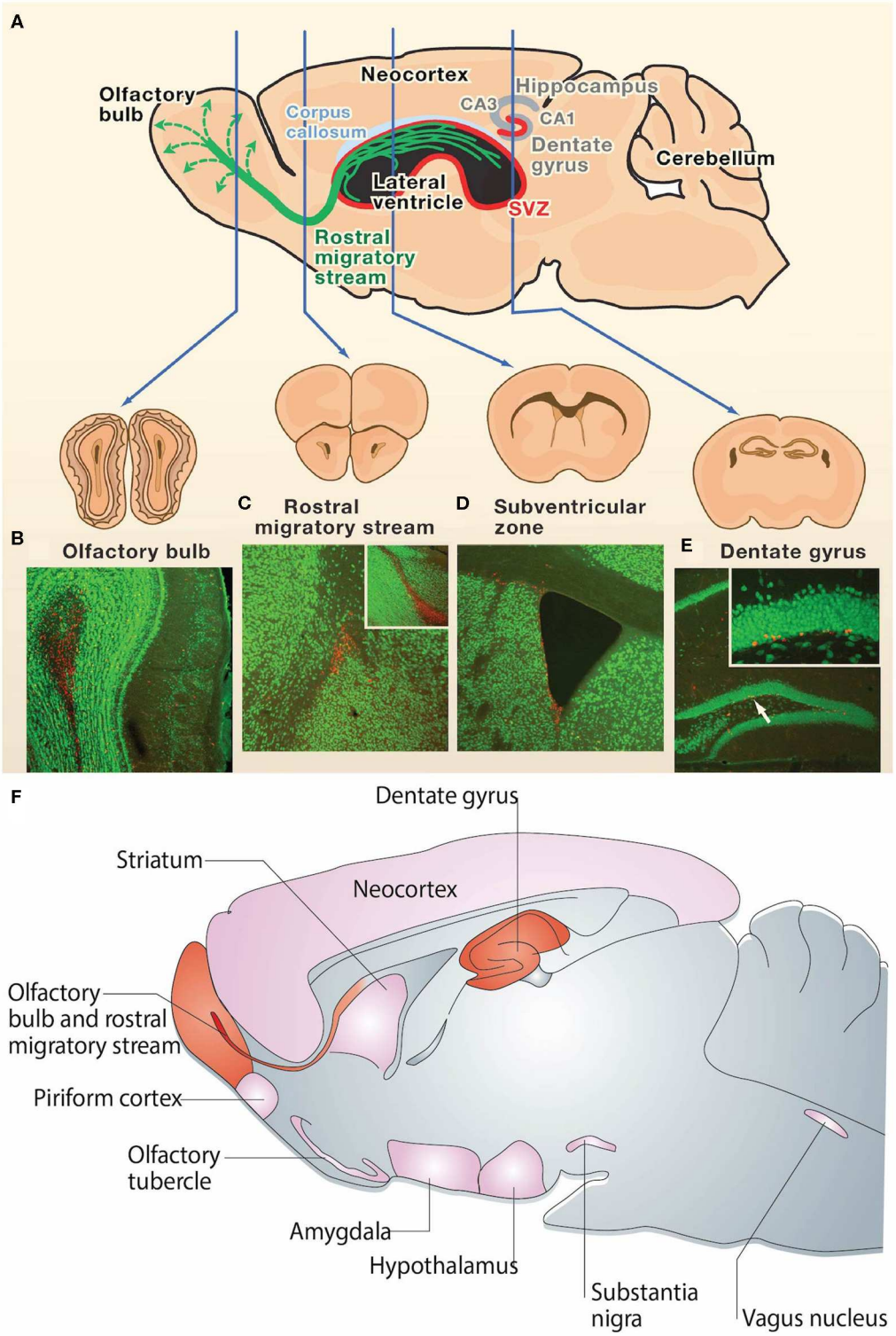

FIGURE 1 | Neurogenesis in the adult rodent brain. **(A)** Neurogenesis occurs post-development in the rodent brain in the subgranular zone of the hippocampal dentate gyrus and the subventricular zone surrounding the lateral ventricles (neurogenic regions depicted in red). Immature neurons born in the subventricular zone migrate along the rostral migratory stream (green) to the olfactory bulb. **(B–E)** NeuN (green) and BrdU (red) staining in coronal sections of the **(B)** adult mouse olfactory bulb, **(C)** rostral migratory stream, **(D)** subventricular zone, and **(E)** dentate gyrus. The presence of BrdU is indicative of post-developmental neurogenesis in these 4 regions. The inset in **(C)** is a sagittal view of the rostral migratory stream and the inset in **(E)** is the dentate gyrus at higher magnification. **(F)** Sagittal view of the rodent brain depicting neurogenic regions. Regions in which adult neurogenesis has been repeatedly shown to occur are depicted in red, and regions in which there is controversial evidence of low levels of adult neurogenesis are depicted in pink. Reprinted with permission from Zhao et al. (23) for **(A–E)** and Gould et al. (62) for **(F)**.

necessarily be extrapolated to humans. Further post-mortem and neuroimaging research within the brains of humans and other mammalian species with gyrencephalic brains is therefore needed before conclusions regarding the exact neurogenic mechanisms regulating the adult SVZ in higher-order mammals can be reached.

Controversy Over Human SVZ and RMS

Divergent evidence exists regarding the existence and structure of the SVZ and RMS as the human brain matures. While it appears that the human SVZ continues to exist within the adult brain and retains some level of neurogenic potential (18–22), there are competing findings concerning how the quantity of NPCs produced within the SVZ changes over time. Here we will discuss the unresolved debate over the existence and structure of the RMS, whether NPCs exist within the RMS, and where these NPCs end up if they are indeed being produced and migrating within the adult human brain.

Evidence gathered from analysis of ten necropsied OBs from healthy postmortem human patients supported the notion that NPCs in the human brain migrate from the SVZ to the OB and continuously populate the OB throughout life (92). In the same year, Alvarez-Buylla and colleagues described the structure of the human SVZ as containing ribbon of proliferative astrocytes that surround the wall of the lateral ventricles and are separated from the ependyma by a process-filled gap (18). After analyzing human SVZ specimens from over 100 neurosurgical resections and autopsied brains, they found evidence that these SVZ astrocytes are capable of neurogenesis, but did not find evidence of migrating neuroblasts either within the adult SVZ or moving toward the OB (18). Further analysis by this group of just under 100 SVZ tissue specimens revealed small numbers of proliferating cells in the SVZ, providing further evidence that the adult human SVZ retains neurogenic potential, but again demonstrated no evidence of chains of migrating neuroblasts within the SVZ (93). The Eriksson lab analyzed sagittal forebrain sections gathered from three human patients and described the structure of the human SVZ as a ribbon of astrocytes surrounding the lateral ventricular walls. Additionally, and in contrast to previous research, they described the structure of the human RMS as extending along what they termed a lateral ventricular extension up to the olfactory bulb, and found evidence of large numbers of chains of migrating neuroblasts proliferating within this structure that go on to become mature neurons in the OB (20). In a comment on these findings, the Alvarez-Buylla lab contested that the high levels of proliferation observed in this study were not confirmed using different markers (i.e., Ki67, Tuj1, PSA-NCAM). They additionally disputed the presence of a ventricular extension and questioned the chains of migrating neurons that were claimed to have been found within it (94). The Eriksson lab stood by their findings, stating that their use of meticulous labeling with PCNA, BrdU, and NeuN and their rigorous MRI imaging combined with the use of serial sagittal sectioning made their study advantageous compared with previous studies that had been conducted by other groups. They argued that neurogenesis in the human OB is as robust as that of rodents, but that the human RMS is distinct in that it is oriented in a different plane than that of rodents, another factor that could have caused this structure to have gone unseen in previous examinations (95). A few years later, the Alvarez-Buylla lab analyzed specimens from 10 neurosurgical resections and 50 autopsied brains and found evidence of immature neuronal cells migrating in the human SVZ and RMS during early development, highlighting that they found an absence of evidence for such migration in older children and adults. They additionally demonstrated that neuroblasts from the SVZ migrate to both the prefrontal cortex and the OB during the first 18 months of life, after which SVZ neurogenesis drastically decreases (19). While this study did provide evidence for the presence of migrating neuroblasts in the juvenile RMS, it did not demonstrate a high volume of these migrating cells as was reported by Curtis and colleagues in 2007 (14, 19). Additional evidence gathered from the Yang lab's analysis of tissue samples from six adult human patients supported the findings that neuroblasts are present in the human SVZ and RMS throughout adulthood, but do not exist within the adult OB (21). Recently, single-cell RNA sequencing was used to identify pools of NPCs in adult human olfactory neuroepithelium gathered from patients undergoing endoscopic nasal surgery, providing further evidence for the continued presence of adult neurogenesis in the human brain (96).

Controversial evidence has also emerged regarding the migration of NPCs from the SVZ to regions of the brain other than the OB. The Frisen lab has gathered evidence demonstrating that neuroblasts produced in the SVZ mature into interneurons and integrate into the neighboring striatum, a process that continues to occur throughout adulthood (97). However, evidence gathered by the Yang lab contrasted these findings and showed that neuroblasts from the SVZ do not integrate into the striatum as mature interneurons (98). Other groups have suggested alternative sources other than the SVZ from which new neurons in the striatum may arise, including parenchymal astrocytes (14, 99).

As research collectively demonstrates, it remains unknown whether neurogenesis exists within the adult human SVZ, the extent to which this process occurs if it does exist, and the fate of potential NPCs arising from this region. Much of the divergent evidence presented above persists due to challenges associated with performing research on human tissue, as well as variation in the techniques and reagents utilized for tissue handling and staining. Many of these studies had small sample sizes and grouped together specimen samples gathered from heterogeneous human groups. Some of these studies analyzed brain tissue from healthy humans while others analyzed tissue from patients that possessed a wide variety of diseases and disorders prior to death. Patients who possessed non-neurological diseases and disorders may still have had altered or disrupted function of the SVZ. It is also important to consider how the levels of adult neurogenesis could intrinsically vary between different individuals, and how factors such as gender, exposure to various environmental factors, and lifestyle choices including diet and exercise could affect this phenomenon. The individual variability of subjects utilized in these studies makes it difficult to extrapolate staining from a handful of specimens to the entire human population.

SVZ Responses to Acquired Brain Injury

There have been multiple reports of altered SVZ neurogenesis and modified migration patterns of SVZ-derived neuroblasts in response to various forms of acquired brain injury in rodents, non-human primates, and humans. These studies, discussed below, provide examples of attempted intrinsic repair mechanisms utilizing endogenous SVZ NPCs to replace lost or damaged neurons following acquired brain injury in adult mammals.

Experimental stroke induced by middle cerebral artery occlusion (MCAO) in adult rats caused increased proliferation of NPCs in the SVZ (100). These new neurons migrated to the damaged striatum where they matured into spiny neurons and formed synapses with existing striatal neurons (100). This process can continue for up to 4 months following MCAO (101). These NPCs appear to be generated in the SVZ rather than in the striatal parenchyma, and migrated in chains, guided by blood vessels, from the SVZ toward the injured striatum (102–104). Experimental stroke in rodents modulates the gene profiles and electrophysiological characteristics of NPCs within the SVZ, specifically by inducing hyperpolarized resting membrane potentials and increasing expression of tyrosine hydroxylase, both of which are markers of neuronal maturity (105). Additionally, neuroblasts generated post-stroke migrate to the injured cortex, although to a lesser extent than the migration toward the striatum (106, 107). External factors such as environmental enrichment further increase neurogenesis in the rodent SVZ post-stroke (108). Neuroblast migration through the RMS is facilitated by release of the soluble signaling molecule Slit1 from neuroblasts and detection by the Robo2 receptors of glial tube astrocytes (109). Neuroblasts overexpressing Slit1 migrated farther into a stroke-induced lesion compared to control neuroblasts in the mouse brain (110). These neuroblasts matured into striatal neurons, integrated into striatal circuitry, and improved functional recovery following MCAO (110).

Increased production and altered migration patterns of SVZ-derived neuroblasts have also been observed in rodent models of disease other than stroke. For example, augmented SVZ NPC production has been observed in a mouse model of Huntington's disease (HD) (111). These SVZ-derived neuroblasts migrated to the injured striatum, but did not demonstrate cell maturity or synaptic integration (111). Simultaneous lesioning of dopaminergic neurons in the substantia nigra and infusion of the transforming growth factor (TGF)-alpha into the striatum caused increased NPC production in the SVZ and migration of these cells toward the striatum (112). Immunoreactivity of cells for BrdU and tyrosine hydroxylase or BrdU and the dopamine transporter indicated that these NPCs reaching the striatum may have differentiated into dopamine neurons (112). Increased SVZ neurogenesis has also been reported in the rodent brain following experimental demyelination (113), targeted lesioning (114–117), lateral fluid percussion brain injury (118, 119), and controlled cortical impact brain injury (120–122). Additionally, global cerebral ischemia in adult macaques caused increased numbers of immature neurons in the SVZ (123).

Altered NPC production and neuroblast migration has also been observed following acquired brain injury in adult humans. Augmented neurogenesis has been reported post-stroke in regions surrounding infarct cortical tissue (124). Damaged tissue from postmortem stroke patients contains increased numbers of cells immunoreactive for Ki67, DCX, TuJ1, and TUC-4 compared to tissue from healthy controls (124). A later case study demonstrated increased neurogenesis following cerebrovascular infarction in the aging brain, showing the accumulation of NPCs, identified via positive staining for Nestin, Musashi, Sox2, PSA-NCAM, and SSEA-4, adjacent to the SVZ as well as in the area of brain injury (125). Increased NPC production has been observed in brain tissue of adult humans following subarachnoid hemorrhage caused by a ruptured aneurysm (126). Here, NPCs were identified by increased expression of nestin, vimentin, Sox2, Musashi1, Musashi2, and immunoreactivity for both Ki67 and Musashi2 in hemorrhage patients compared to controls (126). This study did not determine where these cells were generated and whether they matured into functional neurons (126). Increased proliferation of cells expressing PCNA, TuJ1, and GFAP in the SVZ has also been observed in adult patients with HD (127), and further research using carbon-14 dating approaches provided evidence that SVZ-derived neuroblast migration to the striatum is reduced in patients with HD (97). However, evidence of altered NPC production and neuroblast migration in the human brain following the onset of injury or neurodegenerative disease remains controversial. For example, there is divergent evidence regarding altered NPC production in the SVZ of patients with Parkinson's Disease (PD), with some research claiming decreased NPC production in PD (128) and others failing to find any difference between PD patients and healthy controls (129). Similar to the research describing human adult neurogenesis that was discussed in section Controversy Over Human SVZ and RMS, these studies have small numbers of heterogeneous patients and were conducted in limited postmortem tissue samples. The current available research makes it difficult to generalize these findings to the human population and form definitive conclusions regarding the extent of altered neurogenesis in the human brain following the onset of injury and neurodegenerative disease.

These studies conducted across species highlight the potential of the SVZ to act as an endogenous source of replacement for lost or damaged neurons in response to diverse acquired brain injury and neurodegenerative disease arising in the adult brain. Observations of attempted endogenous neuroregeneration increased interest in the potential to harness endogenous stem cells as a tool to promote brain repair (36, 130). These examples of inherent repair mechanisms within the brain have been the inspiration for the biomaterial and tissue engineering strategies that attempt to manipulate or augment this endogenous neurogenesis that will be discussed in this review.

CURRENT STRATEGIES TO AUGMENT AND REDIRECT ENDOGENOUS NEUROGENESIS

Here we will review the experimental approaches that currently exist to augment and redirect SVZ neuroblast migration,

including various pharmacological approaches and acellular biomaterial scaffolds. We also discuss a tissue-engineered RMS, a newly developed technology that is predicted to facilitate sustained redirection of endogenous neuroblasts from the RMS and into neuron-deficient regions.

Pharmacological Approaches: Neurotrophic Factors and Signaling Peptides

Neurotrophic factors (NTFs) are proteins important for regulating the growth, survival, and plasticity of neurons (131). Increased SVZ neurogenesis has been observed following administration of NTFs to the uninjured and injured rodent brain (**Table 1**). For example, intraventricular administration of epidermal growth factor (EGF) (134, 135), fibroblast growth factor 2 (FGF-2) (135), erythropoietin (EPO) (140), or nerve growth factor (NGF) and EGF (139) increased SVZ NPC proliferation and/or migration away from the SVZ neurogenic niche in the SVZ of the uninjured rodent brain. This augmented proliferation of SVZ NPCs was also observed after administration of NGF in the form of eye drops (144). The introduction of various NTFs can also further amplify the increased SVZ neurogenesis that occurs following acquired brain injury such as experimental stroke. Augmented proliferation of SVZ NPCs and migration of these cells toward injured brain regions has been observed following intraventricular infusion of EGF (136), EGF and EPO (137) (**Figures 2A–H**), EGF and FGF-2 (138), VEGF (147), or BDNF (133) into the post-stroke rodent brain. These effects have also been observed after intranasal administration of TGF-1 (146) or subcutaneous (SC) administration of granulocyte-colony stimulating factor (G-CSF) (141), stromal derived factor 1 (SDF-1) (132), or angiopoietin 1 (Ang1) (132) into the post-stroke rodent brain. These studies collectively provide evidence that administration of various NTFs can augment NPC production and neuroblast migration in rodent models of experimental stroke. However, the majority of these studies fail to examine the long-term survival and integration of neuroblasts into regions of injury. While the majority of these studies did provide evidence of short-term functional recovery as a result of NTF administration (132, 133, 137, 141, 146, 147), research demonstrating long-term functional outcomes resulting from these treatment strategies is lacking. It also remains unknown if NTFs will need to be repeatedly administered in order to continually promote functional recovery over time. While the ability of NTFs to augment NPC production and neuroblast migration are essential first steps to promoting endogenous replacement of lost neuronal populations, the current studies utilizing these treatment strategies do not provide compelling evidence that NTF administration alone is an effective method to promote long-term neuronal recovery following stroke in rodents. Additionally, it is important to note that different ligands of the EGFR receptor have different effects on the fate and migratory capacity of SVZ NPCs following brain injury. For example, the ADAM17/tumor necrosis factor alpha (TNF-alpha)/EGF receptor (EGFR) pathway is upregulated following brain injury and has been shown to exert an anti-neurogenic/pro-gliogenic effect on SVZ NPCs (148, 149) and to inhibit neuroblast migration into regions of injury (150). This is in contrast to the evidence presented above demonstrating a neurogenic effect on SVZ NPCs and increased migration into injured brain regions resulting from EGF administration following brain injury (136, 137). This evidence highlights the possibility that certain receptors may have diverse and complicated effects on neurogenesis in the injured brain, such as occurs with the EGFR.

Administration of signaling peptides is another pharmacological approach used to augment NPC production within the SVZ. A wide variety of cytokines have been used to modulate NPC proliferation and differentiation *in vitro*, including interleukin (IL)-1, IL-6, IL-10, IL-15, Leukemia inhibitory factor, and TNF-alpha (151). Importantly, cytokines modulate NPC proliferation *in vivo*. For example, intracortical administration of prokineticin 2 (PROK2) into the cortex of uninjured mice caused an increase in BrdU-positive cells in the cortex (**Figures 2I–K**), while IP administration of PKRA7 (a PROK2 antagonist) caused a decrease in migration of neuroblasts from the SVZ to the injured cortex following blunt force TBI (145). Additionally, intraventricular administration of IL-15 (142) or an NOS inhibitor (143) increased proliferation of SVZ NPCs. Similar to the administration of NTFs, these results of these experiments demonstrate that signaling peptides can augment NPC production within the SVZ of the injured rodent brain. However, the majority of these experiments have not investigated whether these signaling peptides are efficacious at augmenting NPC production within the SVZ following brain injury, leading to a current lack of evidence indicating whether these cytokines have the ability to promote functional recovery post-injury. Further research is needed before conclusions can be drawn regarding the ability of signaling peptide administration to induce neuronal replacement or promote functional recovery following brain injury.

Importantly, NTFs have been utilized in human clinical trials as a treatment strategy to offset neuronal degeneration resulting from neurodegenerative disease. NTFs including ciliary neurotrophic factor (CNTF), BDNF, insulin-like growth factor 1 (IGF-1), NGF, glial cell line-derived neurotrophic factor (GDNF), and neurturin (NRTN) have been delivered via subcutaneous, intrathecal, intraventricular, and IP routes of administration to patients with amyotrophic lateral sclerosis (ALS), PD, Alzheimer's Disease, and peripheral neuropathies (152). However, although there have been over 3 dozen clinical trials investigating the efficacy of neurotrophic factor administration as a treatment for human neurodegenerative disease, none have demonstrated efficacy at preventing neuronal degeneration and death, indicating that perhaps NTF administration alone may not be beneficial to treat human neurodegenerative diseases (9, 152). One reason may be that the effect of NTF introduction appears to be transient. NTFs may need to be repeatedly administered to continually offset neuronal degeneration occurring in the human brain, making this technique repetitively invasive. Additionally, the optimal dosing and the best timing window in which to administer NTFs following the onset of injury or

TABLE 1 | Pharmacological approaches to augment proliferation and/or migration of SVZ-derived NPCs in the uninjured and injured rodent brain.

Neurotrophic factor or signaling peptide (alphabetical)	Administration	Species	Type of injury	*In vivo* treatment effect	Evaluation method	References
Ang1	SC administration for 7 days beginning 1 day post-injury	Mice	Experimental stroke (MCAO)	Increased DCX-positive neuroblasts in the injured cortex	DCX	(132)
BDNF	IV administration for 5 days beginning 1 h post-injury	Rats	Experimental stroke (photothrombotic)	Increased DCX-positive neuroblast migration from the SVZ to the ipsilateral striatum but not to the ischemic cortex	DCX	(133)
EGF	IV administration for 6 days	Mice	Uninjured	Increased SVZ NPC proliferation; increased neuroblast migration away from LV walls; differentiation into BrdU/NeuN-positive neurons and BrdU/S100-positive glial cells	lacZ reporter gene, [^{3}H] thymidine, BrdU, NeuN, S100	(134)
EGF	IV administration for 14 days	Rats	Uninjured	Increased SVZ NPC proliferation; increased BrdU-positive cells in the striatum 4 weeks post-infusion	BrdU	(135)
EGF	IV administration for 7 days beginning 2 days post-injury	Mice	Experimental stroke (MCAO)	Increased DCX/BrdU-positive SVZ NPC proliferation; migration of DCX/BrdU-positive cells to the injured striatum and maturation into PV-containing interneurons	DCX, BrdU, PV	(136)
EGF and EPO	IV administration of EGF for 7 days followed by EPO for 7 days beginning 4 days post-injury	Rats	Experimental stroke (focal PVD)	Increased BrdU-positive NPC migration from the SVZ to the injured cortex; differentiation into BrdU/NeuN-positive neurons and BrdU/GFAP-positive glial cells in the injured cortex	BrdU, NeuN, GFAP	(137)
EGF and FGF-2	IV co-administration for 3 days beginning 1 day post-injury	Rats	Experimental stroke (MCAO)	Increased BrdU-positive SVZ NPC proliferation	BrdU	(138)
EGF and NGF	IV co-administration for 4 days followed by single infusion of NGF 4 days later	Mice (Aged)	Uninjured	Increased Ki-67-positive SVZ NPC proliferation	Ki-67	(139)
EPO	IV administration for 6 days	Mice	Uninjured	Increased migration of BrdU-positive NPCs from the SVZ to the OB; increased BrdU/TH-positive neurons in the OB	BrdU, TH	(140)
FGF-2	IV administration for 14 days	Rats	Uninjured	Increased SVZ NPC proliferation; increased BrdU-positive cells in the OB 4 weeks post-infusion	BrdU	(135)
G-CSF	SC administration for 15 days beginning 1-h post-injury	Rats	Experimental stroke (MCAO)	Increased BrdU-positive SVZ NPC proliferation; subset of cells was BrdU/NeuN-positive	BrdU, NeuN	(141)
IL-15	Single IV administration	Mice	Uninjured	Increased BrdU-positive and DCX-positive SVZ NPCs	BrdU, DCX	(142)

(Continued)

TABLE 1 | Continued

Neurotrophic factor or signaling peptide (alphabetical)	Administration	Species	Type of injury	*In vivo* treatment effect	Evaluation method	References
NAME (NOS inhibitor)	Single IV administration	Rats	Uninjured	Increased BrdU-positive cells in the SVZ, RMS, and OB	BrdU	(143)
NGF	Single administration in the form of eye drops	Rats	Uninjured	Increased Ki-67-positive SVZ NPC proliferation	Ki-67	(144)
PKRA7 (PROK2 antagonist)	IP administration for 4 days beginning 1 h post-injury	Mice	Blunt force TBI	Decreased BrdU-positive neuroblast migration from the SVZ to the injured cortex	BrdU	(145)
PROK2	Single intracortical administration	Mice	Uninjured	Increased BrdU-positive cells in the cortex	BrdU	(145)
SDF-1	SC administration for 7 days beginning 1 day post-injury	Mice	Experimental stroke (MCAO)	Increased DCX-positive neuroblasts in the injured cortex	DCX	(132)
TGF-1	IN administration 2 and 24 h post-injury	Mice	Experimental stroke (MCAO)	Increased BrdU-positive cells in the SVZ and injured striatum; increased BrdU/DCX/NeuN-positive cells in the striatum	BrdU, DCX, NeuN	(146)
VEGF	IV administration for 3 days beginning 1 day post-injury	Rats	Experimental stroke (MCAO)	Increased BrdU-positive SVZ NPC proliferation	BrdU	(147)

SVZ, subventricular zone; NPC, neural precursor cell; OB, olfactory bulb; IV, intraventricular; IP, intraperitoneal; IN, intranasal; SC, subcutaneous; LV, lateral ventricle; EGF, epidermal growth factor; FGF, fibroblast growth factor; EPO, erythropoietin; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; TGF, transforming growth factor; G-CSF, granulocyte-colony stimulating factor; VEGF, vascular endothelial growth factor; SDF, stromal derived factor; ang, angiopoietin; PROK2, prokineticin 2; IL, interleukin; NAME, N^{ω}-nitro-methyl-L[D]-arginine-methyl ester; NOS, nitric oxide synthase; PVD, pial vessel disruption; MCAO, middle cerebral artery occlusion; PV, parvalbumin; TH, tyrosine hydroxylase; DCX, doublecortin.

degeneration within the human brain is still being investigated (152, 153). Further investigation into dosing, timing and route of administration, and patient population are needed to determine whether NTFs are efficacious treatments to alleviate the effects of neurodegenerative disease in the human brain.

Acellular Biomaterial Strategies

Research suggests that transplanting a biomaterial scaffold *without* the addition of NPCs into an area of injury may support the surrounding brain tissue and promote axon regeneration and neurite formation (154). Several studies have transplanted biomaterials into injured brain regions with the goal of augmenting neuronal regeneration via repair of local, damaged brain cells within these areas of injury (11) and enhancing the effectiveness of cell transplantation and drug delivery (155). There are several reviews describing the ability of these biomaterials to bridge neuronal lesions, promote cell growth, and stimulate axonal outgrowth post-transplantation into regions of neuronal injury (10–12, 37, 154). Just as with biomaterials designed to deliver NPCs to regions of injury, these acellular scaffolds can be coupled with NTFs to further enhance neuronal support and regeneration upon transplantation (11, 37, 154, 156). These experiments have used biomaterials to repair local neuronal damage, but have not sought to manipulate endogenous neurogenesis to aid in potential repair.

Of particular interest to this review are biomaterial methods that have been developed to enhance *endogenous* neuronal regeneration and/or redirect *endogenous* neuroblasts from the SVZ (**Table 2**). These approaches are unique in that they attempt to promote neuronal regeneration via manipulation of the brain's intrinsic NPC sources, rather than relying on extrinsic NPCs for repair. For example, biomaterials and nanotechnology have been used as a strategy to enhance the delivery of NTFs into the brain (169, 170). While the use of these technologies *in vivo* has been limited to rodent models, they show promise for enhancing the beneficial effects of NTFs in application to neurodegenerative disorders. In this approach, biomaterials coupled with NTFs are injected into a region of injury to enhance endogenous NPC proliferation and neuroblast migration from the SVZ. Experiments utilizing this approach have demonstrated that coupling NTFs with biomaterials is advantageous over *in vivo* administration of NTFs alone for augmenting endogenous SVZ NPC production and neuroblast migration post-injury. For example, increased proliferation of SVZ NPCs occurred following injection of hydrogels encapsulating IGF-1 (159), hepatocyte growth factor (HGF) (159) (**Figures 3A–E**), EGF (161), or EPO (162) into the post-stroke rodent brain. The

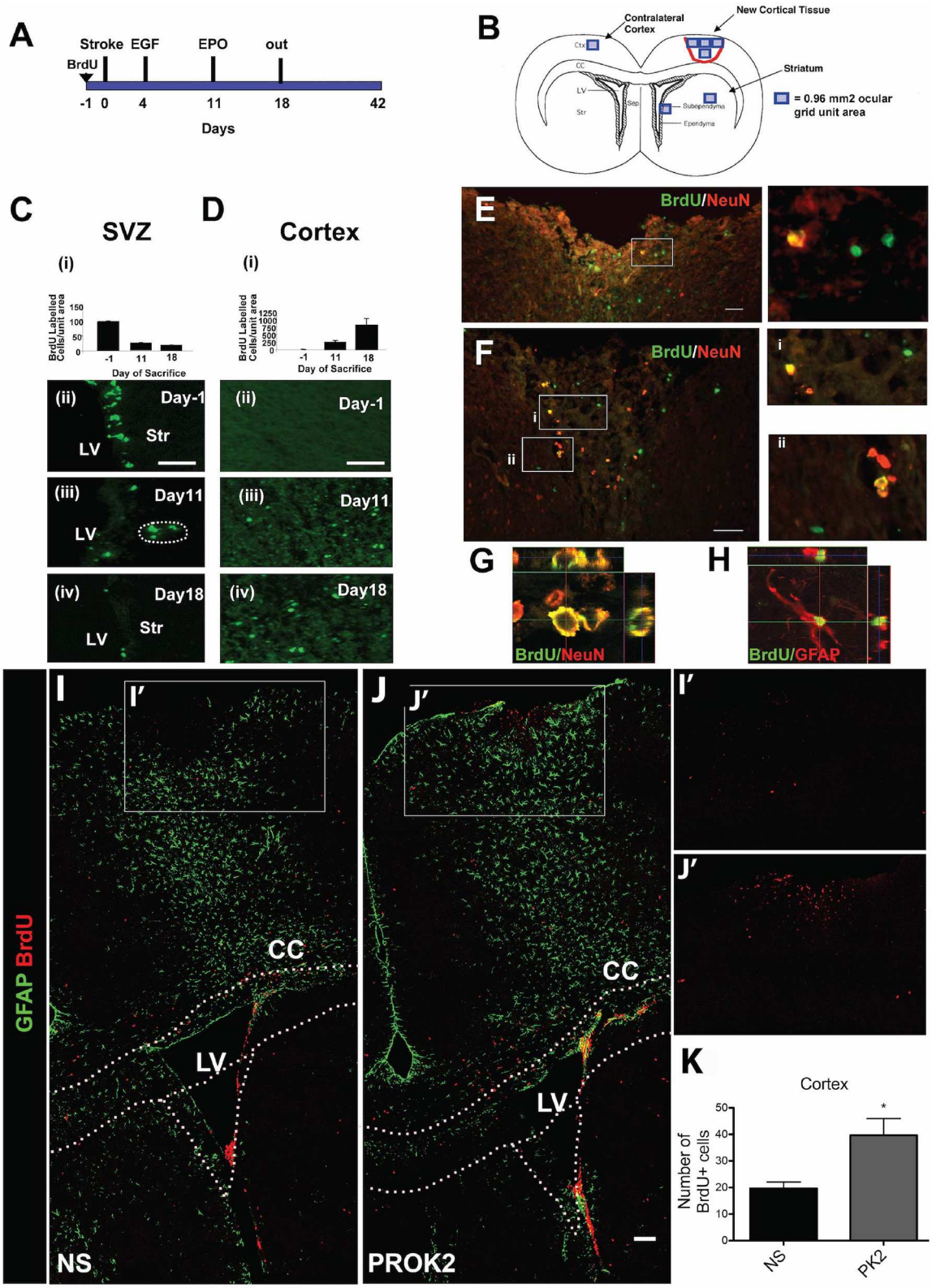

FIGURE 2 | Pharmacological and signaling peptide approaches to augment endogenous NPC proliferation and migration. **(A–H)** Intraventricular infusion of EGF followed by EPO and **(I–K)** injection of recombinant Prokinectin 2 (PROK2) into the injured cortex. **(A)** Experimental design utilized by Kolb et al. (137). Rats received

(Continued)

FIGURE 2 | BrdU injections 1 day prior to stroke, followed by EGF on days 3 and 4 following stroke and EPO on day 11 following stroke. **(B)** Schematic illustration depicting the four regions (indicated by squares) in which BrdU-positive cells were counted. **(C,D)** Quantification and corresponding fluorescent images depicting the number of BrdU-positive cells in the SVZ (over the 0.96 mm^2 area indicated in **B**) and cortex (over the 3.84 mm^2 area indicated in B) 1 day before stroke (−1) and 11 and 18 days post-stroke following growth factor administration. BrdU-positive cells decreased in the SVZ and increased in the cortex over time. **(E,F)** Fluorescence images depicting BrdU and NeuN double-positive cells in the injured cortex. Insets depict higher magnification images of NeuN/BrdU cells. Confocal images of **(G)** BrdU/NeuN double-positive neurons and **(H)** BrdU/GFAP double-positive astrocytes in the injured cortical tissue. In the experimental design utilized by Mundim et al. (145), mice were administered BrdU for 2 days prior to the injection of either recombinant PROK2 or saline into the cortices of uninjured mice. **(I,J)** Confocal microscopy images and **(K)** quantification reveal a greater quantity of BrdU-positive cells (red) in the cortex following PROK2 administration compared to saline (NS). Scale bars **(C,D)** 50 microns; **(E–G)** 30 microns; **(K)** 100 microns. Ctx, cortex; cc, corpus callosum; LV, lateral ventricle; Str, striatum; NS, normal saline. Reprinted with permission from Kolb et al. (137) for **(A–H)** and Mundim et al. (145) for **(I–K)**. $^*p < 0.05$.

hydrogels encapsulating HGF or EPO also increased migration of neuroblasts into the stroke-injured region (159, 162). Biomaterial approaches are also being developed to promote endogenous neuroblast migration by intercepting the SVZ with implanted hydrogel scaffolds encapsulating NTFs or signaling cues. These constructs are designed to divert migrating neuroblasts from the SVZ into the scaffold and deliver them to alternate locations throughout the brain. For example, implantation of biomaterial hydrogels encapsulating GDNF (160), a BDNF-mimetic (168), graphene coated fibers (166), or laminin 1, aprotinin, NGF, and VEGF (158) spanning from the SVZ into the injured cortex caused NPCs to divert from the SVZ and migrate into the biomaterial implant tract. In other examples, an injectable self-assembling peptide amphiphile carrying the migratory bioactive Tenascin-C derived peptide sequence E_2Ten-C PA (167) or an injectable self-assembling peptide encapsulating BDNF (157) elicited the diversion of endogenous neuroblasts from the SVZ/RMS and into the injection tract. An N-cadherin-containing gelatin sponge also enhanced neuroblast migration toward regions of injury in the neonatal mouse brain, mimicking the N-cadherin-mediated adhesion interactions between radial glial fibers and neuroblasts during developmental and post-injury migration (165). Another approach builds off of the observation that endogenous neuroblasts can travel along blood vessel scaffolds when migrating to injured brain regions (102, 103, 105, 171–173). This method constructs scaffolds out of laminin, which is a major component of the basement membrane of blood vessels (174). Transplantation of a laminin-rich porous sponge (164), an injectable laminin-rich hydrogel (104) (**Figures 3F–I**), or a laminin tract with no coupled biomaterials (163) into the injured rodent brain effectively enhanced neuroblast migration into lesioned regions. Collectively, these technologies have demonstrated the rich potential for enhancing and redirecting migration of endogenous neuroblasts. Implantation of a biomaterial scaffold intercepting the SVZ and spanning toward a region of injury may offer greater spatial control over the migration path of neuroblasts emerging from the SVZ than other methods that have been previously designed to augment and redirect neuroblast migration, indicating that this may be the most promising strategy for neuroblast redirection to specific neuron-deficient regions of the brain. Importantly, biomaterial scaffolds that are designed to mimic certain mechanisms with which neuroblasts migrate endogenously throughout the brain (i.e., endogenous signaling mechanisms and endogenous scaffolds) may be particularly effective at eliciting neuroblast migration into regions of injury. Further investigation of survival, maturity, and synaptic integration of redirected neuroblasts, as well as the ability of these scaffolds to promote functional recovery following injury, is needed before the efficacy of these biomaterial methods can be fully determined.

Living Scaffolds

"Living scaffolds" are a tissue engineering regeneration strategy based on the use of living cells that are encapsulated within a preformed three-dimensional structure and can be designed to facilitate axonal pathfinding, replace circuitry, or direct neuronal cell migration (40, 175, 176). Our laboratory has developed the first implantable living scaffold that emulates the glial tube of the RMS (**Figure 4**). These tissue-engineered rostral migratory streams (TE-RMSs) are fabricated by promoting the self-assembly of astrocytes seeded into hydrogel microcolumns that spontaneously re-configure into networks of bipolar, longitudinally-aligned bundles (38, 39) (**Figures 4A–B**). These bundles provide the scaffolding network through which immature neurons can migrate (**Figure 4C**). TE-RMSs are fabricated in hydrogel microcolumns with a diameter of <350 microns in which cells self-align into cable-like structures of process-bearing astrocytes enriched in GFAP and measuring 50–150 microns in diameter (38, 39) (**Figures 4E–J**). We have created TE-RMS scaffolds up to 3 cm long, with no theoretical limit to the length (**Figures 4K–L**). This tissue engineering approach leverages the brain's natural mechanism for sustained neuronal replacement by replicating the structure and function of the RMS, setting this technology apart from biomaterial strategies that have been created to enhance endogenous neuronal replacement throughout the brain.

The TE-RMS has the potential to act as a living "implantable highway" to reroute immature neurons from the SVZ to diverse brain regions, offering an approach to manipulate neuronal migration throughout the brain via the brain's own mechanism for neuronal replacement throughout adulthood. We predict that this technology will be particularly advantageous above other biomaterial methods due to the hypothesized ability of neuroblasts to actively communicate with the living astrocytes comprising our scaffold, replicating the Slit/Robo signaling and numerous other cellular interactions that facilitate neuroblast migration along the endogenous RMS (8, 177, 178). Unlike most cell transplant techniques, TE-RMSs are designed to be stable, long-term implants that will provide sustained delivery of neuroblasts to neuron-deficient regions over time. That is, the

TABLE 2 | Acellular biomaterial strategies to augment proliferation and/or migration of SVZ-derived NPCs in the injured rodent brain.

Biomaterial approach (alphabetical)	Administration or implantation strategy	Species	Type of injury	*In vivo* treatment effect	Evaluation method	References
β-peptide hydrogel (self-assembling) encapsulating BDNF	Implanted to intercept the SVZ and span toward the cortex	Mice (transgenic NestinCre-ER^{T2}:R26eYFP to fluorescently label SVZ NPCs)	Implant tract	GFP/DCX-positive cells in implanted hydrogel; GFP/NeuN-positive cells and GFP/Syn1-positive cells in and at the end of the hydrogel	GFP (permanently labeled in SVZ progeny), DCX, NeuN, Syn1	(157)
Fibrinogen hydrogel containing laminin 1, aprotinin, NGF, VEGF	Implanted to intersect the RMS and span toward the striatum	Rats	Implant tract	DCX-positive cells diverted from the RMS, migrated along implant tract, and entered into ventral striatum (seen at 4-5 weeks post-implantation)	DCX	(158)
Gelatin hydrogel containing IGF-1	Injected into the striatum near the SVZ 11 days post-injury	Mice	Experimental stroke (MCAO)	Increased DCX-positive SVZ NPCs compared to injection of IGF alone	DCX	(159)
Gelatin hydrogel containing HGF	Injected into the striatum near the SVZ 11 days post-injury	Mice	Experimental stroke (MCAO)	Increased migration of DCX-positive neuroblasts from the SVZ to the injured striatum compared to injection of HGF alone	DCX	(159)
Gelatin-HPA (with and without CMC-Tyr) encapsulating GDNF	Implanted to intercept the SVZ and span toward the cortex	Rats	Implant tract	DCX-positive cells migrated along implant tract at 7 but not 12 days following implantation	DCX	(160)
HAMC hydrogel containing EGF or PEG-EGF	Placed epi-cortically over stroke region 4 days post-injury	Mice	Experimental stroke (induced by ET-1)	Increased Ki-67/DCX-positive SVZ NPC proliferation	Ki-67, DCX	(161)
HAMC hydrogel containing EPO	Placed epi-cortically over stroke region 4 and 11 days post-injury	Mice	Experimental stroke (induced by ET-1)	Increased Ki-67/DCX-positive SVZ NPC proliferation; increased NeuN-positive neurons in the injured cortex	Ki-67, DCX, NeuN	(162)
Laminin tract	Injected spanning from the RMS to the lesion 5 days post-injury	Rats	Cortical lesion induced by injection of ibotenic acid	Increased DCX-positive cells along the length of the tract as well as in the lesion compared to control injection; lesion contained DCX/GFAP-positive cells and DCX/NeuN-positive cells	DCX, GFAP, NeuN	(163)
Laminin-rich injectable hydrogel	Injected into the injured striatum 10 days post-injury	Mice	Experimental stroke (MCAO)	Increased DCX-positive neuroblasts migrating on laminin-containing hydrogel compared to control hydrogel; neuroblast chain migration on laminin hydrogel only	DCX	(104)
Laminin-rich porous sponge	Implanted into injured cortex 3 days post-injury	Mice	Cryogenic cortical injury	Increased DCX-positive and GFAP-positive cells within lesion site	DCX, GFAP	(164)
N-cadherin-containing gelatin sponge	Implanted into the injured cortex 3 days post-injury	Mice (neonatal)	Cryogenic cortical injury	Increased DCX-positive neuroblasts in injured region compared control; increased SVZ-derived NeuN-positive neurons in the injured cortex 28 days post-injury	DCX, NeuN	(165)
PEM-PCL electrospun scaffold (graphene coated)	Implanted to intercept the SVZ and span both dorsally and ventrally	Rats	Implant tract	DCX-positive neuroblasts diverted from the SVZ and migrated along the scaffold	DCX	(166)

(Continued)

TABLE 2 | Continued

Biomaterial approach (alphabetical)	Administration or implantation strategy	Species	Type of injury	*In vivo* treatment effect	Evaluation method	References
Peptide amphiphile (self-assembling) carrying a Tenascin-C signal (E_2Ten-C PA)	Injected to intersect the RMS and span toward the neocortex	Mice	Implant tract	DCX-positive cells diverted from the RMS and migrated along implant tract (seen at 7 days post-injection)	DCX	(167)
PCL electrospun scaffold encapsulating BDNF-mimetic	Implanted to intercept the SVZ and span toward the cortex	Rats	Implant tract	DCX-positive neuroblasts diverted from the SVZ and migrated along the implant tract toward the cortex; SMI32-positive neurites observed in the scaffold 21 days post-implantation	DCX, SMI32	(168)

SVZ, subventricular zone; NPC, neural precursor cell; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; HAMC, hyaluronan and methylcellulose; EGF, epidermal growth factor; PEG-EGF, poly(ethylene glycol)-modified EGF; EPO, erythropoietin; HPA, hydroxyphenylpropionic acid; CMC-Tyr, carboxymethylcellulose-tyramine; GDNF, glial cell-line-derived neurotrophic factor; ET-1, endothelin-1; PCL, poly ε-caprolactone; PEM, polyethyleneimine; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; VEGF, vascular endothelial growth factor; Syn1, synapsin 1; DCX, doublecortin; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; MCAO, middle cerebral artery occlusion.

stable structure of the TE-RMS is predicted to reliably redirect the migration of endogenous NPCs from the SVZ to injured and/or degenerated brain regions over time. The characteristic diameter of these scaffolds is suitable for diffusion-based mass transport from the host vasculature, providing benefits for acute and chronic survival of TE-RMS implants within the brain. We hypothesize that this advanced technology will act as an exogenous migratory stream, enabling endogenous neuroblasts to navigate from one region to another, mature into appropriate end-target neuronal phenotypes, and synaptically integrate with host circuitry.

The overall success of the TE-RMS as an implantable technology relies on its ability to replicate *all* known functional aspects of the RMS—that is, to direct neuronal migration, support maturation, and potentially prime immature neurons for integration into circuitry via bidirectional communication with the astrocytes comprising the living scaffold. Studies to date have shown *in vitro* chain migration of immature neurons through the TE-RMS, but not through biomaterial (collagen) control columns (40) (**Figure 4D**). Future studies in our laboratory will quantify the expression of astrocytic surface markers of cells comprising the TE-RMS and compare that with surface expression of astrocytes within the endogenous RMS. We will then evaluate the ability of this implantable microtissue to direct and sustain immature neuronal migration, influence cell fate determination, and promote neuronal regeneration throughout diverse brain regions in adult rodents. Future implantation studies will evaluate subjects for at least 6 months to assess the chronic fate of the new neurons as well as the fate of the glial cells comprising the TE-RMS. Importantly, future studies will explore the ability to fabricate these constructs with astrocytes derived from adult human mesenchymal stem cells [for examples, see (179)]. The ability to fabricate the TE-RMS from an adult human stem cell source will lay the foundation for a new approach to create patient-specific autologous implants, minimizing the risk for rejection after transplantation.

CHALLENGES TO OVERCOME AND POTENTIAL FUTURE APPLICATIONS

There are notable challenges to overcome to successfully utilize biomaterial and tissue-engineered technologies to harness endogenous NPCs for repair purposes (**Figures 5A–G**). To be effective treatments to offset neuronal loss, these technologies must deliver a sufficient quantity of NPCs to neuron-deficient brain regions (**Figures 5B,C**). Redirected NPCs must also exhibit proper neuronal differentiation, maturation, and integration with local neuronal circuitry (**Figures 5E–G**). Additionally, consideration must be given to how a diseased neuronal environment may affect the biomaterials that compose these technologies, and how the long-term presence of these scaffolds within the brain may alter the environment and affect neuronal and/or glial function (**Figure 5D**). The biocompatibility of all foreign materials must be confirmed, the timing of implantation and degradation must be determined, and rigorous testing, imaging, and analysis will be necessary as research progresses toward clinical utilization of these technologies.

The majority of the tissue-engineered scaffolds discussed in this review rely on the presence of NPCs within the SVZ to function (**Figure 5B**). For these technologies to translate into potential treatments for neurodevelopmental disorders, neurodegenerative diseases, and various brain injuries, they must reliably deliver large numbers of endogenous NPCs over long distances into neuron-deficient regions at a rate sufficient to combat neuronal loss (**Figure 5C**). This inherently requires that the SVZ contains a large enough NPC precursor population to supply sufficient cell numbers at the time of scaffold implantation, regardless of the age of the patient. Whether neuronal proliferation in the human SVZ becomes altered in the injured or degenerated brain remains unknown. As discussed previously, while some research has provided evidence that SVZ NPC proliferation and neuroblast migration are increased in the injured and degenerated human brain (124, 125, 127), these findings remain controversial. Increased SVZ proliferation would

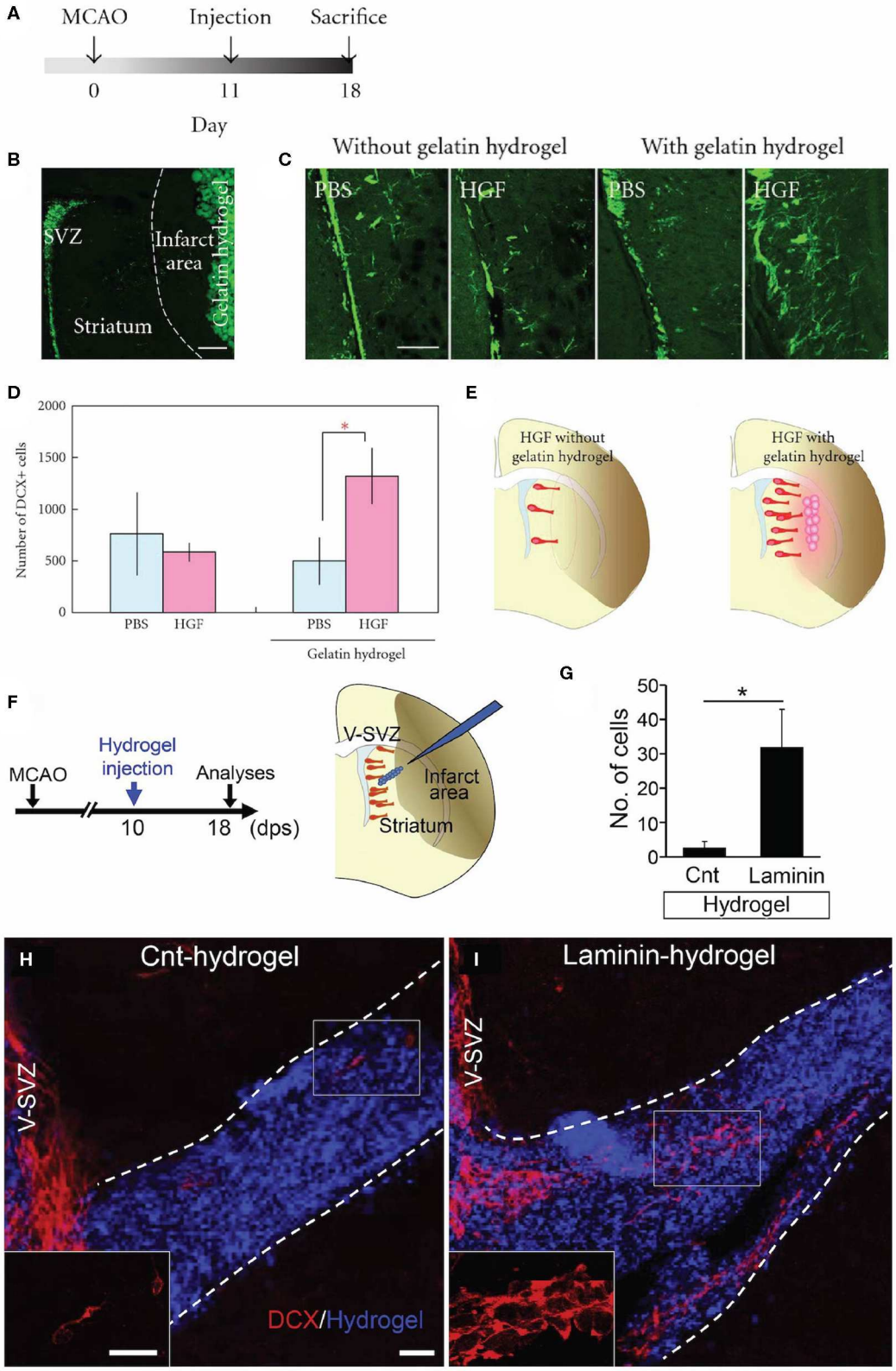

FIGURE 3 | Acellular biomaterial approaches to redirect endogenous NPC migration to regions of injury. Experimental results from *in vivo* implantation of **(A–E)** HGF-containing gelatin hydrogel or **(F–I)** laminin-rich hydrogel. **(A)** Experimental design utilized by Nakaguchi et al. (159). **(B)** DCX-positive (green) cells in the SVZ and

(Continued)

FIGURE 3 | gelatin hydrogel injected into the striatum of a coronally-sectioned rat brain. **(C)** Coronal brain sections depicting a higher quantity of DCX-positive cells (green) in the striatum elicited by injection of HGF-containing hydrogel compared to a PBS-containing hydrogel or gelatin hydrogel alone. **(D)** Quantification of DCX-positive cells in the ipsilateral striatum (seen at least 50 microns from the ipsilateral SVZ). **(E)** Schematic illustrating that the HGF-containing hydrogel was more efficacious at recruiting new neurons to the injured striatum compared to injection of HGF alone. **(F)** Experimental design utilized by Fujioka et al. (104). Schematic illustrates the injection of a self-assembling laminin-rich hydrogel into the striatum following experimental stroke. **(G)** Quantification and **(H,I)** confocal microscopy images of DCX-positive cells (red) migrating along hydrogels with and without laminin (blue). A greater quantity of DCX-positive cells is seen migrating along the **(I)** laminin hydrogel compared to **(H)** the hydrogel without laminin (Cnt-hydrogel). Scale bars **(B,C)**: 200 microns; **(E)**: 20 microns. Reprinted with permission from Nakaguchi et al. (159) for **(A–E)** and Fujioka et al. (104) for **(F–I)**. $^{*}p < 0.05$.

increase the pool of NPCs that could be harnessed for redirection, providing tissue-engineered scaffolds the necessary cell source for diversion from the SVZ into neuron-deficient regions. However, if injury or disease impairs proliferation within the SVZ, then the capacity of these scaffolds to deliver NPCs to atrophied regions would be diminished. If the SVZ lacks NPCs, then scaffolds must be modified to induce NPC proliferation within this niche such that it contains sufficient cell numbers or be combined with a complimentary strategy to increase SVZ capacity. Current knowledge regarding how neurogenesis is regulated in the rodent SVZ (78, 80) will instruct future research investigating how endogenous neurogenesis may be regulated in the human SVZ. Mechanisms instructing human SVZ neurogenesis will be critical to uncover in order to understand how neurogenesis in the human SVZ could be upregulated simultaneously with the implantation of these technologies. If the number of proliferative cycles for SVZ NPCs turns out to be limited, causing injury-induced increases in NPC production to be offset by long-term reductions in neurogenesis later in life, then strategies to create or induce an infinite *in vivo* stem cell niche should be explored. Additionally, if future research reveals a complete lack of neurogenesis in the adult human brain, then the tissue-engineered scaffolds discussed herein can be modified to include their own pool of NPCs. That is, a tissue-engineered "neurogenic niche" can be included to provide an NPC source for sustained delivery into a lesion by the TE-RMS. Such a transplantation strategy will be designed to slowly deliver the encompassed NPCs into neuron-deficient regions, making this approach advantageous compared to traditional exogenous cell transplantation techniques that overwhelm neuron-deficient regions with large numbers of new neurons simultaneously.

Additionally, the success of these technologies relies on the ability of neuroblasts directed from the SVZ to regions of injury or degeneration to differentiate into subtype- and region-specific appropriate phenotypes (**Figure 5E**). There is preclinical evidence that enhancing SVZ neuroblast migration into a striatal lesion following MCAO in mice leads to differentiation of neuroblasts into destination-appropriate striatal projection neurons, integration into existing circuitry, and improved functional recovery (110). However, despite this evidence, the capacity for SVZ neuroblasts to assume diverse phenotypes appropriate for replacement and repair throughout the brain, and whether these processes will become altered in the diseased or injured human brain, remains unknown. A more comprehensive understanding of the cellular and molecular controls over neuronal differentiation both during development and in the adult brain is required to understand if this will be possible (180). Scaffolds may need to be modified to encompass additional signals and cues that will promote successful neuronal migration, maturation, differentiation, and integration into various brain regions. Modification may be required for each specific phenotype of neuron that needs to be generated. Here, our TE-RMS scaffolds have the potential to be further engineered to provide astrocyte-based cues to direct the time course of neuronal differentiation and subtype specification. We envision that these scaffolds may need to be designed to contain specialized factors and/or cues depending on which area of the brain requires NPCs and the neuronal subtype requiring replacement. If these technologies are modified to include additional soluble factors (such as NTFs suspended in the ECM encapsulation), it will be critical to determine the dose, total inclusion volume, and release rate over time. In addition, appropriate methods that allow for accurate *in vivo* phenotype identification of neurons need to be developed and validated to ensure that newly migrated cells develop into mature, region-appropriate neurons. Thus, in addition to investigating whether these technologies are capable of modulating proliferation and migration of cells within the SVZ, it is important that future research will be directed at whether these technologies have the ability to direct cell fate, as this will be a true determinant of whether these technologies can successfully promote functional neuronal replacement.

In addition, appropriate integration and connectivity of neurons will be essential for proper function within new brain regions (**Figures 5F,G**). Strategies to promote appropriate axonal extension and proper axon pathfinding of introduced neuroblasts may need to be built into these technologies or introduced in parallel with implantation of tissue-engineered scaffolds. In this respect, scaffolds that promote gradual introduction of neuroblasts, mimicking development-like processes, may be more efficacious than introduction of large numbers of neuroblasts all at one time. Indeed, if neuroblasts are introduced gradually over time, then conditions may be more favorable for proper assimilation into local networks. However, this will need to be rigorously evaluated and verified. Additionally, gradual introduction of NPCs into specific brain regions may not be efficacious if long-distance connections need to be simultaneously re-established within the brain. In this case, other complementary technologies may be used in parallel to repair lost connectivity. For example, our laboratory has previously developed micro-tissue engineered neural networks (micro-TENNs), which are implantable living scaffolds composed of neurons and preformed long-projecting axonal tracts encased within hydrogel microcolumns designed to recapitulate the

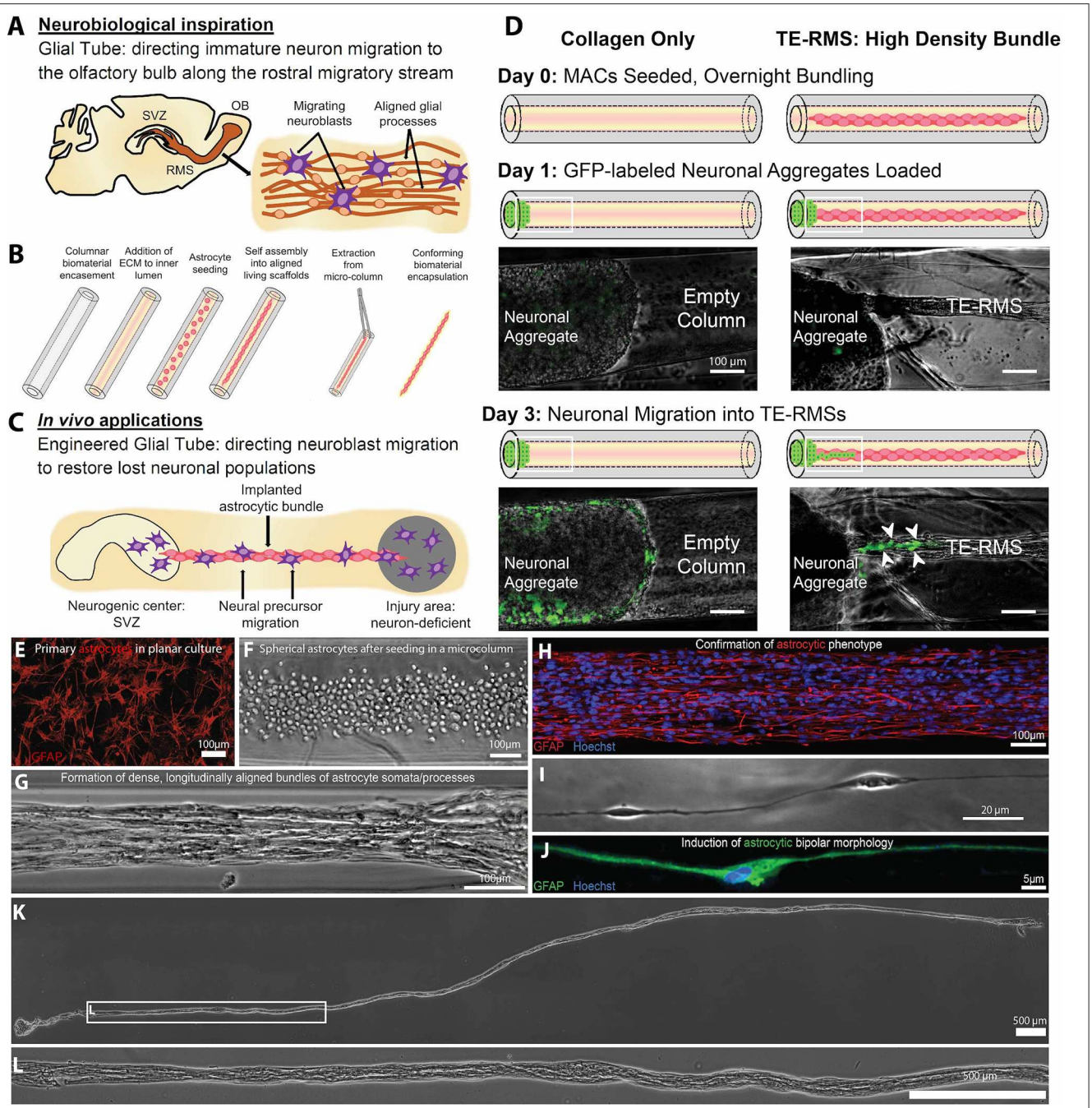

FIGURE 4 | A tissue-engineered rostral migratory stream (TE-RMS) for directed neuronal replacement following brain injury. Schematic illustration of **(A)** the rodent rostral migratory stream comprised of aligned astrocytes, **(B)** the TE-RMS fabrication process, and **(C)** predicted *in vivo* applications of this technology. **(D)** GFP-transduced cortical neuronal aggregates were seeded at one end of hydrogel columns containing either fully formed TE-RMSs or collagen only. Immature neurons migrated out of cortical aggregates and along TE-RMSs, but not along acellular collagen-only columns. White arrows indicate migrating immature neurons. Fluorescence microscopy and phase contrast images showing **(E)** the GFAP-positive astrocytes with stellate morphology used to fabricate TE-RMSs, **(F)** the high density of astrocytes shortly after seeding into a collagen-containing microcolumn during TE-RMS fabrication, **(G)** self-assembly of astrocytes into dense longitudinal bundles during TE-RMS formation, **(H)** maintenance of astrocyte alignment and bipolar morphology post-extraction from the microcolumn, and **(I,J)** individual aligned astrocyte processes within microcolumns. **(K,L)** Phase contrast images demonstrating that centimeter-scale TE-RMSs (>1.5 cm) maintain integrity when extracted from the hydrogel microcolumn. Scale bars **(D–H)** 100 microns; I: 20 microns; **(K,L)**: 500 microns. Adapted with permission from O'Donnell et al. (40) for **(A–D)** and Winter et al. (39) for **(E–L)**.

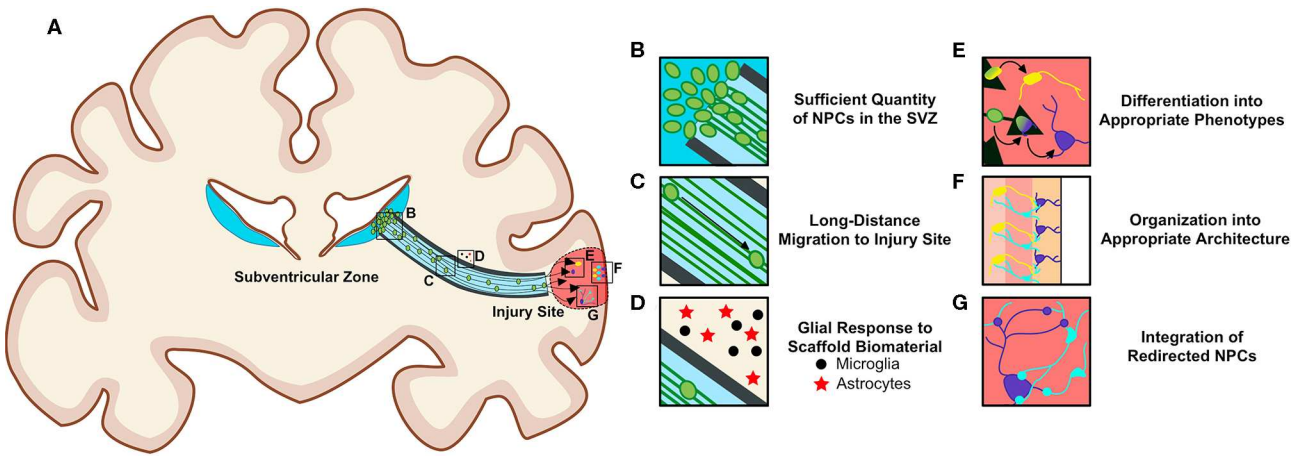

FIGURE 5 | Requirements for the successful redirection of endogenous neuronal stem cells with biomaterial and tissue-engineered scaffolds. **(A)** Schematic depicting a nondescript tissue-engineered scaffold implanted into a gyrencephalic brain spanning from the SVZ to a region of injury. In order for scaffolds to successfully re-direct endogenous NPCs, there **(B)** must be a sufficient quantity of NPCs in the SVZ, **(C)** neuroblasts must migrate long distances within the scaffold, and **(D)** biomaterials composing the scaffolds should not elicit a detrimental immunogenic foreign body response from local and/or recruited glial cells surrounding the implant. Redirected NPCs must **(E)** differentiate into appropriate phenotypes, **(F)** arrange into appropriate architecture, and **(G)** integrate into existing circuitry once they reach their new destination within the brain. Scaffolds may need to be adapted to promote maturation into multiple neuronal phenotypes if differentiation cues at destination are insufficient. Consideration must also be given to how a diseased environment may affect the health of the redirected NPCs. Long-term biocompatibility of the scaffolds with surrounding brain tissue, appropriate degradation rate, and optimal time window for implantation must also be considered. Original figure created by Dayo Adewole, Department of Bioengineering, University of Pennsylvania.

structure and function of specific neuronal-axonal pathways (176, 181–186). Parallel implantation of micro-TENNs, which are designed to reconstruct damaged or lost long-distance axonal circuitry via synaptic integration within host tissue, and TE-RMSs may synergistically act to replace local neurons and re-establish long-distance axonal connections across damaged brain regions. Moreover, implantation of scaffolds may be more effective when combined with other therapies to provide injury-specific rehabilitation of individual brain regions.

In addition to the establishment of proper integration, another challenge will be determining the effect that the environment may have on the neuroblasts that migrate to regions of injury or degeneration. Even if implanted scaffolds successfully facilitate neuroblast relocation to degenerated and/or diseased brain areas, it is still unknown how these aberrant environments may affect the health and integrity of these cells (36). There will likely be many signals from the local neuronal environment that could impair beneficial differentiation and integration of redirected neuroblasts. For example, previous research has demonstrated that transplanted fetal mesencephalic dopaminergic neurons into the brains of human patients with Parkinson's Disease developed alpha-synuclein-positive Lewy bodies (187). Therefore, it could be assumed that *endogenous* neuroblasts redirected to the substantia nigra in patients with PD may also develop Lewy bodies. It is highly plausible that neuroblasts redirected to areas of injury and degeneration could take on aberrant phenotypes caused by signals that are present in these diseased environments. The addition of neuroblasts to certain regions of the brain could also cause additional abnormal functioning of the brain microenvironment, leading to seizures, pain, or new neurological deficits.

Consideration must also be given to whether the presence of foreign biomaterials in the brain will cause changes in inflammation or aberrant functioning of resident glial cells (**Figure 5D**). Research has demonstrated that the process of inflammation and the phenotype of microglia and macrophages are critical in determining the extent of regeneration that can occur post-injury in the CNS (188). It is important to consider how microglia and macrophages influence inflammation in the brain after injury, and how tissue-engineered technologies may need to be modified such that they secrete factors to facilitate glial cell phenotypes that will not be detrimental to the integration of newly migrated neuroblasts into local circuitry (188).

Timing of implantation will be another important determinant of whether these technologies will effectively replace lost neuronal populations. Introduction of scaffolds in the acute phase following injury and/or degeneration may limit further degeneration by promoting regenerative effects prior to major cell loss or damage. However, introduction of scaffolds immediately or soon after injury may expose these implants to a hostile microenvironment induced by the effects of resident inflammation and necrosis, potentially altering the ability of scaffolds to integrate, the time course of scaffold degradation, and the ability of scaffolds to redirect neuroblasts and promote cell survival. Therefore, if the goal is neuronal replacement, then scaffold implantation may be the most successful when introduced during a sub-acute or intermediate phase following brain injury and/or degeneration after factors contributing to acute toxic and detrimental environments have had a chance to subside (175). Delaying scaffold implantation until the secondary injury environment has stabilized may maximize the regenerative and restorative capability of these technologies. Determination

of the optimal time window for scaffold implantation following each specific injury or disorder will be critical to appropriately select patients most likely to benefit from these therapies.

Importantly, it is also unknown whether biomaterial and tissue-engineered scaffolds discussed herein have long-term compatibility with human brain tissue. Consideration must be given to how specific biomaterials may react to the brain microenvironment *and* how the brain microenvironment may react to the presence of foreign biomaterials. These technologies must be rigorously tested before implantation into the human brain can become conceivable. Longitudinal implantation studies must be conducted in multiple animal models, likely including gyrencephalic species to at least approach the neuroanatomy and scale of the human brain. These studies should be repeated by multiple research groups to ensure replicability. Scaffold removal would likely cause undesirable tissue damage, so it may be beneficial to design scaffolds to ultimately biodegrade rather than requiring removal. Consideration must therefore be given to when and how scaffolds will biodegrade, and whether the byproducts will be safe for the brain to metabolize and excrete. The biomaterial components of our living scaffolds, including the TE-RMS, are designed to chaperone delivery and then be gradually reabsorbed, leaving only the anatomically-inspired living component (38, 39, 176, 181–186). TE-RMS scaffolds may require optimization of ECM-based encapsulation techniques to maximize stability and integration while minimizing the microtissue footprint.

There are additional challenges associated with the clinical implementation of regenerative medicine products, particularly biomaterial-based devices and tissue-engineered medical products. Standardized protocols need to be developed for scaffold implantation that take into consideration the timing, location, and logistics of implantation, and can be adapted to diverse patients with heterogeneous injuries and/or degeneration. Additionally, it will be critical to optimize scaffold dimensions, delivery devices, and injection rate to ensure preservation of brain tissue surrounding the implant. Each component composing various scaffolds will need to undergo certification of biocompatibility and lack of any cytotoxic sub-components. Studies will be conducted to examine possible toxicity and immune reactions resulting from the long-term presence of these materials in the human brain. All biomanufacturing procedures must adhere to the FDA current good manufacturing practice (cGMP) regulations. Rigorous regulatory requirements will include quality assurance protocols, including health and potency following storage and distribution of biomanufactured products, particularly those that include live cells. There will be stringent preclinical and clinical testing requirements to ensure product safety and efficacy that will require significant time and expense to achieve.

Finally, the development of reliable, non-invasive imaging methods would be useful to allow for visualization of scaffolds within the brain post-implantation. Once in the brain, it will be critical to visualize them at regular intervals to ensure they are degrading appropriately (if relevant) and not causing aberrant effects. Ideally, imaging methods should have the capacity to elucidate whether the scaffolds are working to appropriately redirect endogenous neuroblasts to neuron-deficient brain regions. As these challenges indicate, there are many milestones to overcome before tissue-engineered technologies will be safe for delivery into the human brain.

As the development of biomaterial and tissue engineering strategies to redirect endogenous neuroblasts advances, uncertainty regarding the existence of the adult human SVZ and RMS raises questions regarding whether these technologies *realistically* have the potential to redirect endogenous neuroblast migration in the human brain. If future research reveals that there is a very low quantity of endogenous NPCs that exist within the adult human SVZ, then biomaterial scaffolds designed to redirect endogenous NPCs are unlikely to promote neuronal repair on their own. Complementary strategies such as gene therapy or stem cell implants to rejuvenate the SVZ may be explored in parallel (189–193). Alternatively, future research may reveal that the human SVZ contains a sufficient quantity of NPCs to be redirected but that the RMS is non-existent or ineffective at eliciting neuronal migration within the adult brain. If lack of a functional RMS is the only factor preventing neuroblast migration, then perhaps replacement of an RMS-like structure is all that would be needed in order to elicit neuroblast migration out of the SVZ in the adult brain. We recognize that implantation of these technologies will most likely need to be coupled with a significant increase in endogenous neurogenesis in the human SVZ in order for endogenous neuroblasts to be successfully redirected. However, lack of knowledge regarding the existence and function of the SVZ and RMS within the adult human brain should not prevent the field from pursuing the development of technological repair strategies that build off of the existence of endogenous NPCs in the adult human brain.

With this in mind, we predict that tissue engineering and biomaterial strategies that redirect endogenous neuroblasts throughout the brain have the potential to serve as beneficial treatments for a variety of neurodevelopmental and neurodegenerative diseases as well as acquired brain injuries. We recognize that discussion of potential clinical utilization of these strategies is currently quite speculative. However, it is important to acknowledge the diverse potential applications envisioned for human patients while these technologies are still in the process of being developed, as the intended clinical applications will certainly influence key design decisions. Examples of diseases and disorders across the human lifespan for which these technologies may eventually be applicable are outlined in **Table 3**. A wide variety of developmental disorders such as fetal alcohol syndrome disorders (194), high-functioning autism spectrum disorders (195, 196), Tourette syndrome (197), attention-deficit/hyperactivity disorder (198–201), 22q11.2 deletion syndrome (202), childhood absence epilepsy (203), and hypomyelination with atrophy of the basal ganglia and cerebellum (204, 205) are characterized by thinning of specific brain regions. It is possible that implantation of tissue-engineered scaffolds into the developing brains of infants and children leading to controlled redirection of endogenous neuroblasts into relevant neuron-deficient

TABLE 3 | Human diseases, disorders, and injuries that are characterized by neuronal loss in focal brain regions.

Injury, disease, or disorder	Brain region lacking neurons	Relevant references consulted
Developmental disorders		
Fetal alcohol syndrome disorders	Cortical thinning in the bilateral middle frontal lobes, lateral and inferior temporal and occipital lobes, pre- and post-central areas	(194)
High-functioning autism spectrum disorders	Cortical thinning in the left temporal and parietal cortices	(195, 196)
Tourette syndrome	Cortical thinning in frontal and parietal lobes (specifically in ventral sensory and motor regions)	(197)
Attention-deficit/hyperactivity disorder	Cortical thinning in frontal, parietal, and temporal lobes (specifically in pars opercularis, medial temporal cortices, medial and superior prefrontal and precentral regions)	(198–201)
22q11.2 deletion syndrome	Cortical thinning in superior parietal cortices, right parietooccipital cortex, and bilateral pars orbitalis	(202)
Childhood absence epilepsy	Cortical thinning in left orbital frontal gyrus and bilateral temporal lobes	(203)
Hypomyelination with atrophy of the basal ganglia and cerebellum	Small putamen and caudate nucleus; cerebellar atrophy	(204, 205)
Neurodegenerative diseases		
Parkinson's disease	Degeneration of substantia nigra pars compacta	(206, 207)
Huntington's disease	Degeneration of striatal medium spiny neurons in the caudate and putamen	(208, 209)
Amyotrophic lateral sclerosis	Atrophy of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord	(210, 211)
Acquired brain injury		
Focal ischemic stroke	Various localized brain regions	(212–216)
Focal traumatic brain injury	Various localized brain regions	(217–221)

regions has the potential to partially combat gray matter deficiencies that occur in these diverse developmental disorders. If redirected neuroblasts are able to successfully mature and integrate into functional neuronal circuits within neuron-deficient regions, then perhaps this could mitigate some of the developmental defects that are characteristic of these disorders. While introduction of neuroblasts into gray matter-deficient regions would not be a cure for these disorders, it may offset or lessen some of the symptoms that arise specifically due to gray matter loss in specific developing brain regions.

There are also several neurodegenerative disorders which may benefit from the implantation of the tissue-engineered scaffolds discussed herein. Although disorders of the aging brain are complex and not fully understood, it is known that certain neurodegenerative disorders such as PD (206, 207), HD (208, 209), and ALS (210, 211) are partially characterized by atrophy and neuronal loss in specific brain regions. While there is evidence that neuronal degeneration in PD, HD, and ALS is not confined to these specific regions (206, 208, 209, 222), the most *prominent* and *persistent* neuronal degeneration resulting from these disorders is focal. Recent research has turned to tissue engineering strategies as a means to promote functional brain recovery following injury and degeneration (12, 175, 176, 223). Neurodegenerative disorders characterized by region-specific degeneration, including PD, HD, and ALS, may benefit from implantation of tissue-engineered scaffolds designed to redirect neuroblasts from the SVZ and explicitly deliver them into degenerating regions. The TE-RMS scaffolds being developed by our laboratory, which are specifically designed to provide sustained delivery of immature neurons over time, may prove to be a beneficial strategy to offset the continual degeneration that occurs as these diseases progress. If neurons could be continually replaced at the same rate as they are degenerating, then perhaps this could prolong the function of deteriorating brain regions and lessen some of the cognitive and behavioral phenotypes associated with neurodegenerative disorders.

Biomaterial and tissue-engineered scaffolds may also be advantageous treatments for acquired brain injuries that occur throughout the human lifespan, specifically for focal ischemic stroke and focal traumatic brain injury (TBI). Ischemic stroke can

occur throughout the brain, leading to localized tissue damage in various regions containing diverse neuronal populations (212, 213). The human populations at the greatest risk for ischemic stroke are the elderly, fetuses, and newborn infants (214, 215). While less common, acute ischemic stroke also occurs in children and young adults (216). Focal TBI may also occur at any age throughout life (217–221). Similar to the application of these technologies in neurodevelopmental and neurodegenerative disorders, tissue-engineered scaffolds could be used to redirect neuroblasts from the SVZ to regions that have been damaged due to focal ischemic stroke or focal TBI, thereby providing a potential treatment option to offset neuronal loss resulting from these injuries. These examples illustrate the wide range of clinical applications across the human lifespan that could arise from the successful redirection of endogenous neuroblasts via biomaterial and tissue-engineered scaffolds.

As discussed above, there remain several questions regarding how these tissue-engineered technologies would function in the aberrantly developing, degenerating, and injured brain. One concern would be that redirection of neuroblasts could lead to thinning of the brain regions from which these cells were diverted. This might be especially concerning in application to developmental disorders given the immature status of the brain. Perhaps the biggest challenge in understanding the effect that these technologies would have in the context of neural injuries and disorders is that we do not fully understand the mechanisms that regulate the injuries and disorders themselves. Our predictions regarding how these technologies will function could turn out to be accurate in the healthy brain but unreliable in certain instances of the diseased brain. It is possible that these engineered scaffolds could induce unique *in vivo* effects for each clinical application. There are significant challenges to overcome before these technologies can be used clinically.

CONCLUSION

The presence of neurogenic niches within the adult brain provides endogenous NPCs that can potentially be harnessed to add neurons in cases of developmental disorders or replace neurons that have been lost due to injury or neurodegenerative disease. There have been substantial efforts to harness these cells for neuronal replacement purposes. The pharmacological, biomaterial, and tissue engineering strategies discussed herein that attempt to augment and/or redirect endogenous neuroblast migration from the SVZ have the potential to offset neuronal loss in diverse regions throughout the brain. Current research has provided evidence that these biomaterial and tissue engineering strategies will have the potential to promote neuronal repair following diverse afflictions including acquired brain injury, developmental disorders, and neurodegenerative diseases that arise throughout life. We predict that the most efficacious strategy to augment and redirect endogenous NPC replacement will build upon the brain's intrinsic mechanisms for neuronal replacement and will have the ability to promote sustained neuronal delivery over time. There are still many challenges to overcome—from ensuring biocompatibility of scaffolds to confirming that they can sufficiently promote neuroblast maturation and synaptic integration—before these newly developed technologies will be able to successfully redirect endogenous NPCs to promote neuronal repair.

AUTHOR CONTRIBUTIONS

EP conducted literature review and drafted the manuscript. JO'D, HC, and DC provided significant guidance on manuscript content and critically reviewed the manuscript for important intellectual content. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Potential of Chitosan and Its Derivatives for Biomedical Applications in the Central Nervous System

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It is well known that the central nervous system (CNS) has a limited regenerative capacity and that many therapeutic molecules cannot cross the blood brain barrier (BBB). The use of biomaterials has emerged as an alternative to overcome these limitations. For many years, biomedical applications of chitosan have been studied due to its remarkable biological properties, biocompatibility, and high versatility. Moreover, the interest in this biomaterial for CNS biomedical implementation has increased because of its ability to cross the BBB, mucoadhesiveness, and hydrogel formation capacity. Several chitosan-based biomaterials have been applied with promising results as drug, cell and gene delivery vehicles. Moreover, their capacity to form porous scaffolds and to bear cells and biomolecules has offered a way to achieve neural regeneration. Therefore, this review aims to bring together recent works that highlight the potential of chitosan and its derivatives as adequate biomaterials for applications directed toward the CNS. First, an overview of chitosan and its derivatives is provided with an emphasis on the properties that favor different applications. Second, a compilation of works that employ chitosan-based biomaterials for drug delivery, gene therapy, tissue engineering, and regenerative medicine in the CNS is presented. Finally, the most interesting trends and future perspectives of chitosan and its derivatives applications in the CNS are shown.

Keywords: chitosan, chitosan derivatives, central nervous system, drug delivery, tissue engineering, regenerative medicine

INTRODUCTION

The central nervous system (CNS) consists of the brain, spinal cord, and retina, which are composed of more than 100 billion individual nerve cells surrounded by bone structures (Payne et al., 2019). The CNS has long been recognized as immune-privileged, attributed to the blood brain barrier (BBB) and the lack of lymphatic vessels within the parenchyma (Engelhardt et al., 2016). Nevertheless, the CNS is unable to generate robust adaptive immune responses (Ransohoff and Brown, 2012). In the absence of immediate or long-term medical care, this situation could lead

to permanent damage or death following a severe nervous system injury (Weil et al., 2008). The treatment of CNS diseases gets further complicated by the BBB, which acts as a shield for foreign substances including therapeutic molecules (Huang et al., 2017). Potential treatments against neurodegenerative disorders are considered difficult to implement because of the limited access to the CNS and the aggressiveness of surgical interventions (Tysseling and Kessler, 2017).

It is now known that central axons are capable of regenerating after injury, but their success is highly dependent on their local environment (He and Jin, 2016; Tedeschi and Bradke, 2017). The composition of the microenvironment is defined by the presence of reactive neural cells. Astrocytes and microglia secrete biomolecules as cytokines, chemokines and growth factors in response to insults. These cells display a big heterogeneity (in morphology, function, and gene expression) and have been associated with both beneficial and detrimental regenerative outcomes on CNS injury (Anderson et al., 2014; Karve et al., 2015). It is also known that the adult CNS possesses neural stem cells with the ability to differentiate into neurons and glia. However, these stem cells need a neurogenic microenvironment to achieve migration and differentiation (Gáge and Temple, 2013). Recent advances in biomaterials have encouraged the search to overcome these challenges, either on their own or as vehicles for stem cell, genetic material, or bioactive molecule delivery (Führmann and Shoichet, 2018). These biomaterials can have a natural or synthetic origin. Natural biomaterials often present good biocompatibility, biodegradability, and cell adhesion but can exhibit some disadvantages as poor mechanical properties or trigger an immune response. Their synthetic counterparts are often easier to chemically modify and have low immune responses but may contain toxic substances (Lim and Spector, 2017; Wang Y. et al., 2018). Thus, natural and synthetic biomaterials are frequently used together to exploit the advantages of both, resulting in products with the desired characteristics for each application.

Currently, chitosan is one of the leading natural biomaterials for CNS applications, both in its natural form or as a modified derivative. In biomolecules delivery, it stands out for its penetration enhancement ability and mucoadhesive capacity, which make it a great material for nose-to-brain approaches (Rassu et al., 2016; Yu et al., 2019). In tissue engineering and regenerative medicine, chitosan and its derivatives have shown to promote axonal regeneration, anti-inflammation, and to successfully deliver neurotrophic factors and cells with a consequently functional recovery (Wang Y. et al., 2018). In this way, chitosan-based biomaterials have become increasingly popular to use, alone or in combination with other molecules. This review offers an overview of the physicochemical and biological properties of chitosan and its derivatives. These are useful for different applications, focusing on the delivery of therapeutic molecules and regenerative approaches in the CNS. A literature review was performed through online platforms as PubMed, ScienceDirect, and the National Library of Medicine (clinicaltrials.gov), considering only the works published in the last 5 years. This review aims to show the reader the current trends and limitations of this biopolymer in biomedical applications directed toward the CNS.

CHITOSAN

Chitosan is a polysaccharide mainly composed of D-glucosamine and, in a lower proportion, N-acetyl-D-glucosamine units randomly β-(1-4)-linked. It can be obtained by deacetylation processes of chitin, which has been recognized as the second most abundant polysaccharide in nature, after cellulose. Even though the main source of chitin is crustacean shell, recent technologies have made possible the obtention of chitin and chitosan from other sources like insects and microorganisms (Peniche et al., 2008; Zargar et al., 2015). Particularly, fungal sources have gained increased attention due to some potential advantages like a homogeneous polymer length, a high degree of deacetylation, and high solubility (Ghormade et al., 2017). In general, there are two types of processes to obtain chitosan: chemical and biological. The chemical method is the most commonly performed at an industrial scale, using strong acid and alkaline treatments (El-Knidri et al., 2018). Biological methods involve microorganisms and enzymes (Arbia et al., 2013), but despite the efforts to achieve scalable enzymatic deacetylation, the high crystallinity of chitin remains the main obstacle (Jaworska and Roberts, 2016).

The source and obtention process of chitosan are important factors to consider according to the desired application. These factors define the final product characteristics. For biomedical applications, its purity, molecular weight (Mw), crystallinity, and deacetylation degree (DD) are of great importance (Nwe et al., 2009). These factors deeply correlate with chitosan's mechanical and biological properties. Aranaz et al. (2009) reported the relationship between the physicochemical properties of chitosan and its behavior in biomedical applications. This will be further detailed for each biomedical application described in this work.

The increasing interest in chitosan as a biomaterial is due to its natural origin and several biological properties: biocompatibility, non-toxicity, non-allergenicity, and biodegradability, as well as its antifungal, antibacterial, antioxidant, anti-tumor and anti-inflammatory activities. Besides, it has been recognized as an immunoadjuvant, anti-thrombogenic and anti-cholesteremic agent (Younes and Rinaudo, 2015; Kim, 2018). It also possesses high versatility, so it can be used in many physical forms as fibers (and nano-fibers), gels, sponges, beads, films, particles (and nanoparticles), membranes and scaffolds (El-hefian et al., 2011; Rebelo et al., 2017). All these properties make chitosan adequate for many biomedical applications as drug delivery, gene delivery, tissue engineering, and regenerative therapies, among others. However, when it is used on its own, it has poor mechanical properties in wet conditions and low solubility at pH > 7.0. This situation has led to the search of different strategies to overcome chitosan deficiencies by its combination with other materials or through changes in its superficial structure.

It is important to highlight that chitosan is a polycationic polymer, this attribute is conferred by the protonation of D-glucosamine which forms a positively charged moiety (NH_3^+) at neutral/physiological pH (Muanprasat and Chatsudthipong, 2017).

Despite the versatility that this characteristic gives to chitosan, cationic polymers have been reported as neurotoxic and CNS damage inducers (Li and Ju, 2017). The neurotoxicity has been associated with chitosan's particle size through inflammasome activation (Bueter et al., 2011). However, the evaluation of chitosan's neurotoxicity is still limited and not detailed. On the other hand, chitosan and its derivatives have also been reported as neuroprotective over different neuronal disorders including Alzheimer's and Parkinson's disease, sclerosis, stroke, and injury, among others (Pangestuti and Kim, 2010; Hao C. et al., 2017; Ouyang et al., 2017).

CHITOSAN DERIVATIVES

The molecular structure of chitosan's units contains an amino/acetamido group at C-2, a secondary hydroxyl group at C-3 and a primary hydroxyl group at C-6 (**Figure 1A**). So, the improving modifications that have been developed for this polymer make use of these groups by grafting other molecules. Some of these modifications consist of carboxyalkylation, thiolation, sulfation, phosphorylation, esterification, graft copolymerization, and cross-linking strategies (Mourya and Inamdar, 2008; Muñoz and Zuluaga, 2017). These modifications confer new and unique properties to the obtained products. For example, chitosan has been grafted with heparin to increase its anticoagulant and angiogenic properties, and to increase its affinity for growth factors (Skop et al., 2019). It has also been grafted with laminin-derived peptides to facilitate the attachment of neurons and neurite outgrowth (Kuo and Chiu, 2011). Many molecules can be grafted to improve the application of chitosan to the CNS but have not been evaluated yet. Dicarboxylic acids contain two binding sites that can lead to the crosslinking of chitosan polymeric chains and offer an antioxidant environment. In the same way, hydroxycinnamic acids possesses an important antioxidant activity. For example, the release of ferulic acid into the lesion site of traumatic brain injury (TBI) has shown to effectively protect further secondary injury through the inhibition of neurological oxidative stress (Dong et al., 2015). Nevertheless, these acids have mainly been grafted to chitosan to modify its physicochemical characteristics as solubility, thermal stability, or rheological properties, and have not been widely studied into the CNS (Liu et al., 2017).

Among the most commonly used modifications of chitosan for biomedical applications directed to CNS are carboxymethylation, N-trimethylation, and thiolation (**Figures 1B–D**, respectively). These modifications confer new properties to chitosan, as solubility and mucoadhesiveness, converting these biomaterials into proper substrates for biomolecule delivery. Chitosan graft copolymerization is also widely used for CNS application, because it allows to obtain polymers with controlled structures and activities. These are defined by the graft characteristics, including the structure of the molecule, its length, and number (or binding degree). Copolymerization is widely used for the elaboration of tailor-made scaffolds (Mourya and Inamdar, 2008). In addition to the graft attributes, the binding site also plays an important role in the final properties of chitosan-based biomaterials. Ding et al. (2014) produced 6-O-sulfated chitosan and observed a strong effect of the sulfate site in promoting the neural differentiation of mouse embryonic stem cells.

Carboxymethyl Chitosan (CMC)

The introduction of carboxyalkyl groups into the structure of chitosan, as carboxymethyl, has been developed mainly as a strategy for increasing chitosan's solubility. The reaction occurs either at the C6 hydroxyl group or at the NH_2 moiety, giving N-CMC, O-CMC or N,O-CMC as products. These derivatives are amphoteric polymers that produce water-soluble compounds with pH-dependent solubility, water retention

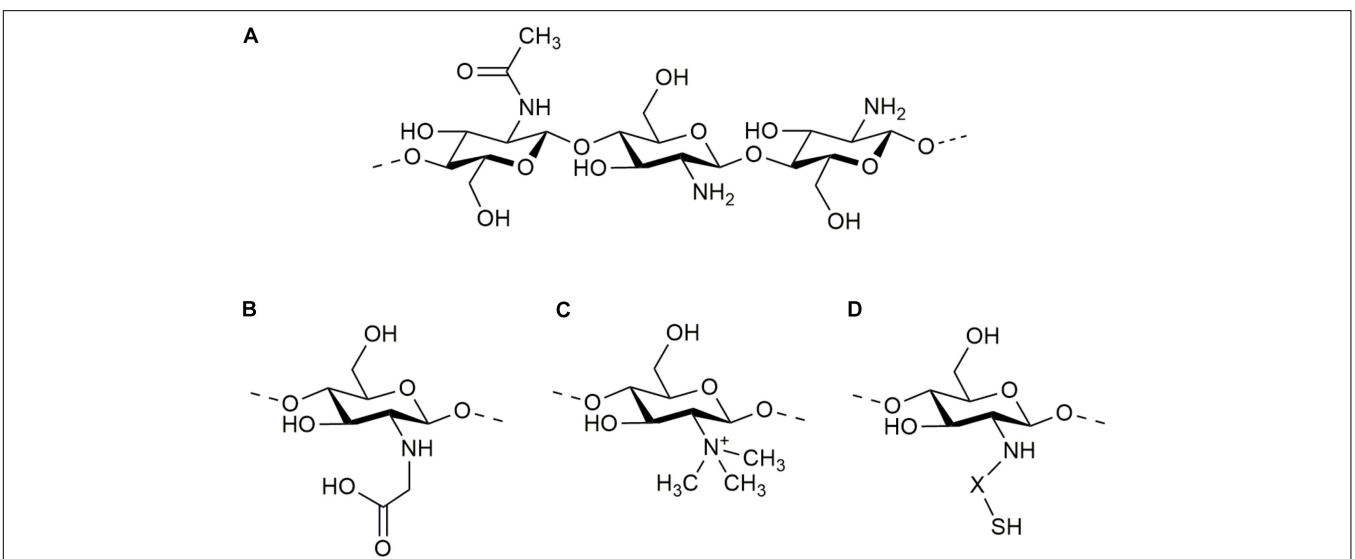

FIGURE 1 | Molecular structure of chitosan **(A)** and some of its derivatives: N-carboxymethyl chitosan **(B)**, N-trimethyl chitosan **(C)**, and thiolated chitosan **(D)**.

properties, biodegradability, biocompatibility, and antioxidant activity (Muñoz and Zuluaga, 2017; Shariatinia, 2018; Xu et al., 2018). Therefore, these amphoteric polymers can be loaded with hydrophobic drugs and display strong bioactivity (Upadhyaya et al., 2014). Moreover, the presence of the functional –OH, $–NH_2$, and –COOH groups in its structure gives the possibility of being easily modified. For example, CMC has been crosslinked with alginate and agarose to be used as a scaffold for stem cell *in situ* differentiation into functional neurons and supporting neuroglia (Gu et al., 2016). CMC has also been employed to enhance the efficacy of active constituents with poor solubility and bioavailability, and increase brain drug concentration (Ding et al., 2016; Liu et al., 2018). However, Wahba and collaborators developed a galantamine delivery system, against Alzheimer's disease, attaching galantamine to ceria-containing hydroxyapatite as well as ceria-containing CMC-coated hydroxyapatite nanocomposites. They found that the CMC coating delayed the *in vitro* release for galantamine and nanoceria (Wahba et al., 2016).

N-Trimethyl Chitosan (TMC)

Methylation consists in the introduction of various alkyl groups at the amino groups of chitosan. The most common product of these reactions is TMC, which is considered one of the strongest mucoadhesive polymers due to its cationic nature (M Ways et al., 2018). That is why it has been used for brain-targeting drug delivery, showing great potential in nose-to-brain applications (Kumar et al., 2013; Meng et al., 2018; Pardeshi and Belgamwar, 2018). Another promising application of TMC is its use to treat brain tumors. For example, Turabee and his team found that the addition of TMC to a pluronic F127 hydrogel increased the biological activity of docetaxel against U87-MG cells. The pluronic F127-TMC/docetaxel hydrogel was evaluated *in vivo* employing BALB/c nude mice and showed sustained release of docetaxel with tumor suppression (Turabee et al., 2019). Similarly, Sedeky et al. (2018) observed a significant improvement in cytotoxicity of Piperine-loaded TMC nanoparticles on human brain cancer cell line Hs683.

Thiolated Chitosan

Thiolation is the reaction of primary amino groups of chitosan with coupling reagents that contain thiol groups (thioglycolic acid, 2-iminothiolane, cysteine, and thiobutylamidine). This product has high permeation, mucoadhesion, higher solubility at physiological pH and displays *in situ* gelling properties (Sreenivas and Pai, 2008). These properties present thiolated chitosan as a good substrate for drug delivery to the brain, mainly used as nanoparticles (Patel et al., 2012, 2013; Singh et al., 2016; Sunena et al., 2019). In this way, Patel et al. (2013) studied brain uptake of cyclobenzaprine HCl-loaded thiolated chitosan nanoparticles on Swiss albino mice after intranasal administration and observed that thiolation of chitosan reduced trans-mucosal toxicity and enhanced the bioavailability. The *in situ* gelling ability makes thiolated chitosan suitable not only for nose-to-brain applications but also for the elaboration of scaffolds. However, it has not been widely used for neural tissue engineering. For this purpose, methacrylamide chitosan has been thiolated, giving as products porous and biodegradable scaffolds that are suitable for cell growth and neural stem cell differentiation in 3D (Yu et al., 2007; Leipzig et al., 2011).

Grafting Copolymerization of Chitosan

Frequently, chitosan is grafted with other polymers to reach copolymerization. The graft polymer is selected by its chemical, mechanical or biological properties and the copolymerization results in a chitosan-based product with added characteristics. For example, polyethylene glycol (PEG)-grafted chitosan derivatives have increased solubility over a wide range of pH and have shown enhanced mucoadhesion (Bhavsar et al., 2017). In this way, 2-O-PEGylated chitosan has been used for the elaboration of siRNA-carrying nanoparticles that target the brain to treat neurodegenerative diseases (Malhotra et al., 2013a). Other polymers that have been grafted to chitosan for CNS application are gelatin (Gao S. et al., 2014), poly lactic-co-glycolic acid (PLGA) (Tong et al., 2017), poly (3,4 ethylenedioxythiophene) (PEDOT) (Wang S. et al., 2018), alginate, and agarose (Gu et al., 2016), among others.

CHITOSAN-BASED DELIVERY SYSTEMS TO CNS

For many years, the increasing incidence of neurodegenerative disorders and the lack of functional treatments have encouraged the search for new therapeutic approaches to counteract CNS diseases. The administration routes directed to the CNS mainly consist of systemic administration, nose-to-brain, and direct injection into the brain parenchyma or cerebrospinal fluid. However, it remains challenging to find effective treatments. One of the main reasons for this is the BBB, which separates the brain from the blood supply and distinguishes between the molecules that can and cannot cross through itself. The BBB allows the entry of nutrients and hormones but restricts other external materials. Therefore, most of the therapeutic molecules are unable to cross and access to the CNS from the bloodstream, following systemic administration (Chatterjee et al., 2019). This situation has led to the development of different strategies for aiding therapeutic molecules to permeate the BBB and to get access to the brain. Dong elaborated a review article providing an overview of the current strategies to enhance drug delivery to the brain (Dong, 2018). According to it, permeability enhancers, active transporters, viral vectors, nanoparticles, and exosomes have been proposed for aiding therapeutic molecules to cross the BBB after systemic administration (Choi et al., 2008; Dong, 2018; He et al., 2018). For direct administration into the brain parenchyma or cerebrospinal fluid, implantable devices have emerged as effective delivery systems that avoid systemic concerns (Stewart et al., 2018). However, most of these strategies have the disadvantage of bearing low drug concentrations or being invasive. Therefore, the use of carriers/vehicles and non-conventional administration routes have emerged as a new approach for facilitating the delivery of therapeutic molecules to the brain (Upadhyay, 2014; Bonferoni et al., 2019). In this way, nose-to-brain administration has also made use of viral vectors,

exosomes, and nanoparticles to achieve less invasive and more effective treatments (Jain, 2012; Belur et al., 2017; Dong, 2018; Li et al., 2019). This information is summarized in **Figure 2**. Nevertheless, the use of biocompatible carriers is encouraged to prevent unwanted effects and achieve high and sustained local drug delivery (Chen et al., 2019).

Drug Delivery to CNS

Chitosan possesses a lot of advantages as a brain-targeted drug carrier. Coupled with its capability to penetrate the BBB, it also can control release, adhere to mucus, and open tight junctions of the nasal membrane. These abilities favor its application in nose-to-brain drug delivery strategies (Mohammed et al., 2017; Yu et al., 2019).

Another advantage of chitosan is its versatility, it can be used for drug delivery purposes as microspheres, capsules, hydrogels, conjugates, nanoparticles, films, beads, or tablets (Ali and Ahmed, 2018). However, nanoparticles have gained special attention in this field due to their capability to protect drugs from degradation during administration (Tzeyung et al., 2019). Chitosan nanoparticles have shown to enhance the brain targeting efficiency and, therefore, to improve the therapeutic potential of drugs (Md et al., 2013; Nagpal et al., 2013). Chitosan has also been used as a nanoparticle coating, to grant drug-loaded nanoparticles with a net positive charge and facilitate cellular internalization (Varan and Bilensoy, 2017).

For drug delivery, it has been reported that the use of low Mw chitosan increases the encapsulation efficiency (Yang and Hon, 2009), reduces cytotoxicity and increases the degradation rate of nanoparticles, properties that have been also associated with higher DDs (Sarvaiya and Agrawal, 2015). On the other hand, the penetration in the mucin layer and the mucoadhesion strength of chitosan increase when the Mw is higher (Rassu et al., 2016). It is worth mentioning that these properties are influenced when chitosan is functionalized, and they depend on the added molecules. For example, Kuo and collaborators, recently developed chitosan-PLGA nanoparticles grafted with anti-aldehyde dehydrogenase and sialic acid for brain tumor-targeted delivery of curcumin (Kuo et al., 2019). They promoted the BBB permeation through N-acetylglucosamine. However, the targeting of the delivery system was improved with the addition of sialic acid and the anti-aldehyde dehydrogenase by directing it to the membrane of glioblastoma cells and brain cancer stem cells.

Administration routes of therapeutic molecules to the CNS	Improving strategies	Advantages	Disadvantages	References
Systemic	Permeability enhancers	Transient and localized BBB opening	Side effects	Dong, 2018 ; He et al., 2018; Choi et al., 2008
Systemic	Active transporters	Ability to cross the BBB	Small molecules and low dose	Dong, 2018
Nose-to-brain	Viral vectors	Non-invasive** and high gene transfection	Safety concerns and limited crossing of the BBB*	Dong., 2018; Belur et al., 2017
Nose-to-brain	Exosomes	Ability to cross the BBB* and non-invasive**	Pharmacokinetics*, loading procedures, and lack of specific biomarkers	Dong, 2018; Li et al., 2019; Zhuang et al., 2011
Nose-to-brain	Nanoparticles	Ability to cross the BBB*, non-invasive**, and targeted delivery	Limited by changes in the BBB* and variability in the absorbed dose**	Dong, 2018; Jain, 2012; Ong et al., 2014
Direct injection to brain parenchyma or cerebrospinal fluid	Implantable devices	Minimal systemic exposure	Invasive and difficult administration	Stewart et al., 2018

*After systemic administration.

**After nasal administration.

FIGURE 2 | Advantages and disadvantages of the current strategies to enhance therapeutic molecules delivery to the CNS.

The use of chitosan and its derivatives for drug delivery to the brain has been employed for developing treatments against many neurological disorders, mainly for Parkinson's and Alzheimer's diseases. Other studies have been guided to treat conditions like depression, schizophrenia, migraine, brain tumor, general anxiety disorder, epilepsy, pain, viral and bacterial infections, and so on (**Table 1**). However, at the time of writing this article, only one of these studies has been taken to clinical trials (Ruppen, 2015). In that clinical research, a nasal ketamine spray with chitosan was evaluated in comparison with oral morphine to treat pain in cancer outpatients but no results have been reported yet.

Gene Therapy

Gene therapy has been set as a form of drug delivery, where cellular machinery is modulated to produce a therapeutic effect (Blau and Springer, 1995). As in drug delivery systems, some of the most remarkable difficulties to direct this technology toward the CNS consist of low BBB permeability, brain heterogeneity, invasive or inefficient routes of administration, and dosing (Joshi et al., 2017). Different types of vectors have been used to overcome these limitations, being the viral ones the most employed (Choudhury et al., 2017). Nevertheless, human infections and immune response caused by viral vectors have led to the search for safer vectors. The aforementioned polycationic property of chitosan confers the polymer the capacity to establish strong electrostatic interactions with negatively charged molecules, like DNA and RNA.

To this day many chitosan-based systems for gene delivery have been employed. Mao et al. (2010) reviewed the formulation factors that affect siRNA and DNA delivery and transfection efficiency. They highlighted that the transfection efficiency depends on many parameters and concluded that intermediate values of Mw and DD of chitosan form complexes of intermediate stability and efficient transfection. Chitosan derivatives have also been employed for this purpose. Specifically, for therapeutic gene delivery to the brain, PEGylation has shown to enhance biocompatibility and stability of siRNA loaded complexes (Gao Y. et al., 2014). Moreover, PEG plays the role of a linker between chitosan and targeting peptides, which

TABLE 1 | Chitosan drug delivery systems for brain targeting reported in the last 5 years.

Drug	Presentation	Application	Administration route	References
Pentamidine	Chitosan coated niosomes	Parkinson's disease	Intranasal	Rinaldi et al., 2019
Methotrexate	Chitosan hydrogel nanoparticles	Antineoplastic agent	Intravenous	Pourtalebi-Jahromi et al., 2019
Carbamazepine	Chitosan coated lipid nanoparticle formulation	Epilepsy	Oral	Ana et al., 2019
Rotigotine	Chitosan nanoparticles	Parkinson's disease	Intranasal	Tzeyung et al., 2019
Doxorubicin/erlotinib	Chitosan liposomal nanoparticles	Glioblastoma	–	Lakkadwala and Singh, 2019
Docetaxel	TMC hydrogel	Glioblastoma	Intracranial injection	Turabee et al., 2019
Risperidone	Chitosan lipid nanoparticle	Schizophrenia	Intranasal	Qureshi et al., 2019
Pramipexole dihydrochloride	Chitosan nanoparticles	Parkinson's Disease	Intranasal	Raj et al., 2018
Galantamine	Chitosan nanoparticles	Amnesia/Alzheimer	Intranasal	Sunena et al., 2019
Selegiline	Chitosan nanoparticles	Parkinson Disease	Intranasal	Sridhar et al., 2018
Temozolomide	Nano lipid chitosan hydrogel	Antineoplastic agent	Intranasal	Khan et al., 2018
Cyclovirobuxine d	Chitosan nanoparticles	Cerebrovascular disease	Intranasal	Wei et al., 2018
Diazepam	Chitosan mucoadhesive microemulsion	Status epilepticus	Intranasal	Ramreddy and Janapareddi, 2019
Tapentadol hydrochloride	Chitosan nanoparticles	Pain	Intranasal	Javia and Thakkar, 2017
Rivastigmine hydrogen tartrate	Chitosan mucoadhesive microemulsion	Cholinesterase inhibitor	Intranasal	Shah et al., 2018
Ribavirin	Chitosan microparticle agglomerates	Viral infection	Intranasal	Giuliani et al., 2018
Huperzine A	Lactoferrin-conjugated TMC surface-modified PLGA nanoparticles	Alzheimer's disease	Intranasal	Meng et al., 2018
Ropinirolle-detran sulfate	Chitosan mucoadhesive neuro-nanoemulsion	Parkinson's disease	Intranasal	Pardeshi and Belgamwar, 2018
Zolmitriptan	Chitosan mucoadhesive nanoemulsion	Migraine	Intranasal	Abdou et al., 2017
Desvenlafaxine	PLGA-chitosan nanoparticles	Depression	Intranasal	Tong et al., 2017
Selegiline hydrochloride	Thiolated chitosan nanoparticles	Depression	Intranasal	Singh et al., 2016
Quetiapine fumarate	Chitosan microemulsion	Schizophrenia	Intranasal	Shah et al., 2016
Rasagiline	Chitosan glutamate nanoparticles	Parkinson's disease	Intranasal	Mittal et al., 2016
Ropinirole hydrochloride	Chitosan mucoadhesive nanoparticles	Parkinson's disease	Intranasal	Jafarieh et al., 2015
Buspirone hydrochloride	Thiolated chitosan nanoparticles	General anxiety disorder	Intranasal	Bari et al., 2015
Doxepin hydrochloride	Chitosan-glycerophosphate-PEG thermoreversible biogels	Depression	Intranasal	Naik and Nair, 2014
Buspirone	Chitosan mucoadhesive microemulsion	General anxiety disorder	Intranasal	Bshara et al., 2014
Donepezil	Chitosan nanosuspension	Alzheimer disease	Intranasal	Bhavna et al., 2014
Levodopa	Chitosan nanoparticles	Parkinson's Disease	Intranasal	Sharma et al., 2014

form complexes with nucleic acid and enhance the cellular uptake of chitosan nanoparticles (Malhotra et al., 2013b; Jiang et al., 2014).

Despite the versatility of chitosan, nanoparticles have been the preferred candidates to counter different neurological disorders. Among these disorders are glioblastoma (Malmo et al., 2013; Danhier et al., 2015; Xu et al., 2015; Van Woensel et al., 2016, 2017), medulloblastoma (Kievit et al., 2015), Parkinson's disease (Peng et al., 2014), Alzheimer's disease (Gao Y. et al., 2014; Rassu et al., 2017), and multiple sclerosis (Youssef et al., 2019). Even viral infections, like HIV-infected brain, have been a target for this therapeutic strategy (Gu et al., 2017). Recently, the search for less invasive strategies has guided the development of novel formulations for nose-to-brain gene delivery. For example, Rassu et al. (2017), made chitosan-coated solid lipid nanoparticles carrying BACE1 siRNA for intranasal application against Alzheimer's disease. Similarly, Van Woensel et al. (2017) formulated siRNA targeting Gal-1 loaded chitosan nanoparticles for intranasal delivery in mice, obtaining remarkable changes in the tumor micro-environment of glioblastoma multiforme. Moreover, Sánchez-Ramos and his collaborators designed a chitosan-Mangafodipir intranasal nanocarrier system for the delivery of siRNA or dsDNA. They employed anti-eGFP siRNA and reported the effectiveness of the nanoparticles for reducing GFP mRNA expression in Tg GFP+ mice along different brain zones (Sanchez-Ramos et al., 2018). These advances suggest an imminent overcoming of the difficulties that limit the CNS-directed gene therapy.

CHITOSAN-BASED MATERIALS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE IN CNS

The design of different chitosan-based biomaterials for tissue engineering and regenerative medicine in CNS aims to facilitate neural cell adhesion, proliferation, and differentiation. These biomaterials can be used as scaffolds to mimic the natural extracellular matrix and microenvironment for better *in vitro* approaches or tissue replacement. Thus, these polymeric materials can be useful to overcome the limitations of cell therapy. For *in vitro* applications, the conformation of the biomaterials must present good biocompatibility and porous structures that favor 3D cell growth. Regenerative medicine requires biomaterials that offer mechanical support for growing neurites. Biological support is also required to lead the processes to tissue restoration through stem cell differentiation and integration into the surrounding healthy tissue (Boni et al., 2018). Moreover, it is important to cause a minimal inflammatory response when implanted. In this way, properties as biocompatibility, biodegradability, mechanical strength, architecture, and cell-adhesion capacity become crucial for biomaterial success.

Gnavi et al. (2013) made a review article detailing the characteristics of chitosan-based scaffolds for nervous system regeneration. They highlighted that the physicochemical properties of chitosan (and modified chitosan) can be easily manipulated to design specific structural features for the scaffolds. According to the required structure and properties for tissue restoration, the biomaterial scaffolds for CNS regeneration can be classified into two types: hydrogels and biodegradable scaffolds (Wang Y. et al., 2018). Chitosan hydrogels can be obtained by physical or chemical crosslinking. The physical associations, like ionic bonding and hydrogen bonds, provide unstable structures while the chemical associations formed by covalent bonds give place to uniform properties. For faster hydrogel biodegradation, it is recommended the use of labile bonds that can be broken under physiological conditions (Pellá et al., 2018). On the other hand, *in situ* gelling can be achieved by physical interactions, providing the advantages of cell delivery without previous geometrical shape preparation of hydrogels and with a less invasive implantation process (Shariatinia and Jalali, 2018). For application in the CNS, hydrogels have been obtained from chitosan (Chedly et al., 2017), its derivatives as CMC (Xu et al., 2018) and chitosan lactate (Nawrotek et al., 2017), and mixtures with other polymers like gelatin (Gao S. et al., 2014). Biodegradable scaffolds are mainly structured by freeze-drying but can be also obtained by electrospinning, solvent evaporation, supercritical carbon dioxide, and 3D printing (Croisier and Jérôme, 2013; Wang Y. et al., 2018; Sun et al., 2019). For porous scaffolds, many chitosan-blends have been made by combining different biodegradable materials, like gelatin (Wang et al., 2017), collagen (Yan et al., 2019), and PEDOT (Wang S. et al., 2018), among others. One of the principal advantages of these scaffolds resides in having stabilized porous structures that can be designed with different size ranges and mechanical properties (Xu et al., 2017).

For non-CNS tissue engineering applications, Mw and DD have been associated with biodegradability and viscosity. Higher Mw gives delayed biodegradation when implanted, and more viscous biomaterials. DD values between 65 and 82% give faster biodegradation (Rodriguez-Vazquez et al., 2015). It is worth mentioning that there is huge variability in the main chemical properties of the starting chitosans used in the reviewed studies, including Mw from 1 (Yao et al., 2018) to 550 kDa (Chedly et al., 2017) and values of 75–95% of DD (Feng et al., 2014; Tseng et al., 2015). Moreover, none of the reviewed CNS application studies in tissue engineering and regenerative medicine evaluates different Mw or DD in their starting materials. Many of the studies do not detail these two important chemical characteristics of their starting chitosan. Otherwise, the main variation in these works consists of using different polymeric blends and ratios as starting materials.

Implanting chitosan-based biomaterials in the CNS provides a way to its poor regenerative capacity through the reconstruction of lost tissue and reconnection of neuronal processes. Although, the incorporation of stem cells and biomolecules into these scaffolds has emerged as an additional strategy to enhance regenerative therapies (Ricks et al., 2014). In this way, biomaterials assist cell therapy as delivery vehicles that promote cell survival and engraftment. Another advantage of the combination of both research areas is that the implanted cells can be separated from the host damaged tissue. Thereby, biomaterials provide an independent microenvironment for cell

differentiation and proliferation, which does not occur in the natural response to damage (Wang Y. et al., 2018).

Beyond the aforementioned physicochemical properties of chitosan that make it a suitable biopolymer to make biodegradable scaffolds and hydrogels, chitosan has neuroprotective properties. Anti-neuroinflammatory activity, suppression of β-amyloid and acetylcholinesterase formation, and anti-apoptosis effects have been reported (Pangestuti and Kim, 2010; Hao C. et al., 2017). These neuroprotective effects promote an adequate microenvironment for cell proliferation in some CNS damage processes.

Chitosan-Based Scaffolding

Over the past few years, many strategies for increasing cell adhesion, differentiation and viability on chitosan-based scaffolds have been implemented (**Table 2**). Different mixtures of biopolymers with chitosan have been employed for modulating the micro-structure of the scaffolds and their properties. For example, collagen copolymerization has proven to promote cell affinity through its arginine-glycine-aspartic acid sequence which is recognized by transmembrane integrins (Kuo and Yeh, 2011). In the same way, polylactic acid copolymerization gives rise to materials with better mechanical properties and it has been cataloged as a perfect synthetic polymer to elaborate composite materials with chitosan (Ebrahimi-Barough et al., 2015; Hoveizi et al., 2015). On the other hand, Abasi et al. (2019) recently developed bionanocomposites of polyaniline-chloride/chitosan and observed that physical factors of the scaffolds (as electrical conductivity and morphology) have a bigger influence in cell-substrate interactions than molecular affinity. Also, Sung et al. (2015) studied the behavior of Neuro-2a cells over flat, micro-, and nano-textured chitosan substrates, and found that cellular adhesion increases over flat chitosan surfaces. Given that the design of the internal structure and surface of the scaffolds is determinant for cell adhesion and proliferation, Sun and collaborators printed a collagen-chitosan 3D scaffold with a specific structure. They observed nerve fibers regeneration and functional recovery after its implantation in rats with spinal cord injury (SCI), showing enhanced therapeutic effects compared with the non-3D-printed material (Sun et al., 2019).

The addition of neurotrophic factors into chitosan scaffolds or microspheres, like nerve growth factor (NGF), neurotrophin-3 (NT-3), or fibroblast growth factor-2 (FGF-2), has shown to enhance neurogenesis, neural differentiation, and cell survival (Yi et al., 2011; Skop et al., 2013; Duan et al., 2015; Hao P. et al., 2017). Rao et al. (2018) elaborated NT-3 – chitosan tubes that promoted neuroprotection, neurogenesis, revascularization, and antiinflammation on SCI conditions. After implantation, they observed robust neural regeneration with motor and sensory functional recovery in rhesus monkeys (Rao et al., 2018).

TABLE 2 | Chitosan-based biomaterials for implantation in CNS or neural cell culture reported in the last 5 years.

Composition	Presentation	Application	Model	References
Collagen and chitosan	3D printed scaffolds	Implantation as therapeutic in SCI	Rat	Sun et al., 2019
Chitosan-multiwalled carbon nanotubes	Nanomaterial scaffold	Culture for implantation	*In vitro*	Gupta et al., 2019
Polyaniline-chloride, chitosan, and NGF	Microporous scaffolds	Tissue engineering	*In vitro*	Abasi et al., 2019
Gelatin and glycine-functionalized polypyrrole-coated poly(vinyl alcohol) with chitosan	Scaffold	Culture for implantation	Mice	Naghavi-Alhosseini et al., 2019
PEDOT, chitosan and gelatin	Scaffold	Substrate for NSC research and neural tissue engineering	*In vitro*	Wang S. et al., 2018
Chitosan and PDGF	Scaffold and microspheres	Tissue-engineered spinal cord grafts	*In vitro*	Chen et al., 2018
Chitosan	Scaffold	Implantation in SCI	Rat	Yao et al., 2018
NT-3 – chitosan	Tube	Implantation in SCI	Monkey	Rao et al., 2018
PEDOT and CMC	Conductive polymer layer/Hydrogel	Neural tissue engineering	*In vitro*	Xu et al., 2018
Chitosan and heparin	Scaffold	Culture of stem cells for implantation	*In vitro*	Moore et al., 2018
NT-3 – chitosan	Chitosan particles	Implantation in TBI	Rat	Hao P. et al., 2017
Chitosan	Fragmented physical hydrogel suspensión	Implantation in SCI	Rat	Chedly et al., 2017
Chitosan lactate	Hydrogel	Implantation in SCI	Rat	Nawrotek et al., 2017
Polyacrylamide, chitosan scaffold, and PLGA nanoparticles	Inverted colloidal crystal scaffold	Culture for iPS differentiation into neurons and implantation for nerve regeneration	*In vitro*	Kuo and Chen, 2017
Alginate, CMC, and agarose	Porous 3D scaffold	Tissue engineering	*In vitro*	Gu et al., 2016
Chitosan and polylactic acid	Nanofibrous scaffold	Culture of stem cells for tissue engineering and cell-based therapy	*In vitro*	Ebrahimi-Barough et al., 2015
NT-3 – chitosan	Tube	Implantation in SCI	Rat	Duan et al., 2015
Chitosan	Scaffold	Culture of stem cells for differentiation and implantation in TBI	*In vitro*	Feng et al., 2014

The implantation of chitosan hydrogels constitutes an interesting possibility for CNS restoration. Chitosan hydrogels have proved to provide a suitable micro-environment for axons regrowth and increase the survival rate of damaged neurons in different animal models. These hydrogels have shown remarkable potential in CNS repair, even in the absence of added trophic factors or without a detailed design of its structure (Tseng et al., 2015; Nawrotek et al., 2017). Chedly et al. (2017) elaborated a fragmented physical hydrogel suspension employing unmodified chitosan for its implantation in rat SCI (immediately after the injury). They observed axonal regrowth, modulated inflammatory response, and long-lasting locomotor function recovery (Chedly et al., 2017). Even though more studies employing chitosan hydrogels are required to define their therapeutic potential in different damage models or degenerative diseases, these results provide a tool for future evaluations in combined repair strategies.

Chitosan-Based Materials and Cell Therapy to the CNS

Spinal cord and brain injury, as well as neurodegenerative diseases, are conducted by different biological processes and cause diverse symptoms, though all of them result in neuronal degeneration and cell death. Cell therapies for CNS have attained clinical research in different pathological conditions like stroke, TBI, amyotrophic lateral sclerosis, and Parkinson's disease, showing their contribution to mitigating damage (Watanabe, 2018). However, within damage processes occur extracellular matrix, neuronal, and glial cell loss. This tissue loss results in a hostile environment for transplanted cells and causes deficient engraftment with poor cell viability (Boisserand et al., 2016). In recent years, the incorporation of different biomaterials to cell therapy in CNS has shown to promote cell survival, integration, and differentiation (Führmann and Shoichet, 2018). In this way, chitosan-based biomaterials have been employed in combination with stem/precursor cells to build a way to neuro-regeneration (**Table 3**). The function of these biopolymeric structures is not only to serve as delivery vehicles and cell physical supports, besides they must regulate the biological microenvironment to guide axonal growth and favor the integration of the healthy tissue to the lesion zone (Boni et al., 2018). Some of the most studied cells for CNS repair are the mesenchymal stem cells (MSC), bone marrow mesenchymal stem cells (BM-MSC), neural stem cells (NSC), and neural precursor cells (NPC).

A study reported by Sugai et al. (2015) showed that modified-chitosan microfibers promote neural stem/progenitor cell proliferation *in vitro* but not cell survival after transplantation, contrary to collagen-based microfibers. The authors proposed

TABLE 3 | Chitosan-based biomaterials for CNS cell therapy reported in the last 5 years.

Composition	Cells	Presentation	Application	Model	References
Collagen and chitosan	BM-MSC	Porous scaffold	Implantation in TBI	Rat	Yan et al., 2019
Polyaniline-chloride, chitosan, and NGF	PC12/NIH/3T3	Microporous scaffold	Neural tissue engineering	*In vitro*	Abasi et al., 2019
Chitosan, genipin, heparin, FGF-2, and fibronectin	NPC/genetically modified NPC	Microspheres	Implantation as therapeutic in TBI	Rat	Skop et al., 2019
Poly(ε-caprolactone), chitosan, and polypyrrole	PC12	Nanofibrous scaffold	Neural tissue substitute	*In vitro*	Sadeghi et al., 2019
Chitosan	BM-MSC	Porous scaffold	Implantation in TBI	Rat	Tan et al., 2018
Methacrylamide chitosan, dibenzocyclooctyne-acrylic acid, and laminin azide-tagged interferon γ	NSC	Conduit	Implantation in SCI	Rat	Farrag and Leipzig, 2018
PEDOT, gelatin, and chitosan	NSC	Scaffold	Neural tissue engineering	*In vitro*	Wang et al., 2017
Chitosan	NSC and MSC	Co-spheroids	Implantation in TBI	Zebrafish	Han and Hsu, 2017
Chitosan	MSC from dental pulp	Scaffold	Implantation in SCI	*In vitro*	Zhang et al., 2016
Chitosan	MSC	Scaffold	Implantation in SCI	Rat	Kim et al., 2016
Chitosan, genipin, heparin, fibronectin, and FGF-2	Retinal ganglion cells	Microspheres	Cellular and growth factor delivery vehicle in TBI	Rat	Skop et al., 2016
Chitosan and gelatin	BM-MSC	Scaffold	Implantation in spina bifida	Rat fetuses	Li et al., 2016
Chitosan and collagen	BM-MSC	Scaffold	Implantation in ischemic stroke	Rat	Yan et al., 2015
Chitosan, polylactic acid, NGF, and bGFG	PC12	Scaffold	Neural cell differentiation for transplantation in a MS model	Mice	Hoveizi et al., 2015
Glycol chitosan and DF-PEG	NSC	Self-healing hydrogel	Implantation in neural injury	Zebrafish embryo	Tseng et al., 2015
Methacrylamide chitosan, collagen, IFN-γ, and acrylated laminin	NSC	Conduit	Implantation in SCI	Rat	Li et al., 2014
Chitosan and gelatin	MSC from human adipose tissue	Scaffold	Implantation in TBI	Mice	Gao S. et al., 2014

that the stiffness of chitosan precluded the colonization of other cells, like vascular epithelial cells (Sugai et al., 2015). The stiffness of the scaffolds should be in the range of 0.1–1 kPa for mimicking soft tissue like the brain. It is well known that stiffness has a notorious influence on stem cell response and function (Liang et al., 2019). Moreover, it has been proved that cell size affects the cellular response to matrix stiffness in 3D cultures, especially large cells as many of the human stem cells (Bao et al., 2019). So, in these cell-scaffold strategies, it is very important to consider the starting material and cell population. The main advantage of using chitosan as the starting material for this purpose is its high versatility. Thus, stiffness and other important characteristics that affect cell behavior, as viscoelasticity, porosity, and topography can be easily modulated.

Besides its versatility, chitosan and its derivatives have shown to be a superior substrate for cell therapy in comparison with other polymers. Scanga et al. (2010) showed the capability of adult murine NPC to proliferate and differentiate into the three neural cell types when they were cultured over chitosan hydrogel films. On the contrary, NPC differentiation was not observed over poly(oligoethylene oxide dimethacrylate-*co*-2-amino ethyl methacrylate) or its blend with poly(vinyl alcohol), neither over poly(glycerol dimethacrylate-*co*-2-amino ethyl methacrylate) (Scanga et al., 2010). Kim et al. (2016) observed a better functional improvement in rats with SCI after MSC transplantation over chitosan scaffolds in contrast to PLGA scaffolds. Moreover, they studied intralesional injection of the same cells and compared it with scaffold-based transplantation in rats. They found a higher MSC engraftment when the scaffolds were employed (Kim et al., 2016). The culture of rat PC12 line and human neural stem cells over chitosan has also shown better results in comparison with cellulose acetate or polyethersulfone derived electrospun nanofibers (Du et al., 2014).

The incorporation of trophic factors as FGF-2, NGF, PDGF, and bGFG has also shown to enhance stemness of neural stem cells and favor its differentiation and proliferation when implanted with chitosan scaffolds or microspheres (Hoveizi et al., 2015; Skop et al., 2016; Moore et al., 2018). Recently, Skop and his collaborators designed a cell-scaffold strategy employing a radial glial neural precursor cell line that conditionally secreted insulin-like growth factor I. This cell line was attached to a chitosan-based microsphere scaffold and injected into the lesion cavity of adult rats with TBI. They observed differentiation toward the three neural cell types (neurons, astrocytes, and oligodendrocytes) and improved capacity for neuronal differentiation. These obtained effects led to the recovery of the somatosensory function. However, the presence of insulin-like growth factor I was not associated with a higher cell retention rate or improved cell replacement. So, the way it improves functional recovery must be elucidated in future studies (Skop et al., 2019).

CONCLUSION

One of the main factors that preclude the application of chitosan is its poor solubility and poor mechanical properties. However, this review summarizes the different strategies that have been used to overcome these conditions. The obtention of carboxymethylated, trimethylated, thiolated and other chitosan-grafted derivatives has increased the potential of this biopolymer, allowing for the elaboration of biomaterials that help to counteract neurological disorders. Nevertheless, there is still a lack of knowledge about the relation of the molecular changes and the acquired biological properties of these derivatives, especially within a heterogeneous landscape as the CNS. In this way, the authors suggest continuing with the exploration of grafting molecules that improve the biological properties of modified chitosan. For example, hydroxycinnamic acids have been studied by some of the authors and resulted in interesting bioconjugates for CNS applications.

Chitosan-based biomaterials have shown favorable projection. For drug and gene delivery purposes, chitosan nanoparticles have shown to be the most promising strategy due to its mucoadhesion and increased permeability that suits nose-to-brain applications. In this way, the degradation of the therapeutic molecules is reduced, and it also opens the door to less invasive and more effective administration routes. In order to achieve neuro-regeneration, the transplant of stem cells into chitosan-based vehicles gives an optimistic outlook. Strategies like the addition of neurotrophic factors or even the genetic modification of stem cells have successfully increased differentiation and viability. The observed functional recovery in different chitosan-based regenerative therapies encourages the exploration of new cell-scaffold-biomolecule configurations. Despite the wide variety of designed compositions and functions, many factors are implied in cell behavior and, until now, there is not a recipe to elaborate adequate chitosan-based biomaterials that fulfill all the requirements for neuro-repair and to transfer these strategies to clinical trials. Even so, the *in vitro* and *in vivo* studies carried out around the world are helping to understand the biological processes involved in neuro-repair and the effect of chitosan biomaterials on them. The authors suggest that future works with chitosan targeting the CNS must intermix the already suggested strategies and propose novel interdisciplinary approaches to attain translation into the clinical level.

AUTHOR CONTRIBUTIONS

DO-H, AC-A, JM-G, UG-P, and JM-D equally contributed to the literature search, writing and correcting of this review manuscript.

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REVIEW
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Neurorestoration Approach by Biomaterials in Ischemic Stroke

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Ischemic stroke (IS) is the leading cause of disability in the western world, assuming a high socio-economic cost. One of the most used strategies in the last decade has been biomaterials, which have been initially used with a structural support function. They have been perfected, different compounds have been combined, and they have been used together with cell therapy or controlled release chemical compounds. This double function has driven them as potential candidates for the chronic treatment of IS. In fact, the most developed are in different phases of clinical trial. In this review, we will show the ischemic scenario and address the most important criteria to achieve a successful neuroreparation from the point of view of biomaterials. The spontaneous processes that are activated and how to enhance them is one of the keys that contribute to the success of the therapeutic approach. In addition, the different routes of administration and how they affect the design of biomaterials are analyzed. Future perspectives show where this broad scientific field is heading, which advances every day with the help of technology and advanced therapies.

Keywords: neurorestoration, repair, biomaterials, stroke, cell therapy

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BACKGROUND

Stroke is one of the most important health problems worldwide. Ischemic stroke (IS) constitutes 85–90% of the casuistry among the types of stroke and is the leading cause of disability in people over 65 years of age worldwide (Ghuman and Modo, 2016). Due to the epidemiological importance and the big socio-economic expenditure involved, it is priority advance in its prevention, control, and treatment (Kalaria et al., 2016; Benjamin et al., 2017). The ischemic injury is caused by an interruption of blood supply in one or more cerebral blood vessels triggering a set of dynamic processes that affect all brain cells and extracellular matrix (ECM) deteriorating the "glioneurovascular niche" (Boisserand et al., 2016).

The pathophysiology of IS lies in the restriction or reduction of the supply of oxygen, glucose, and nutrients in the affected brain area. The ischemic cascade begins while there is arterial obstruction causing accidental cell death of core cells damaging tissue irreversibly. This process is accompanied by events of glutamate excitotoxicity, oxidative stress, and neuroinflammation, which affect the homeostatic functioning of the neurons in the affected tissue. The combination of all of them induces permanent brain lesions (Taylor et al., 2008; Thundyil and Lim, 2015; Thornton et al., 2017). However, there are regions near the nucleus or ischemic penumbra (IP) that have had access to a collateral blood circulation, being able to partially counteract the energy deficit (Fisher and Albers, 2013; Gavaret et al., 2019).

This review will briefly address the limitations and consequences that arise after the stroke, the endogenous repair mechanisms activated by the brain damage itself, how to enhance these mechanisms through tissue engineering and the incorporation of exogenous cells or growth factors.

STROKE STAGE

The pathological picture of IS is aggravated by anatomical and metabolic limitations of the central nervous system (CNS) itself: the glucose and glycogen deposits of the brain are only able to cover the brain's energy requirements for a brief period and the selective nature of the barrier hematoencephalic (BBB) limits the rate of transfer of molecules from the bloodstream to the brain, restricting access to the necessary substrates for cellular metabolism (Lipton, 1999; Bang et al., 2009).

Therefore, the time factor is decisive to minimize the extent of damaged brain tissue around the core. The period in which it is possible to reduce the impact of IS (therapeutic window) ranges from re-perfusion to 6–24 h, which is very restricted (Crunkhorn, 2018). The positive feedback mechanism of bioenergetic failure, oxidative stress, and inflammatory reaction after IS lead to an adverse microenvironment, incapacitating potentially recoverable cells, to resume their functions. Consequently, it causes damage to the ECM, accumulation of extracellular fluid (Baeten and Akassoglou, 2011), and activation of microglia, macrophages, and astrocytes (Denes et al., 2007; Lalancette-Hebert et al., 2007).

Oligodendrocytes and damaged neurons produce a change in the chemical composition of the extracellular medium that serves as a chemotactic stimulus for microglia and astrocytes. Glial cells alter the pH of the medium and produce an exacerbated inflammatory response by secreting pro-inflammatory cytokines, tumor necrosis factor (TNF-alpha), and interleukin (IL1) (Minami et al., 1992; Lambertsen et al., 2005; Dugue and Barone, 2016). Furthermore, they require a long period to phagocyte and degrade the wastes of dead cells. However, it has been shown that microglial activation can maintain and support neuronal survival by secreting anti-inflammatory and neurotrophic factors (Streit, 2002; Harry et al., 2004). In several studies, it has been shown that microglia promote neurogenesis, guiding neuroblasts to the site of injury (Ziv et al., 2006; Fitch and Silver, 2008; Thored et al., 2009).

In addition to the immune response, astrocytes are activated, modifying their phenotype (reactive astrocytes) to express a series of inhibitory factors, such as cytokines and chemokines, converting the damaged area into a region of restricted transit of molecules and axonal cone growth (Wieloch and Nikolich, 2006; Fitch and Silver, 2008; Paixão and Klein, 2010). Besides, reactive astrocytes begin to synthesize large amounts of chondroitin sulfate proteoglycans, forming a fibrous and acellular membrane, known as a glial scar, which acts as a physical barrier (Busch and Silver, 2007; Yoshioka et al., 2010). This rapid reaction of the microglia and astrocytes has in order to contain the damage and prevent it from spreading, quickly sealing the open path.

SPONTANEUS NEUROREPARATION PROCESS

In the first instance, it is necessary to distinguish between the concepts of repair and regeneration. The first of these refers to the replacement of lost cells in damaged tissue with new cells suitable for the niche; while the second refers to the replacement of injured tissue with homologous tissue, which does not occur in the brain (Modo and Badylak, 2019).

After the pathological events, scientific evidence of the spontaneous activation of endogenous repair processes of the damaged area in the ischemic brain that function as compensatory mechanisms has been described (Arvidsson et al., 2001, 2002; Lindvall and Kokaia, 2015). Among them we can highlight two, the neurogenesis and angiogenesis processes.

Neurogenesis

Neurogenesis is defined as the process by which new neurons are formed from precursors, located in specific areas known as neurogenic niches, from where they migrate, differentiate, and integrate into their destiny to become functional neurons (Ohab and Carmichael, 2008). Despite that the subventricular zone (SVZ) is not the only neurogenic niche in the adult brain, it is the main source of precursors that reach the ischemic zone. The transient and spontaneous increase of parents is produced by a shortening of the cell cycle, beginning at 2 days and reaching the maximum in 2 weeks after the beginning of the damage returning to its basal levels at 6 weeks after it (Zhang et al., 2001; Thored et al., 2006; Zhao et al., 2008).

It has been described that neuroblasts, which physiologically migrate via the migratory rostral route (MRV) to the olfactory bulb, are redirected to the injured area (Arvidsson et al., 2001; Ming and Song, 2005; Ohab and Carmichael, 2008). Ectopic migration begins 3 or 4 days after damage ischemic and remains up to 4 months after it. The redirection is produced by stimuli sent from the ischemic zone through two routes: through changes in the composition of the cerebrospinal fluid (CSF) or through the diffusion of signals through the blood vessels (Christie and Turnley, 2012; Lindvall and Kokaia, 2015). Factors involved in the redirection of neuroblasts, such as brain-derived neurotrophic factor (BDNF), stromal cell-derived factor-1 (SDF-1α) and its CSCR4 receptor, monocyte chemoattractant protein-1 (MCP-1), and metalloprotease (MMP-9) matrix released by neuroblasts themselves (Thored et al., 2006; Bagley and Belluscio, 2010).

Angiogenesis

During the IS, some brain areas are supported by access to collateral flow from pre-existing anastomosis. After ischemic damage, the reduction in blood flow leads to both acute and chronic vascular remodeling. This vascular repair process adds to that of neurogenesis to promote the recovery of damaged tissue (Thored et al., 2006).

In recent years, the data obtained from magnetic resonances in experimental models of ischemic damage have revealed vascular

remodeling processes in both acute and chronic phases. This process has been observed due to an increase in cerebral blood volume (CBV) in its late phase induced by spontaneous stimulation of angiogenesis (Arai et al., 2009; Carmeliet and Jain, 2011; Liu et al., 2014). Cerebral vasculature has been defined as a key factor in the progress of pathological processes and in homeostasis. The bidirectional connection between the nervous system (NS) and the vascular system is strongly established in the CNS (Xu et al., 2017). On the one hand, the NS depends on the integration, communication, and functionality of the different vascular cellular phenotypes for their metabolic and nutritional support; and in turn, the vascular system requires nerve innervation for different regulatory mechanisms, such as vasodilation and vasoconstriction (Uhrin, 2019).

Cerebral angiogenesis is closely regulated by mediating angiogenic factors and the local microenvironment. Recently, these factors have been shown to play an essential role in endothelial cell migration, cell identity, and growth and the regulation of BBB; being involved, in addition, in the alignment of vessels-nerves and nerves-artery in the brain (Arai et al., 2009; Carmeliet and Jain, 2011; Liu et al., 2014). Among these angiogenic factors, the following stand out: (i) the endothelial growth factor (VEGF) that stimulates angiogenesis through VEGF-2 receptors; (ii) the netrins that act as bifunctional signals of attractant or repellent guidance depending on the receptors expressed by the different cell types; (iii) fibroblast growth factor (FGF) that maintains vascular integrity; and (iv) platelet-derived growth factor (PDGF) that is crucial for the maturation and functioning of blood vessels (Carmeliet and Jain, 2011).

Therefore, the angiogenesis process has been postulated as a key restorative mechanism in the response to an ischemic event that participates in functional recovery.

Despite the spontaneous stimulation of neurogenesis and angiogenesis triggered by the body itself to restore the damaged area, there are very few precursors that manage to reach the target; and even less, to mature and repopulate the area (Arvidsson et al., 2002). This failure may be due to the inflammatory environment (Kahle and Bix, 2013), to the deficit of functional connections, and the necessary trophic support (Ming and Song, 2015). Therefore, the recovery of neural function depends, for the most part, of the ability of nearby unaffected neurons to generate new synapses, which is known as neuronal plasticity (Wieloch and Nikolich, 2006; Paixão and Klein, 2010).

POWERING NEUROREPAIR PROCESS

The chronification of gliosis and inflammation in the twilight zone makes endogenous repair strategies difficult (Wieloch and Nikolich, 2006). This is the perfect time to establish strategies that enhance and complement endogenous repair mechanisms in order to partially rebuild the tissue damaged and restore neurological function (Arai et al., 2009; Fisher and Albers, 2013; Liu et al., 2014; Thundyil and Lim, 2015). The development of tissue engineering in the brain with ischemic injury has positioned itself as a great promise to overcome these limitations and replace tissue loss (Modo and Badylak, 2019).

Why Use Biomaterials?

Ischemic brain injury causes a reduction in brain volume (atrophy) that includes the elimination of ECM (Moreau et al., 2012). This is a current challenge for the effective treatment of stroke. Therefore, a support structure such as bioscaffolds is required. Biomaterials are natural or synthetic 3D polymer networks (natural or synthetic ED polymer networks) that provide a suitable environment for cells to survive, proliferate, and differentiate, facilitating the formation of ECM (Ghuman et al., 2016) and for cells to be able to restore their function. These two facts are keys to neurorestoration.

The first biomaterial utility is to offer structural support in an injury that leads to loss of parenchyma, thus facilitating the invasion of the different support molecules and the new endogenous cells. This support allows these to overcome the glial scar generated and penetrate the lesion (Meng et al., 2014; Modo et al., 2018).

As for its second utility, in addition to supporting the physical migration of cells, it is also necessary that inductive signals from the biomaterial be produced to initiate migration and cell invasion. Therefore, biomaterials are being widely used as controlled releases of drugs, cells, and exogenous molecules. The advantage of this fact is that they are carriers of the bioactive molecules up to the therapeutic target, being able to control the rate of release (Massensini et al., 2015).

In addition, biomaterials can act as a protective barrier for these molecules against the adverse microenvironment that exists in ischemic tissue. This protection supposes an increase of the effectiveness of the treatment in the target, although it is not eternal, since when the biomaterial degrades, its protection ceases.

Criteria to Take Into Account to Define Your Design

It is important to consider the chemical and mechanical properties that the biomaterial presents, since the success of its functionality and the fate of the transplanted bioactive molecules will depend on them.

Biocompatibility

The first issue to highlight is that it is biologically accepted by the host tissue, producing a minimal immune and inflammatory response and that, in addition, it is able to maintain its benefits during its useful life (Mitragotri and Lahann, 2009; Wang, 2013).The long-term biocompatibility of the material with the Host tissue marks the effectiveness of implantation. The degree of astrocyte and microglial reaction that may appear around the biomaterial is used in *in vivo* studies to terminate the degree of biocompatibility (Fournier et al., 2003).

Biodegradation

The degradation rate of biomaterials is one of its most important chemical properties, since it allows the release of the bioactive molecules it contains and the structural remodeling of the neural network. There are different formats of presentation of the biomaterial according to the polymerization process used; for example, hydrogels are usually designed for slow degradation,

helping or favoring exogenous cells to develop their own ECM (Mano et al., 2007). However, the higher their biodegradation rate, the more likely it is that a rejection reaction will occur. Therefore, it is convenient to find a balance between degradation rate and functionality (Perez-Garnes, 2015).

Functionality

The functionality of the scaffold is defined by its composition, the place of implantation, the route of administration, the fate of the exogenous cells that house and/or the release of the drug, which is achieved through its chemical and mechanical properties.

Composition

Synthetic

One of the most outstanding advantages of synthetic biomaterials is the possibility of obtaining a homogeneous batch production, that is to say, precisely elaborating certain physical-chemical properties (Busscher et al., 2012; Rimondini et al., 2015; Ghuman and Modo, 2016). Uniform manufacturing translates into greater control of their degradation rate, being optimal candidates to be carriers of drugs or small molecules with controlled release after administration. Ultimately, this advantage results in the reduction of the variability in the immune response generated in the host.

Synthetic biomaterials have been widely used for other pathologies but taking into account the characteristics of the brain as host tissue, and its slow rate of degradation; have not been the best candidates to treat the stroke. However, the most widely used synthetic compounds have been polymers of polylactide (PL), polyglycol (PG), polycaprolactone (PCL), and co-polymers of lactide and glycolide (PLGA). This last compound has been used in nanoparticles form, which has positioned it as one of the best synthetic biomaterials to carry substances even in the brain. A recent paper is the one published by Jeong et al. (2019), who encapsulated erythropoietin in PLGA nanoparticles and cholic acid, because it crosses the blood–brain barrier among other advantages.

The formation of bioscaffold can be carried out, by loading the molecules of interest at the site of the lesion itself or by previously cross-linking with the material itself (Yang et al., 2006; Wong et al., 2007; Dash and Konkimalla, 2012).

Another synthetic polymer commonly used is polyethylene glycol (PEG), resistant to protein degradation. A recent study has been published using PEG conjugated urokinase nanogels (PEG-UK) demonstrating that administration of PEG-UK outside the usual therapeutic window could still exert protective effects in permanent middle cerebral artery occlusion (pMCAO) rats through maintenance of integrity of BBB and the inhibition of apoptosis and excito-neurotoxicity (Cui et al., 2020). Authors, as Balasubramanian et al. (2020), have recently published a study based on silicone nanoparticles, with the aim of promoting the migration of endogenous neuroblasts in post-stroke. This type of component has been less used, but it is not less valid and beneficial.

Other studies inspired by natural platelets (PLTs) and their role in targeting adhesion to the damaged blood vessel during thrombus formation have fabricated a biomimetic nanocarrier comprising a PLT membrane envelope loaded with l-arginine and γ-Fe2O3 magnetic nanoparticles (PAMNs) for thrombus-targeted delivery of l-arginine and *in situ* generation of nitric oxide (NO); for the early treatment of IS (Li et al., 2020).

Because the cells are not able to adhere directly to it, recent studies have used combinations of natural compounds such as hyaluronic acid (HA) or gelatin, thus optimizing their characteristics (Sharma et al., 2015; D'souza and Shegokar, 2016). However, synthetic biomaterials have a limited capacity to induce endogenous repair responses, so their majority use has been for prostheses and implants (Koupaei et al., 2015; Yuan et al., 2019).

Natural

Unlike synthetic biomaterials, natural biomaterials are compounds present in the ECM, which increases biocompatibility with the host tissue and the restoration of the adverse microenvironment. The ECM of the nervous tissue constitutes 20% of the cerebral parenchyma and, its functions are directly related to the maintenance of the structure and the cellular signaling (Stabenfeldt et al., 2006; Reing et al., 2009). The objective of the natural scaffolds is to implant in the damaged tissue an ECM "transient or permanent substitute" that facilitates cell growth to form, again, the three-dimensional structure of the tissue to be repaired (Crapo et al., 2012).

The most widespread natural compounds for application in the restoration of tissue defects and improvements in the adverse microenvironment are fibrin, HA-methylcellulose, chitosan, and collagen (Hopkins et al., 2013; Medelin et al., 2018; Osama et al., 2018).

The combination of hyaluronic acid + methyl cellulose (HAMC) has been used for the first time by Gupta et al. (2006) and has been widely used in models of stroke, spinal cord injury, and retinal degeneration (Ho et al., 2019). One of the last published articles has been a study developed by Tuladhar et al. (2020), where they have used this HAMC combination as a vehicle to release cyclosporine and erythropoietin, to promote functional recovery in stroke.

In most cases, they have been used in combination with exogenous cells that enhance endogenous repair mechanisms (Moshayedi and Carmichael, 2013). One of the latest articles published by Fernandez-Serra et al. (2020) is based on a fibroin biomaterial with the same objective, that of recovering post-stroke function, this time encapsulating mesenchymal stem cells. Other compounds, such as alginate, have been used in microspheres (Cui et al., 2013) or recently, to encapsulate cells in combination with synthetic compounds (Islam et al., 2018).

Two of the most abundant compounds in ECM are collagen and HA, which is why their use in biomedicine has been extended in the last decade. Collagen has mechanical resistance and immunogeneity, and fragments derived from active collagen contribute to biological activities such as growth, differentiation, and cell migration, which has facilitated its use in various studies with rodent models in the form of hydrogel (Cross et al., 2010). In the study conducted by Yu et al. (2010) demonstrated in an ischemic mouse model, an increase in cell survival, synapse formation, and an improvement in neural function by implanting a collagen hydrogel combined with neural stem cells (NSCs).

TABLE 1 | Summary of natural and synthetic components of biomaterial.

References	Biomaterial	Composition	Experimental model	Main findings
Meng et al., 2014	Synthetic	Synthetic fibronectin peptide (PRARIY)	Middle cerebral artery occlusion (MCAo) in Sprague–Dawley (SD) rats	- Reduction of infarction size - Significantly functional outcome - Decrease in apoptosis
Modo et al., 2018	Synthetic	Human neural stem cells (HNSCs) on vascular endotelial growth factor (VEGF)- releasing PLGA microparticles	MCAo in SD rats	- Attraction of endothelial cells from the host, establishing a neovasculature interspersed with NSCs
Wong et al., 2007	Synthetic	Poly (ε-Caprolactone) and PLGA polymer	Acute traumatic brain injury (TBI) in SD rats	- Decrease in astrocytic activation - Promotes neural ingrowth - Prevention of the enlargement of the defect
Medelin et al., 2018	Synthetic	Chitlac (A derivative of chitosan)	Primary culture of hippocampal neurons of postnatal (P2–P3) SD rats	- Induces growth and synapse formation *in vitro*
Ju et al., 2014	Synthetic	Hyaluronic acid (HA) hydrogel + PLGA microspheres containing VEGF and Angiopoietin-1 (Ang-1)	MCAo in C57BL/6J mice	- High rate in angiogenesis - Behavioral improvement - Formation a suitable niche for neural restoration
Jeong et al., 2019	Synthetic	Cholic acid-coated poly lactic-co-glycolic acid (PLGA) nanoparticles loaded with EPO (EPO-CA-NPs)	Middle carotid artery occlusion and reperfusion (MCAO/R) technique in rats	-Able to cross the BBB - Reduction in the extent of the infarct volume and cellular apoptosis - Better performance on sensorimotor phenotype than EPO alone
Cui et al., 2020	Synthetic	Polyethylene glycol conjugated urokinase nanogels (PEG-UK)	Permanent MCAO (pMCAO) in adult male SD rats	- Amelioration of the severity of neurological deficits - Decrease in the infiltration of inflammatory cells and the concentration of interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the brain parenchyma - Inhibition of apoptosis and excito-neurotoxicity
Li et al., 2020	Synthetic	Natural platelet (PLT) membrane envelope loaded with l-arginine and γ-Fe_2O_3 magnetic nanoparticles (PAMNs)	Photochemical cortical ischemic stroke in C57BL/6 mice	- Rapid targeting to ischemic stroke lesions - Promotes vasodilation to disrupt the PLT aggregation - Recovery of blood flow
Ghuman et al., 2016	Natural	Extracellular matrix (ECM) purified from porcine urinary bladder (collagen, fibronectin, decorin, laminin)	MCAo in SD rats	- Promotes host cell infiltration - Retention of the hydrogel within the cavity of the lesion - Antiinflammatory properties
Meng et al., 2014	Natural	High-molecular weight HA (HMW-HA) hydrogel	$4 \times 2 \times 2$ mm^3 cortical lesión created in SD rats	- Reduction in glial scar thickness - Decrease in astrogliosis marker GFAP
Meng et al., 2014	Natural	Laminin-incorporated HA (LN-HA) hydrogel	Cortical defects induced mechanically in SD rats	- Support cell infiltration and angiogenesis - Inhibit the formation of the glial scar - Promotes neurite regrowth

(Continued)

TABLE 1 | Continued

References	Biomaterial	Composition	Experimental model	Main findings
Crapo et al., 2012	Natural	ECM purified from porcine tissues (collagen, fibronectin, decorin, laminin)	PC12 cell line	- Stimulation of cell proliferation
Osama et al., 2018	Natural	Silk hydrogel (4% w/v) + mesenchymal stem cells (MSCs)	MCAo in SD rats	- Good space conformity in the ischemia cavity
Moshayedi and Carmichael, 2013	Natural	Hyaluronan-heparin-collagen hydrogel + neural progenitor cells (NPCs)	Photothrombotic ischemia in C57BL/6 mice	- Improvement of NPCs survival into the infarct cavity after stroke
Islam et al., 2018	Natural	Alginate-collagen microspheres containing fibroblast growth factor 2 (FGF-2)	Zebrafish embryos	- Increase in therapeutic angiogenesis
Yu et al., 2010	Natural	Collagen type I + neural stem cells (NSCs)	MCAo in Wistar rats	- Survival of the NSCs engrafts - Synapsis formation - Differentiation of NSCs
Zhong et al., 2010	Natural	Hyaluronan-heparin-collagen hydrogel + NPCs	Cortical photothrombotic stroke in C57BL/6J mice	- Improvement in NPCs survival *in vitro* and into the infarct cavity (*in vivo*)
Sanchez-Rojas et al., 2019	Natural	HA + adipose stem cells (ASCs)	Middle cerebral artery thrombosis with $FeCl_3$ in athymic mice	- Increase in cell proliferation and neurogenesis at subventricular zone (SVZ) - Angiogenesis and less inflammatory reaction to the graft
Tuladhar et al., 2020	Natural	Hydrogel drug depot, comprised of hyaluronan and methylcellulose (HAMC) containing cyclosporine and erythropoietin (CsA + EPO)	Endothelin-1 stroke in male SD rats and male Long-Evans rats	- Long term stability in the brain - Cyclosporine increased plasticity in the striatum while erythropoietin stimulated endogenous NSPCs
Fernandez-Serra et al., 2020	Natural	Silk fibroin hydrogels-encapsulated MSCs	MCAo in adult male CD-1 mice	- Promotes the survival of intracerebrally implanted MSCs - Improvement of functional outcomes over time in the model of cortical stroke

The other natural compound, with similar characteristics, found in the ECM of the CNS is HA. The enzymes responsible for their formation, and therefore, their size and molecular weight are hyaluronan synthases (HAS) (Brecht et al., 1986; Weigel et al., 1997), these are found in the cell membrane of fibroblasts, keratinocytes, chondrocytes, and specialized connective tissue cells. The molecular weight of HA is directly related to its biological functions; specifically, HA of $\geq$60 kDa is attributed with non-stick properties for cells (Brecht et al., 1986). These allow astrocytes to be kept in a non-reactive state and inhibit the formation of glial scar (Lin et al., 2009; Khaing et al., 2011). In addition, their anti-inflammatory properties and their support for cell survival have been demonstrated (Jiang et al., 2014). In an *in vivo* study of mouse model with occlusion of the middle cerebral artery occlusion (MCAO), in which an HA hydrogel with bioactive molecules (VEGF or angiopoietin-1) was implanted, observed good biocompatibility with brain tissue and increased angiogenesis around the implanted hydrogel (Ju et al., 2014). In this line, other authors have combined an HA hydrogel with exogenous cells, neural progenitor cell (NPCs) (Zhong et al., 2010), or adipose-derived stem cells (ASCs) (Sanchez-Rojas et al., 2019), observing a lower infiltration of reactive cells in the biomaterial and an increase in neural precursors in the area of the lesion.

In **Table 1** shows a scheme with the main synthetic, natural, and mixed biomaterial compositions have been described in this review.

CHOOSING ADMINISTRATION ROUTE

The anatomical limitations of the CNS, such as the skull and the BHE, restrict the passage of molecules and their accessibility.

It is necessary to take these particularities into account to determine which route of administration is ideal for each treatment (Bible et al., 2009a). In addition, the chosen route will define the biomaterial format. For example, some brain areas of interest can be found at great depth, covered with functional tissue that should not be altered. In these situations, it is convenient to use the intracerebral route to guarantee the correct implantation in the therapeutic target (Bible et al., 2009b; Ullah et al., 2017). For this route, it is necessary to use cannulas, in the cases of solid biomaterials or Hamilton syringes for the administration of nanoparticles (Ullah et al., 2017), microspheres (Bible et al., 2012), liquid neurospheres or hydrogels, which are administered before polymerization. This last pharmaceutical form has acquired great interest in *in vivo* studies, due to its easy and minimally invasive administration. It is inoculated by a Hamilton and polymerized, approximately, 8 min later; offering a structural 3D network for endogenous cells (Tate et al., 2001).

In the case that the therapeutic target is in the orbitofrontal cortex, the intranasal route is the one that offers the most advantages, due to the excellent conditions of the nasal mucosa for its absorption and the direct connection with the ethmoid bone. Currently, there are more and more studies in which biomaterials are used in neurospheres through this route, due to their effectiveness, safety, and speed (Yongjun et al., 2011; Wei et al., 2013; Yan-hua et al., 2015).

Also, it is possible to administer these low molecular weight nanocomposites through the intravenous route; however, it has been shown that the dose that reaches the cerebral target is insufficient, and therefore, it is necessary to increase the dose and find adverse effects (Tosi et al., 2019).

TABLE 2 | Summary of advantages and disadvantages of the proposed strategies.

Strategy	Advantages	Disadvantages
Synthetic biomaterial (PL), (PG), (PCL), (PGLA)	Degrading rate control Homogeneous production Reducing variability in immune response	Limited ability to induce endogenous repair responses
Synthetic biomaterial combined with natural compounds	Resistance to protein degradation Optimizing repair features	Hydrophobicity superficial
Collagen biomaterial	Contributing to growth, differentiation, and cell migration	Immunogeneity Low mechanical resistance
Alginate biomaterial	Biodegradable Hypoallergenic	Combined with synthetic compounds for greater consistency
Hyaluronic acid biomaterial	Anti-inflammatory properties Support for cell survival biocompatibility Non-stick properties	Easily degradable Possible formation of fibrosis
Biomaterial combined with exogenous cells	Less infiltration of reactive cells into the biomaterial Increase in neural precursors, modulates neuroinflammation, promotes neuroprotection	Possible neoplastic formation Cells do not integrate into the tissue
Biomaterial combined with bioactive molecules	Promoting neuroreparation processes Improves invasion and colonization of host tissue	Its use is restricted to 2–3 weeks

ENHANCING CELLULAR ATTRACTION BY CELL THERAPY AND TROPHIC FACTORS

At present, many studies use the combination of cell therapy or bioactive molecules and biomaterial to improve its invasion and colonization in the host tissue. In addition, the use of these types of exogenous cells or bioactive molecules has been shown to have an effect *per se* on damaged tissue (Lam et al., 2014; Sanchez-Rojas et al., 2019).

Despite the inflammatory reaction produced in the tissue that inevitably occurs when implanted, it has been shown that the effect of transplantation stimulates endogenous neural precursors through chemoattractant signals, promotes neuroprotection, and modulates neuroinflammation (Orive et al., 2009; Dibajnia and Morshead, 2013). As has been shown, stem cells do not integrate into the tissue, so their use is restricted to their trophic potential for 2 or 3 weeks (Modo and Badylak, 2019). Other authors opt for the encapsulation of trophic factors directly, such as VEGF or BDNF (Bible et al., 2012; Guan et al., 2012; George et al., 2018). Both strategies have the common objective of promoting neuroreparation processes (Erba et al., 2010).

After an ischemic event, the glial scar formed isolates the lesion from the rest of the parenchyma. In the study conducted by Zhou et al. (2015) showed that the administration of ASCs significantly suppressed the expression of the ionized calcium binding adaptor molecule 1 (Iba1) marker and glial fibrillary acidic protein (GFAP) marker compared to the control group. On the other hand, focal cavitation produced after the ischemic event is also a handicap for the effective treatment of stroke. Therefore, the use of biomaterials combined with cell therapy facilitates the establishment of a line of communication between the healthy parenchyma-biomaterial-lesion and favors the microenvironment (Martínez-Ramos et al., 2012; Pérez-Garnes et al., 2014).

As already mentioned, the chemical properties of the biomaterial affect its mechanical properties and therefore, the bioactive molecules or exogenous cells that are inside. The stiffness of biomaterials affects cell proliferation and differentiation *in vivo* (De Santis et al., 2011). Biomaterials with intermediate stiffness have been shown to improve cell proliferation (Leipzing and Shoichet, 2009). In this line, mesenchymal stem cells respond differently in gels of different viscosity (Engler et al., 2006). Therefore, it is important to choose the biomaterial format depending on what you want to combine with.

Other considerations to take into account for clinical translation are safety and efficacy. The implementation in the SNC entails safety specifications so that the least number of adverse effects occur. Delivery directed to the target to avoid tissue displacement or the generation

of cavitation is of paramount importance. On the other hand, when performing the intracranial implant, it is necessary to control the speed and intracranial pressure since they could cause bleeding and an exacerbated inflammatory response. In addition, the use of exogenous cells or bioactive molecules produces a proliferative response, so it is necessary to control that no neoplastic growths occur (Eckert et al., 2013; Xu et al., 2017).

Table 2 shows a brief summary with the advantages and disadvantages of the different strategies that have been described in this review.

FUTURE PERSPECTIVES/NEXT STEPS

Scientific advances place exosomes or extracellular vesicles as the new candidates to be used to improve their colonization and integration into tissue. These molecules are much smaller than a cell and have a key role in intercellular communication. These characteristics are sufficient to develop biomaterials in which to encapsulate them, with the advantage not only of being able to encapsulate a high number of exosomes in each microsphere, but to avoid the adverse reactions associated with the stem cells since, at least so far, has been described to have tumorigenic capacity (Chen and Chopp, 2018).

In addition, research is continuing how to deliver neurospheres or nanoparticles to deep and distal areas of the cerebral parenchyma from the intranasal route. Apparently, it is a route of minimally invasive administration that allows direct access to the brain, avoiding the anatomical limitations of the CNS. However, so far, no remains of these biomaterials have been found farther from the orbitofrontal cortex. Perhaps there is an intracerebral circulation that we still do not know today (Shah et al., 2015).

One of the great challenges is in bioprinting. Currently, very advanced 3D printer technology is being developed that has great advantages, such as homogenizing lots of biomaterials. It is possible to manufacture or print many biomaterials of small dimensions with precision and, in addition, all are exactly the same. It is also possible to make homogeneous mixtures of a drug in the biomaterial thread, instead of encapsulating it. The advantage of this method is the control over the mixture, being possible different concentrations of drug in the same biomaterial, or even of several compounds that are degraded simultaneously or staggered. The pharmaceutical forms are being reinvented. On cell therapy, printers have been developed that directly print the cells of interest in a certain position. This technology requires a high degree of sterility and its price is still very expensive. However, there is no doubt that the future is in these techniques (Norotte et al., 2009; Hsieh and Hsu, 2015).

Personalized medicine will be imposed in the future given the variability of brain damage and diseases; and biomaterials can adapt to this new approach. An exclusive design for a specific lesion is possible, with a volume of affection, a location, and very specific particularities.

What is already a certainty today is the safety of many of the components of biomaterials and cells (for example, adipose cells) supported by biomaterials in animals (Zhao et al., 2019; Kupikowska-Stobba and Lewińska, 2020; Otake et al., 2020). These results led us to argue that the gap between animals and humans in this context will be closed soon. Indeed, recent clinical trials have been conducted in Phase IIb to support the use of restorative cells plus natural biomaterials (alginate encapsulates) in neurodegenerative conditions (e.g., Parkinson's disease) that achieve promising results (Snow et al., 2019).

CONCLUSION

Despite the advances in the design, development and manufacture of biomaterials to favor neural restoration and the microenvironment, it is still a great challenge today. Minimally invasive techniques are sought to release cells, trophic factors, or drugs that potentiate spontaneous neuro-restaurant mechanisms. At the same time, it is sought that the biomaterial arrives and/or remains on the therapeutic target; and to be kept there during its degradation causing the least possible inflammatory reaction. Also, that it is colonized by endogenous cells, facilitating access to support cells to the center of the lesion and crossing the glial scar.

Combinations of biomaterials made of natural and synthetic compounds offer the advantages that both provide. And with the development of new forms such as microspheres, nanoparticles, liquid hydrogels that polymerize within a few seconds, or solids offer many possibilities for personalized treatments.

The objective and function to be achieved, the route of administration, and the limitations that exist to design a successful biomaterial must be defined.

AUTHOR CONTRIBUTIONS

All authors substantially contributed to the manuscript design, they critically reviewed it and gave their final approval. NE-G, CN, and JG were in charge of the bibliographic search and writing. NE-G and LS-S-R conceived the structure and specified the content of the article. JB and FR-R contributed with their medical perspective and knowledge, specially regarding the administration route and clinical considerations. LS-S-R addressed the "Future Perspectives/Next Steps" and "Conclusion" sections.

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A Three-Dimensional Alzheimer's Disease Cell Culture Model Using iPSC-Derived Neurons Carrying A246E Mutation in PSEN1

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Alzheimer's disease (AD) is a chronic brain disorder characterized by progressive intellectual decline and memory and neuronal loss, caused mainly by extracellular deposition of amyloid-β (Aβ) and intracellular accumulation of hyperphosphorylated tau protein, primarily in areas implicated in memory and learning as prefrontal cortex and hippocampus. There are two forms of AD, a late-onset form that affects people over 65 years old, and the early-onset form, which is hereditable and affect people at early ages ∼45 years. To date, there is no cure for the disease; consequently, it is essential to develop new tools for the study of processes implicated in the disease. Currently, *in vitro* AD three-dimensional (3D) models using induced pluripotent stem cells (iPSC)-derived neurons have broadened the horizon for *in vitro* disease modeling and gained interest for mechanistic studies and preclinical drug discovery due to their potential advantages in providing a better physiologically relevant information and more predictive data for *in vivo* tests. Therefore, this study aimed to establish a 3D cell culture model of AD *in vitro* using iPSCs carrying the A246E mutation. We generated human iPSCs from fibroblasts from a patient with AD harboring the A246E mutation in the PSEN1 gene. Cell reprogramming was performed using lentiviral vectors with Yamanaka's factors (OSKM: Oct4, Sox2, Klf4, and c-Myc). The resulting iPSCs expressed pluripotency genes (such as *Nanog* and *Oct4*), alkaline phosphatase activity, and pluripotency stem cell marker expression, such as OCT4, SOX2, TRA-1-60, and SSEA4. iPSCs exhibited the ability to differentiate into neuronal lineage in a 3D environment through dual SMAD inhibition as confirmed by Nestin, MAP2, and Tuj1 neural marker expression. These iPSC-derived neurons harbored Aβ oligomers confirmed by Western Blot (WB) and immunostaining. With human iPSC-derived neurons able to produce Aβ oligomers, we established a novel human hydrogel-based 3D cell culture model that recapitulates Aβ aggregation without the need for mutation induction or synthetic Aβ exposure. This model will

allow the study of processes implicated in disease spread throughout the brain, the screening of molecules or compounds with therapeutic potential, and the development of personalized therapeutic strategies.

Keywords: 3D cell culture, Alzheimer's disease, iPSC-derived neurons, disease modeling, personalized therapy

INTRODUCTION

Alzheimer's disease (AD) is a major degenerative disorder of the central nervous system characterized by continuous neuronal loss mostly in the cerebral cortex and the hippocampus, mainly caused by the accumulations of amyloid plaques and neurofibrillary tangles, which leads to pathological changes in the functional organization and the internal structure of the brain and therefore to progressive loss of memory and cognitive impairment (Sanabria-Castro et al., 2017; Reiss et al., 2018). The average life expectancy of patients with this disorder is 8–10 years; however, clinical symptoms are preceded by prodromal symptoms that extend over two decades and eventually cause death (Wilson et al., 2011; Masters et al., 2015).

Most cases of AD (>95%) are commonly diagnosed in people over 65 years of age, known as late-onset form (LOAD), and is the consequence of the failure of the homeostatic networks in the brain tissue to clear the amyloid-β (Aβ) peptide; additionally, there is a less frequent early-onset hereditary form (EOAD; 2–5% of cases), usually caused by an autosomal dominant genetic mutation in three known genes. These genes encode for amyloid precursors protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2), which affect the normal processing of Aβ and cause the development of the disease at early stages (∼45 years; Masters et al., 2015; Cacace et al., 2016). Among these, mutations in *PSEN1* and *PSEN2* genes are highly penetrant, representing approximately 90% of all identified in EOAD and being the most common and usually associated with a very aggressive EOAD, with 221 mutations for PSEN1 reported in the Alzforum database[1] (Ryan and Rossor, 2010; Kelleher and Shen, 2017; Lanoiselée et al., 2017). Altogether, the pathological hallmarks of EOAD and LOAD are mostly similar; hence, it is difficult to distinguish the two AD forms by any other criterion than the onset age (Masters et al., 2015). Hence, AD represents a significant public health problem and represents an increasing clinical challenge in terms of diagnosis and treatment.

Historically, *in vivo* and *in vitro* systems have constituted powerful models to defining the critical disease-related pathophysiology and in exploring novel potential therapeutic approaches (Saraceno et al., 2013; Penney et al., 2020). Despite many aspects of the mechanisms of AD that have been elucidated thanks to the use of these models, specific molecular mechanisms leading to neurodegeneration are still unknown so that neither cure nor therapeutic approaches are also available (Alonso Vilatela et al., 2012; Graham et al., 2017); besides, knowledge gaps remain, due to significant limitations such as human brain physiology complexity, limited availability of human brain tissue, and the lack of *in vivo* and *in vitro* models that reliably recapitulate the disease phenotype (D'Avanzo et al., 2015; Logan et al., 2019). Better and relevant AD platforms are needed to recapitulate particular features of the pathology that cannot be recreated in current AD models. To fill this gap, in the last years, the development of patient-derived AD disease models by generating iPSC from AD patient somatic cells, further differentiated into neural cells, have revolutionized the human *in vitro* models (Penney et al., 2020). The establishment of these culture techniques represents one of the most innovative biomedical advances in this century, mainly because these patient-specific cells contain genetic information from donors, and in consequence, it offers an opportunity to develop physiologically relevant *in vitro* disease models (Yagi et al., 2011; Israel et al., 2012; Mohamet et al., 2014; Sproul et al., 2014; Hossini et al., 2015; Liao et al., 2016; Logan et al., 2019).

Nevertheless, to study more accurately the human brain complexity, 3D cell models, including scaffolds-based systems and scaffold-free systems (e.g., gels and spheroids, respectively; Logan et al., 2019), have emerged as an innovative and advanced alternative driven by their resemblance to some *in vivo* environmental and architecture characteristics, for example, by allowing complex intercellular communication, the formation of complex structures, as well as a better spatial organization, better cell behavior, and specific chemical and physical cues, essential for the study of human brain diseases at cellular and molecular levels. Also, 3D environments can promote better neuronal differentiation and neural network formation (Choi et al., 2014; Zhang et al., 2014; Ravi et al., 2015; Fang and Eglen, 2017).

Consequently, the use of iPSC-derived neurons in 3D cell cultures has been implemented to obtain and employ cells with a specific genetic background of AD patients in a system of higher similarity to the medium *in vivo* that provides a local environment brain tissue-like that promotes AD-like phenotypes such as elevated Aβ production and tau hyperphosphorylation, its aggregation, and accumulation (D'Avanzo et al., 2015; Raja et al., 2016; Gonzalez et al., 2018; Logan et al., 2019; de Leeuw and Tackenberg, 2019).

With the combined approaches offered by these techniques, *in vitro* AD modeling has gained increasing interest for the study of the pathological mechanisms underlying the disease as well as in pharmacological testing platforms and developing therapeutic personalized strategies due to their demonstrated benefits in providing physiologically relevant information and more predictive data for *in vivo* tests (D'Avanzo et al., 2015; Logan et al., 2019; Penney et al., 2020).

Evidence from many laboratories supports those mentioned above; for example, Zhang et al. (2014) modeled AD in a

[1]http://www.alzforum.org/mutations

hydrogel-based 3D culture using human neuroepithelial-like stem cells which were exposed to a synthetic preparation of Aβ-42 oligomers. They resemble the altered p21-activated kinase distribution found in AD patients and demonstrated their utility in studying the Aβ-induced pathogenesis (Zhang et al., 2014). On the other hand, Choi et al. (2014) demonstrated the robust deposition of Aβ and filamentous tau in a hydrogel-based 3D model of AD using genetically modified human neural stem cells which overexpressed mutant APP and PSEN1 (Kim et al., 2015). This model consists of genetically modified cells, not cells with AD genetic background. On the other hand, Raja et al. (2016) recapitulated AD phenotypes such as considerably elevated production of Aβ, Aβ aggregation, and tau protein hyperphosphorylation, apparent after 60 and 90 days in culture, using iPSC-derived organoids carrying AD mutations. These self-organizing AD 3D models efficiently produce AD phenotypes without genetic manipulation or exogenous toxins (Raja et al., 2016). However, self-organizing organoids lack cytoarchitectural structures, and they also have difficulties in controlling their microenvironment and nutrient access, while in hydrogel-based 3D cultures, this is more controllable (de Leeuw and Tackenberg, 2019).

Here, we present the establishment of a novel human hydrogel-based 3D model of AD with iPSC-derived neurons carrying the A246E mutation in the *PSEN1* gene that produces Aβ oligomers that were detected from day 14 of differentiation with no need to induce AD-related mutations or external addition of synthetic Aβ. This work provides guidelines in the development and implementation of early disease models for mechanistic studies, preclinical drug discovery, and the design of personalized therapeutic strategies.

MATERIALS AND METHODS

Human Fibroblasts (HFs) Culture and Human iPSC Generation

Human fibroblasts (HFs) with PSEN1 A246E mutation (Coriell Cat# AG07768, RRID: CVCL_T877) and non-mutated HFs (ATCC Cat# SCRC-1041, RRID: CVCL_3285) were reprogrammed with lentiviral vectors containing Yamanaka's factors (Takahashi and Yamanaka, 2006) with few modifications (Cevallos et al., 2018). HEK293T packaging cell line (ATCC Cat# CRL-3216, RRID: CVCL_0063) was used to generate lentiviral particles. HEK293T cells of 80% confluence were transfected with envelope pCMV-VSV-G (RRID: Addgene_8454), packaging plasmid psPAX2 (RRID: Addgene_12260), and transfer pSIN4-CMV-K2M (RRID: Addgene_21164) encoding KLF4 and C-MYC and pSIN4-EF2-O2S encoding OCT4 and SOX2 (RRID: Addgene_21162) in a 1:3:4 ratio (20 μg total DNA), and a positive transfection control was included, pLENTI-CMV-GFP-puro (RRID: Addgene_17448) encoding GFP. DNA/calcium phosphate precipitates were generated using CalPhos Mammalian Transfection Kit (Clontech, CA, USA), added dropwise to HEK293T cells and incubated for 6–7 h in standard cell culture conditions. Then, cells were washed with PBS and cultured in medium DMEM/F12 supplemented with 10% fetal bovine serum. At 12, 24, and 36 h post transfection, the virus-containing supernatant was collected, filtered through a 0.45-μm-pore-size cellulose acetate membrane, and stored at 4°C until use.

For lentiviral-mediated reprogramming, passage 5–7 HFs were cultured in fibroblast medium consisting of DMEM/F12 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 10 μg/ml of antibiotics–antimycotics (Gibco, Carlsbad, CA, USA) at 37°C and 5% CO_2. When fibroblasts reached 80% confluency, they were harvested using Trypsin-EDTA (Gibco, Carlsbad, CA, USA) and replated at 3×10^4 cells per square centimeter in six-well culture plates and transduced with a combination of O2S and K2M lentiviral vectors. After 20 h of transduction, the medium was discarded and replaced with a fresh fibroblast medium. At the seventh day post transduction (p.t.), transduced cells were harvested by trypsinization and replated at $1.2–2.5 \times 10^4$ cells per square centimeter onto hESC-qualified Matrigel-coated (Corning, Bedford, MA, USA) six-well plates (Corning) and cultured in E8 defined medium (Gibco, Carlsbad, CA, USA; Beers et al., 2012). Between days 20 and 23 p.t., compact rounded colonies with embryonic stem cell-like (ESC-like) morphology were harvested using 0.1% collagenase IV and mechanically isolated and transferred to hESC-qualified Matrigel-coated 48-well plates and maintained in E8 medium with daily media replacement. Selected iPSC colonies were expanded for 10 passages for further analysis.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase activity detection was performed using the colorimetric assay SigmaFast™ BCIP®/NBT (Sigma, St. Louis, MO, USA) following manufacturer's instruction. SigmaFast tablet was dissolved in 10 ml of deionized water, resulting in a ready-to-use solution. The iPSC culture medium was removed, and cells were washed with 1× TBS buffer (Bio-Rad, Richmond, CA, USA), and SigmaFast solution was added and incubated for 20 min at room temperature (RT). Finally, cells were washed with 1× PBS (Bio-Rad, Richmond, CA, USA), and positive colonies were observed and photo-documented with an inverted phase-contrast microscope (Olympus IX71).

RT-PCR Transgene Silencing and Endogenous Pluripotency Gene Expression Analysis

Total RNA was isolated from whole-cell lysates using TRIzol (Invitrogen, CA, USA) reagent, and RNA purification was performed using the RNeasy Mini kit (Qiagen, Germantown, Maryland) according to the manufacturer's instructions and quantified by spectrophotometry with Epoch (BioTek). Reverse transcription was performed using the One-Step RT-PCR (Qiagen, Germantown, Maryland) according to the manufacturer's instructions. To confirm the transgene silencing of iPSC, transgene-specific primers (pSIN4-EF2-O2S: forward 5′ CAGTGCCCGAAACCCACAC 3′; reverse 5′ GCTCGTCA AGAAGACAGGGCCA 3′; 550 bp; and pSIN4-CMV-K2M: forward 5′ CAAGTCCCGCCGCTCCATTACCAA 3′; reverse:

5′ GCTCGTCAAGAAGACAGGGCCA 3′; 551 bp) were used. Furthermore, to confirm endogenous pluripotency gene expression, specific primers (OCT4: forward 5′ GACAGGGG GAGGGGAGGAGCTAGG 3′; reverse: 5′ CCTCCAACCAGT TGCCCCAAACTCCC 3′; 140 bp; and NANOG: forward 5′ GG ACACTGGCTGAATCCTTCC 3′; reverse: 5′ CTCGCTGAT TAGGCTCCAACC 3′; 143 bp) were used. GAPDH (GA3PDH: forward 5′ AAGGTGAAGGTCGGAGTCAA 3′; reverse: 5′ AATGAAGGGGTCATTGATGG 3′; 108 bp) were used as an internal control. PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide (Sigma, St. Louis, MO, USA).

Immunofluorescence Staining of iPSCs and iPSC-Derived Neurons

iPSCs, passages 14–17, were seeded on hESC-qualified Matrigel-coated plates. iPSCs were fixed with 4% PFA (Sigma, St. Louis, MO, USA) for 20 min at RT. Subsequently, cells were permeabilized and blocked with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS and 1% of bovine serum albumin (Sigma, St. Louis, MO, USA) for 1 h at RT. After washing three times for 5 min with TBS buffer containing 0.1% (v/v) Tween-20 (TBST), the cultures were incubated with primary antibodies in the blocking solution at 4°C overnight. The following primary antibodies were used: 1:2,000 SOX2 (Abcam Cat# ab97959, RRID: AB_2341193), 1:400 Nanog (Cell Signaling Technology Cat# 4903, RRID: AB_10559205), 1:1,000 OCT4 (Abcam Cat# ab19857, RRID: AB_445175), 1:1,000 TRA-1-60 (Abcam Cat# ab16288, RRID: AB_778563), 1:1,000 SSEA4 (Abcam Cat# ab16287, RRID: AB_778073), 1:500 Nestin (Abcam Cat# ab18102, RRID: AB_444246), 1:500 MAP2 (Abcam Cat# ab11267, RRID: AB_297885), 1:1,000 Tuj-1 (Abcam Cat# ab215037), and 1:500 Aβ D54D2 antibody (Cell Signaling Technology Cat# 8243, RRID: AB_2797642). Then, cells were washed and incubated for 2 h at RT with 1:1, 000 Alexa 488 anti-mouse (Molecular Probes Cat# A-11017, RRID: AB_143160) and Alexa 594 anti-rabbit (Molecular Probes Cat# A-11012, RRID: AB_141359) or Alexa 488 anti-rabbit (Molecular Probes Cat# A-11070, RRID: AB_142134) and Alexa 594 anti-mouse (Molecular Probes Cat# A-11005, RRID: AB_141372) secondary antibodies. Finally, for nuclei counterstaining, cells were incubated with 1 μg/ml Hoechst 33342 (Thermo Scientific, Rockford, IL, USA) for 10 min and visualized in an inverted fluorescence microscope (Olympus IX71), and image processing was performed with QuickCapture software. For 3D cultures, all incubations were prolonged overnight with gentle rocking, and also washes were prolonged to 20 min with gentle rocking.

3D Cell Culture and iPSC Neural Differentiation

Neural induction of iPSC, passage 14–17, was performed according to a previously reported dual SMAD inhibition protocol (Shi et al., 2012; Qi et al., 2017) with some modifications. First, iPSCs were cultured onto hESC-qualified Matrigel-coated 24-well plates in E8 medium until they reached 80% confluence, at which point the E8 medium was switched to neural induction medium (NIM) consisting of DMEM/F12 supplemented with 5 μM SB431542 (Sigma, St. Louis, MO, USA), 0.1 μM LDN193189 (Sigma, St. Louis, MO, USA), 1× N2 (Gibco, Carlsbad, CA, USA), 0.5× B27 (Gibco, Carlsbad, CA, USA), and 10 μg/ml of antibiotic, with daily medium replacement for 5 days. After 5 days of neural induction, the rosettes formed were dissociated into clumps using 0.1% collagenase IV (Sigma, St. Louis, MO, USA) and replated onto a Matrigel basement matrix (Corning, Bedford, MA, USA). This 3D culture was performed in accordance with Kim et al. (2015) with modifications. A Matrigel basement matrix stock solution was diluted (1:1 dilution ratio) with ice-cold neural differentiation medium (NDM) consisting of Neurobasal medium (Gibco, Carlsbad, CA, USA) supplemented with 15 ng/ml BDNF and GDNF neurotrophic factors (PeproTech, Rocky Hill, NJ, USA), 1 μM PD0325901 (Sigma, St. Louis, MO, USA), and 10 μM Forskolin (Sigma, St. Louis, MO, USA), and then vortexed for 5 s. Immediately, the prepared dilution was transferred into 48-well plates (200 μl in each well); then plates were incubated for 20 min at 37°C to form a 3D gel layer at the bottom of the well. Subsequently, dissociated rosettes (split 1:2) were plated onto the Matrigel matrix basement in prewarmed NDM for neural maturation. The next day, the 3D cell culture was exposed to 5 μM DAPT (Sigma, St. Louis, MO, USA) for 24 h. The 3D cultures were maintained for 2 weeks, with media replacement every 2 days.

Protein Extraction and Western Blot (WB) Assay

The 3D cultures were homogenized with RIPA (Sigma, St. Louis, MO, USA) extraction buffer containing 1 mM protease inhibitor mixture (Sigma, St. Louis, MO, USA) and 1 mM orthovanadate inhibitor (Sigma, St. Louis, MO, USA). After incubation on ice for 10 min, the samples were centrifuged for 10 min at 8,000 *g*. Tris-tricine SDS-PAGE (Bio-Rad, Richmond, CA, USA) was performed as previously described (Reza-Zaldivar et al., 2019), with few modifications to verify the Aβ aggregates' existence. Proteins were resolved on 4–16% gradient gels; subsequently, the proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA); the membrane was fixed in glutaraldehyde (Sigma, St. Louis, MO, USA) at 0.5% for 10 min. After three TBS-T washes, the membranes were incubated overnight at 4°C with 0.5 μg/ml Aβ anti-rabbit antibody and then were incubated with 1:5,000 hP-conjugated anti-rabbit secondary antibody (Vector, Burlingame, CA, USA) for 2 h at RT. Membrane exposure was performed by chemiluminescence using Luminata Forte (Millipore, Burlington, MA, USA) and was visualized by the ChemiDoc™ XRS system and Image Lab 6.0.1 software.

RESULTS

Generation of iPSCs From HFs Harboring A246E PSEN1 Mutation and Control HFs

The development of technologies to reprogram adult somatic cells, including HFs, to iPSCs has made possible the generation

of patient-specific stem cells and has been used to generate several models with inherited neurodegenerative conditions. We established two iPSC cultures, one with PSEN1 mutation A246E (iFLAG; i: iPSC, F: fibroblasts, L: lentivirus, and AG: AG07768 cell line) and the other one without mutations (iFLN; i: iPSC, F: fibroblasts, L: lentivirus, and N: non-mutated), both *via* the HF transduction of Yamanaka's factors containing lentiviral vectors at feeder-free conditions. Except for minor modifications, the overall reprogramming procedure was basically as previously reported by Cevallos et al. (2018). On 3 weeks p.t., we observed colonies with prominent nucleoli, a high ratio of nucleus to cytoplasm, and tightly packed, reminiscent of ESC-like, morphology (Thomson et al., 1998). iPSCs with the greatest amount of previously described qualities were selected and expanded during 10 passages to test for the stability of self-renewal capacity. The representatives of these iPSCs were used for purposes of analysis and neurogenesis (**Figure 1**).

iPSC Characterization

First, we tested the pluripotency of the colonies *via* the assay of the alkaline phosphatase activity (**Figure 2A**) and the expression of representative marker characteristics of ESCs, which included NANOG, OCT4, SOX2, SSEA4, and TRA-1-60 (**Figure 2B**). We demonstrated the activation of endogenous pluripotency-related genes, OCT4 and NANOG, and the silencing of the lentiviral transgene's expression by RT-PCR (**Figures 2C,D**, respectively). The best transgene shutoff and endogenous expression of stem cell genes were used as the criteria of the established pluripotency.

AD 3D Model Establishment

We established hydrogel 3D human models for AD (AD 3D model) and healthy (nonmutated 3D model) neurons. For their establishment, the iPSCs were cultured until 80% confluence. Then the iPSC medium was changed by NIM for 5 days. During the differentiation process, the cells in the periphery of the colonies changed their morphology to a rosette-like appearance. On day 5 of neural induction, we performed immunofluorescence to assess the neural precursor cells' (NPCs) identity by Nestin expression (**Figure 3**). NPCs had a strong proliferative potential and were passaged every 4–6 days to allow population expansion. Afterward, NPCs were subcultured and replated onto the Matrigel matrix basement. By 14 days of differentiation, all 3D neuronal cultures exhibited MAP2 and TUJ1 expression (**Figure 4**). This 3D neural differentiation protocol (**Figure 5**) was efficient in generating neural cells within 14 days, with a simple and economical method in comparison with other works.

Aβ Marker Expression and Aggregation in AD 3D Neuronal Culture

After establishing the AD 3D model, in order to analyze Aβ marker expression and aggregation associated with PSEN1 function and dysfunction in NPCs/early neurons (14-day differentiation), we performed immunofluorescence and a WB analysis. First, we performed immunofluorescent assay with the anti-Aβ antibody D54D2 previously extensively used (for references on its use, visit the Cell Signaling website) to recognize several amyloid isoforms (Aβ37, Aβ38, Aβ39, Aβ40, and Aβ42). We detected immunopositive staining for this anti-Aβ antibody that co-localized with MAP2 (yellow arrows, **Figure 6A**) but was not detectable in neurons lacking the AD mutation. To validate the observed Aβ aggregation, we used a WB test using as a positive control a commercial Aβ (Aβ Ctrl+) preliminarily incubated for 5 days at 37°C to allow Aβ aggregation. As a result, in the proteins from the AD 3D neurons but not from the nonmutated 3D neurons, several protein bands were detected corresponding to monomers and oligomers with a molecular mass ranging from 5 to 50 kDa. The band with a molecular mass of ∼50 kDa corresponding to Aβ oligomers was the main result (**Figure 6B**) evidencing that the Aβ had undergone an oligomerization in the AD patient-derived 3D neuronal model compatible with the previous report of Raja et al. (2016).

DISCUSSION

Evidence from cellular biology, animal models, and genetics suggests that Aβ protein oligomerization and aggregation play a central role in the initiation and progression of AD. However, the specific pathophysiological mechanisms of the toxic Aβ species by which these processes give rise to the pathogenesis of AD continue to be under discussion. With iPSC technology, we can access iPSC-derived neurons from AD patients' somatic cells carrying AD-associated mutations, which have mostly been used to model *in vitro* several neurodegenerative conditions, such as AD (Penney et al., 2020). Here, we established two iPSC cultures derived from fibroblasts of healthy and diseased individuals, the latter carrying the PSEN1 mutation A246E, which showed defined morphology, phosphatase alkaline activity, ESC markers, and gene expression and were further expanded for 10 passages, demonstrating that mutated and nonmutated HFs were successfully reprogrammed and established into iPSC as previously demonstrated (Yagi et al., 2011; Muñoz et al., 2018).

In the last 5 years, *in vitro* models of AD have been improved by using 3D environments (Logan et al., 2019), among which the simplest involves differentiating iPSCs and NPCs into neurons. Hydrogels are cross-linked polymer networks that are easy to use, mechanically similar to the central nervous system tissue; are permeable for nutrients and oxygen; are hydrophilic; and are made from different synthetic and natural materials, such as Matrigel matrix, alginate, and chitosan (Frampton et al., 2011; Hopkins et al., 2013). Here, we did not embed the cells onto the Matrigel matrix basement, but we first made the basement and then seeded the cells onto the Matrigel basement, since, on the contrary, the cells did not survive; however, the cells entered the matrix within 1 day.

Although 3D models represent a substantial step forward in *in vitro* AD modeling, crucial models consisted of the use of genetically manipulated NPCs-derived neurons which overexpressed AD-related mutations in *APP* and *PSEN1* genes

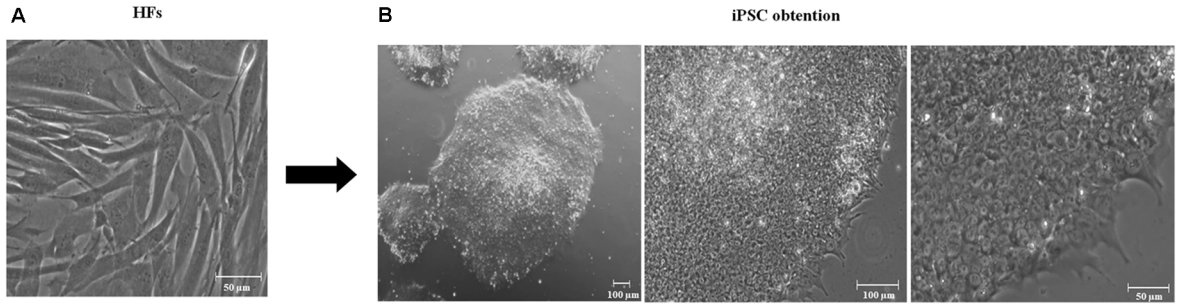

FIGURE 1 | Generation of induced pluripotent stem cells (iPSCs). **(A)** Day 0. Human fibroblast culture. Scale 50 µm, 20× amplification. **(B)** Representative derived iPSCs using lentiviral vectors containing Yamanaka's factors in feeder-free conditions after 10 passages. iPSCs have flat, rounded, and compact morphology, a high ratio of nucleus to the cytoplasm and prominent nucleoli embryonic stem cell-like (ESC-like). From left to right, iPSCs 4× amplification, 100 µm scale; 10× amplification, 100 µm scale; and 20× amplification, scale 50 µm.

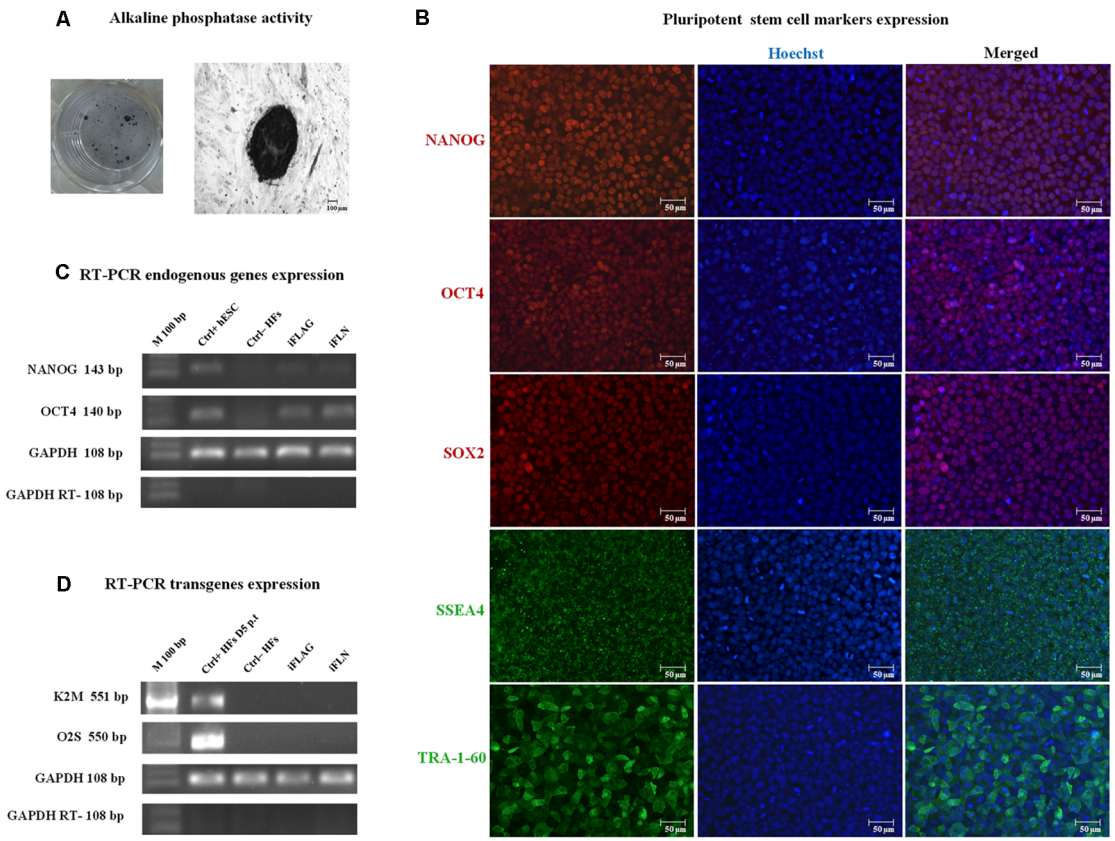

FIGURE 2 | iPSC pluripotency expression. iPSCs were characterized using standard pluripotency assays. Representative images are shown. **(A)** iPSCs showed alkaline phosphatase activity. **(B)** Immunofluorescence staining showed nuclear marker (red) and cell surface (green) pluripotency marker expressions in iPSCs, including NANOG, OCT4, SOX2, and SSEA4, and TRA-1-60. Hoechst nuclear staining in blue. Scale bars 50 µm. **(C)** Expression of pluripotency-related endogenous genes, *OCT4* and *Nanog*, was detected in derived iPSCs by RT-PCR analysis. hESCs were used as a positive control (Ctrl+), and human fibroblasts (HFs) were used as a negative control (Ctrl−) for endogenous pluripotency gene expression. **(D)** Some iPSC clones expressed the transgenes (data not shown), whereas fully reprogrammed iPSCs consistently exhibited lentiviral silencing or attenuation (iFLAG and iFLN). HFs at day 5 p.t. were used as a positive control (Ctrl+ HFs D5 p.t.) for transgene expression, and HFs were used as a negative control (Ctrl− HFs). GAPDH was used as an internal control.

(Choi et al., 2014) and were exposed to exogenous Aβ toxic species (Zhang et al., 2014). Otherwise, self-organizing AD 3D models efficiently produce AD phenotypes without genetic manipulation or exogenous Aβ addition (Raja et al., 2016); however, these self-organizing models lack controlled access of nutrients while the hydrogel-based 3D cultures offer a suitable

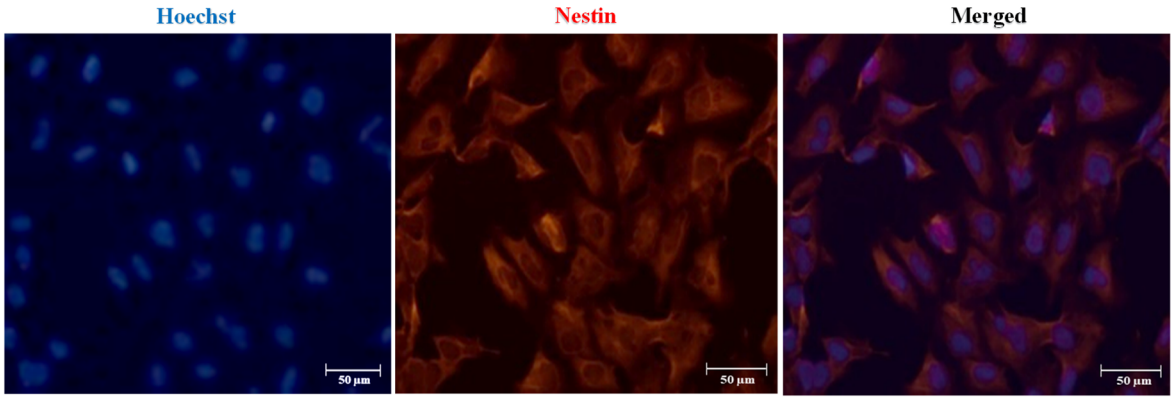

FIGURE 3 | Immunofluorescence staining of neural precursor cell (NPC) marker Nestin. Representative images indicate Nestin expression in NPCs on day 5 post neural induction. Nuclear staining in blue with Hoechst. Scale bars 50 µm.

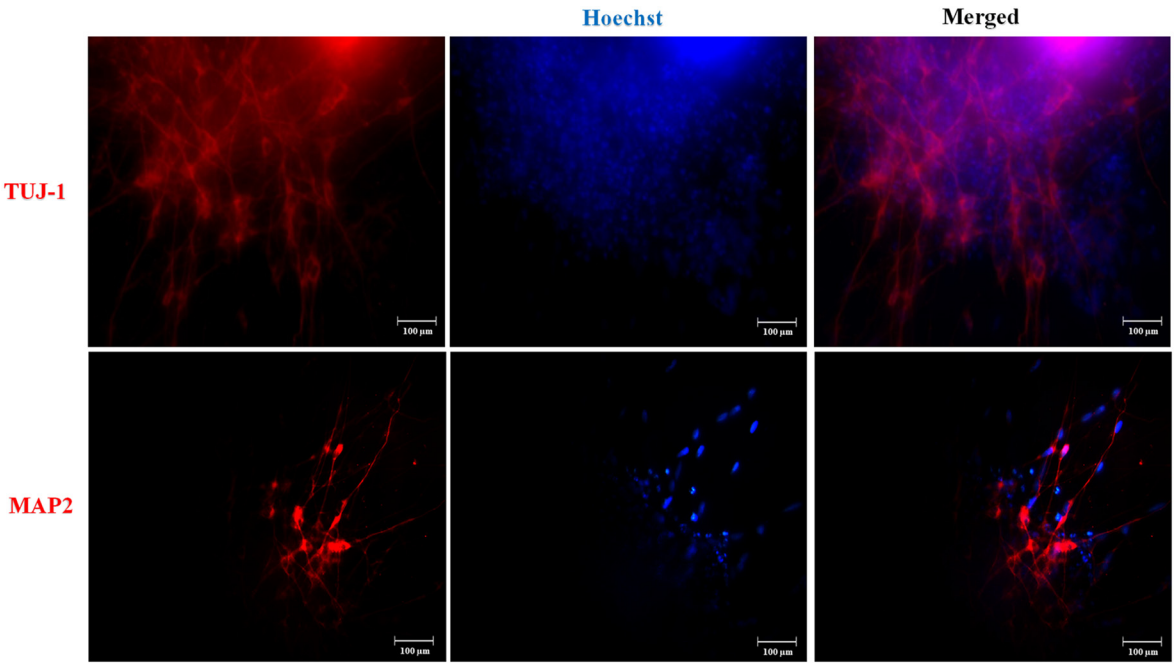

FIGURE 4 | Neuronal marker expression in the three-dimensional (3D) cultures. Representative images are shown. At day 14, we performed immunofluorescence and iPSC-derived neurons in 3D culture expressed TUJ-1 and MAP2 neuronal markers in red. Nuclear staining in blue with Hoechst. Scale bars 100 µm.

stiffness for neural cells and a higher degree of control of the cellular context. Besides, 3D models being suitable in recapitulating brain tissue-like environments showed advantages in reconstituting AD-specific extracellular aggregation of Aβ (Choi et al., 2014). This may be because 3D models increase the number of synaptic contacts, which in turn probably facilitates self-assembly and aggregation of Aβ in neurites. Several aggregation states of self-assembling 4.5-kDa Aβ monomers have been identified: trimers and tetramers of, approximately, 13 and 17 kDa, respectively; 30- to 83-kDa oligomers; and the highest-sized (>100 kDa) insoluble fibrils that were deposited in the brain, giving rise to amyloid plaques (Pryor et al., 2012). In the present work, we show that neurons derived from a patient carrying the pathogenic A246E mutation in the *PSEN1* gene in a hydrogel-based 3D model of AD produced Aβ aggregates with a molecular mass of ∼50 kDa, corresponding to Aβ oligomers, without the need of mutation insertions or exposing the cells to synthetic Aβ, one of the two main pathological features of AD. Furthermore, in this study, we demonstrated by immunofluorescence that AD patient-derived neurons at day 14 of differentiation already showed Aβ protein expression. The presence of oligomers in the absence of amyloid plaques in this

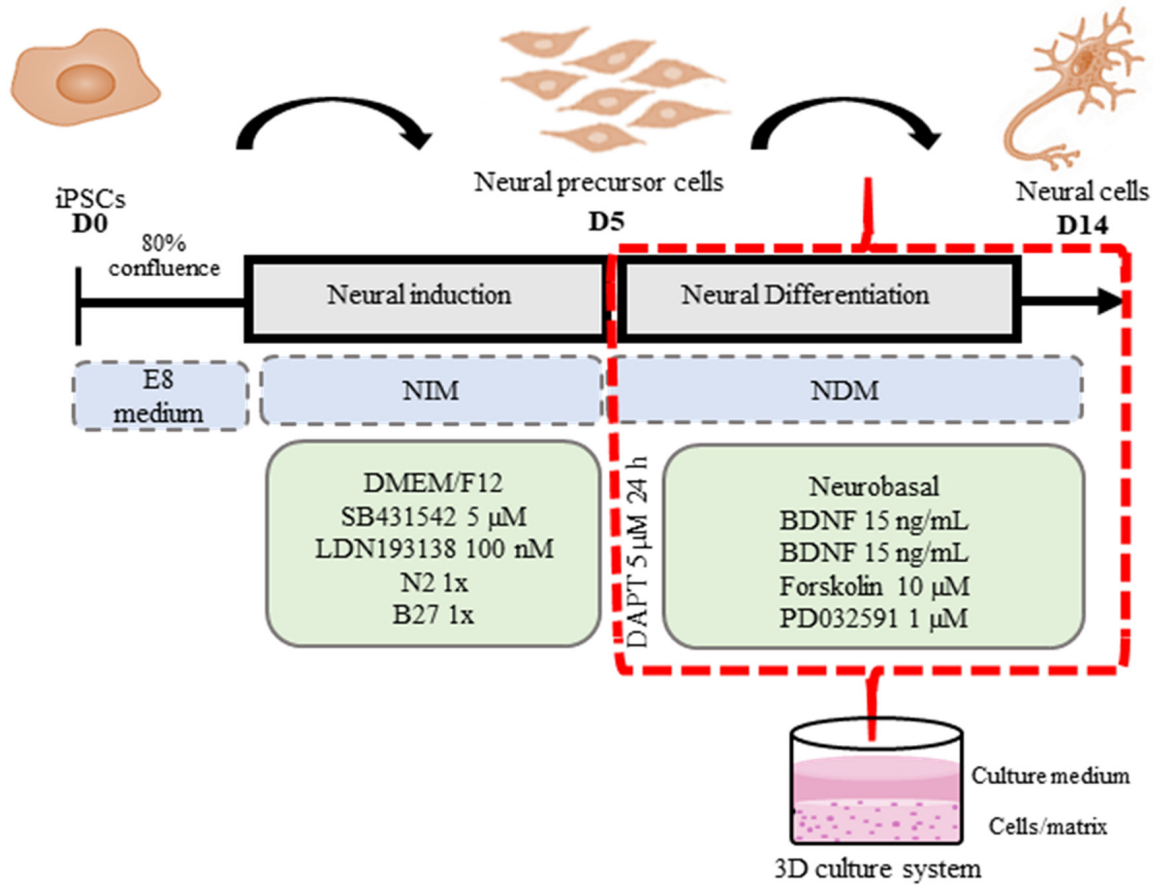

FIGURE 5 | 3D iPSC neural differentiation scheme. iPSCs were plated onto hESC-qualified Matrigel-coated 24-well plates in an E8 medium (D0) until 80% of confluence. Then the E8 medium was replaced by neural induction medium (NIM) for neural induction with daily medium replacement for 5 days. On the fifth day (D5) of neural induction, 3D Matrigel basement matrices were prepared in accordance with Kim et al. (2015) with modifications. A Matrigel basement matrix stock solution was diluted (1:1 dilution ratio) with ice-cold neural differentiation medium (NDM) and then vortexed for 5 s. Immediately, the prepared dilution was transferred into 48-well plates (200 µl in each well); then the plates were incubated for 20 min at 37°C to form a 3D gel layer at the bottom of the well. Subsequently, NPCs were dissociated into clumps using 0.1% collagenase IV and replated (Split 1:2) onto a Matrigel basement matrix in prewarmed NDM for neural maturation. The next day, the 3D cell culture was exposed to 5 µM DAPT for 24 h. The 3D cultures were maintained for 2 weeks, with media replacement every 2 days. At D14, the 3D neural cell cultures were observed and analyzed.

AD 3D model could be due to the short time of culture (14 days of neuronal differentiation), which can be considered an advantage of this model, permitting observation of the initial stages of the Aβ oligomerization in a short period of time (14 days), relevant for the development of prevention strategies. We consider that this 3D modeling is simpler and technically more feasible, thus contributing to the field of AD modeling.

Neural cells are an essential cell type to study the AD context; for that reason, several groups have established multiple *in vitro* iPSC-derived neuron differentiation protocols (Chambers et al., 2009; Shi et al., 2012; Paşca et al., 2015; Qi et al., 2017; Logan et al., 2019). Many of these protocols have focused on differentiating neurons from EOAD and LOAD patient-specific iPSCs and have shown that EOAD iPSCs expressed pathogenic mutation and that these neurons can be consistently differentiated into phenotypical and physiological neurons with amyloidogenic properties and also other key events of the AD pathogenic cascade (Israel et al., 2012; Mahairaki et al., 2014; Hossini et al., 2015). In this work, we obtained neurons derived from mutant and nonmutant iPSCs by dual SMAD inhibition and small molecules that enhance neural fate derivation under feeder-free conditions, as previously reported by Qi et al. (2017) with few modifications. We induced iPSC differentiation into neural cells by blocking the TGF-β and BMP signaling pathways through a dual SMAD inhibition. Then we were able to expand Nestin-positive NPCs for final differentiation into neurons in 3D culture conditions. Neural marker expression is essential, given that it indicates that stem cells ceased their pluripotency and its fate was determined to neural lineage. For that reason, we evaluated neural marker expression in our 3D model where the neural differentiation protocol implemented was successful in generating neural cells positive for MAP2 and TUJ-1 neural markers within 9 days of differentiation from NPCs, using an easy and cheap method to establish our AD

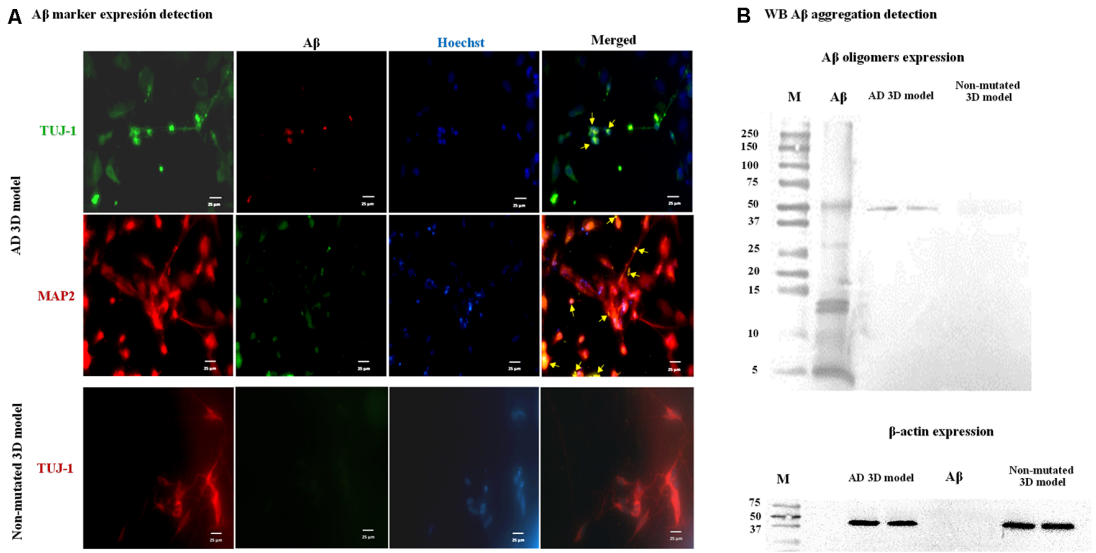

FIGURE 6 | Amyloid-β (Aβ) marker expression and Aβ aggregation in the Alzheimer's disease (AD) 3D model. Representative images are shown. **(A)** Immunofluorescence staining indicates Aβ marker expression in the AD 3D model. TUJ-1 (green), Aβ (red), and MAP2 (red) and Aβ (green). Co-3D model, Aβ marker expression was not detected. TUJ-1 (red) and Aβ (green). Nuclei were stained in blue with Hoechst. Scale bars 25 μm. **(B)** Aβ oligomers were detected by western blot (WB) analysis in the AD 3D model as a protein band with a molecular mass of ∼50 kDa and were absent in the non-mutated 3D model. β-Actin was expressed by mutated and non-mutated cells. These assays were performed at 14 days post neural induction.

3D model and the nonmutated 3D model. Our protocol is cheaper in the sense that we used lower concentrations of neural induction and differentiation molecules as well as fewer supplements that constitute the induction and differentiation media, in comparison with previous reports (Shi et al., 2012; Qi et al., 2017).

While many advances have been made, challenges to creating comprehensive 3D human culture models for AD study and comprehension still lie ahead. Although current AD 3D culture models have successfully recapitulated hallmarks of AD, one of the major challenges is the low optical transparency during high-resolution imaging due to the thick nature of the culture. Another disadvantage of the current 3D cultures is the insufficient maturation and aging of neural cells and also the lack of functional tests such as behavior assessments (Choi et al., 2016).

Despite the unresolved challenges, the research community continues to refine them to facilitate novel insights into AD pathophysiology. Therefore, the application of these AD 3D models may be limited to the early stages of the disease progression.

These results, in accordance with those previously reported, clearly demonstrate that 3D cell culture conditions can accelerate AD pathogenesis in AD 3D models, by promoting local Aβ deposition, whereas in conventional monolayer cell cultures, the secreted Aβ might diffuse into cell culture media and be removed during regular media changes, preventing its aggregation (Choi et al., 2014; Raja et al., 2016). However, even though our AD 3D model only recapitulates oligomer formation, it might be considered as a valid model to study the initial stages of the disease that precede Aβ production and oligomer formation. Moreover, these sorts of results support the Aβ hypothesis of AD that states that the accumulation of Aβ is the initial pathological trigger in the disease. The excess accumulation of Aβ then elicits a pathogenic cascade, including synaptic deficits, altered neuronal activity, inflammation, oxidative stress, neuronal injury, hyperphosphorylation of tau causing neurofibrillary tangles (NFTs), and, ultimately, neuronal death and dementia (Choi et al., 2015), thus contributing to overcoming the limitations and drawbacks previously mentioned.

Taken together, our study indicates that the described hydrogel-based AD 3D culture can model some AD phenotypes, such as Aβ oligomer formation, and provide a valid experimental platform for genetic forms of AD that highlights its potential applications for studying the earliest AD molecular mechanisms underlying the pathology, investigation of the efficacy and potential toxicity of candidate AD drugs, the discovery of new diagnostic biomarkers of AD, and the design of personalized therapeutic strategies; could eventually allow for the identification and treatment of patient-specific alterations underlying the disease; and also would contribute to fill the gap between the results from *in vivo* animal and *in vitro* human models to minimize the failures of clinical trials.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The protocol of reprogramming used was approved by the Ethic Committee of the Institute of Biomedical Research where the experiments on generation have been done. Human fibroblasts (HFs) carrying the A246E PSEN1 mutation were kindly donated by the Coriell Institute for Medical Research; and the HFs without mutation were purchased from ATCC (USA).

AUTHOR CONTRIBUTIONS

The laboratories of the CIATEJ and the UNAM contributed equally to this work. AC-A and KG: funding acquisition. MH-S, ER-Z, RC, AM-A, KG and AC-A: investigation. MH-S, ER-Z, and RC: methodology. AC-A, KG, and AM-A: supervision. MH-S: writing original draft. MH-S, ER-Z, RC, AM-A, KG, and AC-A: writing, review and editing.

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REVIEW
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Chitosan-Based Non-viral Gene and Drug Delivery Systems for Brain Cancer

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Central nervous system (CNS) tumors are a leading source of morbidity and mortality worldwide. Today, different strategies have been developed to allow targeted and controlled drug delivery into the brain. Gene therapy is a system based on the modification of patient's cells through the introduction of genetic material to exert a specific action. Administration of the foreign genetic material can be done through viral-mediated delivery or non-viral delivery via physical or mechanical systems. For brain cancer specifically, gene therapy can overcome the actual challenge of blood brain barrier penetration, the main reason for therapeutic failure. Chitosan (CS), a natural based biodegradable polymer obtained from the exoskeleton of crustaceans such as crab, shrimp, and lobster, has been used as a delivery vehicle in several non-viral modification strategies. This cationic polysaccharide is highly suitable for gene delivery mainly due to its chemical properties, its non-toxic nature, its capacity to protect nucleic acids through the formation of complexes with the genetic material, and its ease of degradation in organic environments. Recent evidence supports the use of CS as an alternative gene delivery system for cancer treatment. This review will describe multiple studies highlighting the advantages and challenges of CS-based delivery structures for the treatment of brain tumors. Furthermore, this review will provide insight on the translational potential of various CS based-strategies in current clinical cancer studies. Specifically, CS-based nanostructures including nanocapsules, nanospheres, solid-gel formulations, and nanoemulsions, also microshperes and micelles will be evaluated.

Keywords: chitosan, brain cancer, brain tumor, nanodelivery, nanoparticles, drug delivery, biodegradable, biomaterials

INTRODUCTION

Cancer is the second most frequent cause of mortality following cardiovascular disease, and has surpassed it in high and middle-income countries (1, 2). Although primary malignant central nervous system (CNS) tumors account for 2% of all cancers, they represent a leading cause for morbidity and mortality worldwide. Malignant CNS tumors are the principal cause of death due to solid tumors in children and the third main cause of death in the 15–34 year age-bracket. The most common presentation of a tumor in the brain is due to metastasis, accounting for 40% of intracranial tumors (3). Overall, CNS cancers represent a therapeutic challenge due to tumor heterogeneity, comprised of multiple distinct sup-populations of cells within the same tumor; each with distinct molecular features and biological responses. Furthermore, genetic and epigenetic alterations affect the progression of the disease as well as response to treatment (4). Treatment of brain tumors includes surgical resection; however, due to the infiltrative nature of some tumors, recurrence at original site and surrounding areas (up to 3 cm of the margin of the primary lesion) is often seen, and may be the primary cause of poor prognosis (5). Following surgery, a scheme based on chemotherapy and radiation is considered the standard of treatment. However, limited benefits are achieved after this multimodal strategy, mainly driven by poor drug tissue penetration and accumulation in targeted areas (6).

Nano-delivery systems have been shown to be a promising strategy against multiple types of cancer (7, 8). The possibility of modulating gene expression or the delivery of specific compounds to regulate different pathways in tumor progression has emerged as a promising alternative for CNS malignancies. Drugs or genes are attached to a variety of compounds, followed by a systemic injection or local administration into the tumor (9, 10). Remarkably, the materials show high specificity and tissue penetration in diseased area when injected locally, decreasing systemic toxicity (11, 12). Anatomical barriers, such as the blood brain barrier (BBB), are major challenges to drug penetration, often resulting in therapeutic failure (13). Nano-size materials used as vectors may serve to overcome this limitation and effectively deliver therapeutics to site of injury (9).

In CNS cancers, one of the main challenges is the administration of chemotherapeutic agents and the successful action of the drug in the desired area. For brain tumors, selective penetration of the BBB is a limiting factor for successful eradication of cancer cells (14). Chitosan (CS) -nanomaterials are local delivery systems that overcome the limitations imposed by the BBB, and allow sustained, controlled and prolonged drug release in specific areas, decreasing the risk of systemic toxicity (15). These systems can also be used for real time tracking of cancer cells when acting as imaging probes for various imaging techniques (fluorescent image guiding, magnetic resonance imaging (MRI), computed tomography (CT), positron-emission tomography (PET) and optical imaging) (16). For diagnostic purposes, when compared with free drugs, CS-nanomaterials increase the stability of contrast enhancing agents and drugs with specific accumulation in target areas. Due to their compact size and protein surface interactions, CS-nanostructure components are able to travel in small blood vessels throughout the body. Upon arrival to the tumor area, CS-nanostructures leave the systemic blood flow through disrupted tumor vasculature, and are concentrated and retained in the tumor area (tumor -homing effect) (17).

In this review, we will highlight the recent advances in CS-based gene and drug delivery systems using nanotechnology for the treatment of brain cancer.

BRAIN TUMORS

Brain tumors are one of the most devastating types of cancer, with the most malignant form having a median survival of ∼15 months. Brain tumors can be primary, meaning they arise from the native cells of the brain, or they can be metastatic, arising from tumors that have spread from other organs. Brain tumors have an annual incidence of about 22 people per 100,000 in the United States, with incidence increasing with age (18). Interestingly, brain tumors as a whole occur more frequently in women, while malignant brain tumors are more common in men, indicating a sex difference in brain tumor biology (18). Out of adult primary brain tumors, approximately one-third are malignant (19). Tumors are typically diagnosed through combined neurological exams, MRI of the brain, CT and PET scans to determine whether the tumor is a metastasis arising from another site in the body, and through tumor biopsy (20).

Gliomas, or tumors arising from glial cells, account for over 75% of malignant adult brain tumors. These tumors are classified by the World Health Organization (WHO) by histopathological features and molecular findings. Diffuse gliomas can be stratified by their cell origin through histological characterization (21). The cell of origin is controversial, with various research studies citing neural stem cells as the source of origin, while others cite glial progenitors; classifications are based on features of glial cells. Astrocytomas present features of astrocytes, the star-shaped glial cells important for brain homeostasis, while oligodendrogliomas express features of oligodendrocytes, the cells that produce myelin. Anaplastic astrocytomas and glioblastomas represent 38% of primary brain tumors (3, 22). Diffuse gliomas are classified by the WHO as oligodendrogliomas (grade II), anaplastic oligodendroglioma (grade III), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and the most common glioblastoma (GBM) (grade IV) (21). Gliomas are further defined by their isocitrate dehydrogenase (IDH) 1/2 mutation status. Mutations in IDH 1 and 2 are extremely common in low grade gliomas and secondary high grade gliomas, or high grade tumors that progress from lower grade tumors (23). However, this mutation is relatively rare in primary GBM (23). Additionally, 1p/19q co-deletion, ATRX loss, and TP53 mutation is profiled in order to fully define diffuse gliomas (21). GBM can be further characterized into four molecular subtypes—proneural, neural, classical, and mesenchymal—based on distinct transcriptional signatures (24).

The current standard of care for GBM involves a combinatorial strategy of surgical resection, chemotherapy, and radiation treatment (25). The addition of the chemotherapeutic

drug, temozolomide (TMZ), to the treatment strategy in 2005 increased median survival of patients from 12.1 to 14.6, signifying the last major change to GBM treatment (25). Recent medical advances including the development of tumor treating fields via the Optune® system have also shown a significant survival benefit, although these treatments do not provide a cure for GBM (26). The current status of brain tumor management results in a significant need for the development of better therapeutic options to improve patient care (6).

NON-VIRAL MEDIATED DELIVERY SYSTEMS

In contrast to viral vectors, non-viral delivery systems are better tolerated, can carry large amounts of nucleic acid and have a higher safety index due to their transient expression compared to stable modifications (27). CS is an organic molecule that is less toxic than other cationic polymers such as polyethyleneimine, polylysine, or polyarginine (27), and is therefore a promising excipient for non-viral gene and drug delivery systems. Non-viral delivery can be divided into physical or chemical methods (27–30).

Physical Delivery Systems

Electroporation: An electrical pulse is applied to the cells to increase the permeability of the cell membrane facilitating uptake of DNA strands (31).

Direct injection of nucleic acids: This method has shown a relative degree of success in some tissues, however, without protection following systemic injection, the plasmid DNA (pDNA) is rapidly broken down by nucleases (31).

Chemical Delivery Systems

Cationic lipids: Lipid-based systems such as FuGene, GenePORTER, Transfast, DOTAP, and Lipofectamine 2000TM are commercially available lipid-based vectors. They are positively charged and encapsulate the anionic nucleic acid to enable cell entry via endocytosis. Lipofectamine 2000TM is the most commonly used reagent and often acts as a positive control in many studies (32).

Cationic polymers: These polymers are positively charged materials that bind electrostatically to negatively charged nucleic acid to form delivery vectors (33). Polymers such as Poly (L-lysine) (PLL), polyethyleneimine (PEI), and Polyamidoamine (PAMAM) dendrimers, have shown promising results pre-clinically, however, toxicity and side effects are often displayed *in vivo* and *in vitro* experiments, ultimately limiting their translational potential (34–36).

Complexation with nucleic acid can reduce the charge of synthetic polymers, for that reason, there is growing concern regarding the degradation and ultimate fate of the construct of non-viral vectors. There is a growing interest in the use of natural biocompatible and biodegradable polymers such as CS (37) which has been used extensively in nucleic acid delivery. CS meets the criteria for a successful non-viral nucleic acid delivery carrier: efficiency in cell uptake, protection of nucleic acids from degradation, efficient unpacking of the genetic cargo, escape from endosomal pathways, and nuclear import (38).

BIODEGRADABLE POLYMER: CHITOSAN

Chitosan is the main derivative of chitin (poly-N-acetyl glucosamine), a linear polysaccharide highly biodegradable and one of the most abundant polymers in nature (second only to cellulose) (11). Partial deacetylation in alkaline conditions of chitin results in the production of CS, a positively charged polysaccharide highly soluble in low pH solutions and poorly soluble in physiological aqueous solutions. CS is present in the exoskeletons of crustaceans (like crabs, lobsters and shellfish), insects and the cellular walls of mycelial fungi with a molecular weight ranging from low (<100 KDa) up to high (>300 KDa) (39). This biomaterial is non-toxic, biocompatible and biodegradable with low allergenicity. It also functions as an antioxidant, hemostatic agent (40, 41); and chelator of elements such as iron, copper and magnesium. CS is cleared by enzymatic hydrolysis mediated by intestinal microorganisms and lysozymes. The main derivatives with medical applicability are N,N,N-trimethyl-CS, N,O-carboxymethyl-CS and O-carboxymethyl-N,N,N-trimethyl-CS (39, 42).

Due to its ability to modulate the inflammatory response, CS has been used for the repair of damaged tissue (wound-healing) by promoting formation of granular tissue after injury (40). Additionally, CS increases the action of neutrophils, macrophages and fibroblasts, ultimately speeding the process of tissue repair. The tissue repair effects of CS are dependent on molecular weight, degree of chemical modification (deacetylation), and CS presentation. Therefore, CS has unique properties that could enhance neuroregeneration by mitigating secondary neuroinflammatory tissue injury. Another strategy for wound healing treatment is through CS-mediated vehicles to deliver growth factors (i.e., FGF, EGF), this option allows for an extended action of the growth factor in the desired location (42).

Antimicrobial action of CS is mediated by its cationic charge that destabilizes the negative bacteria cell membrane, leading to a leakage of inner cellular components (proteins, nucleic acids) and increased permeability in the bacteria cellular membrane impairing nutrient uptake (43). Interestingly, lower concentrations of CS (<0.2 mg/ml) cause bacterial agglutination, while higher concentrations keep them in suspension (40). This biomaterial has broad potency against gram-positive and negative bacteria such as *S. aureus*, *P. aeruginosa*, *P. mirabilis,* and *E. Coli* (43); causing osteomyelitis, cystitis, periodontitis, mucositis, burn, and skin infections among others. The potency of CS biomaterial is dependent on the dose, pH and temperature and on the composition of the polymer (hydrogels, coatings, powder, solution, films, pure, or loaded with different materials) (44).

CHITOSAN-BASED NANOSTRUCTURES FOR BRAIN CANCER TREATMENT

CS multifunctionality and high cargo entrapment efficiency make CS derivatives versatile nanodelivery vehicles. Chitin monomers are linearized under alkaline conditions by deacetylation in the solid state or by the enzymatic hydrolysis of chitin deacetylases (45). The bipolyaminosaccharide structure is composed of a carbohydrate backbone and abundant –OH and $–NH_2$ functional groups that act as readily accessible moieties for functional modifications. This enables tuning for efficient cross-linking, controlled drug release profile, enhanced electrostatic interaction, and increased solubility. The degree of deacetylation and molecular weight ratio of CS-nitrogen to phosphate-cargo make CS a suitable biomaterial that could be utilized for nanoparticle synthesis and nanomaterial fabrication for the delivery of therapeutic agents (46). Amongst the most common nanodelivery systems explored, CS nanoparticles (NPs) have provided a great degree of safety and durability across various pharmaceutical and pre-clinical applications. The inherent cationic nature of CS allows efficient binding to microtubules or motor proteins for cytoplasmic trafficking, increased plasmid or cargo release efficiency mediated by the osmotic pressure in the endosome (caused by influx of hydrogen protons), and finally, low toxicity index due to its biocompatibility and biodegradability across various biological applications (46). Cargo is either complexed or confined inside the CS particle, or dispersed in a CS matrix. These particulate systems can be prepared by cross-linking, cationic salts solvation, emulsification, ionic complexation, or gelation methods by reacting with different functional groups on proteins, antibodies, drugs, DNA/RNA or other pH sensitive moieties (46).

Particles are characterized by their spherical diameter and spatial composition, with **microparticles/microspheres** ranging between 1 and 1,000 μm and **NPs/nanospheres** measuring between 1 nm and >1 μm (47). NPs (and microparticles) are characterized by their constituent components and can be referred to as "**nanocapsules**," a vesicular particulate system with a hollow sphere consisting of an oil or water core (that may include active cargo), and a polymeric shell (48). This structure mediates cargo entrapment in the core or adsorption on the particle surface. Conversely, matricial structures are referred to as "**nanospheres**" and denote particulate systems where the active molecule is incorporated into the polymer network (48). Moreover, "**solid-lipid**" NPs refer to systems that utilize lipids in the solid phase and subsequent emulsification with a surfactant for structure stability (49). This structure is advantageous when delivering cargo that is poorly water soluble. In line with lipid structures are "**nanoemulsions**" created by the mixture of two immiscible liquids stabilized by a surfactant. Single lipid layer derived particles are referred to as "**micelles**" and do not contain an aqueous core (50). **Table 1** summarizes the advantages and disadvantages of the described nanostructures.

CS nanosystems are selected based on multiple factors including cargo polarity, solubility, weight, and rout for optimal administration. CS-coated or CS-formulated particulate systems have proven to be efficient nanocarriers to the CNS. Due to their enhanced membrane adhesive nature, particles carrying genes of interest enable enhanced transfection efficiency to recipient cells. Size and composition of CS nanoparticles are fundamental factors that determine targeting and biodistribution to tumors of various origins (55). Nanosized carriers are suitable for disease models that are hypervascularized, such as brain tumors, and would benefit from the enhanced permeability and retention effect permitting passive diffusion in the intratumoral space; this effect limits off-target toxicity (56).

While the development of CS-based nanocarrier technology for brain tumors have primarily focused on the encapsulation and delivery of chemotherapeutics, we will attempt to highlight current advances in non-viral gene delivery strategies using CS nanoparticles along with some promising strategies of drug and chemotherapeutic based encapsulation approaches used for brain cancer treatment.

TABLE 1 | Advantages and disadvantages of distinct nanostructures.

Morphology	Advantages	Disadvantages	References
Nanocapsules	Rapid absorption of cargo and longer retention time at target site. Low polymer content required for comparable drug loading. Shell prevents direct contact of cargo with environment offering enhanced protection of load from degradation	Aggregation of particles and leakage of cargo	(11)
Nanospheres	Slow and sustained release of encapsulated cargo. Higher efficiency and low toxicity. More readily protects cargo against reticuloendothelial system	Storage by freezing leads to microfibers. Harsh processing conditions required for scaled-up manufacturing	(51)
Solid-lipid formulations	Versatility of cargo incorporation (hydrophilic and lipophilic) and avoidance of efflux (ex: P-glycoprotein) by exporters on cell membrane	Reorganization of crystalline structure during long storage times could compromise cargo release profile. Low loading efficiency due to "burst effect"	(49)
Nanoemulsions	Oil droplet protect cargo from oxidation and hydrolysis in circulation. Efficient self-assembly and solubilization of lipophilic drugs	Rapid release, low stability and lower encapsulation of hydrophilic molecules	(52)
Micelles	Brain cancer targeting moieties to the vasculature widely studied (transferrin receptor integrins) (53) RGD peptides, LRP1 (LDL Receptor Related Protein 1)	Non-modified micelles display impaired penetration through the BBB (sub-therapeutic delivery of treatment load)	(54)

TABLE 2 | Multiple chitosan applications in cancer drug delivery.

Disease model	Morphology	Composition	Preparation	References
C6 glioma cells	Nanoemulsions	Polyethylene glycol	Docetaxel loaded D-α-tocopherol polyethylene glycol succinate 1,000 conjugated CS	(79)
RPMI 2,650 human nasal cell line	Nanocapsules	Lipid-core nanocapsules coated with CS	Simvastatin- loaded poly-ε-caprolactone nanocapsules coated with CS	(80)
C6 glioma cells	Nanoemulsions	Oil kaempferol (KPF) (0.1% w/w) in 16% (w/w) medium-chain triglyceride (MCT) and 5.0% (w/w) egg-lecithin	KPF-loaded nanoemulsion and KPF-loaded mucoadhesive nanoemulsion	(74)
GBM	Scaffolds—polyelectrolyte complexes	CS -polyelectrolyte complex scaffolds	Porous CS- scaffolds	(81)
Human brain cancer stem cells	NPs	CS-PLGA NPs modified with sialic acid (SA)	Curcumin -loaded CS- PLGA NPs modified with SA	(82)
T98G human GBM cell line and human umbilical vein endothelial cells	Nanoemulsion	PLGA NPs (50:50)	5-FU PLGA (50:50) NPs, bevacizumab, were loaded into the scaffold	(83)
GBM	Polymeric NPs	Glycol CS and dextran sulfate NPs	Methotrexate—loaded polymeric NPs based on Glycol CS and dextran sulfate	(84)
Human brain cancer cell line (Hs683)	Piperine micellization	Nanomicelles forming core-shell NPs	Optimum piperine-loaded core-shell NPs	(85)
C6 glioma cells	NPs	Glycol CS NPs	MTX-loaded CS NPs	(86)
Mouse fibroblast cell lines L929	NPs	Core–shell polymeric NPs	Docetaxel-loaded NPs	(87)

CHITOSAN-BASED DELIVERY-SYSTEMS TO BRAIN CANCER

Treatment-resistant brain tumors, such as grade IV gliomas, overexpress epidermal growth factor receptor (EGFR) and galectin-1, leading to chemotherapy resistance. Amplification of EGFR is found in > 50% of GBM cases and presents a logical molecular target for GBM therapy (57). Given the importance of EGFR and its isoforms in brain tumors, several agents have undergone clinical trials in an attempt to target EGFR (i.e., lapatinib, gefitinib) but outcomes have been largely disappointing (58). This is partly due to poor BBB penetration, and the discovery that EGFR receptor blockade is not enough to inhibit downstream signaling, suggesting that EGFR receptor blockade may be activating other pathways that confer cell survival (59). Such a phenomenon would benefit from directed gene silencing. In an effort to examine the silencing efficiency of EGFR and Galectin-1 in U87 human GBM line, CS lipid nanocapsules were complexed with anti-EGFR and anti-galactin-1 small interfering RNA (siRNA) and administered via convection enhanced delivery (CED) (a minimally invasive surgery that placed catheters directly into the tumor bed to deliver pharmaceutical agents) in athymic nude mice (60). Treated groups received concomitant TMZ administration to examine chemotherapy resistance or response after gene silencing. CS nanocapsules carrying EGFR and galectin-1 siRNA significantly increased survival in tumor-bearing mice and decreased gene expression in tumor tissue. While CED administration proved effective in the delivery of CS nanocapsules, another advantage of CS is its mucosal adhesion offering a different administration route. Intranasal delivery has gained momentum in human clinical trials for therapeutic delivery, partly due to its reduced invasiveness and toxicity. CS nanocapsules were delivered intranasally for RNA interference (RNAi) mediated knockdown of galectin-1 in GL261 mouse glioma line, and demonstrated successful nose-to-brain transport of siRNA along with survival benefits when delivered with programmed cell death-1 (PD-1) immunotherapy *in vivo* (61). Data suggests that CS based nanocapsules could effectively translocate across the BBB and deliver nucleic acids to brain cancer *in vitro* and *in vivo* (62, 63).

To circumvent drug delivery limitations to the CNS, poly(lactic-co-glycolic acid) (PLGA) modified CS nanoparticles (CSNPs) were conjugated with Arg-Gly-Asp RGD-linked peptide and loaded with clinically approved paclitaxel (PTX) chemotherapeutic drug for GBM therapy. The PTX-PLGA-CSNP-RGD particle, prepared by emulsion-solvent evaporation, displayed optimal tumor targeting and uptake via integrin receptor mediated endocytosis, induced cell-cycle arrest at G2/M, and increased lung tumor cell death (64). While authors did not address brain tumors in the study, the incorporated RGD linked ligand targets $\alpha_v\beta_3$ integrins on endothelia and is highly expressed in many tumor vasculature beds but largely absent in normal tissue. Hyper-vascularized tumors, such as brain cancers, would benefit from nano-platforms that incorporate integrin targeting strategies for gene or drug delivery. To bypass limitations of chemotherapeutic delivery to the BBB, dual functionalized liposomes were developed to mitigate transportation of doxorubicin and erlotinib to tumor cells. Liposomes were surface modified with transferrin enabling their translocation across endothelial cells lining the blood vessels, and whose surface exhibits high transferrin receptor expression. Additionally, a cell penetrating PFVYLI peptide was coated on the surface to enhance liposomal uptake by U87 human GBM commercial cell line. GBM cells were seeded in PLGA-chitosan

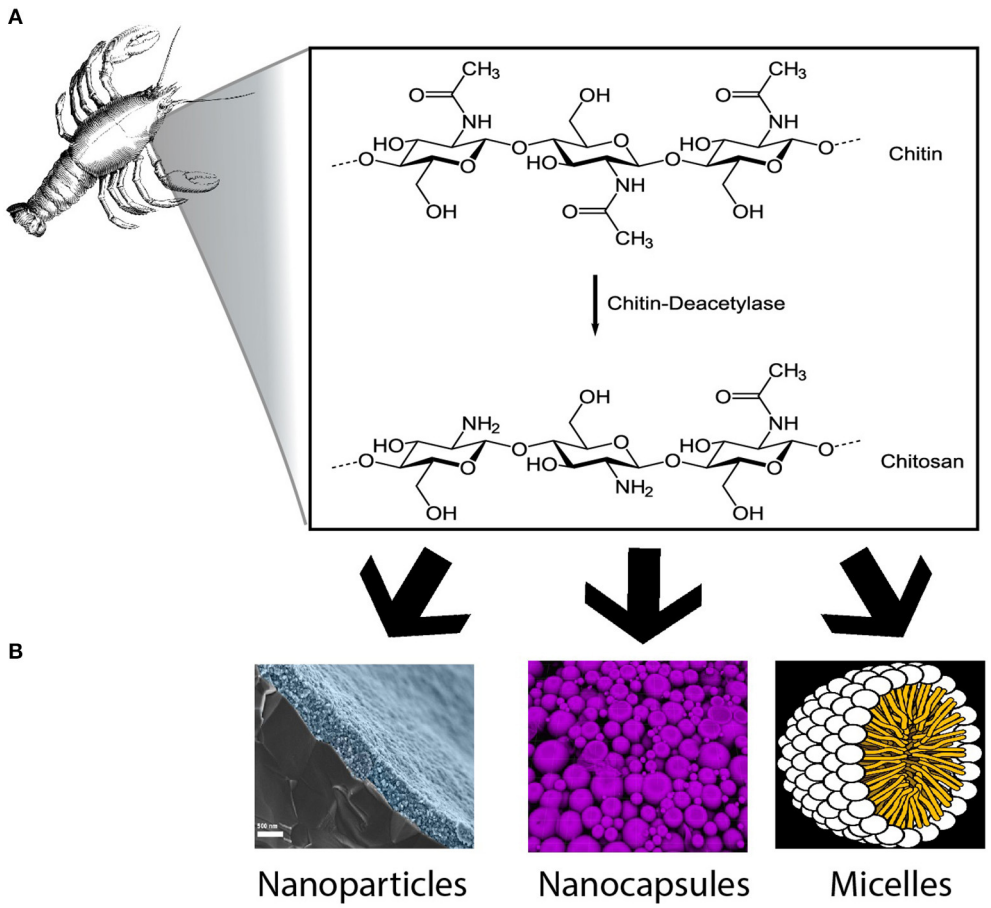

FIGURE 1 | Chitosan based formulations for brain cancer. **(A)** Representation of the chemical conversion from chitin to chitosan. **(B)** Nanoparticles, nanocapsules, and micelles are some examples of chitosan formulations described in this review (Images from **A,B** were obtained from wikimedia commons without any modifications. Chitosan synthesis image by neurotiker, nanoparticles image design by HiguchiJu, nanocapsules image by Stephan Weiss and micelles image by Mariana Ruiz Villareal).

scaffold serving as an *in vitro* porous scaffold 3D platform to study the functional translocation and cellular uptake of coated liposomes. Tumor cells seeded in the PLGA-chitosan scaffold resulted in 52% cell death. This study offers a 3D based platform that acts as a sufficient surrogate to study nanoparticle uptake and translocation in 3D models of brain tumors (65).

Brain targeted chitosan-coated nanoparticles is further shown to enhance particle uptake by human blood-brain barrier cerebral microvessel endothelial cells (hCMECs) via receptor mediated endocytosis. Further evaluation into the mechanisms enabling this translocation revealed a preferential cellular uptake pathway implicating the transferrin receptor with subsequent nanoparticle internalization via receptor-mediated endocytosis (66).

Improved *in vivo* brain pharmacokinetics of conventional GBM chemotherapy, such as TMZ, was shown to be significantly enhanced when polyamidoamine (PAMAM) dendrimer is coated with chitosan and conjugated to TMZ. Chitosan-coated PAMAM conjugated to TMZ improved GBM tumor targeting in U-251 and T-98G cell lines at lower TMZ concentrations. *In vivo* pharmacokinetics exhibited sustained release with a half-life of 22.74 h in chitosan-coated dendrimer compared to free drug (TMZ alone) at 15.35 h. Reported work revealed that chitosan-anchored nanoparticles are sufficient at delivering chemotherapy across the BBB and enhanced tumor cell cytotoxicity *ex vivo* (67).

Similarly, CS nanospheres were constructed by complexing pDNA with CS tripolyphosphate (TPP) and hyaluronic acid (HA) via ionotropic gelation. Ionic gelation permits the formation of sol-gel transition, and TPP stabilizes the complex in biological fluids and decreases particle size (68). Resultant nanosphere (CS-TPP/HA) was evaluated *in vitro* for intracellular delivery of Pseudovirus (pSV)-luciferase (surrogate gene) to neural stem cells and spinal cord slices along with direct injection into the spinal cord *in vivo*. HA signals through CD44 and the receptor for hyaluronan mediated motility (RHAMM) on neural stem cells, regulating proliferation and angiogenesis and mediating the radial migration of spinal cord neurons. CS-TPP/HA resulted in higher gene transfection efficiency, less toxicity, and more retention time of CS nanosphere *in vitro* and *in vivo* compared to PEI or naked-DNA alone, suggesting a viable carrier for gene delivery to neural stem cells using CS nanospheres.

Retinoic acid (RA), a derivative of vitamin A, activates Notch signaling response pathways in glioma initiating stem cells, prompting lineage specific differentiation and arrest at the G0/S phase (69). Strategies to induce cancer stem cell differentiation have been widely used across various malignancies, such as the delivery of bone morphogenetic protein 4 (BMP4) to brain tumor initiating cells for astrocytic induction, rendering cancer stem cells more susceptible to chemotherapy (70). Trimethylated solid-lipid CS formulation was constructed for RA encapsulation to evaluate affinity and delivery of RA to U87 human GBM line (70). N-N-N-trimethyl CS-functionalized (TMC) particles offer increased solubility above native CS solubility threshold (pH $<$ 5.6) (71). The polyelectrolytic cationic nature of TMC improves aqueous solubility across a range of pHs while maintaining efficient cell targeting. TMC solid-lipid particles exhibited significant anticancer effects by inducing apoptosis mediated by the delivery of RA, compared to free RA alone. Trimethylated solid-lipid particles offered enhanced protection from the "burst-effect" and prolonged circulation. Modified solid-lipid CS particles hold great promise for cancer therapy as they can deliver sufficient therapeutic pay-loads, entrap hydrophobic drugs at larger concentrations, and improve drug-release profile.

Nanoemulsions are the product of the mixing of two immiscible liquids into a single phase through the use of a surfactant (72). The resulting size of emulsified spheres lies between 10 and 1,000 nm (72). CS has emerged as an attractive coating in nanoemulsions as a way to treat cancer. In particular, CS nanoemulsions have been used to deliver chemotherapeutics in order to increase drug stability, bioavailability of hydrophobic molecules, or drug uptake using positively charged CS to pass the negatively charged biological membranes. In experiments designed to treat brain cancer, the chemotherapeutic 5-fluorouracil (5-FU) has been entrapped into a CS nanoemulsion in order to increase uptake (73). The created nanoemulsion retained more 5-FU within the core matrix of resulting particles, resulting in a slow-release profile from nanoemulsion over a period of 30 days (73). Despite encouraging results on drug release, this study did not perform any results on glioma cell viability. Other studies have found incorporating CS into a 5-FU nanoemulsion increases mucoadhesive properties, contributing to the feasibility of intranasal application (74). Adding CS to the nanoemulsion in this case increased mucoadhesion and resulted in increased drug release *in vivo* in rats (74). Additionally, the 5-FU-loaded CS nanoemulsion resulted in decreased viability and increased apoptosis in C6 rat glioma cells (74), suggesting this may be a practical alternative way to treat glioma. Based on the results found with increased uptake and slow drug release in chemotherapy-loaded CS nanoemulsions, there is high potential for using these methods in DNA-based therapeutics.

Micelles are particles 10–100 nm in diameter formed by amphiphilic molecules self-assembling by turning hydrophobic compartments inward and hydrophilic compartments outward in solution (75). Similar to nanoemulsions, the addition of CS to micelles is appealing as it allows for increased uptake and bioavailability of hydrophobic compounds. Additionally, the loading of therapeutics into micelles with CS may allow for increased transport across the BBB due to the small, amphiphilic nature of the particles. The use of CS micelles in targeting brain cancer has been limited to the use of chemotherapeutics, but has shown to be relatively effective in drug delivery. When CS-containing micelles are loaded with all-trans RA, there is slow drug release and a significant decrease in the migration of U87 GBM cells compared to the application of free drug (76). Similarly, when loaded with a water-insoluble chemotherapeutic, myricetin, there is increased drug uptake, decreased cell viability and increases apoptosis *in vitro* (77). The use of CS micelles with myricetin also decreased tumor growth *in vivo* compared to free drug and controls (77). Adding conjugated CS to micelles can also target glioma cells based on overexpressed receptors on the cell surface. Conjugating CS to d-α-tocopheryl glycol succinate 1000 (TPGS) and incorporating it into docetaxel-loaded micelles allows for targeting to the transferrin receptors on glioma cells (78). This method of targeting glioma cells for chemotherapy is over 200-fold more effective on C6 rat glioma cell viability than free Docel and also exhibits increased cell uptake and stability *in vivo* over time (78). Based on the findings with CS-containing micelles in chemotherapy delivery, this could be a potential future avenue for DNA technology in CNS cancer.

Table 2 summarizes some examples of CS-based formulations used as a cargo for chemotherapeutic delivery against cancer.

In conclusion, CS has been widely used in several health care materials and extensively studied; chitosan-coated material may offer novel and improved approaches toward the delivery of cancer therapeutics (88). Its biocompatibility and intrinsic characteristics makes it a suitable option to be used as a carrier for brain cancer therapy. Additionally, it is highly available in nature and represents a cost-effective biomaterial for chemotherapeutic delivery to the brain. Based on many pre-clinical studies detailed above, we anticipate that CS will become widely used in upcoming clinical trials and therapeutic development, particularly as a vehicle for previously approved medications and novel DNA gene therapy targeting brain cancer (**Figure 1**).

AUTHOR CONTRIBUTIONS

ML-V, RA, EN, CR-L, and RS-E contributed conception and design of the study. ML-V, RA, EN, and CR-L wrote the first draft of the manuscript and sections of the manuscript. HG-C, AQ-H, WF, and AF contributed reviewing the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Engineering Three-Dimensional Tumor Models to Study Glioma Cancer Stem Cells and Tumor Microenvironment

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Glioblastoma (GBM) is the most common and devastating primary brain tumor, leading to a uniform fatality after diagnosis. A major difficulty in eradicating GBM is the presence of microscopic residual infiltrating disease remaining after multimodality treatment. Glioma cancer stem cells (CSCs) have been pinpointed as the treatment-resistant tumor component that seeds ultimate tumor progression. Despite the key role of CSCs, the ideal preclinical model to study the genetic and epigenetic landmarks driving their malignant behavior while simulating an accurate interaction with the tumor microenvironment (TME) is still missing. The introduction of three-dimensional (3D) tumor platforms, such as organoids and 3D bioprinting, has allowed for a better representation of the pathophysiologic interactions between glioma CSCs and the TME. Thus, these technologies have enabled a more detailed study of glioma biology, tumor angiogenesis, treatment resistance, and even performing high-throughput screening assays of drug susceptibility. First, we will review the foundation of glioma biology and biomechanics of the TME, and then the most up-to-date insights about the applicability of these new tools in malignant glioma research.

Keywords: glioma, tumor microenvironment, stem cell, bioprinting, organoids, organ-on-a-chip, tissue engineering, spheroids

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INTRODUCTION

Tumors are complex systems with dynamic and constant regulation of their different components during initiation, maintenance, and progression. Gliomas, and particularly glioblastomas (GBM), are some of the most comprehensively characterized cancers, and huge efforts have been done in an attempt to overcome the therapeutic plateau existing after current standard, and even experimental therapies. Unfortunately, despite all these efforts, there have not been significant advances in the way we treat our patients, and the cure is far from our current achievements.

Therefore, there is a need to reconceptualize the process in which GBM biology is being studied in order to find meaningful therapeutic approaches. In this setting, tumor microenvironment (TME) is an inevitable masterpiece to consider, as the inherent crosstalk between this and glioma CSCs is a defining driver of GBM heterogeneity, plasticity, and evolution.

Three-dimensional (3D) models, derived completely from patient tissue or incorporating biomaterials, are a new technology that has risen as a potential tool to better recapitulate TME dynamics. We aim to briefly summarize pertinent concepts about glioma biology and the biomechanics of the TME, and then describe recent advances and potential applications of this technology.

GLIOMA CANCER STEM CELLS

Glioma cancer stem cells (CSCs) were first described in early 2000s (Uchida et al., 2000; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004) and were required to fulfill defining criteria of normal stem cells (Uchida et al., 2000; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004). Therefore, glioma CSCs must be able to self-renew and grow tumors resembling its original histopathology. Several models have been suggested to explain CSC maintenance (**Figure 1A**); however, it is most probably that the evolutionary model of the CSCs hypothesis, or an even more holistic understanding, could better serve on this purpose (Chen et al., 2012).

Additionally, there is a lack of uniformity regarding the nomenclature of CSCs, which generates confusion and may redirect the research focus far from the study of CSC biology. While the term *stem cell* is used, this does not necessarily mean that CSCs derive from a distorted canonical stem cell (**Figure 1B**). Regardless of the true cellular origin of CSCs, the use of the term *stem cell* requires that these cells comply with at least functional defining criteria such ability to self-renew and generate different progeny with different hierarchies inside the tumor.

Several enrichment markers of stemness have been suggested to identify CSCs. BMI1, SOX2, NESTIN, OLIG2, NANOG, MYC, and IDI1 (inhibitor of differentiation protein 1), among others, are crucial transcription factors and/or structural proteins required for normal neural stem and progenitor cell (NSPC) function. These markers are shared between glioma CSCs and NSPCs. However, given that conventional methods used for CSC selection (CSC enrichment), such as flow cytometry, are limited in the use of intracellular proteins (as the ones stated above), several surface biomarkers like CD133, CD44, CD15, L1CAM, A2B5, and integrin α6 have been widely used instead. Interestingly, some of these surface biomarkers have been related to glioma cell–microenvironment interactions, which reflect the relationship between TME and glioma biology.

Glioma CSC Markers and Its Interactions With the Tumor Microenvironment

CD133 (Prominin-1)

Human neural stem cells were identified for the first time by Uchida et al. (2000). The group harvested cells from fetal brain tissue and found that the isolated $CD133^{+}$ population was able to fulfill the criteria required to be defined as stem cells. This finding prompted a scientific hunt for brain tumor stem cells, and soon after, CD133 was proposed as the first biomarker for glioma CSCs (Uchida et al., 2000; Hemmati et al., 2003). However, controversies about CD133 reliability raised after two independent groups showed that GBM $CD133^{-}$ cells could also embrace stem cell properties such as self-renewal and differentiation *in vitro* and tumor formation *in vivo* (Beier et al., 2007; Joo et al., 2008; Wang et al., 2008; Wei et al., 2013). Furthermore, $CD133^{-}$ population would tend to grow as

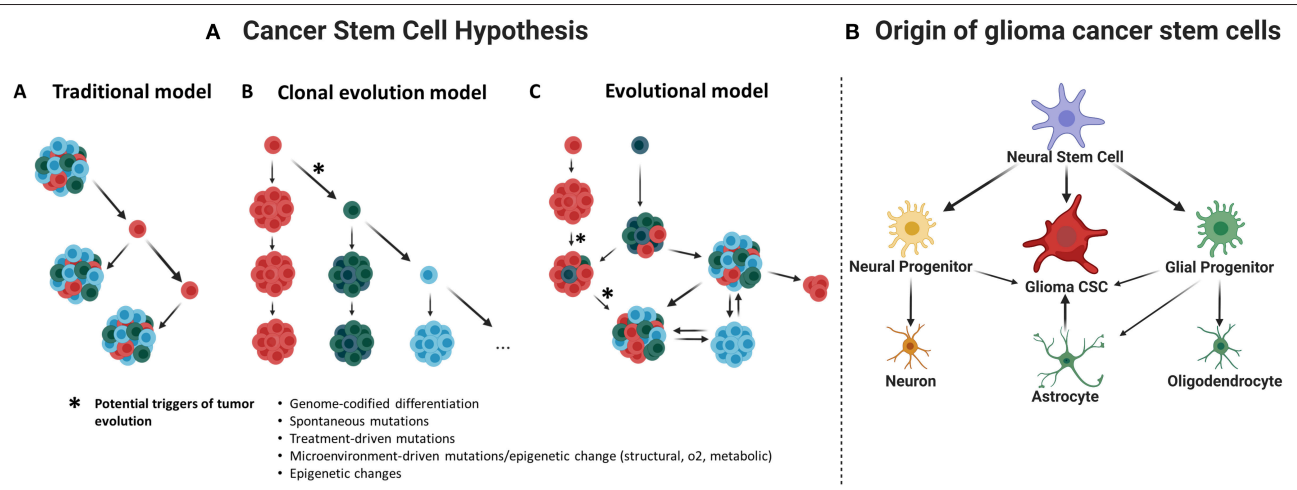

FIGURE 1 | (A) Cancer stem cell hypothesis. This hypothesis suggests that a subset of cancer cells is responsible for tumor initiation and growth, having characteristics such as self-renewal and chemotherapy and radiotherapy resistance. (A) Traditional or hierarchical model. Suggesting the existence of a specific cancer cell population with stem-like properties that function as the tumor initiating cells, this population of CSCs would be sufficient to initiate and drive the tumor growth over time. (B) Clonal evolution model. It is proposed that many clones of CSCs would be functionally equivalent and able to maintain tumor growth; they would remain under constant genetic pressure that can introduce new characteristics and create new clones. (C) Evolutional or stochastic model. Random genetic or epigenetic events can transform any cell to a variety of cancer stem cells at any time within the tumor. This will presuppose a hierarchy of CSCs under constant evolution due to natural selection and genomic instability (Chen et al., 2010). **(B) Origin of glioma cancer stem cells**. Evidence suggests that neural stem cells, astrocytes, or oligodendrocyte precursor cells could be the origin of gliomas.

adherent tumorspheres under conventional *in vitro* conditions and was proven able to give rise to cultures containing $CD133^+$ glioma cells *in vitro* and *in vivo* (Wang et al., 2008; Chen et al., 2010). Overall, it was clear that glioma CSCs could also present as a $CD133^-$ population.

CD133, also known as prominin 1, is a cell surface glycoprotein with five transmembrane domains. Given its superficial location, detection of CD133 may vary depending on several factors such as cell–microenvironment interactions and epigenetic influences. Careful analysis of its informational value is recommended as immediate cell–extracellular matrix (ECM) disassociation, extended *in vitro* culture, and/or equivocal epitope recognition may give rise to false-negative results (Clément et al., 2009; Osmond et al., 2010; Campos et al., 2011).

Although a definitive role for CD133 on glioma CSCs remains elusive, it is clear that the expression of CD133 may vary according to several interactions with the TME. For instance, changes in ECM composition (Logun et al., 2019) or decreased oxygen tension on the TME is related to higher CD133 expression (Platet et al., 2007; Soeda et al., 2009; Musah-Eroje and Watson, 2019) and faster expansion and retained undifferentiation in $CD133^+$ gliomas cells. In the opposite direction, CD133 can lead to activation of PI3K/Akt signaling pathway leading to increased self-renewal and tumor formation (Wei et al., 2013), as well as interleukin 1β signaling-mediated downstream regulation of the TME through increased neutrophil recruitment (Lee et al., 2017).

CD44 (Hyaluronic Acid Receptor)

CD44 is a cell membrane glycoprotein that binds extracellular ligands present in the ECM, such as hyaluronic acid (HA) and osteopontin. These interactions promote cell motility toward ECM through the mechanotransduction involving CD44 linkage to cytoskeletal components (Tsukita et al., 1994).

As $CD133^-$ glioma population was found to display stem cell–like properties, other markers of stemness were sought. The role of CD44 as a surface marker of glioma CSCs has been described by several authors (Tsukita et al., 1994; Anido et al., 2010; Xu et al., 2010); interestingly, CD44 would be the most common shared marked of stemness among CSCs derived from different malignancies (Mooney et al., 2016). CD44 has been associated with GBM aggressiveness through increased invasion and migration (upon binding with HA) (Radotra and McCormick, 1997; Brown et al., 2015), increased proliferation (Monaghan et al., 2000; Feng et al., 2014), and enhanced chemoresistance (Xu et al., 2010).

CD15 (SSEA-1)

CD15, also known as Lewis X or SSEA-1 (stage-specific embryonic antigen 1) is a cell surface carbohydrate antigen. CD15 was first suggested as a marker for glioma CSCs in 2009. Son et al. (2009) found that, in GBM, $CD15^+$ cells possess a 100-fold tumorigenic potential when compared to $CD15^-$ population. Furthermore, all $CD15^+$ cells were also positive for CD133, whereas most of the $CD133^+$ cells were $CD15^+$ as well.

L1CAM

The neural cell adhesion/recognition L1 molecule (L1CAM or CD171) is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily; this protein is normally found during central nervous system (CNS) development. In 2008, Bao et al. (2008) reported that L1CAM supported glioma CSC survival and clonogenicity in $CD133^+$ cells through the regulation of Olig2 and the tumor suppressor p21. Furthermore, L1CAM function in GBM cell migration was determined by the same group; ADAM10 would cleavage L1CAM ectodomain, which then would activate EGFR and integrins (FAK-mediated process) to promote glioma CSC migration (Bao et al., 2008; Yang et al., 2011).

A2B5

A2B5 is a cell surface ganglioside present in glial precursor cells. Ogden et al. (2008) found that this epitope was also present in a sizable population of glioma-initiating cells; even more, most of the $CD133^+$ cells were contained in the $A2B5^+$ population. The authors were able to show that A2B5 renders stem cell properties even in $CD133^-$ population (Ogden et al., 2008). Similar results were also presented by other authors (Tchoghandjian et al., 2010). Sun et al. (2015) showed that $CD133^-/A2B5^+$ population possesses great migratory and invasive potential and hypothesized that this could be characterizing the infiltrative cells of the invasive tumor front leading GBM posttreatment recurrence.

ROLE OF TUMOR MICROENVIRONMENT IN GLIOMA BIOLOGY

TME is a crucial teamster of CSC heterogeneity, plasticity, and evolution (Charles et al., 2010). However, CSCs can reciprocally regulate the microenvironment. Glioma CSCs not only secure self-renewal (Man et al., 2014), malignant proliferation (Fan et al., 2010), and segregation into different tumor cells, but also interact in a multidirectional way with different tumor components such as the ECM, the cellular compartment (cancer-associated fibroblast, immune cells, differentiated neural cells, etc.), and even the blood–brain barrier (BBB) through tumor-derived pericytes in order to establish a favorable niche able to support further malignization and treatment resistance (Cheng et al., 2013). In this section, we will review this reciprocal crosstalk and its implications in glioma treatment behavior and resistance.

Components of Glioma TME

Extracellular Matrix

The ECM constitutes the non-cellular compartment of the TME. This is a 3D molecular network built with water, proteins, and polysaccharides (Frantz et al., 2010). The specific composition of each ECM is driven by a real-time biochemical and biophysical feedback between cells and their surrounding microenvironment (Gattazzo et al., 2014). CSCs are in constant interaction with the ECM via several multifunctional transductors as we reviewed above (CD133, CD44, L1CAM, integrin α6, and others). CSCs are able to give rise to more differentiated cells that can later regulate the production of extracellular components in order to promote

tumor niche homeostasis. It is noteworthy that abnormal ECM remodeling affects endothelial and immune cells, tumor angiogenesis, and drug penetration, thus influencing tumor aggressiveness and progression. Several ECM components, such as integrins, laminins, and cadherins, among others, have been linked to treatment response and patient survival (**Figure 3**) (Ljubimova et al., 2004; Lathia et al., 2012).

Laminins are a family of extracellular T-shaped heterotrimeric glycoproteins consisting of one **α**, one **β**, and one **γ** chain. In vertebrates, different genes codifying for five α, three β, and three γ chain exist. Although 45 combinations are possible, only 18 isoforms have been identified up to date (Laminin isoforms 8 and 9 are some of them). Laminins are usually located on basement membranes, a kind of "ECM residing in the outer layer of the blood vessels," from where they can interact with other ECM molecules or cell receptors. Laminin location is usually α chain–specific, i.e., CNS tissue use to home α2 and α4 laminins (laminins containing an α2 or α4 chain, respectively). They can trigger downstream signaling for different biological processes including migration, adhesion, proliferation, and survival (Durbeej, 2010). In GBM, aberrant overexpression of α4 laminins has been described, and a positive correlation between their expression and tumor grade has been described (Sun et al., 2019). Particularly, laminin isoform 8 (α**4**β**1**γ**1**, or laminin **411** according to the new nomenclature) appeared overexpressed on GBM blood vessels and surrounding healthy tissue and was linked to higher recurrence and shorter survival (Ljubimova et al., 2001; Lathia et al., 2012). Furthermore, inhibition of laminin isoform 8 through CRISPR/Cas9 techniques has proven to suppress Notch pathway, rendering decreased intracranial tumor growth and longer survival in a glioma animal model (Sun et al., 2019). Lathia et al. (2012) showed that α2 laminins provided by perivascular non-CSCs and endothelial cells (ECs) were critical for GBM CSC maintenance and proliferation, promoting glioma CSC radioresistance through enhancing DNA repair. The use of laminin during routine *in vitro* culture of adherent glioma CSCs supports the importance of ECM proteins on glioma CSC biology. Overall, these observations highlight the role of the ECM on glioma treatment response.

Cadherins are surface glycoproteins involved in calcium-dependent cell–cell adhesion. The role of cadherins in glioma progression is not well-understood yet. However, the interaction between CSCs and other cellular components of the TME, such as those forming the white matter tracts that glioma cells used to migrate through or ECs from the BBB, has recently acquired great relevance (Drumm et al., 2019). Although previous studies reported differing results regarding the concentration level of cadherins and glioma cell invasion capacity, it was finally clear that rather than the concentration of cadherins available, the most important factor determining migration and invasiveness in GBM cell lines was the instability and disorganization of cadherin-mediated junctions (Barami et al., 2006). Cadherin E is common in epithelial cancers where, at some point along their evolution, cadherins undergo a process called *switching*. Despite its rarity within the CNS, cadherin E has been found in some GBM tumors. Here, contrary to epithelial tissues, high levels of cadherin E have been associated with aggressive invasiveness (Lewis-Tuffin et al., 2010). Cadherin 11 has been associated with increased migration and proliferation in different cancers. In GBM, cadherin 11 seems to support migration and survival *in vitro* and *in vivo*. Cadherin 11 also serves as a marker of mesenchymal phenotype, GBM subtype that is associated with worse prognosis (Kaur et al., 2012).

Integrins are heterodimeric transmembrane glycoproteins important in cell migration and cell adhesion. Although they are not a component of the ECM, they are key mediators of the interaction between different cellular components and the ECM. For this purpose, integrins function as receptors of laminins and fibronectins. In GBM, integrins are key in many complex processes, such as angiogenesis, tumor invasion, and proliferation (Nakada et al., 2013; Tilghman et al., 2016). The laminin-specific receptor, integrin α6β1, is highly expressed on perivascular glioma CSCs and is critical for their self-renewal and tumor formation capacity (Lathia et al., 2010). Integrin α6β1is also present in ECs from the perivascular glioma niche; upon laminin-binding, it has been shown to inhibit proapoptotic signals mediated by TNFR1, through NF-κB, by increasing cFLIP and XIAP, and promote EC growth (**Figure 2**) (Huang et al., 2012). Integrin α6 has also been reported as an enrichment CSC marker in GBM (Lathia et al., 2010). The invasive behavior of GBM CSCs seems to be mediated by another integrin. Integrin α3 was found overexpressed on glioma CSCs, especially in those leaving the tumor bulk and in those around the perivascular niche. Higher expression of integrin α3 correlated with increased migration and invasion via ERK1/2 signaling (Nakada et al., 2013). Integrin α5β1 is another integrin found in human GBM cells and was related to chemoresistance to temozolomide (Janouskova et al., 2012; Renner et al., 2016). Other integrins have been also reported to be involved in the crosstalk between CSCs and ECM (Haas et al., 2017).

Vascular Compartment: Perivascular and Hypoxic Niches

Similar to neural stem cells located in specific anatomical brain niches: the subventricular zone (SVZ) and the subgranular layer inside of dentate gyrus of the hippocampus (SGZ) (Quiñones-Hinojosa et al., 2006), glioma CSCs are present around the vascular compartment of the microenvironment. Researchers have suggested that this would represent a perivascular niche of glioma CSCs given the presence of $CD133^+$ and $NESTIN^+$ cells surrounding the tumor blood vessels (Calabrese et al., 2007). This statement was supported by the fact that culturing glioma CSCs along with ECs increases CSC proliferation *in vitro*, as well as by the deleterious effect of anti–vascular endothelial growth factor (VEGF) therapies on tumor growth (Calabrese et al., 2007). There is, in fact, an active crosstalk between the vascular endothelium and glioma CSCs. Bao et al. (2006) showed that glioma CSCs can secrete VEGF supporting the local angiogenesis. In turn, EC would produce Notch ligands that are widely known as a key determinant of CSC maintenance and proliferation and even would be able to recruit glioma CSCs and differentiate them into vascular pericytes (**Figure 2**) (Zhu et al., 2011; Cheng et al., 2013). As previously discussed, interactions between α2 laminins from the vascular basement

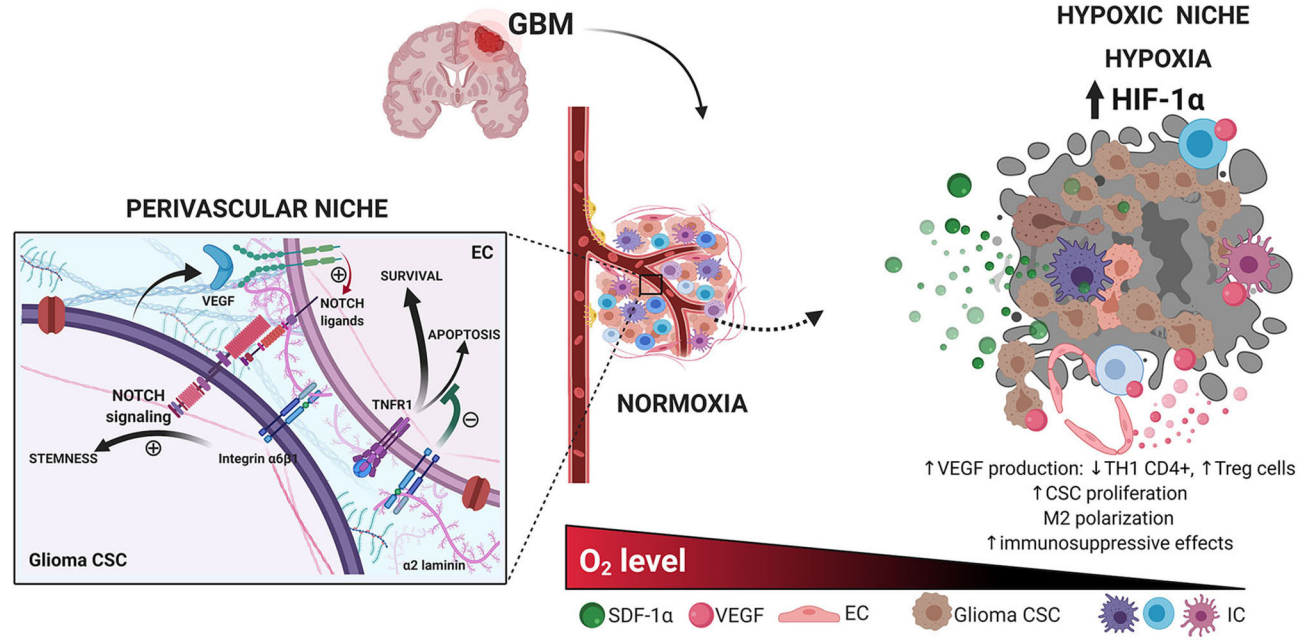

FIGURE 2 | Vascular compartment. Glioma CSCs and endothelial cells interaction in the perivascular niche. Brain CSCs are located within a vascular niche interacting with the surrounded cells. The perivascular niche is critical to maintaining the CSC phenotype. The secretion of growth factor by endothelial, ECM, and hypoxic conditions preserves stem-like characteristic accelerating tumor growth. **Hypoxic glioma niche**. Decreased oxygen tension triggers the expression of HIF-related genes, which in turn increases the production of several factors such as VEGF, decreasing the T_H1 $CD4^+$ cells, and increasing T regulatory cells, macrophage polarization, immunosuppressive profile, and glioma CSC proliferation. Created with BioRender.com.

membrane and the integrin α6β1 present in CSCs surface are important determinants of glioma CSC proliferation and migration. However, it is undeniable that CSCs are also present far from this hypothetical niche. Glioma CSCs from the hypoxic tumor core, as well as those moving away from the infiltrative border of the tumor bulk, are crucial for healthy tissue infiltration and tumor progression.

Hypoxic conditions have proven to facilitate glioma CSC self-renewal. Hypoxia-inducible factor 1α (HIF-1α) and HIF-2α as well as carbonic anhydrase IX, support CSC malignant potential and are correlated with poor patient prognosis (Li et al., 2009; Mohyeldin et al., 2010; Pistollato et al., 2010; Proescholdt et al., 2012; Xu et al., 2018). Thus, a hypoxic niche seems to be another realistic TME with a particular dynamic. HIF-1 would repress core-derived glioma cell differentiation through the suppression of Smad activation (Pistollato et al., 2009a,b), maintaining a higher number of stem cells that express greater levels of the DNA repair protein MGMT (O_6-methylguanine-DNA-methyltransferase) and consequently turn to be more radioresistant (Pistollato et al., 2009a, 2010). Also, low oxygen tension leads vascular ECs within the hypoxic niche to produce several factors, such as VEGF-A, which confer a more aggressive behavior to glioma CSCs and polarize immune cells into an immunosuppressive phenotype, as it is demonstrated by tumor-associated macrophage (TAM) M2 polarization, increased regulatory T cells, and higher rates of $PD\text{-}1^+$ $CD8^+$ T cells, leading to treatment resistance to traditional and modern approaches such as immunotherapy (Escribese et al., 2012; Tamura et al., 2018, 2019a,b) (**Figure 2**).

Cellular Compartment

Apart from the ECM, glioma microenvironment contains a number of cell types as another component of the tumor dynamics. These cells actively interact with glioma cells and the ECM (Wang et al., 2018c); they can secrete several factors triggering different signaling pathways on CSCs, as well as modify ECM composition in response to bilateral tumor cell–non-tumor cell interactions.

Astrocytes play a key role in CNS homeostasis. Astrocytes present in GBM tumor as well as surrounding brain parenchyma are thought to modulate disease progression via displacement and degradation of astrocytic endfeet in the BBB (Watkins et al., 2014). As glioma cell migration has also been reported to happen along the vasculature, these cells could impact migration and invasion. Tumor-associated astrocytes have also been related to malignant transformation of surrounding healthy tissue, as well as resistance to chemoradiation (Chen et al., 2015, 2016; Wang et al., 2018c; Brandao et al., 2019).

Immune cells are an extremely important component of the TME. Myeloid linage cells, such as infiltrating peripheral

macrophages and brain-resident microglia (tumor-associated macrophages/microglia or TAM), represent around 30 to 50% of the tumor mass (Lisi et al., 2017). From this group, around 40% are macrophages, 20% tumor-resident microglia, and the other 40% are myeloid-derived suppressor cells (Gabrusiewicz et al., 2016). Lymphocyte linage cells are also present in the TME. GBM-associated T cells and B cells (tumor-infiltrating lymphocytes or TILs) have been extensively described as having an exhausted phenotype; which correlates with their inability to control disease progression (Ma et al., 2018). This immunosuppressive tumor environment has been referred to as a "cold tumor."

Brain tumor–associated mesenchymal stem cells have acquired relevance recently; these cells play a role in supporting glioma microenvironment (Behnan et al., 2014; Guo et al., 2014; Svensson et al., 2017; Yi et al., 2018). Shahar et al. (2017) showed that a high percentage of them in the tumor population have been correlated with poor clinical prognosis. Although fibroblasts are not a major component in GBM composition, GBM-associated stromal cells closely resemble tumor-associated fibroblasts found in other tumors. They are particularly located in the periphery of the tumor and have been found to enhance tumor growth (Clavreul et al., 2012).

Biomechanics of ECM: Implications in Glioma Behavior

The effect of mechanical interaction on the cells was first proposed by His (1874); however, it was almost completely abandoned until recently. For many decades, research on cellular and molecular biology has been focused on intrinsic cellular biological processes without including biomechanical information about cell–ECM interaction (Paluch et al., 2015). In 1920, Alexander Forbes suggested that the collaborations between different scientific fields, such as biology and physics, will bring a better understanding of the living matter (Forbes, 1920). However, the communication flowed slowly until recent years in which new technologies facilitated access and cross-pollination of a huge amount of human knowledge. Recent multidisciplinary scientific work has led to important advances in understanding the influence of external physical forces in cell behavior, specifically mechanical forces such as tension, elasticity, stiffness, weight, friction, and others.

The process of sensing and responding to mechanical stimuli is known as mechanotransduction (Rice et al., 1973; Herberman, 1981; Wang et al., 1993). Cell migration, differentiation, proliferation, apoptosis, gene expression, and signal transduction (Sharma et al., 2019) are all influenced by mechanical stimuli (Chen and Wang, 2019; Moran et al., 2019).

Mechanical properties of the ECM can induce and maintain the stem-like phenotype in cancer cells. However, the response of cancer cells to the ECM mechanical properties varies between cancer types and even among cellular subpopulations within the tumors. For example, soft matrices induce the expression of self-renewal markers in melanoma CSCs, whereas stiff matrices induce their differentiation, but the opposite occurs in breast cancer (Nallanthighal et al., 2019). Moreover, the stiffness gradient in the TME in breast cancer is associated with specific CSC phenotypes, CSC $CD24^{-}/CD44^{+}$ localized in the tumor edges is quiescent, and CSC $ALDH^{+}$ (more stem) is found in the tumor core (Sulaiman et al., 2018). Indeed, changes in the type and proportion of proteins that constitute ECM can alter its stiffness by modifying the cross-linking ratio, amount of specific proteins, and cell–ECM interactions. Thus, it has been shown that these changes may induce FAK, FGF5, and JKT signaling activation, which contributes to the CSC phenotype (Cazet et al., 2018). Furthermore, mechanical properties in the ECM composition and organization could induce epithelial-to-mesenchymal transition (EMT) in cancer cells, this phenotype confers stem cell–like properties to cancer cells and is associated with chemoresistance and relapse (Singh and Settleman, 2010).

Biophysical Properties of Glioma ECM

The characterization of the mechanical properties of soft tissues in humans represents a great challenge because these are integrated by numerous components. Individual analysis of each of these components has granted insight into tumor mechanobiology; however, under real conditions, these elements work coordinately supporting tumor progression. A roadblock to overcome in order to better understand the TME mechanobiology is that tumor mechanical data obtained from biopsies may not be completely representative, as their value is relative to the location from where the samples were taken.

Young's modulus [force/area, in N/m^2 or Pascals (Pa)] refers to the amount of force needed to deform a substance, and it is commonly used to measure tissue stiffness. Brain tumors have mechanical properties different from those of their surrounding tissue (Chauvet et al., 2016; Pepin et al., 2018). On average, normal brain stiffness is lower than 200 Pa; however, stiffness in gliomas gradually increases accordingly with glioma aggressiveness (World health Organization grade) and ranges from 100 to 10,000 Pa (Miroshnikova et al., 2016). These differences in tumor stiffens have been explained by elevated levels of collagen IV and HA, which turn to be associated with tumor progression.

Lately, a key piece of mechanotransduction has been described. For instance, Chen et al. (2018) described the role of the mechanosensitive ion channel PIEZO1 in glioma. PIEZO1 is a transmembrane protein that locates at various tumor cell regions including focal adhesions. Physical force–induced membrane tension opens the channel to allow ion permeation, leading to a genetic interaction with integrin FAK signaling, which in turn increases ECM proteins production (laminins, HA synthases, etc.) and glioma aggressiveness. Overall, this leads to an increase in tissue stiffening, which further promotes PIEZO1 upregulation in a reciprocal manner promoting glioma invasion and proliferation (Chen et al., 2018).

Other authors have also published results in accordance with the positive relationship between ECM stiffness and glioma aggressiveness. Thus, glioma cells with aberrant expression of EGFR have been shown preference for stiffer microenvironments (Sivakumar et al., 2017), and softness of the glioma tissue positively correlated with higher tumor grade and IDH1

mutation (Pepin et al., 2018). Overall, these findings are a call for more comprehensive studies on ECM–CSC interactions.

Components of ECM and Glioma Aggressiveness

The soft physical consistency of the brain tissue is owed to its ECM composition, which is abundant in proteoglycans such as hyaluronan, tenascin C, brevican, neurocan, and phosphocan (Miroshnikova et al., 2016). CSCs in the brain are exposed to this exclusive microenvironment in which the matrix–cell interaction activates pathways for stem phenotype maintenance, ECM remodeling, and proliferation (Manini et al., 2018). For instance, CD44 is highly expressed in gliomas; this protein interacts with HA to enhance CSC properties by activating NANOG (Pietras et al., 2014; Wang et al., 2017). Integrins are cell-surface proteins that work as transmembrane links between ECM and intracellular cytoskeleton by bidirectional signaling. In cancer, the expression and localization of integrins vary from normal cells; for instance, in GBM, integrin α6 (a receptor for the ECM protein laminin) is overexpressed; the interactions between integrin α6 and laminin regulate CSC distributions and maintenance, as we have mentioned above (Lathia et al., 2010). Integrin α3 is also highly expressed in glioma CSCs, this integrin interacts with fibronectin and laminin and has been localized in the CSC niche, promoting glioma invasion via ERK pathway (Nakada et al., 2013). Integrin α7 is aberrantly expressed in aggressive gliomas and correlates with poor prognosis, is highly expressed especially in glioma CSC subpopulations, and promotes tumor growth and spreading via AKT (Haas et al., 2017).

ECM and Glioma Treatment Response

Heterogeneity and genetic plasticity present in GBM allow for numerous mechanisms of therapeutic resistance. Interaction between glioma cells and the ECM plays a fundamental role as drivers of these two GBM properties. The ECM can induce EMT in CSCs, which confers stem-like properties as well as chemoresistance and radioresistance. Fibulin-3 is an ECM protein absent in normal brain tissue but upregulated in gliomas; this protein activates Notch signaling to promote resistance to apoptosis, chemoresistance, and tumor growth (Hu et al., 2012). Wtn proteins from the ECM confer high chemoresistance and radioresistance to temozolomide (Auger et al., 2006; Han et al., 2017). Additionally, the overexpression of fibrillary proteins in the glioma ECM has been reported as physical barriers against drug dissemination (Shergalis et al., 2018).

Modeling TME to Study Treatment Resistance

As we have previously described, TME characteristics, such as ECM composition and biomechanical properties, as well as its vascular and cellular compartments, clearly influence CSC behavior and treatment response. Thus, it is not surprising that the use of two-dimensional (2D) cell cultures is associated with poor representation of the therapeutic response to chemotherapy and radiotherapy when compared to original tissues or even 3D models (Storch et al., 2010; Luca et al., 2013).

Studies in Radiobiology and Radioresistance

Bauman et al. (1999) pioneered the studies on radiation responses in 3D glioma models. In 1999, the authors used glioma tumor spheroids implanted into a gel matrix of collagen type I to study the effect of radiation on proliferation (Ki67), apoptosis, and invasion. After applying single and fractionated doses of (Pistollato et al., 2010). Co irradiation delivered at 200 cGy/min, they found differences in these variables according to the regional distribution along the spheroid. Cells at the surface of the neurosphere were more affected by radiation, whereas apoptosis and proliferation decrease was minimal or null at the core of the neurosphere. The invasion was affected in a dose-dependent manner, whereas fractionation seemed to confer associated with partial recovery. Taken together, this model showed to resemble qualities of *in vivo* models of malignant gliomas. Despite these results, efforts were not resumed until 15 years later (Jiguet Jiglaire et al., 2014; Yahyanejad et al., 2015) reported on the simultaneous comparison of a 3D spheroid model and an *in vivo* rodent model with regard to response to radiation therapy. They used the small animal radiation therapy platform (X-RAD SmART®) and performed a delivery plan delineating the tumor as gross total volume and the brain as an organ at risk (OAR), planning target volume was equal to GTR (225 kVp at 12 mA, 300 cGy/min). They found that the 3D model could be reliable for radiation efficacy evaluation (Yahyanejad et al., 2015). In this same line, 3D glioma models have been also proven effective in studying glioma radiosensitivity to different types of radiation modalities. Chiblak et al. (2016) used 3D clonogenic survival assays on patient-derived neurospheres and the classical radioresistant U87-MG GBM cell line to study radiosensitivity and measure the relative biological effect (RBE) of photon, proton, and carbon irradiation. The authors found that carbon irradiation RBE ranged from 2.21 up to 3.13 when compared to photon radiation and that the inability to repair double-strain DNA breakdowns after heavy ion irradiation could be a potential explanation for their findings (Chiblak et al., 2016).

In an attempt to represent not only the cell–cell interactions of the TME but also cell–ECM interactions, Jiguet Jiglaire et al. (2014) studied the role of 3D scaffolds based on an HA-rich hydrogel in the screening of radiation and chemotherapy response of commercial or patient-derived glioma cell lines. The 3D model showed good morphological representation when compared to patient-derived tissue specimens. Commercial cell line U87-MG did not show differences in radiation response when 2D and 3D cultures were compared; however, patient-derived glioma cell lines were proven radioresistant when cultured on the 3D model but not in conventional 2D cultures (Jiguet Jiglaire et al., 2014). Interestingly, the authors did not account for the difference in HA concentration between 2D and 3D cultures. Gomez-Roman et al. (2017) showed that, under the same culture conditions, architectural modifications (2D vs. 3D) did not generate differences in radiotherapy resistance. Conventional 2D cultures were compared with 3D cultures using scaffolds of polystyrene, both coated with laminin and using regular serum-free stem cell media. 3D cultures improved the morphological representation including hypoxic gradients characteristic of TME, but this did not represent an

increment in radioresistance. When the 3D culture was enriched with additional laminin, increased radioresistance was evident (Gomez-Roman et al., 2017).

Overall, it is clear that *in vitro* models need to be perfected in order to better represent glioma biology and treatment response; a complex representation of TME biomechanical factors, ECM, and cellular compartments is necessary in order to achieve that goal. Foundations, adequate nomenclature, and applications of traditional and novel 3D models in glioma CSC research will be described in the next section.

THREE-DIMENSIONAL MODELS IN CANCER RESEARCH

Every cell in the human body is immersed in a three dimensional microenvironment that regulates its behavior and potentially, its fate. In this setting, *in vitro* models aiming to understand glioma biology in order to develop effective therapies should ideally mimic the TME. Unfortunately, the traditional methods used for this purpose usually include the use of 2D cell lines cultures, which lack the aforementioned ideal requirement. The 2D approach introduces inherent limitations such as (1) genetic and epigenetic modifications due to the lack of CSC-TME interactions (**Figure 2**) (De Witt Hamer et al., 2008; Luca et al., 2013; Wang et al., 2018c), (2) absence of O_2, nutrients and pH microenvironment gradients (**Figure 2**) (Bristow and Hill, 2008; Mikhailova et al., 2018), (3) lack of physiological inputs from other metabolically active organs such as liver, kidney, etc., and (4) genomic alterations after long-term culture (De Witt Hamer et al., 2008; Torsvik et al., 2014). Additionally, after a successful initial experimental phase involving 2D cultures, the next conventional step is usually carried out through animal studies, which are expensive and time-consuming. Furthermore, animal models have also demonstrated limited chances to translate these data into human outcomes (Shafiee and Atala, 2016).

To overcome these limitations, a great variety of 3D models or biocomplexes incorporating biomaterials and different tumor cells have been studied (Chang et al., 2010; Shafiee and Atala, 2016). Biomaterials are synthetic or natural nontoxic elements that can be engineered to obtain specific physicochemical characteristics; this attribute makes them a perfect fit to create biomimetic platforms able to resemble the 3D TME (Hildebrand Hartmut, 2013). Current technologies allow for recreating controlled patterns and stiffness properties of the ECM, which might provide the required microenvironment for CSCs to mimic their *in vivo* behavior (Shafiee and Atala, 2016). *In vitro* 3D models in cancer research can be classified in spherical cancer models (which include the tumorspheres or neurospheres), organoids, and 3D scaffolds.

Spherical Cancer Models

Spherical cancer models consist of sphere-like structures mainly or totally composed of cancer cells (Friedrich et al., 2009). Due to their easy production, they are the most commonly used 3D *in vitro* model. There are several spherical cancer models described since almost four decades ago; however, their use and nomenclature have been confusing ever since. For instance, the terms *sphere* or *spheroid* have been misused in the literature to refer to cellular aggregates. Although both are a specific type of spherical cancer models, this misuse should be avoided as cellular aggregates differ from spheroids and spheres. Contrary to spheroids, aggregates are not compact enough to allow for manipulation and transfer; they easily detach and have no spherical geometry and probably no cell–cell and cell–matrix interactions, which impact their biological features (Weiswald et al., 2015).

Weiswald et al. (2015) classified the spherical cancer models into four principal types (main features and culture conditions are described in **Table 1** and **Figure 3**, with an emphasis on glioma research). Tumorspheres, or neurospheres in the case of gliomas, are one of the four different types of spherical cancer models. They are proliferations of single-cell suspension of tissue-derived cancer cells, circulating cancer cells, or established cell lines (clonal expansion) and were first described for gliomas by Singh et al. (2003). Tumorspheres are able to maintain CSC multipotency, resemble 3D interactions, and even resemble the tumor gradient of oxygen and nutrients. Thus, they present a quiescent necrotic core and a more proliferative outer layer. Tumor spheroids can be grown in suspension in the regular specific stem cell media or submerged in a gel, which has allowed them to be used as an important tool for high-throughput drug screening (Mirab et al., 2019).

In the gel-embedded systems, cancer cells are surrounded by an artificial matrix to simulate cell–ECM interaction. In this strategy, the biomaterial properties can be modified to imitate ECM mechanical and structural characteristics, which could help resemble the TME. Currently, several commercial matrices such as Matrigel® are commercially available for this purpose. Agarose hydrogels conformed as microwells allowed the size control of tumoroids to evaluate the effect of therapeutic drugs, this technology can contribute to the advancement of personalized medicine (Mirab et al., 2019).

To date, neurospheres are the most common type of tumor spheroid used in glioma research (**Table 2**). Patient-derived neurospheres are grown in enriched EGF/bFGF media under low attachment conditions; when these factors are replaced by serum, glioma CSCs phenotypically change their appearance, loosen cellular adhesions, and turn the neurosphere into a 2D cell culture with decreasing CSC marker expression and telomerase activity (Lee et al., 2006; Claes et al., 2008). Furthermore, it has been reported that neurosphere-derived cells retain their ability to grow diffusely infiltrating tumors, whereas the same glioma cells grown under serum conditions could only produce well-demarcated tumors (Lee et al., 2006; Claes et al., 2008).

Multicellular tumor spheroids (MCTSs, usually known as glioma tumor spheroids) are the second type of spherical cancer model. The initial development of MCTSs was based on the work of Sutherland et al. (1971) dating back to the early 1970s, and its role in glioma research appeared in 1989 with Mashiyama et al. (1989) Despite the model was described a long time

TABLE 1 | Different types of spherical cancer models in cancer biology research.

Spherical cancer model	Origin	Culture conditions
Tumorspheres Other names: • Tumor spheres • Neurospheres	Proliferations of single-cell suspension of tissue-derived cancer cells, circulating cancer cells or stablished cell lines (***clonal expansion***) (Singh et al., 2003) No non-neoplastic cells are present *First described for gliomas by Singh et al. (2003)	Serum-free medium (no FBS) FGF-2 and EGF are required (stem cell medium) (Lee et al., 2006; Claes et al., 2008) Grown in low-attachment conditions (i.e., no laminin-coated plates in case of glioma CSCs) Low seeding density to avoid aggregation and to foster ***clonal expansion***
Multicellular tumor spheroids (MCTS) Other names: • Tumor spheroids	Aggregation and compaction of single-cell suspension from well-stablished cancer cell lines Rarely from single-cell suspension of tissue-derived cancer cells Heterotypic MCTS including CSCs and noncancerous cells have been reported (co-cultures) *First escribed for gliomas by Mashiyama et al. (1989)	Serum-supplemented medium (FBS or FCS) No additional growth factors Grown in non-adherent conditions promoting aggregation of cells Two culture methods: liquid overlay (LOC) and spinner cultures (SPC) (Watanabe et al., 1999). Usually by several weeks The use of U87 cells was described by Bell et al. MCTS size ranges from 400 um to 1000 um after aggregation and compaction (Bauman et al., 1999; Bell et al., 1999, 2001)
Organotypic multicellular spheroids (OMS) Other names: • Organotypic spheroids	Rounding of non-dissociated *ex vivo* fragments directly from surgical specimens (0.3–0.5 mm for glioma tissues) (De Witt Hamer et al., 2008) Maintain stromal components (macrophages and tumor vessels)	Cultured with liquid overlay method until they round up (2 to 5 days) Tumor microenvironment has been shown to be present up to 70 days of culture Improved genomic stability when compared to well-stablished and primary glioma cell lines Cryopreservation of glioma OMS is well-tolerated
Tissue-derived tumor spheres	Remodeling and compaction of partially dissociated (mechanically or enzymatically) tumor tissue No non-neoplastic cells reported inside the sphere	FBS-supplemented or stem cell medium

FBS, fetal bovine serum; FCS, fetal calf serum; FGF, fibroblast growth factor; EGF, epidermal growth factor; CSC, cancer stem cell; ECM, extracellular matrix; MCTS, multicellular tumor spheroid; OMS, organotypic multicellular spheroids. Based on the classification of Weiswald et al. (2015).

ago, it is still a valuable tool to consider for high-throughput screening of several treatments such as radiation, drugs, and nanotherapeutics (Bauman et al., 1999; Yahyanejad et al., 2015; Oraiopoulou et al., 2017; He et al., 2020). Different culture methods and techniques have been developed (Watanabe et al., 1999); but all of them involve seeding an elevated number of cells under non-adherent conditions and promoting their aggregation and compaction. Usually, commercial cell lines are cultured with medium supplemented with serum (such as with U87 or T98G cells) (Bell et al., 1999, 2001; Oraiopoulou et al., 2019), but the use of patient-derived cell lines has also been described in several cancers (Weiswald et al., 2015; Yahyanejad et al., 2015). Even when a main mechanism of spheroid formation is aggregation, these spheroids are not simple cell aggregates; they form a very tightly packaged structure with intermediate junction between adjacent cells, and—as in any spherical cancer model—a differential dynamics is established from the core to the peripheral layer of cells (Bell et al., 1999, 2001).

Organotypic multicellular spheroids (OMSs, also known as organotypic spheroids) are the third type of spherical cancer model. These are rounded, non-dissociated *ex vivo* fragments of tumors obtained directly from surgical specimens. They maintain the non-tumor components such as immune cells and ECM for up to 70 days of culture and have demonstrated more representative GBM genetic profile when compared to primary cell cultures even after several weeks of culture. Although cryopreservation of glioma OMSs has been proven well-tolerated, the limited availability of GBM tissue is a highly restraining factor to introduce this model into regular glioma research (De Witt Hamer et al., 2008).

The last spherical cancer model described by Weiswald et al. (2015) refers to the tissue-derived tumorspheres; however, they have been not described for glioma. All the above mentioned models are described in **Table 1** and **Figure 3**.

Organoids

Organoids are self-organizing, 3D microscopic structures that are derived from individual stem cells growing in an *in vitro* environment. They can recapitulate histoarchitecture and cellular composition, as well as physiological aspects of the mature primary tissue they are derived from Eiraku et al. (2008), Muguruma and Sasai (2012), Lancaster et al. (2013), and Lancaster and Knoblich (2014). In general, organoids can be obtained from adult stem cells (ASCs) or pluripotent stem cells (PSCs) (Tuveson and Clevers, 2019). Although no neural tissue can be obtained from ASCs such as neural stem cells to date, PSC technologies have allowed for the creation of brain organoids from induced PSCs (iPSCs) (Eiraku et al., 2008; Muguruma

TABLE 2 | Current glioma research using 3D platforms.

3D model	Features	Developments and applications
Spheroids	Mirror glioma CSC multipotency Maintain tumor cellular heterogeneity 3D cell-to-cell interactions Biomimetic 3D distribution* • Necrotic core • Inner quiescent layer • Outer proliferative layer Artificial low-adhesion cell growth microenvironment (Velcro type) Inability to organize in tissue-like structures (in case of tumorspheres) Cost-effective/highly reproducible	Drug screening using microfluidics-based chips
Organoids	Created with organoid technology Mini brains resemble non-tumor environment Tumor initiation in mini brains can be obtained by • Genome edition • Glioma CSC transplantation Glioma organoids can derive from pure tumor tissue	Study of gliomagenesis by introducing oncogenic mutations by gene editing strategies in brain organoids Study of tumor progression and Invasion Study of angiogenesis Study of tumor non-tumor interactions (in mini brains developing tumors) Biobank and drug screening for personalized medicine
Scaffolds *Hydrogels*	3D biocompatible polymeric matrices Structured microarchitecture (pores, groves, channels, etc.) Can introduce ECM proteins: HA, etc. Stiffness regulation Biodegradable Smart materials	Study of glioma CSC-vascular niche interactions Study of mechanisms underlying glioma migration Study of the role of ECM stiffness on glioma behavior (simulating diseased and healthy brain tissue) Study of cell-cell interaction
Organ-on a -chip	3D biomimetic system Continue and digitally controlled flow Flow ranges from *m*L to *p*L Tracks and regulates different conditions Interconnects multiple microenvironments	Study of glioma CSC-vascular niche interactions Drug screening using microfluidics-based chips Study of response to magnetic thermal therapy

*3D, there-dimensional, mL, microliter; pL, picoliter; ECM, extracellular matrix; CSC, cancer stem cell; *under certain conditions.*

and Sasai, 2012; Lancaster et al., 2013; Lancaster and Knoblich, 2014). The landmark article published by Lancaster et al. (2013) opened the door to different avenues in developmental and cancer research. The group created brain organoids, also known as cerebral organoids or mini-brains, presenting various discrete but interdependent brain regions. Complying with the definition of organoid, these brain organoids showed a cerebral cortex containing progenitor cells that self-organize and develop into different mature cortical neuron subtypes, as well as a primitive ventricular system and choroid plexus.

Furthermore, despite that developmental biology defines the term "organoid" in this very pure manner, alternative protocols have been described in glioma research, and the term has been adapted to introduce glioma CSCs or even entire pieces of glioma tissue, containing a wide variety of glioma cells, as the origin of tumor organoids. Thus, glioma CSCs or tissue will be treated using organoid technologies in such a way that the newly developed "organoid" will recapitulate the glioma TME rather than a normal brain histoarchitecture. Overall, these different approaches have led to three groups of organoids (**Figure 4**).

Glioma Organoids: From Genetic Bioengineering of Brain Organoids

Genome engineering has been used to generate glioma tumor models in PSC-derived brain organoids or "mini-brains." CRISPR/Cas9 mutagenesis and Sleeping Beauty (SB) transposon-mediated gene insertion have served for this purpose by introducing clinically relevant oncogenic mutations into healthy human cerebral organoids in order to develop glioma tumors (Bian et al., 2018; Ogawa et al., 2018) (**Figure 4**–1).

Ogawa et al. (2018) used human cerebral organoids cultured as described by Lancaster and Knoblich (2014) thus, organoids were grown and matured for 4 months. It was at this point, when the organoids already presented normal cortical structures and markers, that CRISPR/Cas 9 technology was used to mediate homologous recombination of the oncogene $HRas^{G12V}$ into the TP53 tumor suppressor locus. This genomic insertion would simultaneously represent the disruption and truncation of the tumor suppressor gene TP53, as well as the introduction of the oncogene $HRas^{G12V}$, which codes for the expression of RAS protein. Two weeks after this process, transduced cells can be initially observed through tdT and GFP signals, and by 8 weeks, almost 6% of the cells in the organoids are cancer cells. Therefore, this methodology allows for direct observation of tumor initiation, as well as continuous microscopic observations of tumor development. Consistent with other authors, the genetic alteration introduced by Ogawa et al. (2018) led the glioma organoids to show a molecular signature proper of gliomas of the mesenchymal subtype (Friedmann-Morvinski et al., 2012).

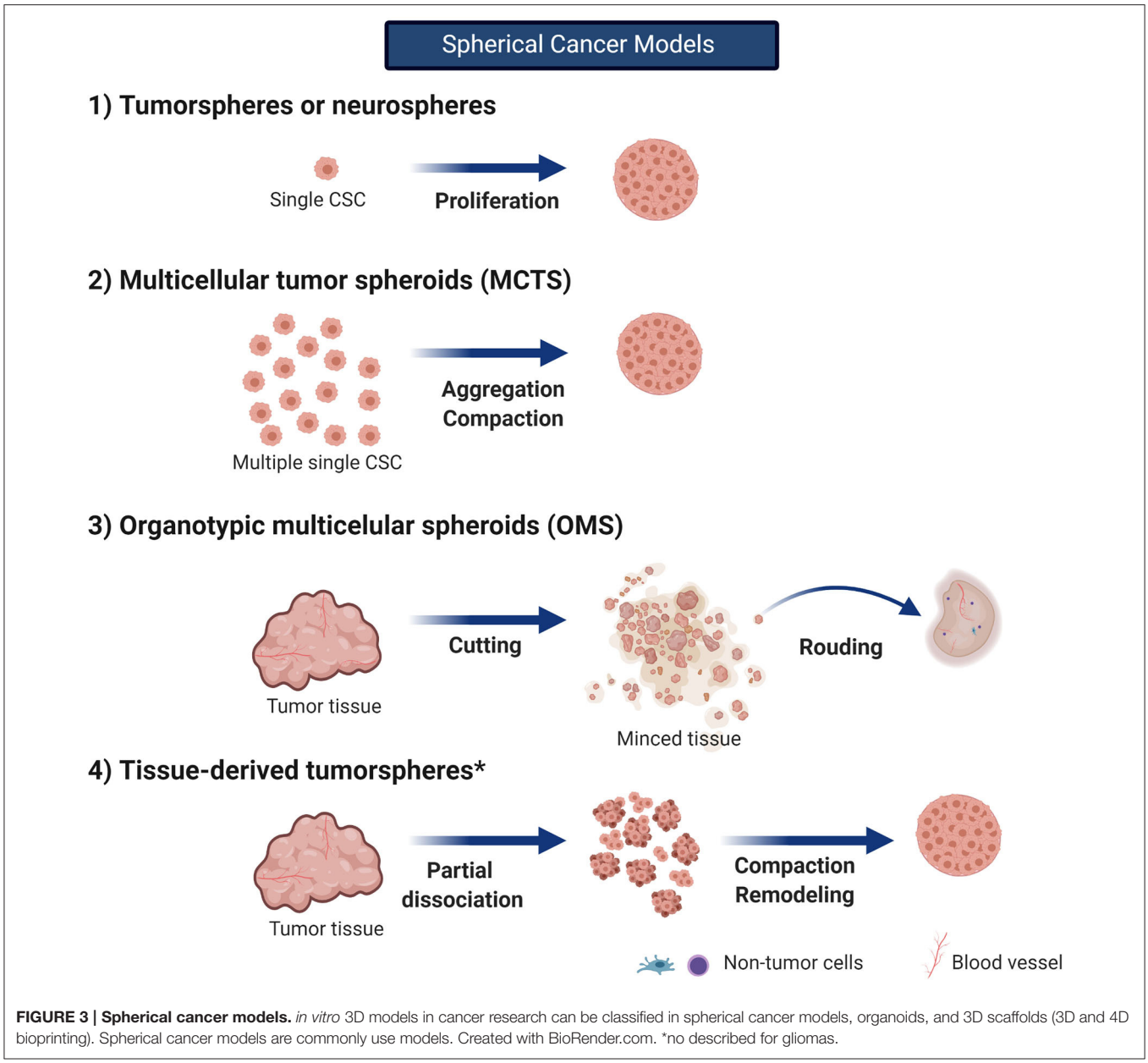

FIGURE 3 | Spherical cancer models. *in vitro* 3D models in cancer research can be classified in spherical cancer models, organoids, and 3D scaffolds (3D and 4D bioprinting). Spherical cancer models are commonly use models. Created with BioRender.com. *no described for gliomas.

The group of Jürgen Knoblich, who initially published the landmark paper on cerebral organoids together with Lancaster, presented a similar approach. Human cerebral organoids were developed from human embryonic stem cells or iPSCs as previously described (Lancaster et al., 2013; Lancaster and Knoblich, 2014). By the end of the neural induction period, around day 11, SB transposon–mediated gene insertion for oncogene amplification and CRISPR–Cas9 technology for tumor-suppressor gene mutation were used to introduce 18 different single mutations or amplifications, and 15 of their most clinically relevant combinations in neuro-oncology. One of the newly developed clusters of organoids (containing three different combinations of genetic aberrations: GBM-1, GBM-2, and GBM-3) presented a glioma signature with particular upregulation of GBM-related genes and phenotype. These organoids proved to be viable and able to expand after heterotopic renal subcapsular engrafting (Bian et al., 2018).

Glioma Organoids: From Inception of Tumor Material Into Brain Organoids

In a similar manner, the development of glioma tumors has been also proven in healthy brain organoids after coculture with GBM CSCs or tumorspheres (da Silva et al., 2018; Ogawa et al., 2018; Linkous et al., 2019) (**Figure 4**–2).

Linkous et al. (2019) developed a cerebral organoid model of glioma called GLICO; they showed that glioma CSCs were able to infiltrate healthy cerebral organoids of different ages by coculturing them for 24 h. Considerable tumor growth was found

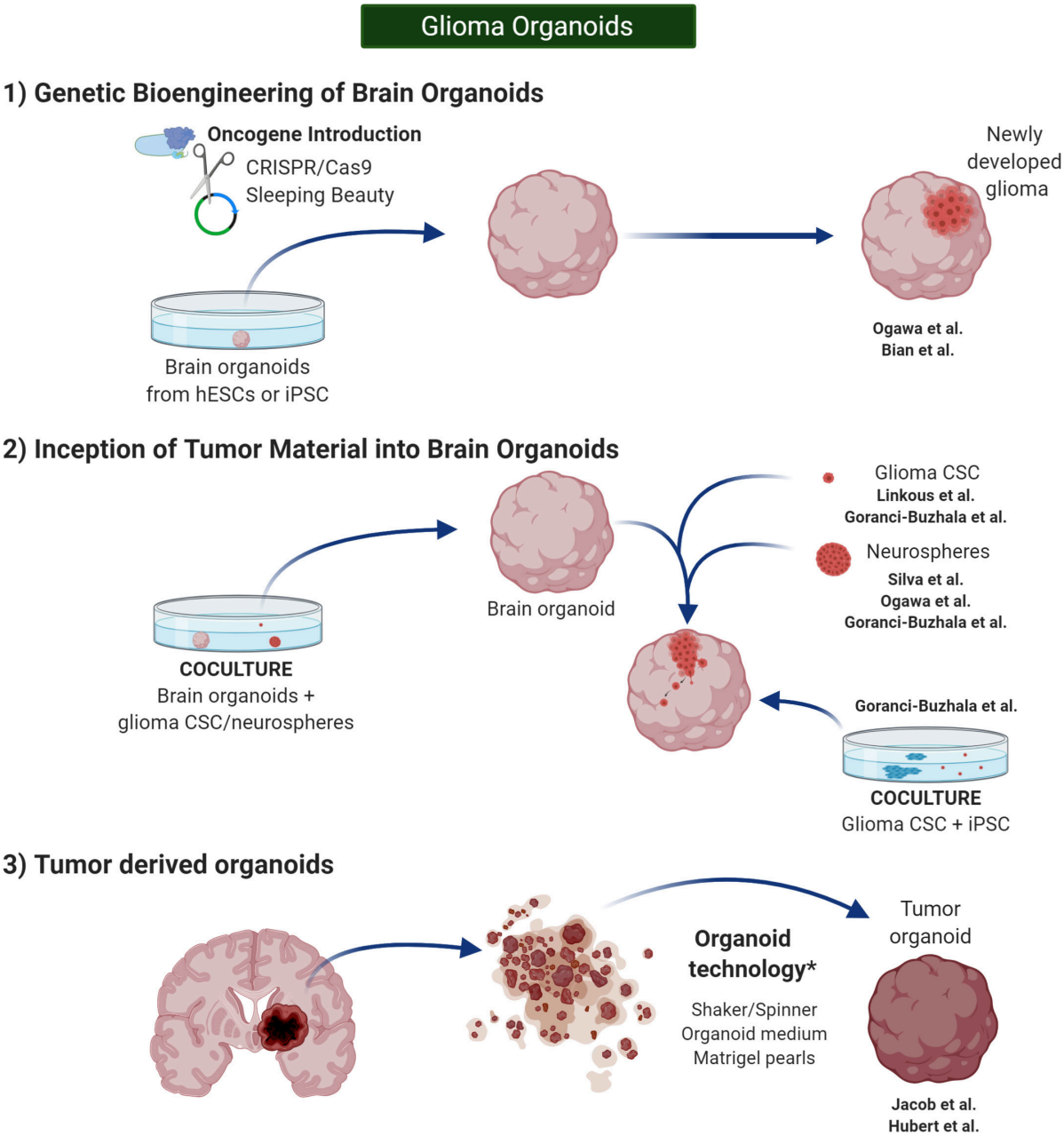

FIGURE 4 | Glioma Organoids. *in vitro* 3D models in cancer research can be classified in spherical cancer models, organoids, and 3D scaffolds (3D and 4D bioprinting). Glioma organoids are produced using protocols similar to the one published by Lancaster et al. (2013) and Lancaster and Knoblich (2014). They need to be cultured under shaking conditions to increase diffusion of the nutrients. Created with BioRender.com.

1 week after coculture, and the resulting tumors resemble original patient tumors genetically, functionally, and morphologically when examined 2 weeks after CSC inception. Apart from the already expected fact that the organoids represent radioresistance and chemoresistance of the primary tumor in a better way than 2D cultures, it was interesting that the non-cancerous microenvironment of the organoids seemed to support the maintenance, viability, and growth of the glioma CSCs (Linkous et al., 2019). Similarly, da Silva et al. (2018) reported the use of early-stage 12-day mouse ESC-derived brain organoids and GBM spheres in order to develop glioma organoids. Coculture of these two elements for 48 h resulted in a 100% rate of spontaneous infiltration of GBM cells into the organoids. The final size ranged from 300 to 800 μm, which would make them pertinent for medium- or high-throughput screening applications (da Silva et al., 2018).

As part of the same effort described in the previous section, Ogawa et al. (2018) studied the oncogenic potential of the glioma cells they had created in human brain organoids by CRISPR/CAS9-assisted mutagenesis, as well as patient-derived glioma CSCs. Similar to da Silva, the authors cocultured spheroids of either of these two types of cells with intact human mature brain organoids. Spheroids from tumor cells created by mutagenesis were able to invade the organoids and represent 30% of them by day 24. Spheroids from patient-derived cells presented different invasion capacity. In general, results from these two approaches correlated with results from *in vivo* experiments using immunocompromised mice (Ogawa et al., 2018).

Recently, Goranci-Buzhala et al. (2020) described three different methods to engineer the interaction between glioma and brain organoids. The authors compared two strategies similar to those described before by Linkous, Ogawa, and da Silva (glioma CSCs + brain organoids as well as glioma neurospheres + brain organoids) and an additional assay able to engineer this interaction as well (by coculturing glioma CSCs and iPSCs to develop organoids under conditions similar to those described by Lancaster et al., 2013; Lancaster and Knoblich, 2014). Thus, by using conventional and novel imaging technologies, the authors showed that the three models allowed analyzing glioma CSC invasion and patterns of invasion when primary and recurrent gliomas were compared. However, they also found that the latter model may not be the most suitable and that glioma CSCs tend to present enhanced tropism for mature brain organoids (Goranci-Buzhala et al., 2020).

Overall, the development of glioma tumors on cerebral organoids, either by mutagenesis or glioma inception, has opened a door to study brain tumor initiation, progression, and treatment. The presence of tumor and non-tumor microenvironments together at the same time allows for the study of the interactions between these two important components. Furthermore, the nature of these two approaches diminishes the need for patient-derived tumor tissue and animal xenotransplantation models to test patient-specific drug responses (Gao et al., 2014; Tuveson and Clevers, 2019).

Glioma Organoids: From Tumor Material Alone

Similar to the advances in other cancers' research, efforts in glioma research have aimed to accurately recapitulate the TME as much as possible. The previous models of organoids offer the possibility to study normal tissue–tumor interactions; however, key elements of the cellular components of the glioma tumor are missing given that only neural and glioma cells are available.

Jacob et al. (2020) used organoid technology to develop glioma organoids derived from tumor tissue, able to preserve cytoarchitecture and maintain different cell–cell interactions (**Figure 4-3**). They cultured the tissue in organoid medium and put it on an orbital shaker in order to increase nutrient and oxygen diffusion. Thus, by the end of the second week, a rounded organoid was appreciated, many of these organoids were able to retain their $CD31^+$ vasculature, and resemble hypoxic niches 300 μm far from these vessels. Robust cellular heterogeneity resembling parental tumors was confirmed by several histological markers. By single-cell transcriptome analysis, the authors determined that both neoplastic and non-neoplastic cell populations (such as lymphocytes, macrophages, and microglia) retain parenteral molecular profiles after 2 weeks of culture. Orthotopic engraftment in an immunocompromised murine model was proven efficient, and aggressive infiltration was appreciated. The organoids were propagated by cutting them into 0.5 mm pieces. Cryopreservation protocols were also developed and optimized, and successful recovery was evident after thawing. Finally, the authors developed a biobank of organoids that allowed for testing different types of treatment *in vitro* (Jacob et al., 2020).

Hubert et al. (2016) reported the first effort to develop organoids for glioma research. The authors developed organoids from tumor tissue by modifying the original protocol described by Lancaster and Knoblich (2014). The group used finely minced patient tissue samples or their dissociated single-cell suspensions for this purpose. As initially described by Lancaster, they embedded the pearls of tissue in Matrigel and cultured them under shaking conditions to develop the organoids. Different from classical neurospheres, organoids grew until 3 to 4 mm after 2 months of culture. Similar to parenteral glioma tumors, the organoids developed a gradient of oxygen and stem cell density, delimiting a hypoxic core with quiescent glioma CSCs and a more oxygenated ring with proliferating CSCs. Xenograft tumors derived from different regions of the organoids (necrotic core and peripheral ring) showed different growth speed. Apart from showing a faster growth after xenotransplantation, cells in the necrotic core demonstrated higher radioresistance (Hubert et al., 2016). Worth to mention is that the culture methods for organotypic spheroids described by De Witt Hamer et al. (2008) were different from the organoid technology used by Hubert et al., which was based on the protocol published by Lancaster et al. (2013), Lancaster and Knoblich (2014).

The contemporary use of patient-derived organoids (PDOs) in general cancer research has led to some lessons: (1) Organoids can be generated from patient specimens; in general, either normal stem cells or CSCs can be used for this purpose (Gao et al., 2014; Bian et al., 2018; Ogawa et al., 2018); (2) organoid cultures can resemble interpatient variations and heterogenic intratumoral profile (Weeber et al., 2015; Tuveson and Clevers, 2019); (3) organoids represent a model to study the initiation, evolution, and drug response of the original brain tumor, allowing the identification of potentially targetable therapy (Hill et al., 2018; Lee et al., 2018; Tiriac et al., 2018). The U.S. Blue Ribbon Panel for the Cancer Moonshot has proposed to use these PDO as a screening tool for patient drug response (https://www.cancer.gov/research/key-initiatives/moonshot-cancer-initiative), and this effort has already shown initial evidence that PDO with specific genetic signature can help to identify a sizable number of patients with improved drug sensitivity.

However, even when PDO has been successful in representing patient therapeutic responses, there is still room for improvement, and as we have previously described, cocultured PDO or *in situ* glioma development in cerebral organoids has been engineered to include non-tumor TME cells such as immune cells (Dijkstra et al., 2018; Neal et al., 2018).

Scaffolds

Scaffolds are 3D materials that provide support and structure to cell cultures; these biomaterials have microscale mechanical properties such as stiffness, porosity, interconnectivity, and structural integrity that can modulate cellular behavior (Mallick and Cox, 2013). For instance, biomaterial stiffness has been proven to affect stem cell differentiation through a number of pathways already described in the literature (Park et al., 2011; Palama et al., 2018; Xiao W. et al., 2019). In general, these properties as well as structural patterns, textures, and angulations can be controlled in an attempt to recapitulate ECM characteristics proper to the specific tissue of interest (Dijkstra et al., 2018). As the glioma TME possesses a distinct ECM composition with a high proportion of fibrillary collagens when compared to normal brain parenchyma (Huijbers et al., 2010; Lv et al., 2016), 3D glioma cultures using 3D collagen scaffolds have been studied with interest. Thus, a higher degree of dedifferentiation was found when compared to 2D cultures as well as a more similar morphology to *in situ* tumor GBM cells was also described. Furthermore, 3D cultures also showed greater resistance to alkylating agents with a high regulation of MGMT (Lv et al., 2016). Different scaffolds created with other relevant tumor ECM components such as HA as well as with synthetic materials have also been described (Erickson et al., 2018; Chaicharoenaudomrung et al., 2019; Xiao W. et al., 2019).

Conventional fabrication technologies used in scaffolds, such as the previously described, involve the use of physicochemical methods such as electrospinning (for polymers and biological materials), temperature-induced phase separation, and others (Lv et al., 2016; Erickson et al., 2018; Chaicharoenaudomrung et al., 2019; Xiao W. et al., 2019). In these cases, after the scaffold has been produced by the physicochemical procedures, the cells will be included in a posterior step as cell suspensions aiming to localize and home within the biocompatible scaffold.

Solid free-form (SFF) technologies, on the other hand, have recently positioned as one of the most relevant advances made in scaffolds fabrication. Among SFF technologies, 3D bioprinting has become a toolbox for a more tailored fabrication, allowing for better mimicking of the TME that now can include the tumor and non-tumor cells, together with biological ECM components such as macromolecules, and biomaterials.

3D Bioprinting

3D bioprinting requires the use of bioinks to be deposited layer by layer, guided by a computer-aided design (Hospodiuk et al., 2017; Matai et al., 2020). There exist two types of bioinks: the first refers to soft biomaterials loaded with living cells (scaffold-base bioink), and the second refers to cells bioprinted without an exogenous biomaterial (scaffold-free bioink) (Dai et al., 2016). In the latter type of bioink, cells are grown up to small neotissues that are three-dimensionally distributed during the bioprinting process and will later fuse and mature to a more complex structure (Hospodiuk et al., 2017). Even when it is possible to create biosimilar acellular scaffolds using 3D bioprinting and later include a cellular component using the top-down method (two-step fabrication), this approach carries several limitations including inadequate reproducibility, cell density control, and spatial distribution control. Furthermore, the possibilities for high-throughput use are also limited (Tasoglu and Demirci, 2013). For this reason, one-step biofabrication techniques such as inkjet-based, microextrusion, and laser-assisted bioprinting are preferred (Asghar et al., 2015; Knowlton et al., 2015) (**Figure 5**). With these techniques, cells are located inside the 3D bioprinting while they are fabricated, thus reducing user input errors (Knowlton et al., 2015).

In glioma research, 3D bioprinting has been developed using one-step biofabrication techniques. Hermida et al. optimized a 3D model including alginates, ECM proteins such as collagen-1 and HA, plus U87-MG GBM cells, as well as stromal cells using the extrusion technique, and demonstrated to better represent therapeutic response when compared to 2D cultures (Hermida et al., 2019). Dai et al. (2016) described a similar approach, by using porous gelatin, alginate, and fibrinogen to simulate the ECM, mixed with U87-MG cells, the group bioprinted a 3D model of GBM where CSCs reach an 87% of survival and high rates of proliferation immediately after bioprinting. Furthermore, glioma cells were able to turn into a more differentiated neural cell population and the vascularized component of the model. In addition, higher chemoresistance was found in the 3D model when compared to 2D culture of glioma cells. (Dai et al., 2016) Wang et al. (2018a) used extrusion-based bioprinting technology to create a 3D glioma model to investigate the vascularization potential of patient-derived CSCs. Interestingly, a gel of gelatin, alginate, and fibrinogen was also used, and cell viability after impression was similar to the one reported by Dai et al. (2016) (86.27% $\pm$ 2.41%). Compared with cells grown in suspension, angiogenesis-related genes, *in vitro* vascularization potential, and stemness properties were more demarcated in the 3D model. Also, cellular ultrastructure in the 3D model showed more microvilli, mitochondria, and rough endoplasmic reticulum when compared to cells grown in suspension (Wang et al., 2018a).

In order to use 3D bioprinting to study the interaction between glioma CSCs and other non-tumor cells, Heinrich et al. (2019) developed a 3D-bioprinted mini-brain consisting of GBM cells and macrophages. The authors found that glioma cells communicate with macrophages, and trigger TAM polarization as described before in patients' tissue. Furthermore, macrophages would promote the EMT of GBM cells as evidenced by an increased expression of vimentin (Vim) and nestin (Nes), as well as a significant loss of E-cadherin (Cdh1). Consequently, higher glioma cell progression and invasiveness were noted in the mini-brains. When therapeutics target this intercellular communication, diminished tumor growth was recorded (Heinrich et al., 2019).

Similar approaches studying cell–cell and cell–ECM interactions in glioma CSC behavior and therapeutic response have also been reported (Dai et al., 2016; van Pel et al., 2018; Wang et al., 2018a,b, 2019; Haring et al., 2019; Heinrich et al., 2019; Mirani et al., 2019). Thus, current efforts in 3D printing for glioma research have focused on generating a better understanding of glioma biology, tumor angiogenesis, invasion, malignant transformation, drug susceptibility, and screening. These models are very promising in glioma research as they

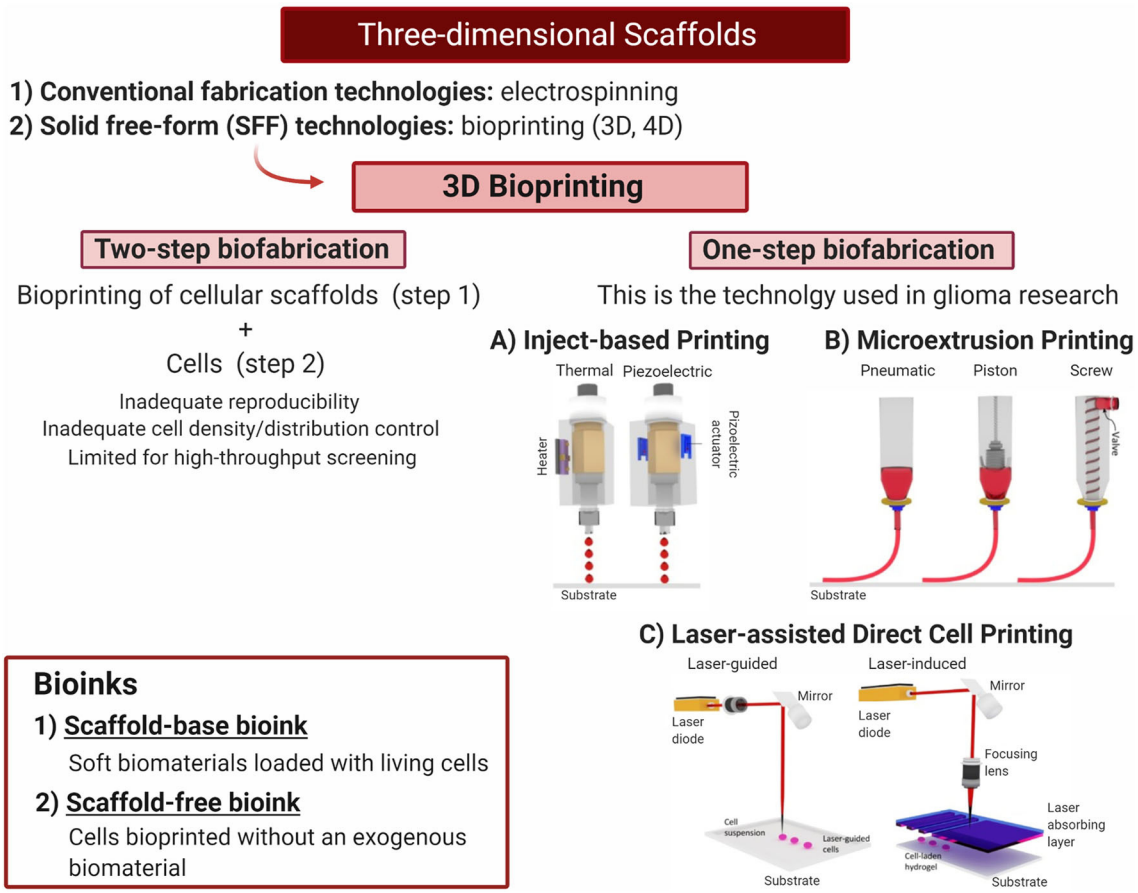

FIGURE 5 | Three-dimensional scaffolds—one-step biofabrication techniques. Bioprinting is a highly promising tool to generate 3D microenvironments combining different biomaterial and cell lines to evaluate tumor growth and progression to generate new therapies. Current glioblastoma research has been developed using one-step fabrication techniques. **(A)** Thermal and piezoelectric inkjet printing. **(B)** Microextrussion printing. **(C)** Laser-assisted direct cell printing. Adapted with permission from Tasoglu and Demirci (2013). Created with BioRender.com.

offer the possibility to manipulate and select specific factors to be studied according to any particular research question. The work of Heinrich et al. (2019) shows that it is possible to include more than one cell type within the gels, which allow for studying cell–cell interactions. We envision that this technology will help to dissect and understand in much more detail the very complex network of communications between different cell types. Additionally, as the manipulation of the physical properties of the gels is feasible, this will allow inquiring how the physical properties of the ECM affect glioma biology and test processes such as mechanotransduction. Overall, the development and refinement of this technology are highly relevant in the understanding of glioma CSC biology.

ADVANCES IN GLIOMA RESEARCH USING 3D MODELS

The use of 3D biomaterials used to simulate ECM mechanical properties and cell–ECM interactions has led to a deeper understanding of the mechanobiology underlying tumor malignancy, cancer cell migrations, and resistance to therapies. Furthermore, the previously described developments have allowed generating even more complex technologies to better study the relationship between not only the TME but also the interaction with the whole human body.

Organ-On-a-Chip

Organ-on-a-chip is a new technology that combines tissue engineering technologies with microfluidics to develop artificial systems that can recreate organ functions, organ interactions, and human physiology (Zhang et al., 2018). Yi et al. (2019) showed that an organ-on-a-chip GBM model that matched the clinical outcome after concurrent chemoradiation with temozolomide exhibited patient-specific sensitivity against specific drugs combinations. The interaction within the perivascular niche has also been studied using this technology, suggesting that glioma CSCs located around the vasculature and presenting with the lowest motility are most probably of the proneural subtype, and those with the highest invasiveness are most probably classified in the mesenchymal subtype; which further supports the role of the tumor niche on intratumor heterogeneity and consequent treatment response (Xiao Y. et al., 2019). Studies regarding GBM response to magnetic hyperthermia have been also carried out in a similar way (Mamani et al., 2020).

Four-Dimensional Bioprinting

Four-dimensional (4D) bioprinting is emerging as the next generation for biofabrication technology. Different from 3D bioprinting, which is static, 4D bioprintings introduce the use of stimuli-responsive biomaterials that can be modified in a time-depended manner (fourth dimension) in an attempt to mimic the physiological activities proper of any living microenvironment (Ashammakhi et al., 2018; Truong et al., 2019; Yang et al., 2019). 4D bioprinting has been used for drug screening, drug delivery, and vascularization models; therefore, this technology could help in the comprehension of glioma progression and therapy (Gao et al., 2016; Ruskowitz and DeForest, 2018).

CONCLUSIONS AND FUTURE PERSPECTIVES

The use of preclinical 3D models represents an opportunity to better understand glioma biology, as well as to perform high-throughput screening able to accelerate the selection of the most effective and personalized therapy for individual patients. To maximize the advantages of these models, they should rigorously represent most factors characterizing the TME, having in mind not only its cellular and non-cellular components but also the biomechanics underlying their interactions. Thus, it will be important to differentiate the characteristics we must represent in order to simulate the different tumor regions such as the core, the external layers, and even the surrounding healthy tissue that gliomas CSCs will inevitably infiltrate. Modeling each of these different regions will be fundamental to better study the heterogeneous CSC phenotypes, behaviors, and treatment responses; which in turn will be crucial to find a clinically relevant alternative for glioma patients.

AUTHOR CONTRIBUTIONS

HR-G and KA-E reviewed the literature and wrote the first draft of the manuscript. HR-G created the figures. PS, AQ-H and DT critically revised the manuscript. HR-G, KA-E, AQ-H and DT worked on the study conception and design. All authors analyzed the data, drafted the manuscript, and read and approved the final version of this work.

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Nanoparticles for Stem Cell Therapy Bioengineering in Glioma

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Gliomas are a dismal disease associated with poor survival and high morbidity. Current standard treatments have reached a therapeutic plateau even after combining maximal safe resection, radiation, and chemotherapy. In this setting, stem cells (SCs) have risen as a promising therapeutic armamentarium, given their intrinsic tumor homing as well as their natural or bioengineered antitumor properties. The interplay between stem cells and other therapeutic approaches such as nanoparticles holds the potential to synergize the advantages from the combined therapeutic strategies. Nanoparticles represent a broad spectrum of synthetic and natural biomaterials that have been proven effective in expanding diagnostic and therapeutic efforts, either used alone or in combination with immune, genetic, or cellular therapies. Stem cells have been bioengineered using these biomaterials to enhance their natural properties as well as to act as their vehicle when anticancer nanoparticles need to be delivered into the tumor microenvironment in a very precise manner. Here, we describe the recent developments of this new paradigm in the treatment of malignant gliomas.

Keywords: biomaterials, nanotechnology, nanoparticles, stem cells, glioma, bioengineering, targeting, surface functionalization

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INTRODUCTION

Gliomas are a dismal entity, associated with poor survival and high morbidity. The current standard of care has reached a therapeutic plateau even after combining maximal safe resection and chemoradiation (Stupp et al., 2005; Cantrell et al., 2019). In this setting, stem cell therapies have risen as a promising therapeutic approach for gliomas; however, there still exist crucial drawbacks holding its pass to an extensive acceptance in clinical applications. The development of nanomedicine is a parallel phenomenon with potential deep implications in the way stem cells will be introduced into human glioma therapy. Stem cells can be engineered using this nanotechnology in different ways in order to increase our understanding about their biology, improve stem cells antitumor properties, and synergize them with other approaches such as chemotherapy, radiation, thermotherapy, etc. (Kim et al., 2011; Mangraviti et al., 2016; Karlsson et al., 2019; Kozielski et al., 2019; Tian et al., 2020). We aim to provide an overview of the foundations of stem cell therapy and nanoparticles to then explore the potential synergy between these two, through an up-to-date analysis of the benefits of coupling both therapeutic approaches.

GLIOMAS

Gliomas are the most common and devastating primary brain tumors, representing approximately 75% of these. According to the World Health Organization (WHO), gliomas are classified in

four histological grades (I–IV), being the glioblastomas the corresponding WHO grade IV tumor. Glioblastoma (GBM) is the most common and aggressive among all gliomas, accounting for 57.3% of the tumors in this group, with around 12,500 new cases diagnosed every year only in United States (Cantrell et al., 2019; Ostrom et al., 2019). Glioblastomas present a median overall survival of 15 months and a 5-year survival rate of only 4.6% even after maximal therapy (Cantrell et al., 2019). Furthermore, most of the patients diagnosed with gliomas of lower grade, such as astrocytomas and oligodentrogliomas grade II and III (anaplastic), will eventually progress and perish because of the disease. Overall, these facts are just the translation of the need to develop novel therapeutic approaches able to help extend survival and improve the quality of life of patients with the diagnosis of glioma.

Limitation of Current Therapies

The current gold standard for the treatment of gliomas includes surgery and chemoradiation. Maximal safe resection is advised in all cases regardless of the WHO grade, given that the overall survival is positively correlated with the extent of resection (EOR) (McGirt et al., 2008, 2009; Chaichana et al., 2014a,b,c; Mahato et al., 2018; Mampre et al., 2018; Marenco-Hillembrand et al., 2020; Suarez-Meade et al., 2020). However, surgery is not curative in any case. Chemotherapy and radiation are required for high-grade gliomas. Anaplastic astrocytomas and oligodendrogliomas (grade III gliomas) will require chemoradiation depending on clinical parameters and tumor molecular characteristics (Caccese et al., 2020). Glioblastoma tumors require postoperative radiotherapy, with concurrent and adjuvant chemotherapy. Unfortunately, despite this multidisciplinary treatment, gliomas will inevitably recur due to their infiltrative nature and high treatment resistance (Cantrell et al., 2019). By the time of surgery, it is estimated that glioma cells have already migrated beyond the macroscopically identifiable tumor, and thereafter, these cells will ultimately seed local recurrence around the surgical cavity (75–80% of cases) and/or non-local recurrence in the reminder 20–25% of cases (Brandes et al., 2009; Chamberlain, 2011; Drumm et al., 2019).

A subset of gliomas cells have been pinpointed as the culprit of this recurrence. The glioma cancer stem cells (CSCs) are a subgroup of malignant cells with the potential of self-renewal, forming tumors that resemble the original pathology, as well as high resistance to current chemotherapeutics and radiation (Singh et al., 2003; Galli et al., 2004; Beier et al., 2007; Li et al., 2009; Cheng L. et al., 2013; Dahan et al., 2014). These cells migrate beyond the macroscopic tumor, infiltrating apparent normal brain parenchyma by the time of surgery and survive even after receiving high-dose radiation and chemotherapy (Li et al., 2009; Lathia et al., 2012). As these cells migrate beyond the tumor bulk to seed further recurrence, a therapeutic strategy able to track these newly developed microscopic glioma foci to deliver antitumor cargoes is of utmost importance. In this setting, the use of neural and mesenchymal stem cells (MSCs) as a therapeutic armamentarium against gliomas represents a potential avenue to achieve this goal and alter the treatment paradigm of this dismal cancer (Pendleton et al., 2013; Li et al., 2014; Smith et al., 2015; Mangraviti et al., 2016).

STEM CELLS AS ELEMENTS OF THERAPY FOR MALIGNANT GLIOMA

Stem cells have risen as a promising therapeutic option for the treatment of malignant gliomas, as they would be able to migrate and home into glioma tumors, including microscopic tumor foci, which harbor the potential to seed future recurrence (Brown et al., 2003; Nakamura et al., 2004; Kim et al., 2005, 2018; Sonabend et al., 2008; Thu et al., 2009; Yong et al., 2009; van Eekelen et al., 2010; Amano et al., 2011; Choi et al., 2011; Kleinschmidt et al., 2011; Ryu et al., 2011; Altanerova et al., 2012; Jiao et al., 2012; Kosaka et al., 2012; Cheng Y. et al., 2013; Huang et al., 2013, 2014; Lee et al., 2013; Balyasnikova et al., 2014; Li et al., 2014; Mooney et al., 2014b; Bryukhovetskiy et al., 2015; de Melo et al., 2015; Martinez-Quintanilla et al., 2015; Morshed et al., 2015; Park et al., 2015; Cheng S. H. et al., 2016; Kim S. J. et al., 2016; Kim S. M. et al., 2016; Liu et al., 2016; Mangraviti et al., 2016; Muroski et al., 2016; Meca-Cortes et al., 2017; Portnow et al., 2017; Hsu et al., 2018; Lang et al., 2018; Pavon et al., 2018; Tirughana et al., 2018; Zhang et al., 2018; Huang R. Y. et al., 2019; Tanrikulu et al., 2019; Allahverdi et al., 2020; Jabbarpour et al., 2020). Stem cells are relatively easy to grow *in vitro* and can be bioengineered to deliver a wide range of antitumor payloads such as proteins, oncolytic viruses, prodrugs, small interfering RNA (siRNA), and nanoparticles (Brown et al., 2003; Nakamura et al., 2004; Kim et al., 2005, 2018; Sonabend et al., 2008; Thu et al., 2009; Yong et al., 2009; van Eekelen et al., 2010; Amano et al., 2011; Choi et al., 2011; Kleinschmidt et al., 2011; Ryu et al., 2011; Altanerova et al., 2012; Jiao et al., 2012; Kosaka et al., 2012; Cheng Y. et al., 2013; Huang et al., 2013, 2014; Lee et al., 2013; Balyasnikova et al., 2014; Li et al., 2014; Mooney et al., 2014b; Bryukhovetskiy et al., 2015; de Melo et al., 2015; Martinez-Quintanilla et al., 2015; Morshed et al., 2015; Park et al., 2015; Cheng S. H. et al., 2016; Kim S. J. et al., 2016; Kim S. M. et al., 2016; Liu et al., 2016; Mangraviti et al., 2016; Muroski et al., 2016; Meca-Cortes et al., 2017; Portnow et al., 2017; Hsu et al., 2018; Lang et al., 2018; Pavon et al., 2018; Tirughana et al., 2018; Zhang et al., 2018; Huang R. Y. et al., 2019; Tanrikulu et al., 2019; Allahverdi et al., 2020; Jabbarpour et al., 2020).

Stem cells are undifferentiated cells with capacity of self-renewal and differentiation by definition. They can mature along symmetric and asymmetric replication processes. The later type of cell division will result in different hierarchies within stem cell niches, which will now include *progenitor cells*; these are daughter cells retaining the same stem cells properties but with a *de novo* limited differentiation ability (Young et al., 2014).

Stem Cell Classification

Stem cells can be designated according to their developmental status as *adult*, *fetal*, or *embryonic stem cells*. Their differentiation potential can further define them as *totipotent*, *pluripotent*, or *multipotent stem cells*. *Totipotent stem cells* are only found during the very first days of life just after fecundation and have the

capacity to derive into any type of human cells, including placental tissues. Once the embryo has reached the blastocyst stage, cells contained inside the inner cell mass are defined as *pluripotent,* as they can differentiate into any cell of all three germ layers but no placental tissues (Takahashi and Yamanaka, 2006; Qiao et al., 2018; Andres et al., 2019; Klimanskaya, 2019). Eventually, these *pluripotent stem cells* will restrict their differentiation potential to only one of the three germ cell layers and thereafter will be defined as *multipotent stem cells*, which can actually be harvested from most of the organs of the human body (Takahashi and Yamanaka, 2006; Qiao et al., 2018; Andres et al., 2019; Klimanskaya, 2019).

Stem cells used in glioma therapy are usually *multipotent* cells obtained from adult or fetal organs. In particular, neural stem cells (NSCs), mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs) are the most common multipotent stem cells used with this purpose (Aboody et al., 2000; Portnow et al., 2017). It is noteworthy, however, that *pluripotent* cells such as the induced-pluripotent stem cells (iPSC) or embryonic pluripotent stem cells have also been described in cell therapy against glioma (Parker Kerrigan et al., 2018; **Table 1**).

Development of Stem-Cell-Based Glioma Therapy

The use of stem cells in glioma therapy relies on their tumor-homing properties. This property was first described in 2000 by Aboody et al. The group presented a seminal paper describing the glioma tropism of *neural stem cells*. The study reported on the capacity of NSC for engrafting into the glioma bulk when intratumor NSC injections were performed, invading normal parenchyma only when tumor cells migrate far from the tumor mass; with this, they also showed the specific NSC ability to track glioma cancer cells infiltrating along healthy tissue. NSCs were also proven to migrate toward glioma tumor masses when implanted distally to these, through ipsilateral,

TABLE 1 | Classification and major features of stem cells reported in glioma therapy.

Stem cell	Defining criteria	Source/Niche	Linage
Pluripotent stem cells: capacity to differentiate into any cell of all three germ layers			
Embryonic stem cells (ECSs)	Markers of pluripotency as found in ICM cells: **Transcription factors:** Oct4, Nanog, Rex-1 **Cell surface markers:** SSEA-3, SSE-A4, TRA-1-60, TRA-1-81, alkaline phosphatase	Blastocyst Morula Growth-arrested embryos Somatic cell nuclear transfer Single blastomere	As defined by pluripotency
Induce pluripotent stem cells (iPSCs)	Essentially the same than ECS markers	Reprogrammed adult somatic cells—usually fibroblasts or skin cells.	As defined by pluripotency given than iPSC are functionally equivalent to ECS
Multipotent stem cells: capacity to differentiate into cells of one of the three germ layers			
Neural stem cells (NSCs)	**Positive:** GFAP, CD133, CD184, and Nestin, Sox1, Sox2, and Pax6 **Negative:** CD271, CD44, CD24 **Immune profile:** Absent HLA II	*Subependymal zone* (SEZ)—lining the lateral ventricles *Dentate gyrus* of the hippocampus *Obtained from fetal and adult mammals	Neurons Oligodendrocytes Astrocytes Ependymal cells
Mesenchymal stem cells (MSCs)	MSC must comply with ISCT criteria: **Positive (>95% +):** CD105, CD73, CD90 **Negative (<2% –):** CD45, CD34, CD14 or CD11b, CD79α, or CD19, HLA DR Adherence to plastic in standard culture conditions *In vitro* differentiation to osteoblast, adipocytes, and chondroblasts. **Immune profile:** Absent HLA II	**Adult tissues:** adipose tissue, bone marrow, peripheral blood, dental pulp, ligamentum flavum, synovium, endometrium, sweat glands, and milk **Fetal tissues:** umbilical cord, umbilical cord blood, Wharton jelly, amniotic fluid, chorionic villi, and placenta	Osteoblast Adipocytes Chondroblast *Differentiation into ectodermal and endodermal linages has also been reported
Hematopoietic stem cells (HMSs)	**Negative:** CD45R/B220 (B cells), Gr-1 (granulocytes), Mac-1 (macrophages), Ter-119 (erythrocytes) and CD4/CD8 (lymphocytes)—for phenotypic enrichment. **Positive:** Sca-1, c-Kit, CD150 *They appear as side population in dye exclusion assays due to the high expression of MDR pumps	**Adult tissues:** bone marrow, peripheral blood **Non-adult tissues:** umbilical cord blood, yolk sac, liver, spleen	Hematopoietic cells

IMC, inner mass cells; MDR, multidrug resistance.

contralateral, and intraventricular NSC injections (Aboody et al., 2000). These abilities and the possibility of being bioengineered to secrete antiglioma cargoes turned NSC into a promising glioma treatment, able to track and tackle this infiltrative malignant tumor. Importantly, NSC showed to retain their stem cell properties and had been proven non-tumorigenic (Snyder et al., 1992). In 2017, the same group published the first phase I clinical trial where NSC-based antiglioma therapy was proven safe; the proof of concept of NSC tumor homing was also demonstrated by the group (Portnow et al., 2017). Unfortunately, despite the encouraging role of NSC in glioma therapy, limited availability of human NSC (hNSC) as well as ethical concerns regarding its use encouraged researchers to seek alternatives sources of stem cells.

Mesenchymal stem cells (MSCs) were first described by Friedenstein more than 50 years ago (Friedenstein et al., 1968, 1970, 1974). He initially isolated MSC from rodent bone marrow and proved that they were able to differentiate into mesenchymal tissue (adipogenic, chondrogenic, and osteogenic differentiation). MSCs ended up being an alternative to the difficult-to-obtain NSC, as they are abundant in several adult and fetal tissues such as bone marrow (BM-MSC) (Friedenstein et al., 1968, 1970, 1974), adipose tissue (A-MSC) (Zuk et al., 2001; Katz et al., 2005; Wagner et al., 2005), umbilical cord (UC-MSC) (Girdlestone et al., 2009), umbilical cord blood, Wharton jelly (Erices et al., 2000; Zeddou et al., 2010), endometrium (Meng et al., 2007), dental pulp (Agha-Hosseini et al., 2010), ligamentum flavum (Chen et al., 2011), etc. (Kassis et al., 2006; Miao et al., 2006; Roubelakis et al., 2007; Poloni et al., 2008; Patki et al., 2010; Ma et al., 2018). MSCs are easy to harvest and isolate even from adult individuals, which would allow for using patient-derived MSC as autografts in glioma patients, thus avoiding ethical dilemmas as well as fears about immune-mediated allograft rejection. In this context where MSCs could be isolated from a variety of tissue sources, cultured following different methodologies, and be defined by using different surface markers, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a standard set of minimum criteria defining MSC for both laboratory-based scientific investigations and preclinical studies (Dominici et al., 2006). Thus, every study currently under development should follow these guidelines in order to assure a better cell homogeneity among different laboratories and greater reproducibility.

The first report on the use of MSC in the treatment of gliomas came from Nakamura et al. In 2004, the group demonstrated that MSCs also possessed glioma-homing properties by proving rat-derived BM-MSC homing in a rat glioma model. In 2005, Nakamizo et al. were able to replicate the findings using human BM-MSC on a murine model harboring glioma xenograft derived from commercial human cell lines (U87, U251, and LN229). Furthermore, both groups were able to bioengineer the MSCs to deliver antitumor cargoes. To date, several reports on the use of MSC as key elements for glioma stem cell therapy have been published with exceptional promising results (**Table 2**; Brown et al., 2003; Nakamura et al., 2004; Kim et al., 2005, 2018; Sonabend et al., 2008; Thu et al., 2009; Yong et al., 2009; van Eekelen et al., 2010; Amano et al., 2011; Choi et al., 2011; Kleinschmidt et al., 2011; Ryu et al., 2011; Altanerova et al., 2012; Jiao et al., 2012; Kosaka et al., 2012; Cheng Y. et al., 2013; Huang et al., 2013, 2014; Lee et al., 2013; Balyasnikova et al., 2014; Li et al., 2014; Mooney et al., 2014b; Bryukhovetskiy et al., 2015; de Melo et al., 2015; Martinez-Quintanilla et al., 2015; Morshed et al., 2015; Park et al., 2015; Cheng S. H. et al., 2016; Kim S. J. et al., 2016; Kim S. M. et al., 2016; Liu et al., 2016; Mangraviti et al., 2016; Muroski et al., 2016; Meca-Cortes et al., 2017; Portnow et al., 2017; Hsu et al., 2018; Lang et al., 2018; Pavon et al., 2018; Tirughana et al., 2018; Zhang et al., 2018; Huang R. Y. et al., 2019; Tanrikulu et al., 2019; Allahverdi et al., 2020; Jabbarpour et al., 2020).

On the other hand, not all are in agreement, as there has been a risen controversy in which some authors have described that MSCs could eventually support glioma tumor growth. Different types of MSCs such as BM-MSC, A-MSC, and UC-MSC have been associated with these proglioma effects through increased proliferation, cancer cells migration, angiogenesis, transition to epithelial–mesenchymal phenotype, and decreased glioma apoptosis (Iser et al., 2016; Ridge et al., 2017); however, this adverse phenotype would vary on a differential basis depending on specific glioma tumors (Breznik et al., 2017). In this same line, brain tumor-derived MSC (BT-MSC) have also been described in mouse- and human-derived glioma tumors supporting glioma microenvironment (Behnan et al., 2014; Guo et al., 2014; Svensson et al., 2017; Yi et al., 2018). In support to these findings, Shahar et al. showed that higher percentages of human BT-MSC directly correlate with worse patient prognosis (Shahar et al., 2017). Overall, these data would suggest that stem cell therapy should be carefully selected in future translational efforts.

In order to improve different aspects of stem cell therapies against glioma, different approaches have been studied. The use of nanoparticles for stem cell bioengineering is one of these potential approaches and will be discussed in the following section.

NANOPARTICLES AS ELEMENTS OF THERAPY FOR MALIGNANT GLIOMA

Nanoparticles (NPs) are defined as nanomaterials sized between 1 and 100 nm in at least one of their external dimensions, which confer them a high surface/volume ratio (European Commission, 2011). Due to this small size, they present significantly different properties when compared to conventional materials of non-nanometric scale. The optical, magnetic, electronic, and biological properties of these nanomaterials can be tuned by size, shape, surface modifications (functionalization), or even by combining them with different materials in order to create new heterostructured nanoparticles (Thimsen et al., 2014; Lee et al., 2017).

In Nano-oncology, nanoparticles represent an important diagnostic and therapeutic tool, as they can be designed to interact with most biological system with great precision and specificity. This is possible due to their particular physicochemical characteristics and the possibility of making them able to target a specific tissue, specific cell types, or a specific cellular compartment (targeted functionalization) (Portney and Ozkan, 2006). The potential benefits of these nanomaterials in

TABLE 2 | Stem cell therapy in glioma.

Stem cell type	Delivery routes	Applications
Neural stem cells[a,b]	• Intravascular: vein (Brown et al., 2003) • Intracranial (Mooney et al., 2014b; Morshed et al., 2015) • Intraventricular • Intracerebral (Altanerova et al., 2012; de Melo et al., 2015)	**Prodrug activating enzymes**: CD (Portnow et al., 2017), rCE, and hCE1m6 (Mooney et al., 2014b), HSV-TK (Uhl et al., 2005; Tamura et al., 2020) **Oncolytic viruses**: CRAd-S-pk7 (Morshed et al., 2015) **Cargo proteins**: IL-4 (Benedetti et al., 2000), IL-12 (Ehtesham et al., 2002), PF-4 (Lee et al., 2003), TRAIL (Balyasnikova et al., 2011), PEX (Kim et al., 2005), BMP4 (Liu et al., 2016), TSP-1 (van Eekelen et al., 2010) **Nanoparticles** (Mooney et al., 2014b) • Stem cells tracking: FE-Pro (Thu et al., 2009), FTD (Kim S. J. et al., 2016) via MRI, MSN (Cheng S. H. et al., 2016) via SPECT/CT • Payload release: SD (Muroski et al., 2016)**, MSN-Dox (Cheng Y. et al., 2013)
Mesenchymal stem cells		
Adipose-derived	• Intranasal (Balyasnikova et al., 2014) • Intracranial (Altanerova et al., 2012; de Melo et al., 2015)	**Prodrug activating enzymes**: yeast CD (Altanerova et al., 2012), HSV-TK (Meca-Cortes et al., 2017)[c], (de Melo et al., 2015) **Oncolytic viruses**: ICOVIR17 (Martinez-Quintanilla et al., 2015) **Cargo proteins**: TRAIL (Balyasnikova et al., 2014; Tanrikulu et al., 2019) **Oligonucleotides**: miR-4731 (Allahverdi et al., 2020)
Bone marrow-derived	• Intratumoral (Kosaka et al., 2012; Lee et al., 2013): alginate microencapsulated (Kleinschmidt et al., 2011) • Intracarotid (Yong et al., 2009)	**Prodrug activating enzymes**: CD (Kosaka et al., 2012), HSV-TK (Amano et al., 2011) **Oncolytic viruses**: Delta24-RGD (Yong et al., 2009), CRAd (Sonabend et al., 2008)*** **Cargo Proteins**: IL2 (Nakamura et al., 2004), INF-B (Park et al., 2015), TRAIL (Choi et al., 2011), BMP4 (Li et al., 2014; Mangraviti et al., 2016) **Oligonucleotides**: miR-124 (Lang et al., 2018; Lee et al., 2013) and miR-145 (Lee et al., 2013), miRNA-584-5p (Kim et al., 2018) **Nanoparticles**: •Gene therapy: MTN (TRAIL) (Huang R. Y. et al., 2019) •Intrinsic MSC modification: IO MNP (improve MSC homing) (Huang et al., 2014) •Stem cell tracking: MNP (Huang et al., 2013), FTD (Kim S. J. et al., 2016) via MRI. NIR675 (Kim S. M. et al., 2016) via near-infrared imaging
Human placenta-derived	• Intratumoral (Lee et al., 2013)	**Cargo proteins**: NK4 (Jabbarpour et al., 2020) **Oligonucleotides**: miR-124 and miR-145 (Lee et al., 2013) **Nanoparticles** • Stem cell tracking: PEG-SPIO (Hsu et al., 2018) via MRI
Umbilical cord-derived	• Intratumoral (Lee et al., 2013) • Intravascular: tail vein (Pavon et al., 2018)	**Cargo proteins**: IL12 (Ryu et al., 2011) **Oligonucleotides**: miR-124 and miR-145 (Lee et al., 2013) **Nanoparticles** • Stem cell tracking: MION-Rh (Pavon et al., 2018)*
Amniotic membrane-derived	• Intratumoral (Jiao et al., 2012)	**Direct antiglioma properties**: increased apoptosis (Jiao et al., 2012)
Hematopoietic progenitor cells	• *In vitro* (Bryukhovetskiy et al., 2015)	Migration in an *in vitro* model (Bryukhovetskiy et al., 2015)

CD, cytosine deaminase; rCE, rabbit carboxylesterase; hCE1m6, modified human carboxylesterase; MSN-Dox, doxorubicin loaded-mesoporous silica nanoparticles; FE-Pro, ferumoxide–protamine sulfate complex; FTD, FerraTrack Direct; NIR675, NEO-LIVE, Magnoxide 675 nanoparticles; MSN, mesoporous silica nanoparticles; MTN, magnetic ternary nanohybrid; IO MNP, iron-based magnetic nanoparticles; MNP, mesoporous nanoparticles; NK4, hepatocyte growth factor antagonist; PEG-SPIO, polyethylene glycol-superparamagnetic iron oxide; PF-4, platelet factor 4.

**MSC were found to promote tumor growth.*

***2u magnetic disks.*

****Only specified as human MSC.*

[a] *GMP production and scale-up of these cells have been performed (Tirughana et al., 2018).*

[b] *First-in human studies have been performed assessing safety of intracranial injection (Portnow et al., 2017).*

[c] *Modified MSC produced by CRISPR/Cas9.*

medicine have led some of them to obtain Food and Drug Administration (FDA) approval to be investigated under several clinical protocols (**Table 3**).

Targeted Functionalization

In order to achieve a targeted distribution at a cellular or even intracellular level, NPs can be functionalized via active targeting. Active targeting is achieved by different methods; a method called *ligand targeting* works by coating the nanoparticles' surface with one or more ligands such as transferrin, epidermal growth factor (EGF), folic acid, arginyl-glycyl-aspartic tripeptide (RGD) peptide, hyaluronic acid, antibodies, and others (Ruiz-Garcia et al., 2020). These ligands allow NPs to bind specific "receptors" differentially expressed only in certain cancerous blood vessels and/or tumor cells, thus leading to a precise cellular internalization (Maier-Hauff et al., 2007; Kim et al., 2010; Wegscheid et al., 2014; Cheng Y. et al., 2016; Shen et al., 2017; Yu et al., 2017, 2019; Hua et al., 2018; Daniel et al., 2019; Denora et al., 2019; Dufort et al., 2019; Kefayat et al., 2019; Kunoh et al., 2019; Luque-Michel et al., 2019; Rego et al., 2019, 2020; Ruan et al.,

TABLE 3 | Advances in the uses of nanoparticles in glioma therapy and diagnosis.

Use	Experimental setting and nanoparticle type
Nanocarrier: Drug bioavailability/therapeutic efficacy enhancer • Usually loaded with drugs such as doxorubicin or biological agents such as siRNA (Yu et al., 2017) • Usually functionalized with ligands of common GBM membrane proteins	Preclinical: • *In vivo*: PLGA-NP (Sousa et al., 2019; Ye et al., 2019; Caban-Toktas et al., 2020; Chung et al., 2020), RGD-NP (Ullah et al., 2020), oleic acid NP (Wang H. et al., 2020) • *In vitro* only: PLGA-NP (Luque-Michel et al., 2019; Ferreira et al., 2020; Roberts et al., 2020), ethyl arachidate (TPLN) (Alves et al., 2020), FONP (Daniel et al., 2019), pSiNPs
Standalone therapy	Preclinical • *In vitro*: Selenium NP (Xu et al., 2020), MNP (Shen et al., 2017) • *In vivo*: MP (Cheng Y. et al., 2016; Kim et al., 2010)
Drug sensitizer	Temozolomide: direct attenuation on EGFR and MET signaling, through delivered miRNAs (Meng et al., 2020)
Imaging technologies enhancer	Fluorescence: USPION (Denora et al., 2019)
Magnetic hyperthermia	*Clinical* • Phase I and II: SPION (Maier-Hauff et al., 2007; Wegscheid et al., 2014) *Preclinical* • *In vivo*: SPIONa (Rego et al., 2019, 2020; Shi et al., 2019)
Sonodynamic therapy	Preclinical • *In vivo*: (Liang et al., 2020)
Radiotherapy enhancer	Charged particles • Proton (Martinez-Rovira et al., 2020) • Helium • Carbon • Oxygen Photon therapy (X-rays) • *In vitro* only: AuNP (Kunoh et al., 2019) • *In vivo*: FA-AuNC (Kefayat et al., 2019), PEGylated-AgNP (Zhao et al., 2019), PEGylated-liposome (Hua et al., 2018), AGulX (Dufort et al., 2019)*
Photodynamic therapy enhancer	Preclinical • *In vivo*: 5-ALA (Wang X. et al., 2020), AuNS (Zhu et al., 2020)[a], ICG (ZhuGe et al., 2019)
Immunotherapy enhancer	Functionalization with anti-PDL1 (Ruan et al., 2019; Zhang et al., 2019)

*SPION, superparamagnetic iron oxide nanoparticles; USPION, ultrasmall SPION; PLGA, poly-lactide-co-glycolic acid; PEG, polyethylene glycol; RGD, arginyl-glycyl-aspartic tripeptide; TPLN, FONP, fluorescent organic nanoparticles; pSiNPs, porous silicon nanoparticles; terpolymer-lipid nanoparticles; MP, permalloy magnetic particles; FA-AuNC, folic acid gold nanoclusters; AuNP, gold nanoparticles, AgNP, silver nanoparticles; AGulX, gadolinium-based nanoparticle; 5-ALA, 5-aminolevulinic acid; AuNS, gold nanospheres; ICG, indocyanine green; HSPA5, heat shock protein A5r4t. *Theranostic NP: Possesses diagnostic and therapeutic functions. [a]Improve CT/MRI imaging and also works as radiosensitizer by AuNS properties and loading an HSPA inhibitor.*

2019; Shi et al., 2019; Sousa et al., 2019; Ye et al., 2019; Zhang et al., 2019; Zhao et al., 2019; ZhuGe et al., 2019; Alves et al., 2020; Caban-Toktas et al., 2020; Chung et al., 2020; Ferreira et al., 2020; Kazmi et al., 2020; Liang et al., 2020; Martinez-Rovira et al., 2020; Meng et al., 2020; Roberts et al., 2020; Ullah et al., 2020; Wang H. et al., 2020; Wang X. et al., 2020; Xu et al., 2020; Zhu et al., 2020; see **Table 3** for examples of *ligand targeting* in glioma research).

Another active targeting method to increase functional specificity of NPs that are used as gene delivery systems is the *transcriptional targeting*, which can occur at a transcriptional or posttranscriptional level (Golombek et al., 2018). Here, the delivered gene includes a tumor-specific promoter (highly functional only in cancer cells), which will secure a well-localized expression of the transgene, limited to occur only inside the cancer cells of interest. Posttranscriptional regulations of the product encoded by the exogenously delivered gene are achieved by controlling RNA splicing, RNA stability, and initiation of the RNA translation once it is present in the cancer cell (Maier-Hauff et al., 2007; Kim et al., 2010; Wegscheid et al., 2014; Cheng Y. et al., 2016; Shen et al., 2017; Yu et al., 2017; Hua et al., 2018; Daniel et al., 2019; Denora et al., 2019; Dufort et al., 2019; Kunoh et al., 2019; Luque-Michel et al., 2019; Ruan et al., 2019; Rego et al., 2019, 2020; Shi et al., 2019; Sousa et al., 2019; Ye et al., 2019; Zhang et al., 2019; Zhao et al., 2019; ZhuGe et al., 2019; Alves et al., 2020; Caban-Toktas et al., 2020; Chung et al., 2020; Ferreira et al., 2020; Kazmi et al., 2020; Liang et al., 2020; Martinez-Rovira et al., 2020; Meng et al., 2020; Roberts et al., 2020; Ullah et al., 2020; Wang H. et al., 2020; Wang X. et al., 2020; Xu et al., 2020; Zhu et al., 2020).

To date, several nanoparticles have shown to be effective in improving different aspects of traditional and novel cancer therapeutic approaches, to the point that several nanocarriers and nanoradiotherapy enhancers are being studied in phase II and III clinical trials (Maier-Hauff et al., 2007; Kim et al., 2010; Wegscheid et al., 2014; Cheng Y. et al., 2016; Shen et al., 2017; Yu et al., 2017; Hua et al., 2018; Daniel et al., 2019; Denora et al., 2019; Dufort et al., 2019; Kunoh et al., 2019; Luque-Michel et al., 2019; Rego et al., 2019, 2020; Ruan et al., 2019; Shi et al., 2019;

TABLE 4 | Rational for a combinatorial approach using nanoparticles and stem cell therapy in malignant glioma.

Rational for nanoparticle-based stem cell therapy in malignant glioma	
Drawbacks of using nanoparticles alone	• Big nanoparticles could be engulfed by the phagocytic mononuclear system (macrophages/lymphocytes) depending on their size
	• Necrotic central core and histological heterogeneity predispose to uneven intratumor biodistribution of nanoparticles
	• Infiltrative cells leaving the tumor bulk are unlikely to be tracked by nanoparticles
Potential advantages of using combine therapy	• Stem cells can transport bigger nanoparticles, increasing nanoparticle loading capacity
	• Stem cells could transport big nanoparticles through the BBB
	• Stem cells could better deliver therapeutic nanoparticles into the hypoxic central glioma core where treatment-resistant CSC locate
	• Stem cells could track and deliver their cargo to CSC leaving the tumor bulk. These CSC have been pinpointed as culprits of future tumor recurrence

BBB, blood brain barrier; CSC, cancer stem cells.

Sousa et al., 2019; Ye et al., 2019; Zhang et al., 2019; Zhao et al., 2019; ZhuGe et al., 2019; Alves et al., 2020; Caban-Toktas et al., 2020; Chung et al., 2020; Ferreira et al., 2020; Kazmi et al., 2020; Liang et al., 2020; Martinez-Rovira et al., 2020; Meng et al., 2020; Roberts et al., 2020; Ullah et al., 2020; Wang H. et al., 2020; Wang X. et al., 2020; Xu et al., 2020; Zhu et al., 2020). In the next section, we will briefly review the role of nanoparticles as a standalone therapeutic approach for glioma tumors (**Table 3**), and then, we will review in detail the role of nanoparticles as a tool to further improve stem cell therapy (**Table 4**).

Classification

Nanoparticles can naturally occur in the environment mediated by biological or geological processes (Sharma et al., 2015), or as incidental by-product of human activities such as smelting or other processes involving the generation of metal fumes (Gonzalez-Pech et al., 2019). In addition, nanoparticles can be artificially synthetized and engineered (Kus et al., 2018). Given the wide variety of existing NPs, classification criteria are also abundant. We present the classification of NPs according to their origin and structure, as they will help understand the terminology used to describe NPs used in cancer research.

Classification of Nanoparticles Based on Its Origin

Organic

Organic nanoparticles are based on natural compounds such as lipids, glycosides, peptides and others, as well as synthetic organic molecules (Romero and Moya, 2012; Tzeng et al., 2016; Kus et al., 2018; Karlsson et al., 2019; Kozielski et al., 2019; Tian et al., 2020). These organic elements can arrange themselves in three-dimensional (3D) structures (Euliss et al., 2006), which is one of the main characteristics that differentiate organic from inorganic nanoparticles, as inorganic NPs do not form these 3D structures in any case (Romero and Moya, 2012). Furthermore, due to the weak interactions that hold many organic NPs together, they present a dynamic nature that allows, for example, for fusion and generation of larger structures depending on external conditions (Romero and Moya, 2012).

Organic nanoparticles display highly desirable characteristics in the biomedical field (Hussein Kamareddine et al., 2019). They have a dynamic nature and are able to respond to environmental variations in temperature, pH, and UV radiation (Jagannathan et al., 2006; Affram et al., 2017; Hussein Kamareddine et al., 2019). Furthermore, they can easily cross biological barriers and are considered less toxic due to its biodegradability; therefore, they are ideal as drug or gene delivery systems (Jagannathan et al., 2006; Hussein Kamareddine et al., 2019). Liposomes, vesicles, micelles, polymeric NPs, and dendrimers are all among the most common organic nanoparticles (for specific characteristic and applications, see **Figure 1**); however, among all of them, polymeric NPs are probably the most relevant in cancer research.

Polymeric NPs, also known as polymeric nanospheres, are commonly defined as solid polymer particles with matrix type structure, where a cargo can be embedded within the polymer matrix or included in the surface (Reis et al., 2006). Based on its origin, polymeric NPs can be classified as natural or synthetic. The first group contains NPs such as chitosan, which is a widely available natural cationic carbohydrate polymer approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for drug and gene delivery and tissue engineering in humans (Lara-Velazquez et al., 2020). The second group, or synthetic polymer-based nanoparticles (SP-NPs), is the most relevant in medicine, as they can be easily synthesized and their properties can be tailored according to specific needs.

SP-NPs are prepared using synthetic polymers (Romero and Moya, 2012), which can be classified in *polyesters* such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(caprolactone) (PCL), and poly(lactic-co-glycolic acid) or (PLGA); *polyalkyl alcohols* such as polyvinyl alcohol or PVA; and *polyethers* such as poly(ethylene glycol) (PEG) and poly(propylene glycol) (PPG) (Ranganathan et al., 2018). Currently, there are around 15 FDA-approved nanomedicines based on SP-NPs, 6 of them are used in cancer therapy (Bobo et al., 2016; Farjadian et al., 2019). Up to date, glioma research based on SP-NPs has been mainly focused on the development of more effective delivery systems, able to cross the blood brain barrier and specifically target the cancer cells (Ambruosi et al., 2006; Hua et al., 2011; Jiang et al., 2011; Guo et al., 2013; Bishop et al., 2016; Tzeng et al., 2016; Karlsson et al., 2019; Kozielski et al., 2019). This includes the generation of hybrid systems using booth synthetic polymers and natural compounds (Agrawal et al., 2015; Cook et al., 2015; Alex et al., 2016; Wang et al., 2017; Qi et al., 2020), as well as smart nanoparticles able to react according to the surrounded conditions or to specific stimulus (Soppimath et al., 2005; McNeeley et al., 2009; An et al., 2015; Mangraviti et al., 2015; Gao et al., 2016; Ye et al., 2019).

Nanoplatforms used as Nanocarriers in Cancer Therapy

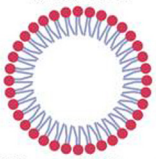
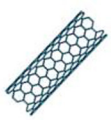
Nanoplatforms	Features
Liposomes  100 nm - 5 um	• Spherical polymeric vesicles • Shell is made of a lipid bilayer • Ability to carry hydrophobic and hydrophilic molecules • Easily manipulated during their synthesis process (T° or pH sensitive, to allow for controlled release) • Long circulation time and good diffusion properties
Micelles  20 nm - 200 nm	• Spherical polymeric vesicles that can be functionalized for better targeting • Shell is made of lipids or another amphiphilic material • Hydrophobic interior and hydrophilic exterior (can carry protected insoluble drugs/materials) • Can be T° or pH sensitive, to allow for controlled release
Polymeric Nanoparticles 	• Built by either natural or synthetic polymers • Hydrophobic core with large loading capacity • Hydrophilic shell for protection and customized targeting (through surface modification) • Can carry hydrophilic and hydrophobic molecules and macromolecules (nucleic acids, proteins, etc.) and release them in a controlled manner • Good safety profile and natural metabolism
Dendrimers  5 nm - 10 nm	• Tree-like nanoparticle, containing extensive branching and multivalent functional groups • Can enclose different therapeutics inside its structure. • Can simultaneously contain imaging materials and targeting molecules • By selecting a particular polymer, a controlled degradation of its branches can regulate the release of the therapeutic molecules
Metal Nanoparticles  ~1 nm - 150 nm	• Self-assembled of metallic atoms such as gold or iron (magnetic) • Can be modified (through surface modifications) to achieve better targeting • Gold: biologically inert → low toxicity/high biocompatibility . Surface plasmon resonance properties, so visible in the region of light spectrum • Iron: strong magnetic properties →allows for magnetic hyperthermia (thermal ablation therapies) and advancing MRI imaging (supermagnetic iron oxide nanoparticles (SPIONs))
Carbon Nanotubes  ~3 nm - 6 nm	• Carbone nanotubes (CNT) are allotropic form of carbons related to the fullerene family, used in diagnosis and cancer treatment • High surface are but its needle shape allows for easily penetrate cells • Penetrate tumor by enhanced permeability and retention (EPR) • Exceptional thermal properties → Thermal ablation (cancer treatment) • Good loading capacity (to carry chemotherapeutics).

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FIGURE 1 | Nanoplatforms used as nanocarriers in cancer therapy.

Inorganic

Inorganic nanoparticles present unique physicochemical properties (optical, magnetic, etc.), inertness, high stability, and easy functionalization, which give them different advantages when compared to organic NPs. Due to their cellular internalization ability and low immunogenic response, these nanoparticles were initially used as drug and gene delivery systems (Xu et al., 2006; Evans et al., 2019). Different types of elements and inorganic compounds based on metals [metal NP (mNP)], metalloids, or non-metals such as gold, silver, iron, magnesium, silicon, and others are differentially arranged and/or combined in order to display specific properties. Thus, there are some particular groups of NPs such as *magnetic nanoparticles*, which are usually based in a core of iron oxide mNP with a large magnetic momentum under an external magnetic field, which allow its use as MRI contrast enhancer and thermotherapy agents (Maier-Hauff et al., 2007; van Landeghem et al., 2009; Wegscheid et al., 2014). *Plasmonic nanoparticles* refer to mNPs such as gold (Au) or silver (Ag) NPs presenting with surface plasmon resonance (SPR), meaning that NP free electrons can be excited by electromagnetic fields (UV or infrared light) and resonate, creating the possibility to sense these changes (biosensors), produce heat (photothermal ablation/therapy), or create technologies such as surface-enhanced Raman spectroscopy (SERS) (Kaur et al., 2016; Chen et al., 2018; Liu et al., 2018). *Quantum dots (Qdots)* are another group of inorganic NPs, usually smaller than 50 nm; these semiconductor NPs efficiently produce bioluminescence once excited by UV light, which has led them to be used in single cell and *in vivo* imaging (Xu et al., 2006; **Figure 1**).

Carbon Based

These nanoparticles are predominantly composed by carbon, and their discovery revolutionized diverse scientific fields (Cha et al., 2013; Patel et al., 2019). Carbon-based nanomaterials have outstanding properties like high mechanical strength, thermoelectrical conductivity, and flexibility (Cha et al., 2013). These nanoparticles include fullerenes (carbon nanotubes), graphene, and nanodiamonds. Their broad range of properties makes these materials ideal imaging agents for tumor diagnosis (Patel et al., 2019; **Figure 1**).

Classification of Nanoparticles Base on Their Structure

Single Nanoparticles

Single nanoparticles are made of a single element such as gold, silver, copper, among others, and due to their homogeneity and electrochromic properties, they are widely used in electro-optical applications, energy conversion, and storage (Evans et al., 2019). Diverse systems for synchronized release of multiple drugs for cancer therapy have been designed based on single nanoparticles (Liao et al., 2014).

Heterostructured Nanoparticles

In an attempt to increase the performance and functionality of nanomaterials, heterostructured nanoparticles composed of two or more different materials were created. This technology allowed for the design of advanced NPs with additional properties arising from the synergy of the different materials (Wei and Zhao, 2016). One method to concrete this effort was to coat nanoparticles with one or more layers. Nanoparticles created in this way can be classified as *core–shell* (CS), when a central core (NP) is surrounded by one or more layers of different material [shell(s)], or as *yolk–shell* (YS). when the a movable core is located in a hollow cavity surrounded by a shell (Purbia and Paria, 2015). A *hollow core–shell* structure or *hollow* NP is another term referred to a NP without a core; the resulting empty space inside the shell can then be loaded with drugs, microRNA (miRNA), genes, peptides, and others that can now be released in a controlled manner. *Janus nanoparticles* are a different type of nanomaterials; they possess a tunable asymmetric structure; their surface has two or more regions with different properties, which confer them unique properties as selective reactivity or directional interactions. The field of application is broad and innovative including its use as sensors, self-propelled carriers, or coatings (Agrawal and Agrawal, 2019; **Figure 2**).

Nanoparticles as a Theragnostic Approach in Glioma

Nanoparticles as Radiosensitizers

Cancer tumors frequently contain a chemo and/or radioresistant subpopulation that survives and proliferates after standard treatments, contributing to the recurrence of a more aggressive tumor (Dahan et al., 2014; Yuan et al., 2018). Cancer stem cells (CSCs) represent this treatment-resistant subpopulation, and huge efforts are being focused on developing strategies to make them more amenable to current and novel therapies. The use of radiosensitizers is a potential approach to overcome radioresistance; however, its principal shortcoming is the lack of target specificity, which may lead to low concentrations in tumor tissue and toxic effects in healthy cells.

In this regard, nanoparticles have been tested as radiosensitizers agents and also as radiosensitizers carriers, showing promising results after photon and particle radiation (Caban-Toktas et al., 2020; Chung et al., 2020; Kazmi et al., 2020). For instance, Kunoh et al. developed DNA–gold nanoparticles complexes to work as radiosensitizers; they showed good cellular targeting and being effective in inducing cell death by mitotic catastrophe in glioma CSC after X-ray irradiation (Kunoh et al., 2019). Kefayat et al. also described good performance of folic-acid-coated gold nanocluster in radiosensitizing orthotopic C6 glioma tumor in a murine model (Kefayat et al., 2019). Folic acid receptors are differentially expressed in the luminal side of cancerous blood–brain barrier (BBB) endothelial cells as well as in cancer cells but not in normal tissues, which explain the higher concentration of these NP in glioma tumor when compared to a healthy brain tissue (Kefayat et al., 2019).

Furthermore, in order to target the glioma-resistant population specifically located in the tumor hypoxic niche, Hua et al. developed hypoxia-responsive yolk–shell nanoparticles (liposomes) by encapsulating radiosensitizer hydrophobic drugs [aniopep-2-poly-(metronidazoles)n and doxorubicin (DOX)] in hydrophilic polymers (PEG2000); these NPs were functionalized to target gliomas cells and release its content only under hypoxic

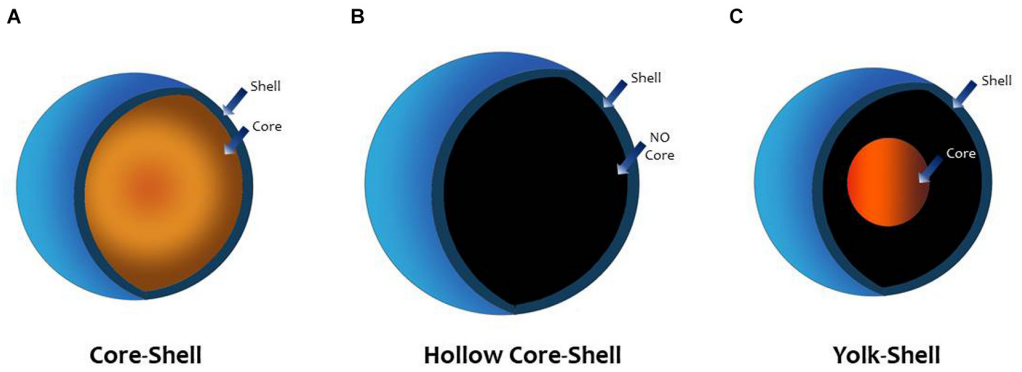

FIGURE 2 | Nanoparticle classification based on its structure. **(A)** *Core–shell* (CS) nanoparticle (NP) possesses a central core (NP) surrounded by one or more layers of different material [shell(s)]. **(B)** *Hollow core–shell* structure or (*hollow* NP) refers to an NP without a core; the resulting empty space inside the shell can then be loaded with drugs, microRNA (miRNA), genes, peptides, and others that can now be released in a controlled manner. **(C)** *Yolk–shell* (YS) NP possesses a movable core that is located in a hollow cavity surrounded by a shell (Purbia and Paria, 2015).

conditions, increasing radiosensitization as shown *in vitro* and *in vivo* after systematic NP administration (Hua et al., 2018; **Table 3**).

Nanoparticles as Nanocarriers

The restricted permeability of the BBB has been one of the biggest challenges in the mission of effectively treating brain tumors. In nanomedicine, not all nanoparticles can efficiently cross this biological barrier despite their size and physicochemical characteristics; consequently, previously discussed strategies, such as *ligand targeting*, or improvements on the *enhanced permeability and retention (EPR) effect* are required.

While *ligand targeting* is an active targeting method and required NPs to be designed with this purpose in mind, *EPR effect* refers to a passive targeting mechanism common to all NPs. EPR effect relies on pathophysiological characteristics of tumor vs. healthy vessels as well as NP size, which is larger than individual conventional chemotherapeutics (usually < 1,000 Da). Due to their relatively larger size, NPs are not able to penetrate normal blood vessels but can easily cross diseased vessels such as those presented in brain tumors, leading to a selected distribution into cancer tissues. NP with diameters of at least 5–10 nm present reduced kidney excretion (by exceeding the clearance renal threshold of 40,000 Da), prolonged blood half-life, and better accumulation in the tissue of interest. For instance, the plasma half-life of doxorubicin increases from 5–10 min to 2–3 days when this is encapsulated into liposomes. In order to achieve better results from the application of organic nanoparticles such as liposomes, micelles, etc., some polymers such as PEG can be used to decrease NP aggregation and opsonization by plasma proteins, thus adding to the improved blood half-life (Hua et al., 2018).

Unfortunately, EPR effect is highly heterogeneous at inter- and intraindividual level, changing over time in the same tumor and even being dissimilar among different brain tumor lesions for the same patient. This, altogether, has led to clinical outcomes that does not match with preclinical results. In order to overcome these drawbacks, additional strategies to enhance BBB disruption and facilitate NP penetration have been applied. These strategies include pharmacological and physical methods such as sonoporation and radiation. Radiation can increase vascular permeability due to increased secretion of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Lee et al., 1995; Park et al., 2001). Thus, Lammers et al. (2007) showed a positive effect in the accumulation of DOX-loaded polymeric NP sized between 5 and 10 nm (31 and 65 kDa, respectively) when tumors were primed with different doses of radiation.

Overall, all the previously described strategies should be carefully weighted when trying to optimize the use of nanoparticles as nanocarriers. In this setting, when applied to an orthotopic glioblastoma model, the use of functionalized biodegradable polymeric nanoparticles coated or loaded with anticancer drug has been able to confer longer survivals in preclinical models (Yu et al., 2019). Among all the different nanocarriers (**Figure 1**), liposomes have been largely used. Preclinical studies using liposomes loaded with doxorubicin or coated with temozolomide showed higher concentrations of these drugs inside the brain when compared with the plasmatic levels; in these same models, survival benefit was also described (Zhao et al., 2018; Li et al., 2019). Noteworthy, liposomal doxorubicin has been clinically used in primary and recurrent high-grade glioma patients, and good biodistribution and decent outcomes were obtained; however, none of the studies were randomized controlled trials (RCTs) and were published just before or after the publication of the Stupp protocol (Fabel et al., 2001; Hau et al., 2004).

Overall, these results point the use of nanocarriers as a promising enhancer of effective therapies for the treatment of patients with glioma (**Table 3**).

Nanomachines

Nanomachines or nanobots are molecular self-propelled nanodevices considered as smart delivery systems that respond to specific triggers (Khawaja, 2011; Jager and Giacomelli, 2015; Saxena et al., 2015; Fu et al., 2017). DNA nanorobots are nanometric devices controlled by an aptamer-encoded logic gate, able to sense specific stimulus such as intracellular pH or cell surface ligands in order to activate and reconfigure its structure for delivery of different payloads. Li et al. reported on a DNA nanorobot created through the DNA origami method; this was programmed to unfold itself upon binding to caveolin molecules expressed in cancerous blood vessel endothelial cells in order to deliver thrombin into tumor vessels. The authors were able to prove this concept in a murine model of breast cancer, successfully inducing intratumorally vascular thrombosis that resulted in tumor necrosis and growth tumor inhibition (Li et al., 2018). This technology is revolutionizing the traditional way of treating different tumors and is a promising strategy to improve prognosis on brain tumor patients. Other novel approach introduced as a promising tool in the armamentarium for the treatment of glioma tumors is the use of stem cells. Along the next section, we will describe how the above-described nanotechnology has been coupled to engineer improved stem cell therapies for the treatment of brain cancer (**Table 3**).

APPLICATIONS OF NANOPARTICLES IN STEM CELL GLIOMA THERAPY

Nanomedicine has extend the reach to several cancer treatment approaches such as radiotherapy, chemotherapy, immunotherapy, and others. In the case of stem cell therapies, improvements in several aspects are clearly needed. In an attempt to consolidate the translational potential of this approach, nanoparticles have been used to enhance safety and efficacy, stem cell tumor homing, and *in vivo* tracking after stem cell delivery. On the other hand, apart from nanoparticle surface modifications performed in an attempt to improve pharmacokinetics and pharmacodynamics parameters, stem cells appear as a reasonable option to overcome the suboptimal penetration, distribution, and retention associated to some nanomaterials when used as therapeutic nanocarriers. The use of stem cells in this context definitely add another option for a more targeted nanoparticle delivery. Thus, the benefit obtained from this combined approach using nanoparticles and stem cells is bidirectional.

Nanoparticles for Stem Cells Genetic Engineering

Stem cells are known by their ability to serve as vehicles of antitumor cargoes. For this purpose, viral gene vectors have been traditionally used to transduce stem cells with a high degree of gene delivery efficiency resulting in constant payload production (anticancer proteins, cytokines, antibodies, viral vectors, etc.). Although newer generations of viral vectors present better safety profiles, these vectors have been associated with immunotoxicity as a response to viral proteins production or potential viral replication. They also would carry the hypothetical risk of uncontrolled viral genome integration and insertional mutagenesis, latent virus activation, and inflammatory responses leading to demyelination or neurodegeneration (Dewey et al., 1999; Mangraviti et al., 2016). In this setting, nanoparticle-based gene delivery represents an attractive non-viral strategy to bioengineer stem cells. Different from commercially available reagents such as Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) nanoparticles may represent a less toxic and more effective approach for gene delivery.

Our group reported on the use of biodegradable polymeric nanoparticles based on poly(beta-amino ester)s (PBAEs) to enable effective BMP4 gene delivery on human A-MSC, allowing for higher transfection rates than those of commercially available reagents. Transfected MSC retained their multipotency and their tumor-homing capacity and were functional, leading to extended survival in a rat orthotopic GBM model (Yong et al., 2009). Huang et al. also reported on the use of nanoparticles for stem cell bioengineering; using hyaluronic acid (HA)-decorated superparamagnetic iron oxide nanoparticles as part of a magnetic ternary nanohybrid (MTN), the group was able to construct tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-secreting human mesenchymal stromal cell (hMSC). Decoration with CD44-binding HA and magnetic forces were used in this approach to increase cellular uptake of MTN. Impairment in tumor-homing properties were not observed (Huang R. Y. et al., 2019). Overall, nanoparticles raise as an option of safe and efficient gene delivery for stem cell; thus, helping stem cell therapy to achieve its maximal therapeutic potential.

Nanoparticles as Stem Cells Payloads

In the treatment of several malignancies, different nanoplatforms acting themselves as anticancer agents (Mooney et al., 2014a) or as carriers for these anticancer drugs (Mooney et al., 2014b) have been delivered locally and systemically. Recent advances in nanomedicine have allowed tuning nanoparticles properties in such a way that crossing the BBB and reaching brain tumors is now possible. However, there is a fine line between three factors: (1) the ideal size that a nanoparticle must have to easily cross the BBB (up to150 nm, optimal passage if < 15 nm) (Gao and Jiang, 2006), (2) being big enough to still be able to carry enough payload, and (3) being small enough to avoid engulfment by the mononuclear phagocyte system but still contain all the necessary ligands to assure specific cancer targeting (Owens and Peppas, 2006).

Furthermore, even if researchers could secure that nanoparticles reach the glioma tumor bulk, there exist other potential drawbacks that are imperative to highlight; they are related to the presence and location of glioma cancer stem cells (1) Nanoparticles are neither able to track infiltrative glioma cells leaving the tumor bulk to colonize distal healthy brain parenchyma nor (2) to reach the necrotic glioma core where blood flow is impaired. These areas do not present an EPR effect, which would facilitate nanoparticles to distribute across other areas of the tumor. Allowing nanoparticles to access the hypoxic central core would be crucial, as the treatment-resistant

subpopulation of glioma cancer cells would predominantly locate in that area (**Table 4**). Even after active targeting strategies including ligand targeting and microenvironment-related targeting (delivering payload depending on pH, temperature, etc.) (Koo et al., 2006; Bernardi et al., 2008; Madhankumar et al., 2009; Hadjipanayis et al., 2010; Wang et al., 2011), nanoparticles alone are still insufficient and would be unlikely to overcome the above-mentioned roadblocks.

In this scenario, coupling stem cell therapy to nanotherapeutics offers the possibility to solve the previously stated dilemma regarding the inadequate distribution of therapeutic nanoparticles to the hypoxic glioma core and distant infiltrative tumor foci. Thus, stem cells could extend the reach for nanoparticles to penetrate these areas. This approach implies nanoparticles to be conjugated to stem cell surfaces or internalized before migrating toward malignant gliomas. Furthermore, the internalization of nanoparticles inside stem cells would allow them to be up to fivefold larger than the usual nanoparticles used in cancer therapy, without entailing problems in crossing the BBB or a higher risk to be engulfed by macrophages or lymphocytes (Koo et al., 2006; Bernardi et al., 2008; Madhankumar et al., 2009; Hadjipanayis et al., 2010; Wang et al., 2011). This increase in the nanoparticles' longitudinal size translates into an approximately 125-fold increase in the nanoparticle load potential (by a volume-based, three-dimensional factor of 5) (Koo et al., 2006; Bernardi et al., 2008; Madhankumar et al., 2009; Hadjipanayis et al., 2010; Wang et al., 2011).

In this same line, the Aboody group demonstrated that *neural stem cells* were able to improve intracranial nanoparticle retention and tumor-selective distribution in an *in vivo* model by coupling huge nanoparticles to NSC surface (Mooney et al., 2014b). Taking advantage from the significant differences in the environmental pH between tumor and healthy tissues, the Aboody group loaded FDA-approved NSC cell with pH−sensitive doxorubicin−loaded mesoporous silica nanoparticles (MSN−Dox); the authors were able to tune nanoparticles properties to delay doxorubicin toxicity, allowing NSC to home into glioma tumors and deliver its payload only after arriving at the acidic tumor microenvironment. The approach led to a significant difference in survival when studied in a preclinical *in vivo* model (Cheng Y. et al., 2013). The same group also evaluated the role of NSC loaded with gold nanorods (AuNRs) to improve plasmonic photothermal therapy (aka thermal ablation), where the nanoparticles help to convert light into heat, aiming to eliminate cancerous tumor cells. The authors found that intratumor injections of AuNR-loaded NSC improved AuNRs distribution inside the tumor bulk when compared to locally injected free AuNRs in a brain metastasis heterotopic *in vivo* model (Mooney et al., 2014a).

The role of *mesenchymal stem cells* as nanoparticle carriers has also been investigated. Polymeric nanoparticles (paclitaxel-encapsulated PLGA nanoparticles) were loaded into BM-MSC. Osteogenesis, adipogenesis (chondrogenesis was not evaluated), and tumor homing were not affected by nanoparticle inclusion. The approach was associated with improved survival in a rat orthotopic glioma model when the modified MSCs were injected in the contralateral hemisphere (Wang et al., 2018). A similar approach to the one described by the Aboody group was performed on modified MSCs by loading them with gold nanoparticles (nanostarts) to improve phototherapy. Although studied in a heterotopic model of prostate cancer, the results support the use of MSC to maximize clinically relevant gold nanoparticles' optical–electronic properties by increasing nanoparticle intratumor distribution (Huang L. et al., 2019).

Nanoparticles to Modulate Stem Cell Tumor Homing

Several *tumor cytokines* and *stem cells surface proteins* have been involved in enhancing MSC migration toward glioma tumors; however, no specific mechanism has been described yet. *Tumor cytokines* such as endothelial cell growth factor (EGF), platelet-derived growth factor (PDGF), VEGF, tumor growth factor β1 (TGF-β1), interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and stromal cell-derived factor 1 alpha (SDF-1α) as well as *stem cells surface proteins* such as CD44, CXC chemokine receptor 4 (CXCR-4), integrin α4, and TGF-β receptors have been associated with increased MSC homing in gliomas (Young et al., 2014; Yamazoe et al., 2015).

The impact on stem cell behavior after being loaded with nanoparticles for different purposes has not been the principal focus of research. However, there already exist reports describing the increase in migration toward cancer cells after loading hMSC with iron oxide nanoparticles. This would be related to the overexpression of EGFR observed after nanoparticle inclusion and the characteristic elevated production of EGF by colon cancer cells used in the *in vitro* Boyden chamber experiments (Chung et al., 2011). Interestingly, the same trend has been observed when human BM-MSC were labeled with ferucarbotran nanoparticles and protamine. Using cellular magnetic resonance imagining (MRI) to track the labeled stem cells, increased BM-MSC migration toward *in vitro* and *in vivo* glioma models was found, and the SDF-1/CXCR4 signaling axis was associated to this phenomenon (Chien et al., 2011).

Nanoparticles for Tracking Stem Cells During Glioma Therapy

Stem-cell-based therapies rely on the ability of the grafted cells to target the organ of interest. In case of malignant gliomas, it is crucial to ensure stem cell tumor homing. In the preclinical setting, conventional methods for tracking migration and final fade of stem cells are traditionally based on bioluminescence imaging; however, poor spatial distribution and lack of translational applicability made necessary to establish a reasonably translational method that can be easily applied in a clinical setup.

Cellular MRI-based tracking technologies have risen as gold standard for non-invasive, real-time monitoring of transplanted stem cells (Kim et al., 2011). This approach would allow the study of stem cell biodistribution, migration, survival, and even differentiation with high spatial resolution and without the need for ionizing radiation. To make this possible, stem cells will require being labeled with magnetic nanoparticles. Although several options exit, magnetic iron oxide nanoparticles such superparamagnetic iron oxide nanoparticles (SPIONs) have been commonly used for this purpose (Cromer Berman et al., 2011).

The conjugation of stem cells and SPIONs has allowed for tracking MSC migration and homing into glioma tumors in a rodent glioma model without compromising such migratory capacity (Wu et al., 2008; Menon et al., 2012). NSCs have also been widely studied in this regard (Spina et al., 1975; Neri et al., 2008). After 1 month of follow-up, it was demonstrated that SPIONs would not impair multipotency, cell survival, or proliferation (Agha-Hosseini et al., 2010). Furthermore, a NSC migration speed of 50–70 μm/day has been calculated after the cells were loaded with ferumoxide (SPION + dextran) (Flexman et al., 2011). Clinically relevant results were those presented by Thu et al. The group showed that loading FDA-approved NSC with ferumoxide–protamine complex nanoparticles did not impaired humor-homing properties in a murine glioma model (Thu et al., 2009; Auffinger et al., 2013). Gutova et al. also reported on similar findings when using ultrasmall superparamagnetic iron oxide nanoparticles (USPIONs) in clinically graded nanoparticles and FDA-approved NSC (Gutova et al., 2013). Currently, different complementary imaging modalities and nanoparticles stem-cell coupling techniques are being studied (Egawa et al., 2015; Cheng S. H. et al., 2016; Qiao et al., 2018).

Even when this approach was first evaluated in the clinical setting around 2006 (Zhu et al., 2006) and has been used in different pathologies and other cancers (de Vries et al., 2005), glioma patients have not yet harnessed the benefit of the clinical applicability of this technology. This could be related to the difficulties in obtaining long-term follow-up of nanoparticle-labeled stem cells, as their self-renewal capacity render less nanoparticle concentration through each replicative cell cycle.

CHALLENGE, POTENTIAL PITFALLS, AND FUTURE PERSPECTIVE

Challenge and pitfalls associated with this relatively novel approach is proper of any disruptive technology. The ethical concerns associated with the use of particular stem cells, while seemingly addressed with modern techniques, need to be further discussed before extensive use can be assumed (Ramos-Zúñiga et al., 2012). Clinical endeavors utilizing stem cells as potential therapeutic tools in glioma patients have already glimpsed relative success. In this setting, careful and individualized selection of specific types of stem cell will be key in in future clinical applications for these patients. For instance, we concentrate our efforts in the application of adipose-derived MSCs, which can be easily obtained from the same patient. Although still in preclinical phase, we expect them to be rapidly bioengineered and used for autologous transplantation, thus allowing for an individualized and expedited process so the patients can therapeutically receive them even at time of surgery.

The introduction of the nanotechnology in stem cell therapies has shown to be beneficial and hopefully will keep turning stem cell therapies into a less worrisome and more controlled therapeutic strategy. To date, we have explored NP for stem cell bioengineering and cell tracking; however, we believe that their malleability allows for further uses such as the ones previously described, alone or in combination, and even for stem cell functionalization (Kim et al., 2011; Bishop et al., 2016; Mangraviti et al., 2016; Wilson et al., 2017a,b; Tian et al., 2020).

Finally, the combination of these therapies should not be limited to only nanoparticles and stem cells; this combined approach will need to explore if further value can be obtained by coupling with additional fields such as radiotherapy, thermotherapy, targeted systemic therapies, focused ultrasound, and other novel diagnostic techniques such as ultrahigh magnetic strength imaging and novel radiotracers in order to maximize its benefits.

AUTHOR CONTRIBUTIONS

HR-G and KA-E screened titles for relevance and abstracted the data from the eligible full text articles. SK, AQ-H, and DT critically revised the manuscript with input from the entire team. HR-G and KA-E created the figures and tables. HR-G, KA-E, and DT worked on study conception and design. All authors analyzed and interpreted the data, drafted the manuscript, and read and approved the final draft.

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Human Dental Pulp Stem Cells Display a Potential for Modeling Alzheimer Disease-Related Tau Modifications

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We present here the first description of tau in human dental pulp stem cells (DPSCs) evidenced by RT-PCR data on expression of the gene MAPT and by immunocytochemical detection of epitopes by 12 anti-tau antibodies. The tau specificity of eight of these antibodies was confirmed by their affinity to neurofibrillary tangles (NFTs) in Alzheimer's disease (AD) postmortem brain samples. We therefore used DPSCs and AD brain samples as a test system for determining the probability of the involvement of tau epitopes in the mechanisms converting tau into NFT in AD. Three antibodies to non-phosphorylated and seven antibodies to phosphorylated epitopes bound tau in both DPSCs and AD NFTs, thus suggesting that their function was not influenced by inducers of formation of NFTs in the AD brain. In contrast, AT100, which recognizes a hyperphosphorylated epitope, did not detect it in the cytoplasm of DPSCs but detected it in AD brain NFTs, demonstrating its AD diagnostic potential. This indicated that the phosphorylation/conformational events required for the creation of this epitope do not occur in normal cytoplasm and are a part of the mechanism (s) leading to NFT in AD brain. TG3 bound tau in the cytoplasm and in mitotic chromosomes but did not find it in nuclei. Collectively, these observations characterize DPSCs as a novel tau-harboring neuronal lineage long-term propagable *in vitro* cellular system for the normal conformational state of tau sites, detectable by antibodies, with their state in AD NFTs revealing those involved in the pathological processes converting tau into NFTs in the course of AD. With this information, one can model the interaction of tau with inducers and inhibitors of hyperphosphorylation toward NFT-like aggregates to search for drug candidates. Additionally, the clonogenicity of DPSCs provides the option for generation of cell lineages with CRISPR-mutagenized genes of familial AD modeling.

Keywords: tau protein, phosphoepitope, Alzheimer disease models, dental pulp cells, neurodegeneration, tauopathies

INTRODUCTION

Alzheimer's disease (AD), characterized by neuronal cell death, is the most widespread and harmful neurodegenerative disease in the world, and it accounts for more than a half of all cases of dementia (1, 2). Despite significant progress in the understanding of this ailment, the neurobiological mechanisms underlying AD are unknown, and no successful cure has been identified (3, 4). *In vivo* and *in vitro* experimental models have been used to study the pathophysiology and explore therapeutic candidates for this disease (5–7). Brain banks have been of utmost importance for a better understanding of AD. Tau protein, a microtubule-associated protein, was discovered over four decades ago (8). Tau is encoded by a single-copy gene (MAPT) with 16 exons located on chromosome 17q21.3 (9, 10). The alternative splicing of MAPT generates six isoforms of tau in the central nervous system (CNS) [(11–17), reviewed in (18)]. In healthy individuals, tau is distributed nonrandomly in the brain (19) with predominant localization to axons (20), dendrites (21), and synapses (22). Tau has also been evidenced in oligodendrocytes (23), in astrocytes (24), and in some nonneural cells, such as skin fibroblasts (25). Tau function and pathogenesis are restricted to the cytoplasm; hence, the meaning of its presence in nuclei (26–28) remains generally understudied. Tau abnormalities are commonly observed in many neurodegenerative diseases including AD, Parkinson's disease, and Pick's disease. A growing amount of evidence suggests that Aβ oligomers in concert with hyperphosphorylated tau (pTau) serve as the major pathogenic drivers of neurodegeneration in AD. The functions of tau are regulated by its phosphorylation on specific serine/threonine and a few tyrosine residues, which disrupts tau association with microtubules in a physiologically normal manner (14). Under pathological conditions, tau is abnormally hyperphosphorylated (29, 30), introducing into tau a molecule with up to fourfold more phosphates (6–8 mol/mol of tau) than those contained in a monomer (∼1.9 mol/mol of tau) (31). Excessively phosphorylated tau monomers lose their affinity for microtubules, self-assembling to form insoluble aggregations or paired helical filaments (PHFs), which are the precursors of neurofibrillary tangles (NFTs) (32, 33) considered a key marker of AD (34). Phosphorylation of tau results in microtubule instability, ineffective transport of molecules and organelles, and incompatibility with neuronal function.

Tau is the predominant constituent of NFTs (35, 36). Antibodies against tau and NFTs (37–39) have allowed NFT characterization in AD pathogenesis (40). However, the process underlying the formation of NFTs is unknown. In normal brains, tau can undergo transient aggregations such as oligomeric structures in the cortex (41, 42) and more advanced PHFs in neurons of hibernating animals (43–45) and fetal brains (46, 47), suggesting the existence of a physiological mechanism of *reversible* aggregation of tau. However, these observations could not be reproduced *in vitro*. The cell models used so far for tau studies are either malignant [neuroblastoma, PC12; (48)] or transgenic (49, 50) cell lines suffering from the consequences of genomic modifications in the epigenetic state different from normal cells with endogenous tau protein.

In the present study, we describe for the first time the presence of an endogenous tau protein in human dental pulp stem cells (DPSCs) evidenced by the detection of its non-phosphorylated and phosphorylated epitopes by a panel of tau-specific antibodies, some of which are used for characterization of tau modifications in the AD. With this discovery and their previously described capacity to develop into neurons in other (51, 52) and our (53) studies, DPSCs demonstrate their potential of modeling tau normal state and possibility of inducing changes mimicking the modifications occurring during neurodegeneration. The principal advantage of DPSCs is their being authentic neuronal cell progenitors. Other tissue origin multipotent stem cells, such as bone marrow (54), adipose tissue (55), umbilical cord blood (56), and spleen and thymus (57), have been shown to develop into cells with neuronal phenotype. However, these cells, collectively defined as "mesenchymal stem cells" [a term coined by Arnold Caplan in 1991 (58)] and approved for extensive use by the International Society for Cellular Therapy position statement (59), have two crucial differences from dental cells, which were discussed in our previous publications (53, 60): (i) they originate from mesenchyme that is developmentally unrelated to the nervous system, and (ii) none of them has been shown to contain tau protein because mesenchyme origin cells do not express this protein *in vivo*. The phenomenon of their conversion *in vitro* into cells with neuronal phenotype might result from the epigenetic plasticity that stem cells possess. As neurons produced from these stem cells lacked tau protein, they can be of utility in regenerative medicine but not in modeling tau-related aspects of neurodegeneration. In contrast, DPSCs are known from embryological (61) studies to stem from ectodermal–neuroepithelial–neural crest lineage producing, among many cell types, cells of the peripheral neural system and glia (62, 63).

Currently, apart from the neurons produced from *pluripotent* stem cells able to model AD (7, 64), neural crest origin *multipotent* stem cells, with DPSCs as the most known representatives, represent the most relevant and easy-to-use cellular system capable of recapitulating *in vitro* the tau aggregation toward NFT-like pathogenicity. This novel experimental strategy, combined with existing *in vitro* AD modeling approaches, can contribute to a better understanding of pathological mechanisms underlying AD and the development of effective therapeutics.

MATERIALS AND METHODS

Isolation and Culture of DPSCs

Deciduous teeth were collected from 7- to 8-year-old male children in a dental clinic in Mexico City. Informed consent was obtained from their parents. The study was approved by the Bioethics Committee of the Biomedical Research Institute at the National Autonomous University of Mexico. The lower primary front teeth that usually erupt first and thus also the first to fall were readily eliminated by odontologists without any surgical procedure and inspected, and those with any visible or suspected abnormality were placed in sterile Hank's balanced

salt solution (Gibco, Thermo Fisher Scientific, Waltham, MA) with 2X antibiotic–antimycotic solution (Anti-Anti; Gibco), transferred in a special transport to the laboratory and processed under sterile conditions within 24 h as previously described (60). Briefly, teeth in the laboratory were repeatedly washed with commercial mouthwash solution (Listerine Cool Mint, Johnson and Johnson, New Brunswick, NJ) and then with 2X Anti-Anti in phosphate-buffered saline (PBS; Gibco). Teeth were mechanically broken with a pincer to expose the pulp, which was minced in a sterile glass Petri dish and digested using a 3 mg/ml solution of collagenase type I (Sigma-Aldrich, St. Louis, MO) in PBS for 60 min at 37°C. Enzymes were inactivated by diluting with Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 medium (1:1 ratio, Gibco) supplemented with 10–20% fetal bovine serum (FBS, Gibco), in a 5% CO_2 environment, until small colonies of spindle-shaped cells appeared. Colonies and single cells were removed from the dish by 5-min digestion with TrypLE Express (Invitrogen, Thermo Fisher Scientific), seeded in T25 bottles, and cultured during no more than five passages before being used. The ability of DPSCs to respond to inducers of the neuronal lineage was tested, as described (53). During this period, healthy tooth bone preserves the normal state of the pulp to be used and the reproducibility of the cells isolated from them and cultured. In our studies, healthy tooth bone preserved the viability of the pulp for at least 30 h as evidenced by the preservation of the proliferation, phenotypic, and stemness characteristics of the obtained and cultured cells during at least five passages of cell populations.

Phase Contrast Microscopy With Immunofluorescence Equipment

An IX71 phase contrast microscope (Olympus, Tokyo, Japan) and QCapture Suite software (QImaging, Surrey, BC) were used for image analysis. A confocal microscope, SP8 (Leica, Wetzlar, Germany), was used for immunocytochemical analyses.

Antibodies

Supplementary Table 1 presents the panel of the 12 antibodies used in this study, indicating the tau protein amino acids implicated in the three types of epitopes that induced them: non-phosphorylated, phosphorylated, and hyperphosphorylated. The antibodies were of the class IgG, except for TG3 (IgM). The tau specificity of the antibodies has been confirmed in earlier (65) and recent (28) studies. The antibody to glycogen synthase kinase 3β (GSK3β) was from EMD Millipore Billerica (MA, USA). Human β-amyloid-specific monoclonal antibody (BAM-10) was from Invitrogen (Thermo Fisher Scientific cat. #MA1-91209). Secondary antibodies used were anti-mouse and anti-rabbit IgGs (Fc mouse and Fc rabbit; see **Supplementary Table 1**).

Immunofluorescence Assays of DPSCs

DPSCs were plated in glass chamber slides and cultured in two passages. The cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature (RT) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at RT. Nonspecific binding was blocked with 1% bovine serum albumin (BSA; MP Biomedicals, Santa Ana, CA) in PBS for 2 h at RT. DPSCs were incubated with the primary antibodies diluted in blocking solution (1% BSA in PBS) overnight at 4°C and then with one of these secondary antibodies for 1 h at RT in the dark: (1) Alexa 488-tagged secondary antibody, (2) fluorescein isothiocyanate-tagged goat anti-rabbit immunoglobulin G, (3) tetramethylrhodamine-tagged goat anti-mouse immunoglobulin G, or (4) cyanine 5-tagged goat anti-mouse IgM. Nuclear counterstaining was done with TOP-RO-3 (Thermo Fisher Scientific) or Hoechst. Rhodamine phalloidin was used for the tetramethylrhodamine-conjugated filamentous actin staining.

Origin and Immunofluorescence of AD Tissue Sections

AD human tissue was obtained from the National Dementia Biobank, Mexico, in accordance with the institutional bioethical guidelines. A representative immunoassay image confirming the affinity of the eight antibodies for AD NFTs (**Figure 1**) was from the studies of the Laboratory of Diagnosis and Investigation of the Mexican National Dementia BioBank (Facultad de Estudios Superiores, Cuautitlán, UNAM, Estado de México, México) (postmortem brains of Braak stages 5 and 6, used at PM 5–8 h, of four females aged 70, 70, 75, 80 years and two males aged 78 and 89 years with AD). Histological sections were prepared, blocked with 0.2% IgG-free albumin (Sigma Chemical Co.) in PBS for 20 min at RT, and then incubated with the primary antibody overnight at 4°C. Tissue slices were then incubated with the secondary antibodies: FITC-tagged goat–anti-mouse IgM or FITC/TRITC-tagged goat–anti-mouse IgG or FITC/CY5-tagged goat–anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove).

For confocal microscopy analysis, immunolabeled AD brain sections of the hippocampus were mounted in an anti-quenching media, VECTASHIELD (Vector Labs, Burlingame), and examined with a confocal laser scanning microscope (TCP-SP8, Leica, Heidelberg), using a 100X oil-immersion plan apochromat objective (NA 1.4). Ten to 15 consecutive single sections were sequentially scanned at 0.8- to 1.0-μm intervals for two channels throughout the z-axis of the sample. The resulting stack of images was projected and analyzed onto the two-dimensional plane using a pseudocolor display of green (FITC), red (TRITC), and blue (CY5). Fluorochromes were excited at 488 nm (for FITC), 530 nm (for TRITC), and 650 nm (for CY5).

RT-PCR Assay

Total RNA was isolated using TRIzol reagent following the manufacturer's instructions (Invitrogen). RNA isolation was followed by DNase I treatment (Invitrogen). RNA was cleaned up using the RNeasy mini kit (Qiagen). Reverse transcription and DNA amplification were performed using the One-Step RT-PCR kit (Qiagen). The PCR primers used were as follows:

GAPDH forward: 5′AAGGTGAAGGTCGGAGTCAA;
GAPDH reverse: 5′AATGAAGGGGTCATTGATGG;
MAPT forward: 5′CCAAGTGTGGCTCATTAGGCA;
MAPT reverse: 5′CCAATCTTCGACTGGACTCTGT;
CD44 forward: 5′CTGCCGCTTTGCAGGTGTA; and
CD44 reverse: 5′CATTGTGGGCAAGGTGCTATT.

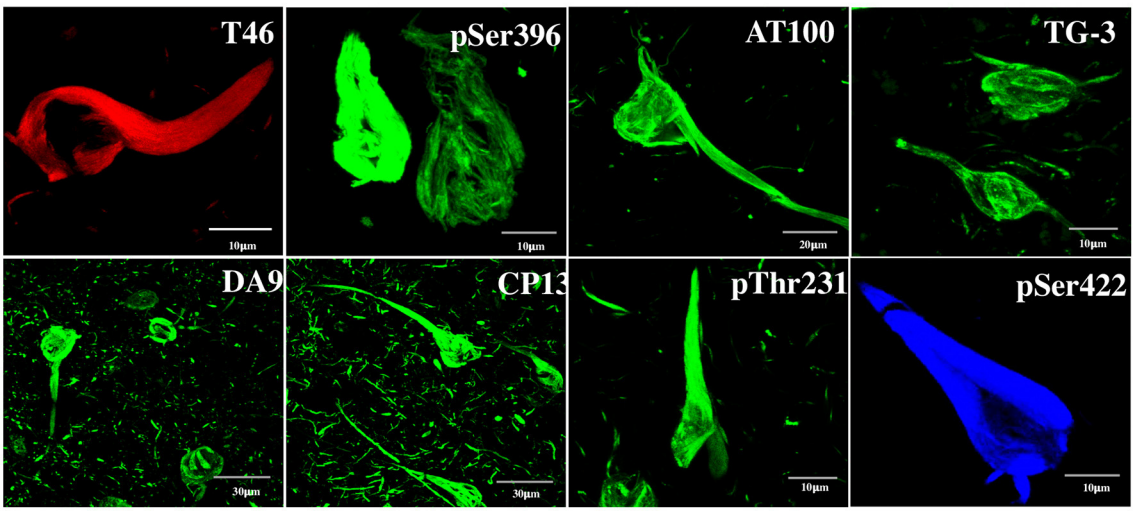

FIGURE 1 | Detection by anti-tau antibodies of NFTs formed in AD brain. Immunofluorescent data showing the affinity of the eight antibodies to postmortem AD brain NFTs, which served as tools for the discovery of tau in DPSCs. The specificity of the antibodies is as follows. DA9, T46: recognize epitopes not requiring phosphorylation; CP13 (aa T231), pT231, pS396, pS422: recognize moderately phosphorylated epitopes; TG3, AT100: recognize hyperphosphorylated epitopes forming NFTs. Channels: red (T46), blue (pS422), green (the rest). For information on the critical amino acids of the tau epitopes recognized by these antibodies, see Materials and Methods.

The reliability of the described results was ensured by the repetitions of the assays and presentation of representatives.

RESULTS

Testing the Specificity of Tau Antibodies in NFTs in the AD Brain

Non-phosphorylated and phosphorylated tau immunoreactivity in NFTs from the AD brain was tested. It was done with two purposes: (a) to serve as a positive control for the tau immunochemical assay in DPSCs and (b) to obtain information about the antigenically functional state of the tau epitopes in NFTs. This shows clearly that each of the tested antibody recognized its epitope in NFTs, evidencing its antigenically active state (**Figure 1**). The antibodies have been used previously in studies of AD at the National Dementia BioBank (Ciencias Biológicas, Facultad de Estudios Superiores, Cuautitlán, UNAM, Estado de México, México; Banco Nacional de Cerebros-UNPHU, Universidad Nacional Pedro Henríquez Ureña; and Neuroscience Research Laboratory, Faculty of Health Sciences, Pontifica Universidad Católica Madre y Maestra Santiago de los Caballeros, República Dominicana).

DPSCs Harbor mRNA of Tau MAPT Gene and Tau Protein, Which Are Phosphorylated by GSK3β and Aβ Precursor Protein (APP)

The major difficulty in the studies of tau in AD brain is that the NFTs represent the advanced stages of the tau aggregation process in the environment of neuronal degeneration. Therefore, neural-lineage cellular systems capable of recapitulating the earlier stages of tau aggregations are needed. Dental stem cells have been shown by several groups to differentiate into cells with neuronal phenotype; however, the major marker of neuronal identity, tau, has not been shown to exist in those neurons (51–53). Therefore, we have undertaken assays to see whether the cells contain endogenous tau detectable by RT-PCR and anti-tau antibodies. **Figure 2A** shows DPSCs cultured in growth-promoting medium supplemented with high (10%) FBS (see Materials and Methods). Under this condition, the cells display properties characteristic of the class of mesenchymal stem cells (59): fibroblast-like morphology; plastic adherence; the markers CD73, CD105, and CD90; and tri-lineage (bone-oriented) differentiation potential (53, 60). We recently reported on the ability of these cells to display under serum-less culture conditions an upregulated Wnt/β-catenin signaling neural crest features (CD271/p75, CD57/HNK1, Sox10, among others), associated with the neural-lineage fate which is evidenced by the expression of neural markers and production of neurotropic factors (see **Supplementary Figure 5**) and the capacity of responding to a neurogenic environment via the transient expression of the neuronal commitment gene Sox2 and then neural marker beta tubulin (53). To complement these neuronal properties of DPSCs, we show here the presence of tau in these cells detected at the gene expression and protein levels. An RT-PCR assay (**Figure 2B**) revealed the RNA copies of MAPT gene known to encode the amino acid sequences of the six tau isoforms in the brain of AD persons (66, 67). The tau protein was immunochemically detected (**Figures 2–4** and **Supplementary Figures 1–4**) by means of

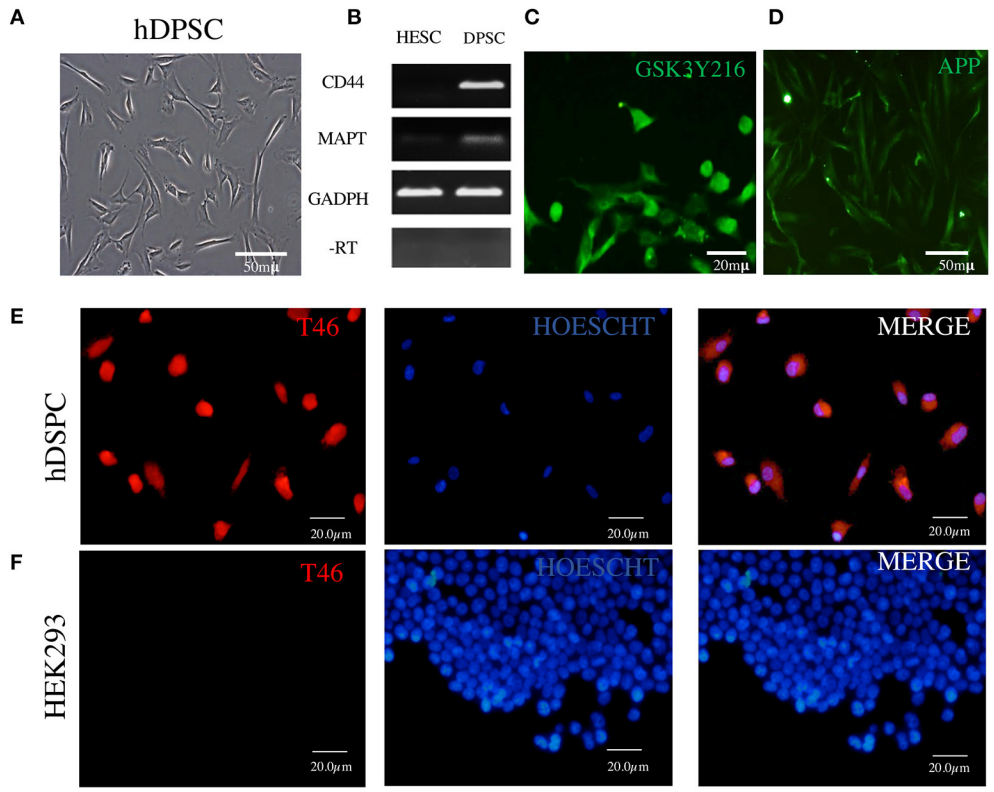

FIGURE 2 | Detection in DPSCs of tau RNA, tau protein, GSK3β, and APP. **(A)** Fibroblast-like morphology of DPSCs in fetal serum-containing medium. **(B)** RT-PCR assay showing cDNA copies of tau MAPT and CD44 RNAs; RNA of human embryonic stem cells (ESCs) used as a negative control and RNA of GAPDH as a positive control. **(C,D)** Immunofluorescence of GSK3β and APP. **(E)** Immunofluorescence of tau protein revealed by T46 antibody in DPSCs. **(F)** HEK293 cells used as a negative control for T46 specificity. The nuclei are stained with Hoechst.

12 antibodies (**Supplementary Table 1**), of which the T231 revealed it as threads seemingly associated with microtubules (**Supplementary Figure 1**).

Collectively, these results underscore the tau in DPSCs as unique neural components involved in normal and pathological cellular processes. Below, we describe in detail the results obtained in these immunocytochemical analysis.

In the immunocytochemical experiments, apart from the staining of tau with antibody, we used counterstaining of nuclei and cytoplasmic filamentous actin with TO-PRO-3 and rhodamine phalloidin, respectively, confirming cellular integrity. In the first round of fluorescence immunostaining experiments, we used three antibodies to non-phosphorylated epitopes localized to exons of three different protein sites (**Supplementary Table 1**).

The assay with antibody T46 (which bound a tangle of AD brain, see **Figure 1**) identified an unphosphorylated epitope in the tau protein as a homogeneously stained component of the cytoplasm and of the nucleus of majority of the cells (**Figure 2E** and **Supplementary Figure 2C**). The specificity of the immunochemical reactions was proven by the *positiveness* of the T46 to tau in AD brain NFTs (**Figure 1**) and the *negativeness* to the irrelevant HEK293 (**Figure 2F**). This result indicated the presence of the immunogenically active state of the T46 epitope in both these tau-containing cells (DPSCs and AD brain). The two other antibodies to non-phosphorylated epitopes, Tau5 (involving exon 9 aa 210 to aa 241 residues) and DA9 (recognizing exon 4 and 5 aa 102 to aa 140 residues) (see **Supplementary Table 1**), showed a similarity to the T46 tau protein homogeneously arranged in the cells (**Figure 3** and **Supplementary Figure 2**). The detection of these three epitopes in N-terminal exon 3 (DA9), central (exon 9), and C-terminal end (exon 13) regions of the protein, along with the expression of the MAPT gene (**Figure 2B**), suggested the presence in DPSCs of the full-length tau protein containing six isoforms previously documented for tau in AD brain (67). The detection in these experiments of tau in nuclei of DPSCs confirmed the previous data (27) but did not add novel information elucidating the role of this tau. The presence in DPSCs of the GSK3β, known to phosphorylate tau (68), and the APP, the source of toxic amyloid peptides, enhancing this kinase activity to the level of hyperphosphorylation and formation of NFTs in AD (69–71).

Moderately Phosphorylated Tau Epitopes Were Active Both in DPSCs and in Postmortem AD Brain

Seven phosphorylated epitopes formed two groups, one consisting of three epitopes (pS396, pS404, and pS42; **Figure 4A**, right side) localized to the C-terminal exon 13 and the other

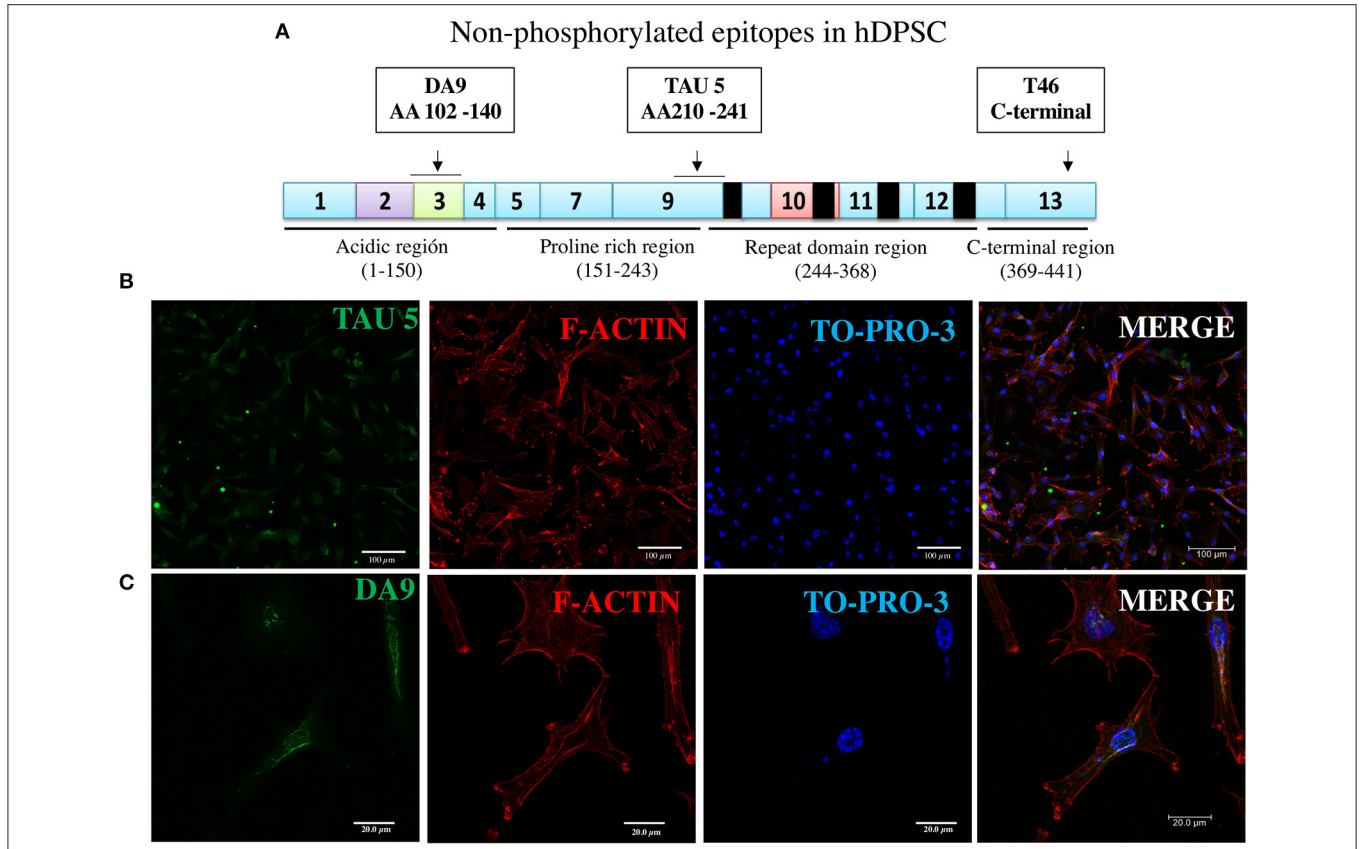

FIGURE 3 | Immunofluorescence of two additional non-phosphorylated tau epitopes. **(A)** Epitopes of TAU5 and DA9 antibodies reveal their non-phosphorylated epitopes localized to N-terminal exon 3 and proline-rich exon 9 regions, respectively. (**B,C**) Immunofluorescent staining of the tau revealed in DPSCs by the antibodies. Nucleic acids and F-actin are counterstained with TO-PRO-3 and rhodamine phalloidin, respectively.

one including the epitopes pT231, pS202 (CP13), and pS235 (MC6) localized to the proline-rich exon 9 site. Of these, the pS396, pS422, pT231, and CP13 showed the ability to detect AD NFTs (**Figure 1**). In DPSCs, the pS396 antibody predominantly bound nuclear tau and only barely the cytoplasmic tau. The immunostaining seen for pS404 and pS422 antibodies revealed a very intense signal in the cytoplasm and in the nuclei with the difference that nuclear tau revealed by pS404 formed approximately 10 small compact bodies per cell, compared to a diffuse distribution of nuclear tau revealed by pS422. Antibodies of the epitopes pT231, pS202 (CP13), and pS235 (MC6) recognized their phosphorylated epitopes in DPSCs via a homogeneous staining of the cytoplasm and the nuclei (**Figure 4** and **Supplementary Materials**).

AT100 and TG3 Distinguished Between the Cytoplasmic and Nuclear Tau but in a Reciprocal Manner

The results of testing AT100 and TG3 (**Figure 4A** and **Supplementary Figures 3A–C**) are of great interest due to their being induced by AD NFTs (38). Consistent with this origin, the AT100 epitope in NFT is considered a hallmark of AD (34), and the antibody is exploited as the most specific reagent in AD immunodiagnostics (37). Consistent with this known feature, the AT100 in this study did not bind to the cytoplasmic tau of DPSCs (**Figure 4A** and **Supplementary Figure 3A**), confirming the absence of its antigenically active epitope in these normal cells. In contrast, TG3 was positive for the cytoplasmic and negative for the nuclear tau (**Figure 4A** and **Supplementary Figures 3B,C**).

Tau in Mitotic Cells

Remarkably, the TG3 antibody was the only one in the panel that labeled tau in mitotic chromosomes at all phases of the mitosis (**Figure 4A**) in agreement with the observation by others (28, 38, 72). We found rare cells at the anaphase–telophase phases showing low tau amounts in recently reformed nuclei (**Supplementary Figure 3C**), which, along with the above shown presence of tau in mitotic chromosomes, suggests that tau appears in mitosis and disappears in the nuclei of interphase cells. **Figure 4C** and **Supplementary Figure 4** present the tau in centrosomes revealed by MC6.

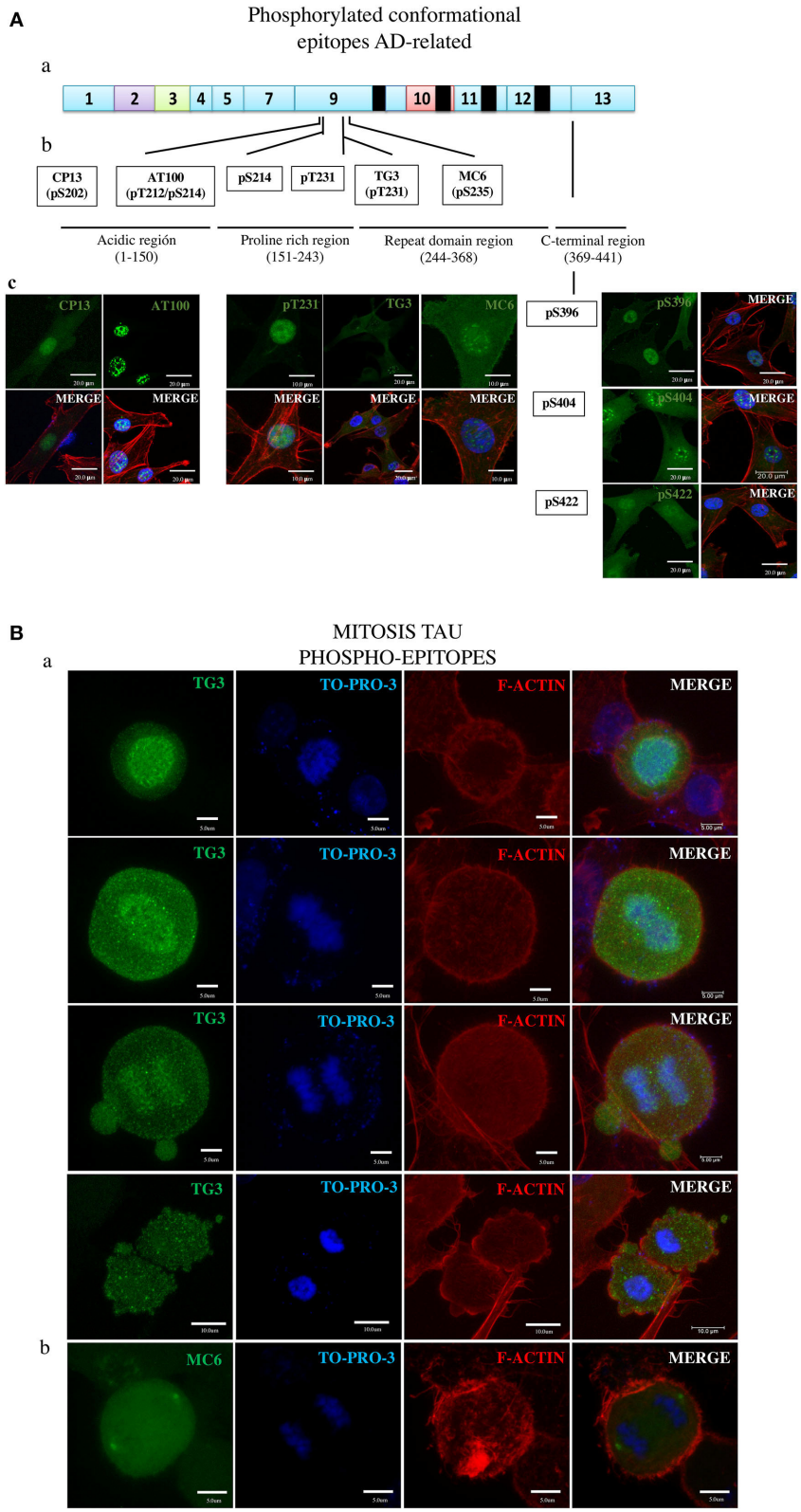

FIGURE 4 | Overall presentation of the tau protein fluorescence revealed in DPSCs by antibodies to phosphorylated epitopes. **(A)** Tau in interphase cells. (ab) Localization of epitopes to tau protein proline-rich exon 9 and C-terminal exon 13 sites. (b) Amino acids involved in the epitopes of the moderately phosphorylated proline-rich (CP13, pS214, pT231, MC6, and pS369) and C-terminal (pS369, pS404, and pS422) epitopes. (c) Imunofluorescence images. **(B)** Tau in mitosis. (a) TG3 reveals tau associated with chromosomes at consecutives phases of mitosis. Counterstaining with TO-PRO-3 and rhodamine phalloidin. (b) MC6 bound to its epitope in the tau associated with centrosomes. (c) Immunofluorescent staining of tau by means of the antibodies.

DISCUSSION

DPSCs Are Ectodermal Neural Crest Origin Tau-Containing Neuronal Precursors

Cellular models able to reproduce different facets of AD pathogenesis are needed to support continued efforts to learn about the disease. *In vivo* processes in neurons are not accessible in live brains, and rare biopsy samples often die in cultures. Majority of the studies of *in vivo* neurodegeneration are done using biopsy material from fixed postmortem brains containing the advanced stages of tau modifications. It is possible to reconstruct the initial steps of pathogenesis via a meta-analysis of independently obtained experimental data, but such reconstructions may not be reliable. Neuronal precursors have been obtained from human induced pluripotent stem cells (51, 52), including cells with mutations that cause familial AD (73) or cells that have been genetically manipulated toward AD pathogenesis (74). The potential differentiation of these cells into neurons is the advanced strategy for recapitulating amyloid β and tau pathology in advanced human neural cell culture models of AD (71). This strategy has recently been improved through the use of 3-D AD models, which have emerged as an advanced alternative to 2-D models (73, 75). The drawbacks of the currently used 3-D models of AD include insufficient maturation and their inaccessibility for routine employment.

The task of these and other novel models is to display *in vitro* the tau modification and to collect novel experimental observations regarding the current view that the hyperphosphorylation-induced tau aggregations and formation of the NFTs are associated with neurodegeneration and that abnormal processing of APP and accumulation of β-amyloid triggers this process in a combination with stress factors as a step toward preclinical drug discovery, which holds potential for personalized therapeutic applications.

DPSCs widely known as mesenchymal cells, now shown to contain tau, have a potential to be a novel easier-to-use cellular system for modeling tau-mediated pathogenesis. The potential relies substantially on the recently demonstrated neural crest identity treats (epithelial morphology and a set of specific markers such as P75, HNK1, and Sox10 displayed in experiments) (60) on their culture in media required for embryonic neural crest cells produced *in vitro* from pluripotent stem cells (76). Under appropriate neurogenesis induction culture conditions, DPSCs develop into cells with neuronal phenotype including axon and dendrite-like outgrowths (51, 52). Nevertheless, since DPSCs do not participate in the innervation of teeth, their neuronal identity was unclear. This study presents two novel features of DPSCs essential for the validation of the neurons they produce and the capacity of these neurons to model tau implication in neurodegeneration: (i) the description of tau provided direct proof for their neural identity, and (ii) the display of the epitopes recognized by a panel of anti-tau antibodies permits the investigation of tau modifications. A conceivable explanation for these neural properties of DPSCs is that the cells are endowed by neural crest with epigenetic plasticity, permitting the reprogramming of dental pulp cells to neural crest stage cells (77) and proceeding to other lineages (78), here to neural lineage of the peripheral system that, as in the DPSC population, is the derivative of the neural crest (79). With these novel characteristics, DPSCs can be used in studies of AD and other tauopathies to compare the functional states of tau in these physiologically normal cells and in NFTs available from postmortem AD brains.

DPSCs as a Novel System for Modeling Normal and Pathological Tau Modifications

This study aimed to examine the properties of tau in DPSCs as a prospective system for modeling the initiation of tauopathy. The findings describe the functional state of the epitopes recognized by 12 antibodies that were frequently employed in studies of tau aggregated forms in postmortem brains of AD patients (see Introduction). First, we obtained reliable data on the presence of tau in these cells at RNA and protein levels; furthermore, it is suggested that the full-length protein is produced considering the detection of tau epitopes in the N-terminal, C-terminal, and central, proline -rich domains containing the residues responsible for the interaction with microtubules. The counterstaining with phalloidin reveals in multichannel immunofluorescent merged images of tau positioning along phalloidin-revealed threads, presumably microtubules. Based on this finding, the prospective aim was to use tau in two types of studies: (a) to test comparatively the functional state of its epitopes in monomer tau of DPSCs and in NFTs of the AD brain samples and (b) to test the propensity of the protein sites in which these epitopes are located to undergo initial steps of aggregation toward NFTs under the influence of currently known *in vitro* inducers of tau phosphorylation aggregation to model the formation of NFT, such as okadaic acid (79, 80) and inhibitors of these processes as drug candidates against neurodegeneration (81). In this study, we describe the results of the first study hypothesizing that the active or inactive state of epitopes (evaluated by the binding/nonbinding of the respective antibody) in monomer tau of DPSCs and in its aggregated NFT form in samples from AD brains would provide information about the mode of the participation of respective tau sites in neurodegeneration. The active state of epitopes in DPSCs and in AD indicated that the phosphorylation and conformational events that activated them were not induced by neurodegeneration. In contrast, the active state of epitope in AD NFTs but not in DPSCs indicated that the epitope was activated by neurodegeneration factors. Based on these assumptions, the study compared the ability of binding of 12 antibodies to their epitopes in DPSCs (normal monomer tau) and AD (pathological NFT) brain samples.

Three antibodies (Dau9, Tau5, T46), whose epitopes do not need phosphorylation, (i.e., are permanently active), were used to standardize the conditions of immunocytochemical assays of other epitopes. These antibodies bound tau in both DPSC (**Figures 2**, **3** and **Supplementary Materials**) and AD brain sample (**Figure 1**); therefore, the tau sites of their locations were beyond the processes converting monomer tau into NTF. The activity of nine antibodies was dependent on phosphorylation. Six out of the nine antibodies (CP13, pS214, T231 in the protein

central "proline rich" exon 9 region spanning aa151 to aa243, and the pS396, pS404, pS422 located in the C-terminal tau aa369–aa441 region; see **Figure 4A**) bound their epitopes in both the normal (monomer) tau in DPSCs and four of them showed their affinity for pathological (aggregated) tau in AD NFTs (**Figure 1**). The results suggested that these phosphorylated epitopes, like the three unphosphorylated epitopes, were phosphorylated and immunogenically active in monomer tau and remained in this state during tau aggregation to form NTFs. The representing these epitopes tau residues (from N- to C-terminal direction: Ser202, Ser214, Thr231, Ser396, Ser404, and Ser422.) are therefore considered by this study to not depend on the process of tau pathological aggregation and formation of NFTs in AD. Antibodies of the two remaining epitopes, AT100 and TG3, showed results that differed from those described above, and displayed properties compatible with the ability of DPSCs to model their relation to AD. Previous studies of these two epitopes have suggested two properties that are relevant to the role of tau in AD. First, these epitopes were generated by immunizing mice with NTFs purified from postmortem AD brains (38) that possessed tau-specific antigenicity (35, 36, 82). Second, the establishment immunogenically active form of these epitopes required preliminary phosphorylation of other epitopes. Specifically, the functionally active state of AT100 epitope depends on sequential phosphorylation of the AT8 epitope residues Ser202 and Thr205, followed by phosphorylation of Thr212 and Ser214, both of which are critical for recognition by AT100 of its epitope (83, 84). Each of these phosphorylation steps was strongly required to prime the phosphorylation of the following it residue; for example, if Ser214 would be abnormally phosphorylated first, the phosphorylation of the preceding residues would be blocked and the epitope would not be active in the AD-related state (83). Existing evidence assumes this priming process of the epitope activation to occur only when neurodegeneration factors induce formation of NFTs thus considering this epitope in NFT as a hallmark of AD and the AT100 antibody as its most specific diagnostic. The fact that AT100 did not bind tau in the cytoplasm of DPSCs but bound AD NFTs provides an independent proof for the diagnostic potential of AT100 antibody. Unexpectedly, the same AT100 had a strong reaction with nuclear tau; however, the origin and the structural state of tau in nuclei, and its relation to tau pathogenesis remain to be elucidated (27).

The active state of the TG3 epitope requires phosphorylation of threonine 231 (see **Supplementary Table 1** and **Figure 4**) by GSK3β, which has been shown to depend on prior (priming) phosphorylation of T235 by CDK5 (85, 86). Importantly, phosphorylation of T231 is vigorously required for the assembly of tubulin into microtubules, and, together with the S214, the T231 is known as a tau phosphorylation site (87) crucial for the Tau–MT interaction (87, 88). Interestingly, tau revealed by pt231 in DPSCs looks as if it co-localizes putative microtubules (**Supplementary Materials, Figure 1**). The most likely explanation for the above-described findings is as follows. The differential immunogenic activation of the epitopes of AT100 and TG3 antibodies in nuclear (AT100), cytoplasmic, and mitotic chromosome-associated (TG3) tau seems to be regulated by the priming mechanism, which is dependent on the peculiar structural contexts of these cell compartments. In normal cells, the contexts keep these epitopes inactive to protect the cell from their pathogenic potential. Factors inducing neurodegeneration alter the structural contexts which activate the priming system and the respective cell component phosphorylation of these AD-related epitopes. The hypothesis is in an agreement with evidence that AT100 and TG3 are specific to NFTs in the AD brain (38, 72), and according to one scenario (65), they are among the epitopes (pT231 → TG-3 → AT8 → AT100 → Alz-50) sequentially phosphorylated in the course of AD pathogenesis. The inclusion in this sequence of the AT100 and TG3 epitopes is remarkable.

In conclusion, this study describes DPSCs as a novel AD modeling system. The results of this initial study suggest that the epitopes of 10 out of the 12 antibodies we used were active in the monomer tau of DPSCs and passed to NFT independent of the factors that induced this pathological tau form. In contrast, the epitope of AT100 was inactive in DPSCs but active in NFTs, thus demonstrating that the active state of its epitope is not formed in the cytoplasm of normal cells and is formed when the tau is hyperphosphorylated and forms insoluble tangles. Examination of the active state of the TG3 epitope in the cytoplasm of DPSCs represents another possible area for future research. Future experiments will address this issue in a more quantitative manner.

The study findings suggest that DPSCs are suitable for detailed systematic investigations of up to 80 tau serine/threonine phosphorylated sites (87). Each site contributes to the dynamics of the protein conformational pattern, and some of them contain epitopes recognized by antibodies. Their binding to the epitopes in DPSCs and in brain samples at different stages of AD can illuminate the basic mechanisms that regulate tau implication in the disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Imelda Lopez Villase; Raul Mansilla Jimenez; Agness Odele Fleury. Instituto de Investigaciones Biomédicas, UNAM, Mexico. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KG: conceptualization, project administration, supervision, and writing – original draft. LT: data curation. LR-G, LT, JL-M, and KG: formal analysis. KG and JL-M: funding acquisition and resources. KG, LR-G, and LT: investigation. LT, LR-G, and JL-M: methodology and visualization. LT, LR-G, JL-M, and MP-H: validation. KG, LT, LR-G, MP-H, and JL-M: writing – review &

editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.612657/full#supplementary-material

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