Faculdade de Engenharia da Universidade do Porto

Effect of immobilized α6β1 synthetic ligands on the behavior of oligodendrocyte progenitor cells (OPCs) in 3D fibrin hydrogels

Joana Catarina Santos Leite

MASTER THESIS

Integrated Masters in Bioengineering

Supervisor: Professor Isabel de Freitas Amaral
Co-Supervisor: Helena Sofia de Azevedo Domingues

September 2015

Resumo

As doenças desmielinizantes, como a esclerose múltipla e a lesão da espinal medula, são condições patológicas devastadoras, levando frequentemente a lesão axonal, deficiência neuronal e perda de função. Embora as células progenitoras de oligodendrócitos endógenas (OPCs) sejam prevalentes nas lesões de desmielinização, estas não são capazes de se diferenciarem em oligodendrócitos mielinizantes devido ao microambiente inibitório local. Apesar dos esforços para desenvolver novas terapias capazes de restaurar até alguma extensão a função neuronal, a regeneração do sistema nervoso central (SNC) continua a ser um desafio devido à complexidade dos seus componentes e dos mecanismos subjacentes ao processo de desmielinização.

O transplante de OPCs surgiu como uma terapia viável para promover a remielinização de axónios não lesados e em regeneração. Ainda assim, o transplante de OPCs sob a forma de suspensões celulares apresenta desvantagens, tais como baixa sobrevivência e baixas proliferação celular e diferenciação celulares. Neste sentido, a combinação da entrega de OPCs com um biomaterial como suporte pode ser vantajosa, nomeadamente para proporcionar um nicho para que as OPCs transplantadas possam sobreviver e diferenciar-se em oligodendrócitos mielinizantes. Devido às suas propriedades, os hidrogéis podem ser concebidos para imitar fisicamente o tecido nativo, proporcionando um microambiente altamente permissivo para a sobrevivência celular e crescimento tecidular. Apesar de vários hidrogéis terem sido desenvolvidos para entrega de drogas e células para o SNC, apenas alguns foram explorados para entrega de OPCs, entre eles a fibrina.

Ambos as OPCs e os oligodendrócitos expressam um repertório limitado de integrinas, entre elas a integrina α6β1. A interacção entre a laminina-211 expressa no SNC e a integrina α6β1 tem mostrado desempenhar um papel importante na sinalização dos mecanismos que estimulam a formação da membrana de mielina pelos oligodendrócitos.

Deste modo, o objectivo da presente dissertação consistiu em desenvolver um hidrogel capaz de incorporar ligandos sintéticos que se ligam especificamente à integrina α6β1, para entrega de OPCs no SNC. Para este efeito, os hidrogéis de fibrina foram funcionalizados com péptidos sintéticos com afinidade reportada para a integrina α6β1: (1) o péptido T1 do indutor angiogénico CCN1 (GQKCIVQTTSWSQCSKS) e (2) o péptido sintético HYD1 (KIKMVISWKG). É esperado que a funcionalização com ligandos para esta integrina melhore a bioespecificidade da fibrina para as OPCs, através da promoção da extensão de processos e da formação de membrana de mielina num microambiente 3-D.

O efeito da concentração de fibrinogénio na viabilidade e diferenciação de OPCs em hidrogéis 3-D de fibrina foi primeiramente avaliado. Embora a concentração de fibrinogénio não tenha afectado significativamente a viabilidade celular, esta revelou um impacto na extensão de processos pelas OPCs, um indicador de diferenciação, com géis de fibrina preparados com concentrações de fibrinogénio intermédias de 4 e 6 mg/mL a mostrarem o maior número de processos por célula, após 9 dias de cultura. Uma vez que a concentração de

fibrinogénio de 4 mg/mL mostrou o melhor desempenho biológico global, esta foi seleccionada para utilização nos estudos de funcionalização.

A funcionalização de fibrina com HYD1, T1, e com uma combinação de HYD1 com T1, que se ligam especificamente à integrina α6ß1, promoveu a extensão de processos pelas OPCs em hidrogéis 3-D de fibrina, sem afectar a formação de membrana de mielina. Uma concentração de 5 μM de péptido foi suficiente para aumentar o número de processos por célula após 5 dias de cultura, em todos os géis funcionalizados. Além disso, a funcionalização com 5 μM de péptidos também levou a valores maiores de comprimento máximo, embora não tenham sido encontradas diferenças estatisticamente significativas. Ainda, o aumento da concentração de péptido para 10 μM ou 20 μM não levou a um aumento da extensão de processos, independentemente do péptido utilizado. Em adição, nenhum efeito aditivo/sinérgico foi observado em termos de impacto na extensão de processos com a combinação dos dois péptidos (5 μM de cada péptido).

Os resultados obtidos sugerem assim que os géis de fibrina funcionalizados com ligandos para a integrina α6ß1 são interessantes candidatos para cultura e diferenciação de OPCs num microambiente 3-D, e, finalmente, para utilização como um veículo para a entrega de OPCs no SNC.

Palavras-Chave: Desmielinização, Células progenitoras de oligodendrócitos, oligodendrócitos, hidrogéis de fibrina, integrina α6ß1, laminina 322, péptidos HYD1 e T1.

Abstract

Demyelinating disorders such as Multiple Sclerosis (MS) and Spinal Cord Injury (SCI) are devastating pathological conditions, often leading to axonal damage, neuronal disability and loss of function. Although endogenous oligodendrocyte progenitor cells (OPCs) are prevalent in demyelinated lesions, they are not able to differentiate into myelinating oligodendrocytes (OLs), due to the local inhibitory microenvironment. Despite the efforts made to develop new therapies capable of restoring at some extent neuronal function, regeneration of the central nervous system (CNS) remains a challenging issue due to the complexity of its components and of the mechanisms underlying the process of demyelination.

Transplantation of OPCs has emerged as a feasible therapy to promote remyelination of spared and regenerating axons. Still, transplantation of OPCs in the form of cell suspensions presents drawbacks such as low cell survival and poor proliferation and differentiation. In this sense, the combination of OPC delivery with a suitable biomaterial-based vehicle may be advantageous, namely to provide a supportive niche for transplanted OPCs to survive and differentiate into myelinating OLs. Due to their properties, hydrogels may be designed to physically mimic the native tissue while providing a highly permissive microenvironment for cell survival and tissue ingrowth. Despite several hydrogels have been developed for cell and drug delivery into the CNS, only a few were explored for OPC delivery, and among them fibrin.
Both OPCs and OLs express a limited repertoire of integrins, among them the α6β1 integrin. The interaction between laminin-322 expressed in the CNS and α6β1 integrin has shown to play an important role in signaling mechanisms that stimulate myelin membrane formation by OLs.

Therefore, the aim of this thesis was to develop a hydrogel incorporating synthetic ligands engaging specifically the α6β1 integrin, for delivery of OPCs into the CNS. For this purpose, fibrin hydrogels were functionalized with synthetic peptides with reported affinity for α6β1 integrin: (1) the T1 peptide of the angiogenic inducer CCN1 (GQKCIVQTTSWSQCSKS) and (2) the HYD1 synthetic peptide (KIKMVISWKG). The functionalization with α6β1 ligands was expected to improve fibrin biospecificity towards OPCs, by promoting process outgrowth and myelin membrane formation, in a 3-D microenvironment.

The effect of fibrinogen concentration on OPC viability and differentiation in 3-D fibrin hydrogels was first assessed, with fibrinogen concentrations ranging from 2 mg/mL to 8 mg/mL Although fibrinogen concentration did not significantly affect cell viability, this was found to impact OPC process outgrowth, an indicator of differentiation, with fibrin gels prepared with intermediate fibrinogen concentrations of 4 and 6 mg/mL showing the highest number of processes per cell after 9 days of cell culture. Since the fibrinogen concentration of 4 mg/mL showed a better overall biological performance, this was selected for use in functionalization studies.

The functionalization of fibrin with HYD1, T1, and with a combination of HYD1 with T1, specifically binding to α6β1 integrin has shown to promote OPC process extension in 3-D fibrin hydrogels, without hindering myelin membrane formation. A peptide input concentration of 5 μM was sufficient to increase the number of cellular processes per cell after 5 days of cell culture, in all functionalized gels. Additionally, functionalization with 5 μM of peptides also led to higher values of maximum length, although statistically significant differences in maximum length were not found. Still, increasing the peptide input concentration from 5 μM to 10 μM or 20 μM did not result in an increase in process extension, regardless of the peptide. In addition, no additive/synergetic effect resulted from the functionalization of fibrin with the combination of the two peptides (5 μM of each peptide), in terms of their impact on process outgrowth.

The results obtained suggest that fibrin gels functionalized with α6β1 ligands are interesting candidates for OPC culture and differentiation in a 3-D microenvironment, and ultimately, for use as a vehicle for OPC delivery into the CNS.

Key-words: Demyelination, Oligodendrocyte progenitor cells, Oligodendrocytes, Fibrin hydrogel, α6β1 integrin, Laminin-322, HYD1 and T1 peptides

Acknowledgements

Esta lista de agradecimentos não é de todo suficiente para agradecer a todas as pessoas que se cruzaram comigo e que de algum modo me ensinaram diferentes formas de viver e que me viram crescer e mudar a todos os níveis. Ao terminar agora mais uma fase importante, não poderia deixar de agradecer a algumas dessas pessoas com especial carinho.

Antes de qualquer outro agradecimento, não poderia deixar de agradecer aos meus pais, por me terem ensinado desde sempre que com coragem e perseverança, é possível ultrapassar todos os obstáculos.

Ao Ivo, por me fazer acreditar que quem não vive desassossegado, não vive sequer. Obrigada por todo o apoio, pelo carinho, pelos telefonemas longos (e tardios) mas, acima de tudo, por me (desa)ssossegares todos os dias.

À Brígida de Sousa, por ser a melhor amiga que alguma vez poderia ter, por mesmo à distância me ter feito sempre acreditar que dias melhores iriam finalmente chegar.

A todos os amigos que conheci ao longo do curso e que de algum modo me marcaram, cada um à sua maneira. Um obrigado especial à Bruna e ao Nuno, por me fazerem sempre acreditar que não estava sozinha com os meus pensamentos e por me mandarem para *A Roda* acalmar quando era preciso.

Um dos meus agradecimentos mais especiais é dedicado à Rita Bento, por me ter ajudado sempre em tudo o que precisei durante a realização do trabalho que deu origem a esta dissertação. Obrigada Rita, por estares sempre disponível para me ajudar com tudo (mesmo quando te era quase impossível). Obrigada pelas horas infindáveis de confocal, sempre acompanhadas de boas conversas e momentos de riso.

Obrigada à Joana Silva, que me deu o impulso e apoio iniciais para que começasse a ambientar-me com o tema do trabalho.

Obrigada também à Ana Rita Ferreira por se ter disponibilizado sempre para me ajudar quando precisei.

Obrigada aos *Salsichinhas*, que fizeram todos os dias com que as *catacumbas* do INEB não parececem tão *sombrias*.

Gostaria de agradecer à Professora Isabel Amaral, que orientou todo este trabalho, por me ter dado a oportunidade de trabalhar neste projecto e por ter estado sempre presente e disponível, mas acima de tudo, por tudo o que me ensinou ao longo deste período. Obrigada também à Sofia Domingues, que co-orientou o trabalho, pela sua disponibilidade em esclarecer todas as dúvidas e por me ter dado a oportunidade de realizar procedimentos que enriqueceram o meu trabalho.

Não poderia terminar sem agradecer a toda a equipa do INEB, especialmente à NeuroTeam, na qual fui integrada, por me ter recebido desde o início com a maior simpatia.

"*I read somewhere how important it is in life not necessarily to be strong…but to feel strong.*"

Chris McCandless, in 'Into the Wild'

Table of Contents

List of figures

List of tables

Abbreviations

BBB	Blood-Brain Barrier
BMP	Bone Morphogenic Protein
CNS	Central Nervous System
CNP	2'-3'-cyclic nucleotide 3'-phosphohydrolase
DS	Donkey serum
ECM	Extracellular Matrix
ERK	Extracellular Signal-Regulated Kinases
FAK	Focal Adhesion Kinase
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GalC	Galactocerebroside
HA	Hyaluronan
IGF	Insulin-like Growth Factor
MAP	Microtubule-associated Protein
MAPK	Mitogen-activated Protein Kinases
MBP	Mielin-basic Protein
MMP	Matrix Metalloproteinases
MOG	Myelin/Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NGS	Normal Goat Serum
NRG	Neuroreguline
NT-3	Neurotrophine-3
OL	Oligodendrocyte
OPC	Oligodendrocyte progenitor cell
PAI	Plasminogen Activator Inhibitors
PDGF	Platelet-derived growth factor
PEG	Poly-(ethyleneglycol)
PGA	Poly-(glycolic acid)
PI3K	Phosphatidylinositol 3 kinase
PKB	(Akt) Protein Kinase B
PLA	Poly-(lactic acid)

PLP Proteolipid Protein

PNS Peripheral Nervous System

ROCK Rho kinase

SCI Spinal Cord Injury

SFKs Src Family Kinases

TGF-β Transforming growth factor

VEGF Vascular-Endothelial Growth Factor

Chapter 1

Introduction

1.1. Demyelinating disorders affecting the Central Nervous System (CNS)

1.1.1. Myelination in the CNS

The myelin-membrane formation is a distinctive characteristic of glial cells in vertebrates. During the myelination process, maturing axons are wrapped by multiple concentric membranous layers of myelin produced either by oligodendrocytes (OLs) in the CNS or by Schwann cells in the peripheral nervous system (PNS). The myelin sheath enables not only the rapid and accurate conduction of electrical impulses along the nerve fiber, but also axonal protection and integrity. Besides, there is a mutual regulation mechanism between OLs and axons: during development, glial cells ensure neuronal cells survival and determine both axolemma domains organization and axons diameter, while in turn, axons provide crucial signals for glial proliferation, survival and differentiation, consequently regulating myelin-membrane formation as well. In adult life, myelinating OLs are responsible for maintaining axons diameter, axolemma organization and also neuronal integrity and function, whereas axonal signals regulate oligodendrocyte progenitor cells (OPCs) differentiation into OLs and maintain myelin integrity [1].

The myelin membrane is composed of a specific set of lipids and proteins, whose assembly must occur at a precise and controlled time and space: the glial cells must associate with the axons at the appropriate developmental time, so signaling mechanisms can be established to deliver newly synthesised myelin-membrane components to the axon [2]. More precisely, proliferative OPCs migrate into the white matter regions, exit the cell cycle and undergo differentiation, turning into mature OLs. At this point, they begin to express a subset of myelin-associated proteins. The mechanism starts with the OLs process extension, followed by targeting and adhesion to axonal receptors. Once this connection is established, myelin components are synthetized and taken to the appropriate sites within the sheath, axons become wrapped and myelin membrane is finally compacted, where the sheaths are almost fused and absent of cytoplasm. Nonetheless, this membrane must be maintained throughout adulthood. This is achieved by a continuous turnover of myelin associated to a high expression of myelin genes, which continues even after the myelination developmental process.

Myelin is composed by approximately 70% lipid and 30% protein. The lipid composition includes cholesterol, phospholipids and glycosphingolipids, while the major proteins present in myelin are myelin basic protein (MBP) and proteolipid protein (PLP), but other proteins such as myelin-associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG) are also present [3,4]. This composition gives it insulating properties, which are crucial for rapid and efficient propagation of neuronal impulses [5].

As myelin formation requires the synthesis of both lipids and myelin specific proteins, a complex cellular mechanism is required to properly synthesize and localize these components [6].

1.1.2. Demyelination mechanisms

A demyelinating disease is any disease of the nervous system in which the myelin sheath of neurons is damaged. When this occurs, axons lose their myelin sheaths as a consequence of OLs death in the site of primary lesion. Furthermore, OLs also undergo apoptosis at considerable distances from the lesion, which leads to the hindrance of action potential propagation by surviving axons and, consequently, to the loss of neuronal function.

The breakdown of the blood-brain barrier (BBB) facilitates the infiltration of macrophages and myelin-specific T-cell lymphocytes into the CNS, which attack the myelin-producing OLs. As a consequence of myelin debris, both microglia and astrocytes are activated. After clearing the debris, macrophages, activated microglial cells and reactive astrocytes work together to secrete growth factors, cytokines and matrix metalloproteinases (MMPs), that degrade ECM components and increase inflammation. These growth factors and cytokines also activate quiescent OPCs, thus increasing their proliferation rate. In addition, OPCs respond to pro-migratory signals provided by demyelinated axons. As a result of these demyelination events, OPCs proliferate and migrate to demyelinated areas, in an attempt to differentiate into myelinating OLs to repair the lesion (**Figure 1**) [7].

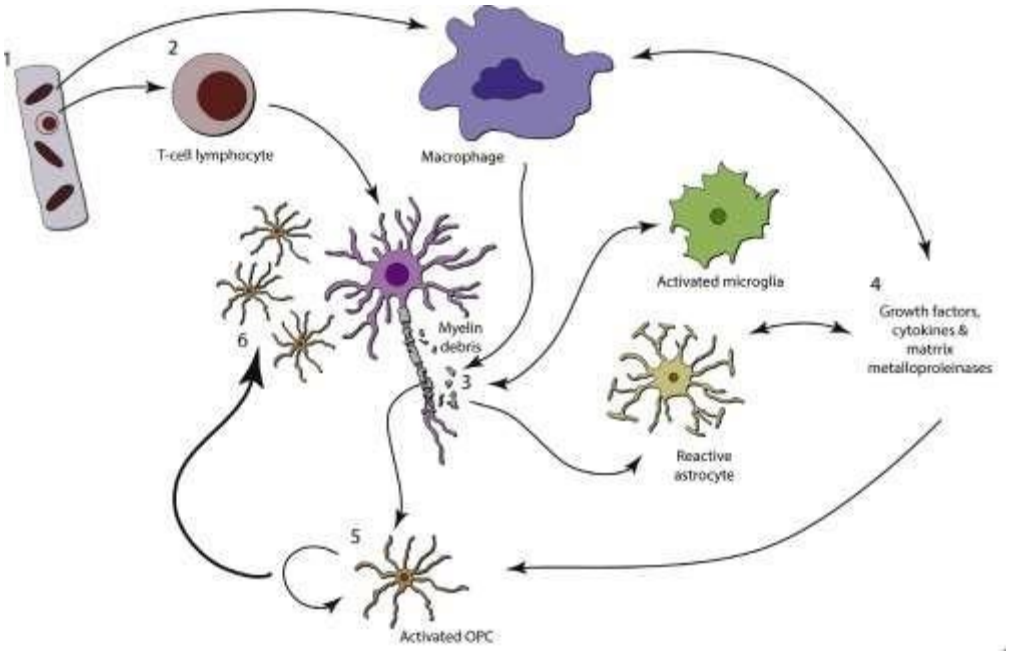


Figure 1. Cellular and molecular events occurring during demyelination. Adapted from: [7]

The main diseases associated to demyelination in the CNS are multiple sclerosis (MS), stroke and traumatic lesions, including spinal cord injury (SCI) and traumatic brain injury (TBI). MS and SCI will be discussed in detail in the next sub-sections.

1.1.2.1. Multiple Sclerosis

MS is the most common nontraumatic disorder of the CNS. It affects around 2.5 million globally, 0.5 million in European Union and 50 000 in Portugal, being more incident in developed countries. MS is a chronic inflammatory demyelinating and degenerative disease, affecting mainly young adults and causing progressive and significant disability. In addition to motor and sensory deficits, there is cognitive deterioration in as many as 65% of patients.

The disease course greatly varies from patient to patient, but it generally presents a relapsing/remitting form, in which episodes of acute demyelination and neurological dysfunction are followed by remyelination and functional recovery. The breakage of the blood-brain barrier leads to the infiltration of macrophages and T-cell lymphocytes, which react against cells expressing myelin-specific antigens, i.e. oligodendrocytes. This leads to destruction of myelin sheaths, axonal demyelination and, consequently, neuronal death [8]. Remyelination occurs spontaneously in the first stages of the disease, when the lesions present an acute nature. However, remyelination is commonly incomplete or not efficient and chronically demyelinated lesions eventually fail to remyelinate. After several episodes, the failure of remyelination leads to augmented axonal degeneration and progressive disability [9].

The only current treatment for MS consists of FDA-approved medications based on anti-inflammatory drugs, which are expected to prevent immune and inflammatory episodes during the relapsing/remitting phase of the disease. However, with the disease progression and accumulated axon damage, these medications become not effective in advanced stages [10].

Remyelination of axons is therefore necessary for functional recovery. Proliferation and migration of OPCs near MS plaques have been the target of several studies, however an inability of OPCs to differentiate has also been reported. This inability may be a result of inhibitory mechanisms or loss of axonal function, since axons normally provide differentiation signals for OPCs.

1.1.2.2. Spinal Cord Injury

Globally, it is estimated that around 250 000 and 500 000 people suffer a SCI. There is no reliable estimate of global prevalence, but the estimated annual global incidence is 40 to 80 cases per million. In the United States, there are approximately 12 000 new cases per year with an additional 232000–316 000 people currently living with SCI [11].

SCI is a result of a lesion caused by a compressive or stretch injury in the spinal cord. It presents complex hurdles to regeneration due to the multifaceted nature of inhibition conditions that occur to cord parenchyma and stroma after trauma. In addition,

from a clinical sight, SCI is aggravated by its heterogeneity in size, shape and extent of injury. The primary injury caused by initial mechanical trauma is often followed by secondary injury events, in which the lesion extends from grey to white matter and may cause blocking of the propagation of action potential along axons [12,13]. This secondary lesion includes several related events such as BBB dysfunction, local inflammation and axonal demyelination, which eventually end up in neuronal death and breakdown of neurological pathways [14]. Furthermore, OLs are very sensitive to SCI injury, thereby undergoing both necrotic and apoptotic cells death [15].

SCI is well characterized by the development of a glial scar by reactive astrocytes, which presents the major hurdle to neuronal regeneration after injury. The glial scar associated with the production of neurite outgrowth inhibitors form an inhibitory microenvironment, thus preventing neuronal function recovery [16].

Current treatments include surgery (when the spinal cord is totally compromised) and methylprednisolone administration, however none of them is effective. Ongoing therapeutic approaches, including the delivery of neurotrophic factors, antagonists of neurite outgrowth inhibitors and modulation of inflammatory response have shown to restore some of the lost functionality [11,16].

1.1.3. The inhibitory microenvironment and remyelination failure

Remyelination requires the generation of new mature OLs, which are believed to derive from a population of adult precursor cells, often referred to as adult OPCs.

In response to injury, local OPCs switch from a quiescent to a proliferative and responsive phenotype. This involves both changes in morphology and upregulation of genes associated with the generation of OLs during development. This is followed by the repopulation of the demyelinated area with OPCs. Once recruited, OPCs must differentiate into functional OLs.

However, the adult CNS has a limited regenerative capacity, due to the fact that, although endogenous OPCs are prevalent in demyelinated lesions, they are not able to differentiate into OLs and remyelinate due to the local inhibitory microenvironment. The formation of a glial scar, composed of astrocytes and connective tissue elements, is one of the barriers to regeneration of CNS axons. The molecular composition of the scar (connective tissue components such as collagen and elastin), the inhibitory molecules (tenascin, semaphorin 3, ephrin-B2, slit proteins and chondroitin sulfate proteoglycans) released by astrocytes and active microglia, as well as genetic factors, sex and age, are all contributing factors for regenerative failure [17,18].

1.2. Transplanted OPCs as a source of myelin-producing cells

1.2.1. OPC differentiation into myelin-producing OLs

OPCs were first identified in the early 80s by Martin Raff and his team as proliferating cells that could differentiate both into OLs or type 2 astrocytes, the reason of their original name O-2A cells Nonetheless, further studies confirmed that in most of the cases their differentiation was associated to an OL fate [19].

During development, the first OPCs are originated from the neuroepithelial precursor cells (NEPs) from the ventricular zone of the spinal cord. The first wave of OPC production starts in the ganglionic eminence and eventually a second and third waves originate from the lateral and caudal ganglionic eminences, respectively, giving rise to adult OLs. OPCs then migrate to other areas, populating the future brain and ultimately myelinating the CNS [20].

The development of myelin-producing OLs involves two interdependent stages. The first one is the differentiation of OPCs into OLs expressing markers of differentiation, including galactocerebroside (GalC) and myelin basic protein (MBP), while in the second stage, these OLs subsequently undergo morphological changes associated with the production of myelin sheaths [21].

Several intrinsic and extrinsic factors are involved in differentiation of OLs and the major ones are summarized in **Table I**.

Downstream signaling of transcription factors that lead to increased expression levels of Olig1 and Olig2 promotes OPC differentiation into OLs and consequently, myelination of the axonal tracts. A factor that controls the formation of oligodendrocytes is platelet-derived growth factor (PDGF) (**Figure 2**). PDGF stimulates the proliferation of OPCs and in its absence, OPCs exit the cell cycle and may differentiate into OLs prematurely. Also, this factor avoids neural stem cells (NSCs) differentiation in other cell types, such as neurons and astrocytes, thereby ensuring an OPC fate [22]. Thyroid hormones, particularly T3, induce proliferation of OPCs and promote their differentiation into OLs by triggering cell-cycle exit, thereby enhancing the morphological and functional changes involved in their maturation [23].

Nonetheless, a balance must be kept between promoters and inhibitors of OL differentiation, so it occurs in a controlled time and space fashion [24].

In the CNS (**Figure 2**), myelination is controlled by three main types of signals: axon- derived signals (e.g. TNFα), ECM (e.g. Fyn signaling) and soluble factor signals (e.g. IGF-1, PDGF, CXCL12, Notch family) and finally, intracellular signaling cascades (e.g. downstream signaling from receptors that increase the expression of Olig1 and Olig2) inside myelinating OLs. Both positive and negative effects should be considered. The control of myelination by ECM will be discussed in detail in the section 1.2.3.

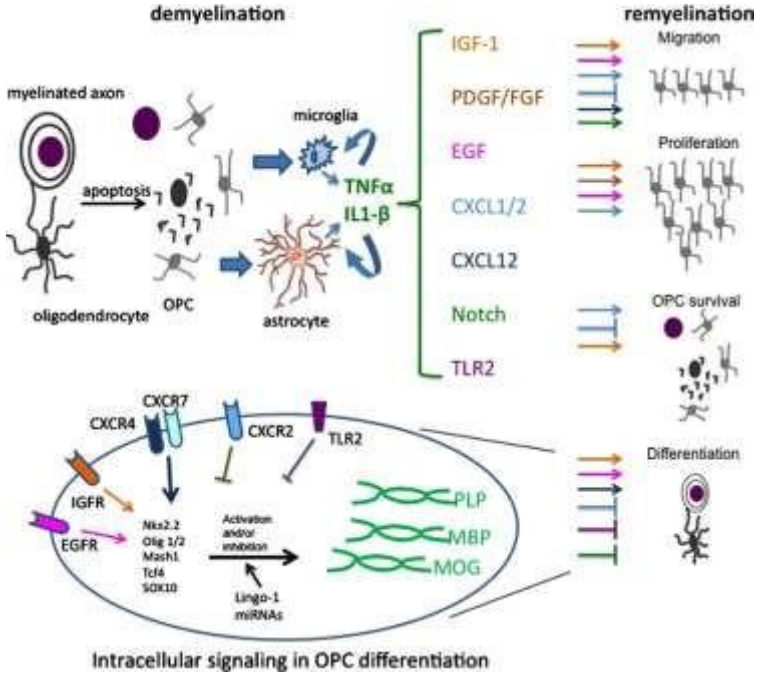


Figure 2. Remyelination mechanisms: role cytokines, chemokines, growth factors, transcription factors and other proteins in OPCs fate. [25]

Table I. Major intrinsic and extrinsic factors controlling the differentiation of OPCs into OLs. Based on: [7]

Extrinsic factors		Intrinsic factors	
T3	Triggers cell-cycle exit.	Olig1	In response to demyelination, promotes the transcription of myelin-specific proteins.
PDGF	Withdrawal of this growth factor acts on intracellular factors to trigger cell-cycle exit.	Olig2	In response to demyelination, regulates Nkx2.2 expression.
IGF-1	Promotes cell-cycle exit and OPC differentiation.	Nkx2.2	- Lineage specification; - Promotes the transcription of myelin-specific genes.
TGF-β1	Promotes cell-cycle exit and OPC differentiation.	Myt1	Controls the expression of myelin-specific proteins.
Sox2	Promotes cell-cycle exit and OPC differentiation.	Fyn*	Activates the transcription of myelin-specific genes.
		PI3K-AKT1	Activates mTOR (final differentiation of OPCs into OLs).

*discussed in detail in sub-section 1.2.3.

In the adult CNS, new OLs are derived from adult OPCs dispersed throughout both grey and white matter and in the subventricular zone (SVZ). Although adult OLs differentiation show slower rates of migration and longer cell cycle times, it presents some similarities with developmental OLs [8]. It was previously mentioned that when myelin is damaged, local OPCs undergo an activation step, thereby switching from a quiescent state to a regenerative phenotype. This activation step involves the upregulation of several genes associated with

OLs differentiation, including Olig2, NKX2.2, MYT1 and Sox2. OPCs are firstly recruited in response to factors released by microglia and astrocytes and start to proliferate. The differentiation phase of OPCs into OLs comprises three major steps: developing contact with the axons, expressing myelin genes and finally producing a myelin membrane and wrapping it around axons [20].

1.2.2. Therapeutic effect of OPCs in remyelination

As previously mentioned, OPCs are glial cells that differentiate into myelinating OLs during embryogenesis and early stages of post-natal life. However, a number of OPCs remain in an undifferentiated state, hence their abundance in the adult brain.

Since local OPCs cannot differentiate due to the inhibitory microenvironment, transplantation of OPCs as emerged as a feasible therapy to promote remyelination of spared and regenerating axons.

Adult OPCs can be induced to proliferate and migrate *in vitro* by the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), which are both upregulated during remyelination [8].

A few studies provided *proof-of-principle* for clinical glial cell transplantation. *Archer et al. (1997)* performed OPC transplantation into shaking dog pups, with PLP mutations that trigger Pelizaeus-Merzbacher disease) and demonstrated the ability of the transplanted OPCs to myelinate axons in the demyelinated lesions of these animals. [26] In 2008, *Windrem et al.* studies showed that transplantation of highly enriched preparations of human glial progenitor cells into multiple sites in mice carrying mutations in MBP enables widespread myelination throughout the CNS and recovery from myelin dysfunction in some cases. The transplanted mice also exhibit prolonged survival [27].

In a study performed by *Keirstead et al. (2005)*, transplantation of human embryonic stem cell (hESC)-derived OPCs into a rat model of SCI resulted in enhanced remyelination and improved motor function. However, enhanced remyelination and locomotor recovery were only observed when OPCs were transplanted at early time points after SCI (7 days after injury). In animals receiving OPCs 10 months after injury, there was no enhanced remyelination or locomotor recovery, possibly due to the presence of inhibitory molecules associated to the astroglial scar [17].

1.2.3. Role of the ECM and OPC integrin receptors in myelin-membrane formation

It is known that the interactions between cells and their surrounding microenvironment are crucial for cell survival, growth and differentiation. The organization of the ECM in the developing CNS is still poorly understood. However, *Colognato et al. (2005)* studies have shown that ECM interactions are relevant for glial cell development [28].

The ECM is a complex association of extracellular molecules, including structural proteins (i.e. collagen), glycosaminoglycans (GAGs) and specially glycoproteins such as fibronectins and laminins, which activate intracellular signal pathways through interactions with integrins [29].

Integrins are transmembrane protein receptors that bind to ECM proteins, thus mediating cellular adhesion and signalling cascades between the extracellular and intracellular environments. When a ligand binds to the extracellular domain of a specific integrin, integrin clustering occurs and the actin filaments as well as signalling proteins are recruited to the intracellular domain of the integrin.

Integrins structure is heterodimeric, thus consisting of two different chains, α and ß subunits. The α chains are responsible for determining ligand specificity, while ß subunits have a cytoplasmatic tail that can bind to several intracellular anchor proteins, such as talin, α-actinin and filamin. These anchor proteins can then bind to actin, thus allowing the actin association to integrins, which leads to integrin clustering and consequently, to the formation of focal adhesions between cells and the ECM [30,31].

Integrins have been demonstrated to be important for myelination. OPCs and OLs express a limited repertoire of integrins, including αvß1, αvß3, αvß5, αvß8 and particularly the laminin receptor α6ß1 integrin. Each one of these integrins is regulated and expressed depending on the differentiation stage since they play different roles in development: αvß1 promotes migration, αvß3 proliferation, αvß5 differentiation and α6ß1 differentiation and survival of new formed OLs [32]. Moreover, α6ß1 integrin has shown to enhance cell survival of myelinating oligodendrocytes and promote myelination of axonal tracts *in vivo* [17,33,34].

It has been previously referred that upon differentiation into OLs, OPCs undergo a phenotype switch, characterized by a dramatic change of morphology and the formation of a large network of branching processes. When OPCs exit the cell cycle in order to differentiate into myelinating OLs, the survival of these developing cells depends on the contact with axonal receptors and on the effect of soluble factors, such as PDGF and neuroregulin-1 (NRG-1). While these factors act as mitogens and inhibit differentiation at earlier stages of development, upon OL maturation they are fundamental for cell survival and differentiation. Hence, the same factors that stimulate proliferation and hinder differentiation may become crucial for survival, by switching their phenotype at the precise time and space where OLs undergo final stages of differentiation. Although the mechanisms underlying this switch are still not well understood, they are thought to be regulated by interactions between integrin receptors and ECM ligands [35]. But how do receptors transmit signals from the ECM to OPCs in order to change their phenotype?

Several downstream effectors and pathways may be involved in ECM signaling for myelin-membrane formation, including Src Family Kinases (SFKs), small Rho GTPases, Focal Adhesion Kinase (FAK), ILK and the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)/Extracellular Signal-Regulated Kinases (ERK) pathways (**Fig. 3**).

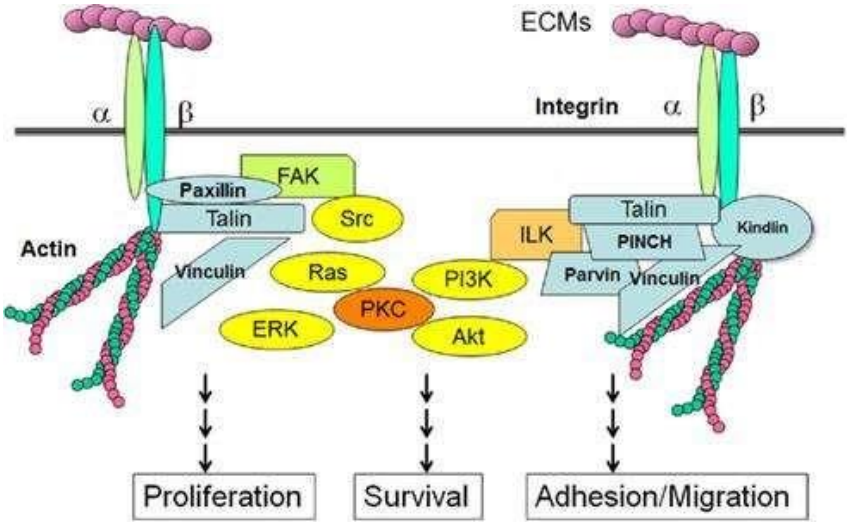


Figure 3. Integrins, adaptor proteins, and signaling pathways. [36]

The signaling cascade begins with integrins and other ECM receptors connecting to the downstream pathways through SRKs. SRKs are non-receptor tyrosine kinases tethered to the inner side of the cell membrane that play an important role in the transduction of external signals into changes in intracellular signaling.

Both Fyn and Lyn belong to the SRKs family and regulate integrin-guided pathways. Lyn associates with the PDGFR-αvβ3 integrin complex and its activation contributes to the proliferation of OPCs. On the other hand, OPC differentiation into OLs depends on Fyn. Fyn is expressed throughout the brain and particularly highly expressed in OLs and its activation and expression levels are upregulated during the final stages of differentiation. It is activated by signals triggered by laminin through integrins (particularly α6β1) [37,38].

SRK pathways regulate the activation of Rho GTPases (e.g. Rho, Rac and Cdc42) through Fyn (**Fig. 4**). After differentiation, Fyn phosphorylates and activates p190RhoGAP, which then inactivates Rho activity, thus causing modifications in OL morphology. Furthermore, Fyn activates both Cdc42 and Rac, which promote differentiation of OLs. Rho GTPases, either through activation or inactivation, regulate the polymerization of actin, and consequently control cytoskeleton structure and cellular motility [39].

FAK is activated in differentiating OPCs and, such as Fyn, it also integrates signals from the ECM, inducing cytoskeleton changes that ultimately contribute to myelination. Both Fyn and FAK pathways mediate laminin interactions with the cytoskeleton.

Laminins are heterotrimeric proteins of the ECM that consist of a α-chain, a β-chain and a □-chain. The trimeric proteins form a cross-like structure that can bind to other cell membrane and ECM molecules, thereby playing an important role in cell adhesion, migration, proliferation and differentiation [40]. Laminins are found in myelinating axon tracts stimulating soluble factors such as PDGF and NRG [38].

The interaction between laminin-211 expressed in the CNS and oligodendrocyte α6β1 integrin has shown to play an important role in signaling mechanisms that enhance the

morphological changes, stimulating the myelin membrane formation by OLs [34]. Studies from *Buttery and ffrench-Constant (1999)*, *Relvas et al. (2001)* and *Colognato et al. (2004)* have shown that laminin-integrin interactions affect oligodendroglial process extension in both length and branching [20,33,38]. Moreover, in a study performed by *Zhao et al. (2009)*, laminin was reported to re-appear in demyelinated lesions that are starting to repair. Laminins potentiate the ability of newly differentiated OLs to survive at limited quantities of trophic factors. On the other hand, fibronectin was shown to reduce the ability of OLs to form branched processes by stimulating OPCs to continue to proliferate [28].

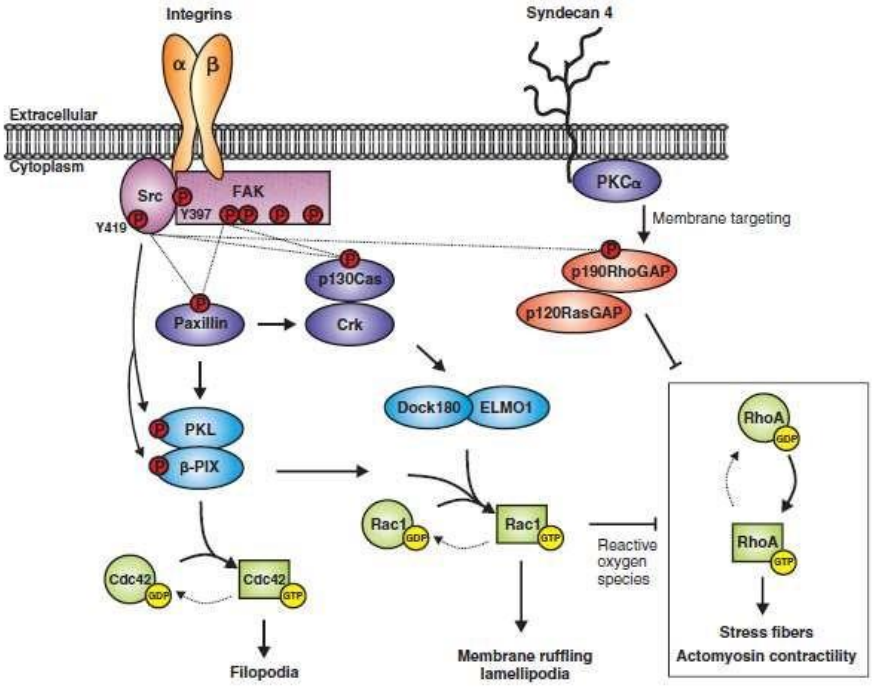


Figure 4. Integrin regulation of Rho-GTPases during stages of cell spreading and process outgrowth. [41]

The previously referred Fyn pathway is also required to enhance PDGF signaling to promote myelin-membrane formation and to change NRG signaling course from a PI3K to a MAPK pathway, this way changing the development stage from proliferation to differentiation. PI3K is thus activated by NRG-1 type III produced by naked axons, subsequently activating the PI3K-AKT1 pathway by growth factors and ECM molecules. This pathway will eventually induce differentiation of OPCs into myelin-producing OLs, as well as cytoskeleton reorganization [42]. In a study from *Colognato et al.* (2002), the activation of ERK1 and ERK2 was enhanced as a response to NRG when OLs were cultured on laminin-2 [38]. Therefore, besides enhancing survival, MAPK/ERK signaling has shown to promote process extension and differentiation of OLs. Furthermore, it has revealed that a switch from a PI3K-dependent to a MAPK-dependent signaling is necessary in order to promote differentiation.

1.2.4. The cytoskeleton in myelin-membrane formation

During OL development, signals relevant to process formation must be transduced into adequate changes in cytoskeletal organization. OPCs have a bipolar morphology, however, as

the OLs mature, they become multipolar through the extension of several processes that eventually extend many orders of branches. Therefore, OL cytoskeleton plays an essential role on their interaction with axons and also in the regulation of branching and process outgrowth [44].

OLs present a complex cytoskeleton architecture, which is characterized by an organized and dynamic network of microtubules and microfilaments, however absent from intermediate filaments. Myelin-constituting proteins were shown to be involved not only in OL differentiation, but also in process extension. In 2006, *Galiano et al.* reported MBP as a microtubule-stabilizing protein in OLs undergoing differentiation [45]. Furthermore, 2'-3'- cyclic nucleotide 3'-phosphohydrolase (CNP) was associated to process outgrowth by copolimerization with tubulin [46].

Microtubules are polarized dynamic structures which are formed by the polymerization of heterodimeric complexes of α-tubulin and ß-tubulin. Microtubule growth and integrity are regulated by microtubule-associated proteins (MAPs) that bind to the microtubules through tubulin domains. Several MAPs can be found in OLs, including MAP1B, MAP4, MAP2c and tau proteins. The latter are specifically present at branching areas and ends of the cellular process extensions. Upon differentiation, there is a decrease in tau phosphorylation, enhancing their binding to microtubules.

Also Fyn kinase interacts with OL cytoskeleton by coupling with tau and tubulin, thus contributing for the initiation of process outgrowth and myelination. Moreover, The OL morphology during differentiation may occur via ECM pathways from integrins to Fyn and finally, to Rho GTPases, which leads to the formation of the motility structures referred [45].

In **Figure 5**, it is possible to observe the differences in branching and process outgrowth associated with different stages of differentiation (OPCs and OLs).

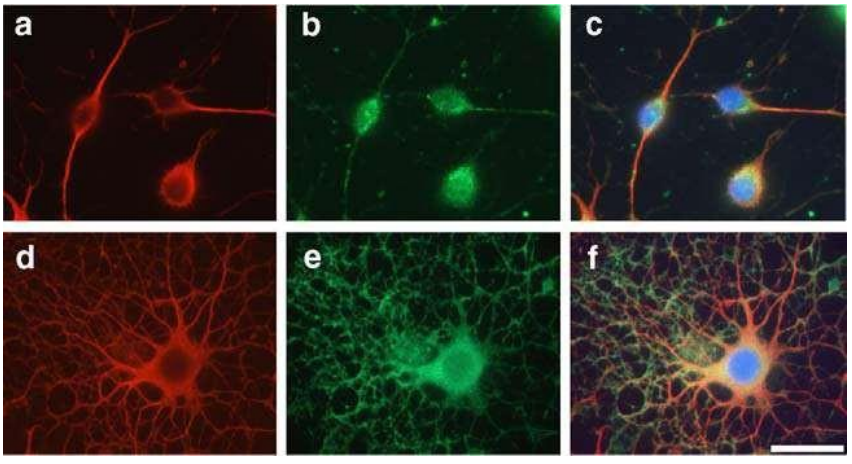


Figure 5. Immunocytochemical localization of microtubules and MBP during OL differentiation. OPCs (a-c) and OLs cultured for 6 days *in vitro* (d-f) derived from rat brain can be observed. Immunofluorescence with antibodies against tubulin (a,d) and MBP (b,e). Nuclei stained with DAPI and merged with tubulin (red) and actin (green) staining(c,f). Scale bar=25 µm. [44]

1.3. Vehicles for OPC delivery to the CNS

1.3.1. Overview

Despite being a source of potential myelin-producing cells, transplantation of OPCs by direct injection of cell suspensions presents drawbacks such as low cell survival and poor proliferation and differentiation [43]. This is mainly due to the lack of mechanical and biochemical support associated to the inhibitory microenvironment generated either by the glial scar or the inflammation mechanisms occurring upon demyelination [8]. Tissue engineering provides an alternative approach to overcome these limitations and thus increase the efficacy of OPC transplantation therapies in the CNS, namely by combining cellular transplantation with 3-D biomaterial based-vehicles. These 3-D matrices may protect transplanted cells from the local hostile tissue environment, thereby creating a supportive niche for OPC survival, proliferation and differentiation.

1.3.2. Hydrogels as vehicles for OPC delivery into demyelinated lesions

Cells can sense and respond to the biophysical properties of the microenvironment through mechanotransductional mechanisms. In particular, adhesion ligands binding to integrins and other cell surface receptors, serve as mechanical transducers between the surrounding environment and the cytoskeleton [44], allowing cells to sense and respond to matrix stiffness. The stiffness of the matrix affects cell spreading, morphology and function, and generally, cells show better *in vitro* behavior when cultured in matrices with mechanical properties similar to those of their native microenvironment.

Therefore, apart from being biocompatible and biodegradable (by cell-secreted proteolytic enzymes, ideally) the engrafted biomaterials should have mechanical properties closely matching those of the target tissue.

Hydrogels have proven to fulfill these requirements, as these can be prepared to present different stiffnesses (by varying the polymer concentration and the cross-linking degree). Furthermore, hydrogels are hydrophilic polymer networks with high water content (similar to the ECM) and high permeability, allowing exchange of oxygen, nutrients and metabolites, thus providing a highly permissive microenvironment for cell survival and growth. Finally, hydrogels can be decorated with biochemical or biophysical cues (cell adhesive or growth factor binding ligands) to better mimic the native ECM [34].

In situ forming hydrogel systems are of particular interest in tissue engineering applications. Injectable hydrogels are likely the most investigated materials for local drug and cell delivery to the CNS. It was previously referred that hydrogels already present a set of advantages that make

them suitable for these applications. In addition, injectable hydrogels have the ability to provide a minimally invasive, localized and filling platform for therapeutic purposes. Upon injection into the spinal cord tissue, these polymers undergo a rapid transition from liquid to gel phase, thereby adapting to the specific lesion site and ultimately integrating with the native tissue.

Also, like any hydrogel-based scaffold design for the CNS, injectable hydrogels can be formulated to present mechanical properties closely matching those of the spinal cord ECM. [47].

A list of hydrogels used for neural tissue engineering is presented on **Table II**. The mechanism of gelation is indicated and the main advantages/disadvantages discussed.

Table II. Hydrogels used for neural tissue engineering: mechanisms of gelation and advantageous (+) and disadvantageous (-) characteristics. Adapted from: *Li X. et al. (2012)* [48]

Hydrogel	Gelation mechanism	Advantages and disadvantages
Polyacrylamide	Chemical crosslinking	Adjustable mechanical properties (+) Control of architecture and chemical composition (+) Non-immunogenic (+) Non-biodegradable (-) Non-permissive (-) Neurotoxicity (-)
NeuroGel™ poly[N-2 (hydroxypropyl)methacrylamide] (PHPMA) gel modified with a RGD sequence[49]	Chemical crosslinking	Biocompatible (+) Non-immunogenic (+) Mechanical properties (+) Non-citotoxicity (+) Non-biodegradable (-)
PEG [50]	Photocrosslinking; chemical crosslinking	Highly biocompatible (+) Non-immunogenic (+) Neuro-protective (+) Highly hydrophilic (-) Non-biodegradable (-)
Modified PEG (with poly(lactic acid) (PLA) and poly(glycolic acid) (PGA))[51]	Photocrosslinking; chemical crosslinking	Highly biocompatible (+) Non-immunogenic (+) Neuro-protective (+) Biodegradable (-) Highly hydrophilic (-)
Collagen [52]	Chemical crosslinking (pH, temperature and ionic strength sensitive)	Highly biocompatible (+) Injectable (+) Enzymatically degradable (+) Possibly immunogenic (-) Weak mechanical properties (-)
Fibrin [53]	Enzymatic crosslinking	Highly biocompatible (+) Injectable (+) Chemical modifiable (+) Incorporation of bioactive signaling molecules (+) Enzymatically degradable (+)
HA [54]	Chemical crosslinking	Highly biocompatible (+) Injectable (+) Non-immunogenic (+) Enzymatically degradable (+) Chemically modifiable (+) Highly hydrophilic (-)
Agarose [55]	Thermosensitive	Injectable (+)

		Non-neuronal permissive (-) Lacking of cell binding domain (-) Non-naturally enzymatically degradable (-)
Alginate [56]	Ionic crosslinking	Easy crosslinking (+) Injectable (+) Inhibition of protein adsorption (-) Non-enzymatically degradable (-)
Chitosan [57]	Ionic (polyanions) and/or chemical crosslinking, thermosensitive	Highly biocompatible (+) Easy modification (+) Injectable (+) Enzymatically degradable (+) Only soluble in dilute acid (-) Inflammatory response (-)
Methylcellulose [58]	Thermosensitive	Injectable (+) Limited protein adsorption (-) Non-enzymatically degradable (-)
Matrigel ® [59]	Thermosensitive	Injectable (+) Highly biocompatible (+) Enzymatically degradable (+) From mouse tumor cells (-) Immunogenic (-) Doubt about exact composition (-)
Polysialic acid (PolySia)-based Hydrogels [60]	Diepoxyoctane crosslinking	Bioresorbable (+) Non-immunological (+) Highly specific degradation (+)
Xyloglucan [61]	Thermosensitive	Injectable (+) Difficult production (-) Enzimatically degradable by glycoside hydrolases (GH)
Peptide hydrogel: - PuraMatrix™ (Peptide RADA 16) - ß-sheet forming ionic selfcomplementary peptide [62] - Self-assembling peptide amphiphile (PA) [63,64]	pH, temperature and ionic strength sensitive	Injectable (+) Highly biocompatible (+) Low mechanical properties (-)

Although hydrogels have already been used for cell delivery to the CNS [30], at the best of our knowledge, only two studies using hydrogels for OPC delivery into the CNS were reported to date. *Li et al. (2013)* developed an injectable biocompatible hydrogel based on thiol-functionalized hyaluronic acid (HA-S) and thiol-functionalized gelatin (Gtn-S), which were crosslinked by poly-(ethylene glycol) diacrylate (PEGDA). Transplant of OPCs within these hydrogels showed enhanced survival and differentiation into OLs, as well as the ability to remyelinate demyelinated axons [65]. In the same year, *Asmani et al.* performed the first study reporting fibrin hydrogels as a suitable 3-D support for culture and differentiation of OPCs derived from endometrial stromal cells, which not only allows cell-cell interaction, but also mechanical stimulation similar to that of ECM. This study will be discussed in detail in the section 1.4.2. [66]

1.4. Fibrin hydrogels as versatile vehicles for cell delivery

1.4.1. Fibrin structure

Fibrin is a FDA-approved hydrogel with widespread clinical use since it was first purified in large quantities in the 1940s. Due to its role in hemostasis and wound repair, fibrin has been used extensively in hemostatic materials, such as fibrin sealants (known as fibrin glue) and wound dressings [67].

As a naturally occurring clotting agent in human body, fibrin is biocompatible and susceptible to proteolytic degradation mediated mainly through plasmin and MMPs. In addition to its important role in hemostasis, fibrin serves as a provisional matrix for tissue repair following injury, constituting a key regulator of wound healing.

Fibrin sealants include fibrinogen (fibrin zymogen form) obtained from human pooled plasma by cryoprecipitation of fresh-frozen plasma or stored plasma [(Tisseel and Artiss (Baxter) and Evicel (Johnson & Johnson)] as well as bovine thrombin containing $CaCl_2$ and antifibrinolytic agents (aprotinin) [68].

Additionally, fibrin can be prepared from the patient's own blood and used as an autologous scaffold without the potential risk of foreign body reaction and infection or, alternatively, from recombinant fibrinogen and thrombin [68].

Fibrin may thus be obtained from fibrinogen polymerization by the serine protease thrombin and then cross-linked by factor XIIIa in the presence of calcium chloride ($CaCl_2$) to form a protein mesh.

Fibrinogen molecules, the circulating precursors of fibrin monomers, are dimeric glycoproteins consisting of two sets of polypeptide chains, Aα-, Bß- and γ, which are bound together by disulfide bridges (**Fig. 6).** Each molecule contains two outer D domains and a central E domain, connected to each other by a coiled-coil segment.

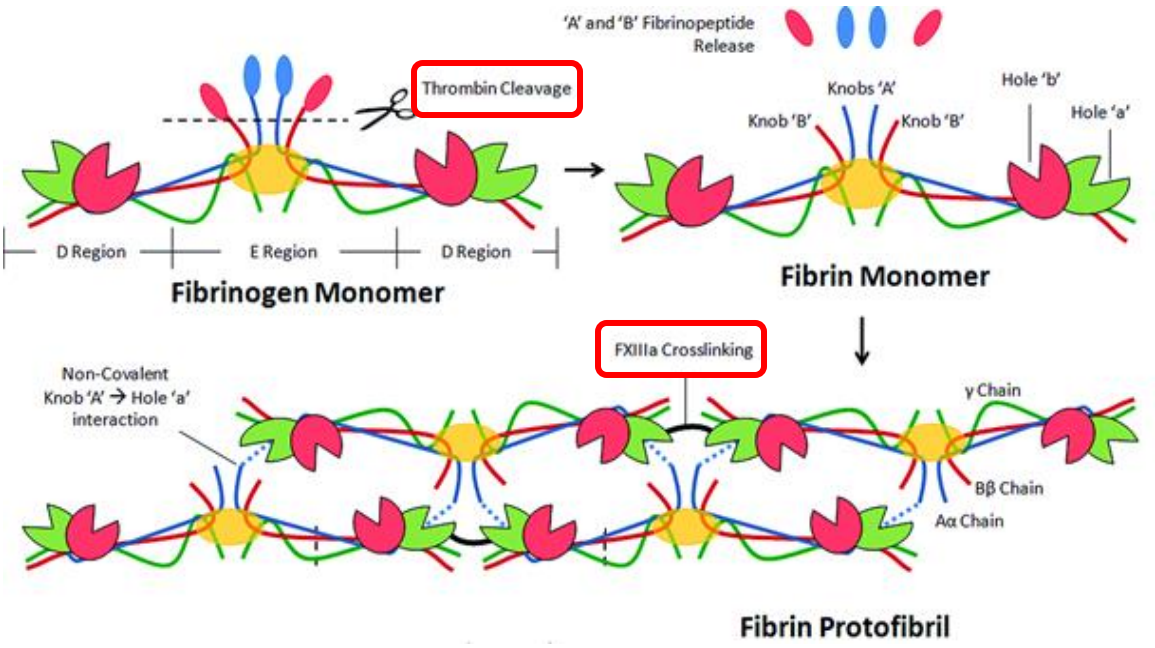


Figure 6. Scheme representing fibrinogen structure, its conversion to fibrin and thrombin-mediated conversion of the factor XIII to XIIIa. [69]

During the coagulation process, polymerization of fibrinogen molecules, which will eventually originate fibrin, initiates when thrombin cleaves the fibrinopeptide A (FPA) from fibrinogens Aα-chains. Associations between D and E domains allow the formation of double-stranded fibrils, that together with branching processes, form a clot-like network. This assembly promotes antiparallel alignment of γ-chain regions, which are covalently crosslinked into γ- dimers by the plasma transglutaminase IIIa (active form of factor XIII), in the presence of $CaCl_2$ (Figure 6). The inactive form, factor XIII, circulates in the blood with fibrinogen and becomes activated by thrombin cleavage. The factor XIIIa then stabilizes the fibrin gel by crosslinking of glutamine residues within the fibrin network to lysine residues, thereby forming the 3-D protein network referred above. Cells can then be directly embedded in the 3-D network upon gel formation.

The properties of fibrin hydrogels can be controlled by two main variables. The first one is the concentration of fibrinogen and thrombin, which affect the fibrin network structure and mechanical properties of the gels obtained. Normally, when the fibrinogen concentration is increased, the resulting gels are dense and turbid (composed of thick fibers). If fibrinogen concentrations are maintained constant, varying only the concentrations of thrombin, lower concentrations lead to the formation of fibrin hydrogels with thick fibers, few branch points and large pores, while in opposition, higher concentrations result in a less turbid formation of tightly packed thin fibers. Moreover, gel stiffness is also affected by fibrinogen and thrombin concentration, mainly by fibrinogen concentration than thrombin's and normally with greater matrix stiffness corresponding to higher concentrations (**Table III**). The other variable consists in using protease inhibitors to prevent the degradation of fibrin by cell- secreted proteases [70]. Protease inhibitors that have been added to fibrin hydrogels in order to slow proteolysis include the serine protease inhibitor aprotinin and pharmacological MMP inhibitors [68].

Table III. Effect of fibrinogen and thrombin concentration on fibrin hydrogels mechanical properties. [68]

Fibrinogen concentration	Thrombin concentration
↑ Fibrinogen concentration ↓ • Fibrin gels dense and turbid, with thick fibers; • Greater stiffness; ↓ Fibrinogen concentration ↓ • Fibrin gels soft and permissive, with thin fibers; • Lower stiffness.	↑ Thrombin concentration ↓ Fibrin gels with tightly packed thin fibers and less turbid ↓ Thrombin concentration ↓ Fibrin gels with thick fibers, few branch points and large pores;

1.4.2. Fibrin as a 3-D matrix for the differentiation of OPCs

Fibrin mechanical and biological properties make it an attractive biopolymer to be used as a cell carrier. This fibrous, non-globular protein polymerizes *in situ* without adverse effects on co-transplanted cells and with gelation times that allow the use of minimally- invasive procedures for the implantation of the cell-loaded matrix. Moreover, fibrin structural and mechanical properties can be easily adjusted by varying the concentration of fibrin components in fibrin polymerizing solution to prepare suitable environments for OPC culture and neurite outgrowth [71,72]. Fibrin gels obtained with low fibrinogen concentrations are more permissive to cell infiltration, however these are too soft and thus do not present enough mechanical strength and robustness. In this sense, a fibrin scaffold should be both permissive allowing cell infiltration and regeneration and hold appropriate mechanical properties to a specific application [68].

The advantages of using fibrin as a scaffold include the extremely elastic resistance to deformation without breaking and the presence of natural ligands for cell adhesion (two pairs of RGD sites and a pair of AGDV sites), proteolytic enzymes (e.g. plasmin), growth factors (e.g. FGF-2, FGF-5 and FGF-7, vascular endothelial growth factor (VEGF-B), PDGF-AB, PDGF- BB, PDGF-DD, TGF-ß1, TGF-ß2, BMP-2 and BMP-2/7 and neurotrophin-3 (NT-3) and brain- derived neurotrophic growth factor (BDNF)) [73], ECM proteins (e.g. fibronectin), and protease inhibitors (including aprotinin, □-aminocaproic acid, plasminogen activator inhibitors PAI-1 and PAI-2, α2-macroglobulin and thrombin activatable fibrinolysis inhibitor) [68]. Recently, *Asmani et al. (2013)* performed the first study reporting fibrin hydrogels as a suitable 3-D support for culture and

differentiation of OPCs derived from endometrial stromal cells, which not only allows cell-cell interaction, but also mechanical stimulation similar to that of ECM. In this study, hydrogels with three different fibrinogen concentrations (2 mg/mL, 3 mg/mL and 4 mg/mL) were tested. Comparing the three gels, it was concluded that the hydrogel with a fibrinogen concentration of 3 mg/mL provided the best 3-D scaffold to mimic spinal cord environment. The analysis of OPCs cell body and the process extensions confirmed the interaction between cells and the fibrin scaffold, thereby indicating that OPCs had successfully attached and incorporated into the matrix [66].

1.4.3. Fibrin functionalization with α6β1 integrin ligands

In opposition to synthetic hydrogels, fibrin is not a passive and non-interactive cell delivery matrix, thus allowing specific binding and functionalization with cell delivery domains and growth factors in order to enhance their bioactivity and specificity [67].

Peptide adhesion domains covalently incorporated within 3-D fibrin matrices can greatly increase the bioactivity of the matrix. In addition, the immobilization of these peptides by substrates for factor XIIIa seems to provide an effective approach for fibrin gels functionalization. *Schense et al.* (2000) incorporated peptides from laminin and N-cadherin either alone or in combination with fibrin hydrogels. In this study, each gel was modified with a different bi-domain peptide, containing a factor XIIIa substrate sequence in one domain and a different bioactive peptide in the other domain, which included RGD, IKVAV, YIGSR and RNIAEIIKDI (derived from the ECM laminin) and HAV (derived from N- cadherin). Neurite extension *in vitro* was enhanced when exogenous peptides were included, with an improvement reaching 75%. When tested *in vivo*, the fibrin derivative containing laminin-derived peptides induced an 85% enhancement in the regeneration of the rat dorsal root compared to non-modified fibrin gels. Therefore, these results demonstrated the possibility to enhance the bioactivity of fibrin gels by the enzymatic incorporation of exogenous oligopeptide domais of morphoregulatory proteins (e.g. laminin, N-cadherin) [74].

As previously referred, growth factors might also enhance the bioactivity of fibrin hydrogels. *Taylor et al. (2004)* developed a heparin-based delivery system (HBDS) to immobilize NT-3 within fibrin gels, thus allowing the release of NT-3 in a controlled manner, as a therapy for SCI. The HBDS system consisted of an immobilized linker peptide which sequesters heparin within fibrin gels and then, the sequestered heparin binds NT-3, thereby avoiding its diffusion. Fibrin gels containing the HBDS system with NT-3 stimulated neural outgrowth from chick dorsal root ganglia up to 54% compared to unmodified fibrin. In a preliminary *in vivo* study, fibrin gels containing the HBDS and NT-3 demonstrated increased neural fiber density in spinal cord lesions when compared to unmodified fibrin at 9 days [75]. *Sakiyama-Elbert and Hubbell (2000)* have previously reported the development of an affinity- based drug delivery system designed to slow the diffusion of heparin-binding growth factors from fibrin gels. These systems were used to

deliver high affinity heparin-binding growth factors such as bFGF [76], but also low affinity neurotrophins, such as nerve growth factor (β- NGF) [77].

The tethering of α6β1 ligands (peptides HYD1 and T1) to fibrin was recently explored by our lab [78].

The angiogenic inducer CCN1 (cysteine-rich 61) is a ligand for several integrins, including α6β1. CCN1 interaction with α6β1 was shown to mediate adhesion of fibroblasts, endothelial cells and smooth muscle cells, and the T1 sequence within the domain III of CCN1 (GQKCIVQTTSWSQCSKS) defined as the critical binding motif for α6β1 [79].

HYD1 (KIKMVISWKG) is a biologically active integrin-targeting synthetic peptide comprised of D-amino acids that interacts with α6β1 and α3β1 integrins (non-engaged as well as pre-engaged integrin complexes), which, when immobilized, supports the adhesion of tumor cells [80]. When added in the soluble form, HYD1 reversibly blocks tumor cell migration on laminin-322, resulting into cytoskeletal remodeling, yet stimulating signaling through the activation of FAK and ERK [81].

Functionalized hydrogels were obtained binding bi-domain peptides with the α6β1 ligands (HYD1 or T1) at the carboxyl terminus and the factor XIIIa substrate domain (NQEQVSPL) at the amino terminus to fibrin gels, through the action of factor XIIIa (**Figure 7**). This strategy is reported to be effective for the binding of peptides into fibrin with retention of biological activity. Fibrin hydrogels incorporating immobilized HYD1 or T1 synthetic peptides were evaluated in terms of ability to promote migration of embryonic stem (ES)-derived neural stem progenitor cells (ES-NSPCs) as well as neurite extension from rat sensory neurons. Immobilization of T1 and HYD1 efficiently promoted outward migration from ES-NSPC neurospheres. While functionalization of fibrin with 20 μM of bidomain HYD1 peptide led to a 2.4-fold increase in neurosphere outgrowth area, a 2.2-fold increase was obtained when fibrin was functionalized with 40 μM of bidomain T1 peptide. Maximal outgrowth distance on these gels was also found to be significantly higher than that observed with unmodified fibrin. Blocking against α6 and β1 integrin subunits significantly inhibited cell outgrowth from neurospheres on T1/HYD1-functionalized fibrin gels, indicating that HYD1/T1 ability to promote NSPC migration in fibrin gels is partially mediated through α6β1 integrin. Immobilized HYD1 also showed to be efficient in promoting neurite extension from rat E18 dorsal root ganglia (DRGs), independently of the input peptide concentration tested [78].

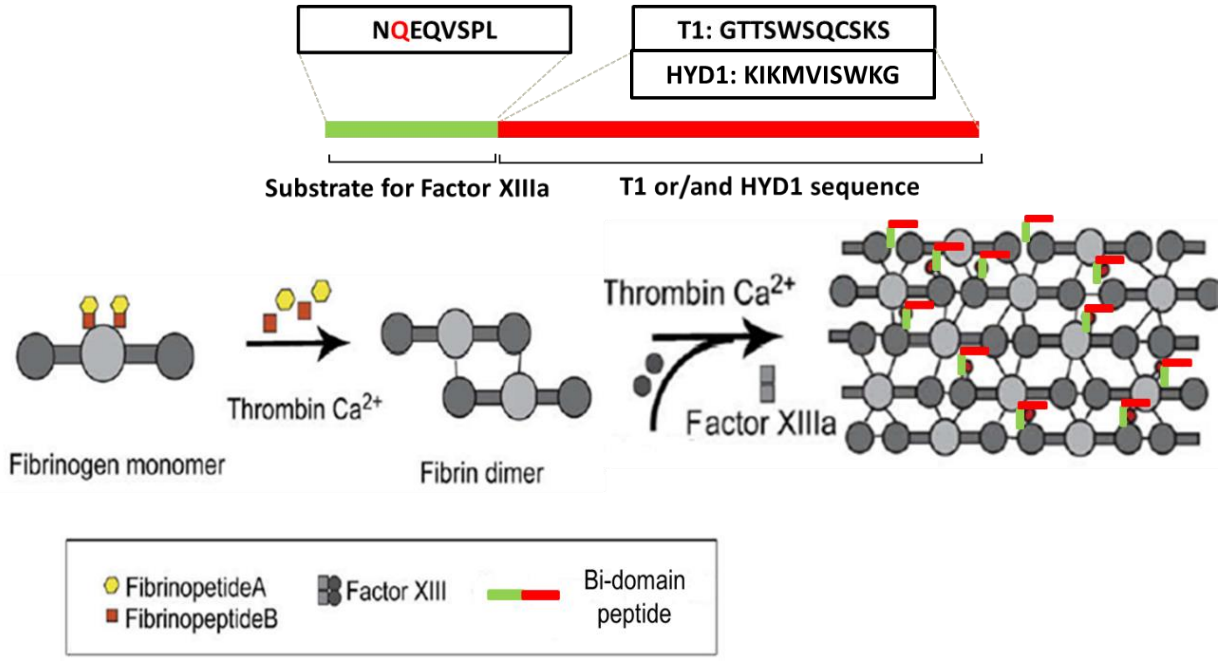


Figure 7. Functionalization of fibrin hydrogels with bi-domain peptides (HYD1, T1) with α6β1 ligands at the carboxyl terminus and the factor XIIIa substrate domain (NQEQVSPL) at the amino terminus, through the action of factor XIIIa. Adapted from: [82]

Objective

Remyelination of CNS lesions caused by demyelinating disorders such as MS and SCI is still a challenging field. Natural remyelination mechanisms are not efficient in advanced stages of demyelination, eventually preventing neuronal function recovery. This is mainly due to the inhibitory microenvironment that prevents progenitor cells from differentiating into new myelinating OLs. In this sense, cell-based therapies have emerged as a promising alternative to remyelinate spared axons. The first studies on cell transplantation were performed back in the early 80s, demonstrating that transplanted glial cells are capable of remyelinating the CNS following their introduction into local injuries. Based on these approaches, many studies have been performed since then using different cell types, such as OPCs, Schwann cells, olfactory ensheathing cells, NSC lines and ESC-derived glial precursors. All of them had shown some progress in remyelinating the CNS lesions. However, cell transplantation by direct injection in the form of cell suspensions frequently leads to low cell survival and poor proliferation and differentiation. Therefore, combination of cell transplantation with a biomaterial-based scaffold can greatly improve the efficacy of the therapies. Although many hydrogels have already been used for CNS regeneration therapies, to date, only a few studies (*Li, et al. (2013)*; *Asmani et al. (2013)*) reported the development of hydrogels specifically designed for the delivery of OPCs into the CNS.

The objective of this Master thesis is to assess the effect of immobilized α6β1 synthetic ligands on OPC behavior in 3D fibrin hydrogels. The functionalization of fibrin with synthetic peptides specifically binding to α6β1 integrin is expected to improve fibrin biospecificity towards OPCs, namely by promoting cell survival, process extension and myelin membrane formation *in vitro*, and ultimately enhance cell survival of transplanted/recruited OPCs and their ability to remyelinate axons *in vivo*.

Chapter 2

Materials and Methods

2.1. Cell Culture

2.1.1. Mixed glial cell (MGC) cultures: dissection, plating and culture of neonatal rat cortices

Mixed glial cell cultures were obtained by dissecting and isolating the brain cortices of P1-P2 (1-2 post natal days) Wistar Han rat pups, and adapted from the the protocols developed by *McCarthy and deVellis* (1980) [83] and *Chen* (2007) [84]. Pups were sacrificed by decapitation with large scissors and the heads were placed in a Petri dish containing cold Hank's Balanced Salt Solution (HBSS) (Gibco, Life Technologies) + 1% penicillin/streptomycin 10000 U/mL. By holding the nose portion of the head, the skin was cut along the midline towards the nose and then, the skull was cut starting at the foramen magnum towards the nose and opened, followed by two lateral cuts made until the base of each skull. The two sides of the skull were fold back, the brain was scooped out into chilled HBSS supplemented with 1% penicillin/streptomycin 10000 U/mL and the cerebellum removed. Each brain was divided along into two cerebral hemispheres and exposed. The meninges were removed under a Leica S6 E Greenough stereomicroscope to finally isolate the cortex. Meninges-free cortices were placed in a clean Petri dish containing HBSS on ice (1 ml per brain). The steps are illustrated in **Figure 8**.

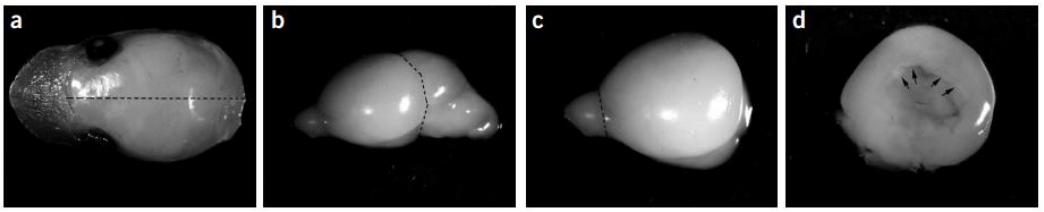


Figure 8. Steps of the dissection of the neonatal rat cortex, from the skull cut (a) until the cortex is meninges-free and totally isolated (d). Adapted from: [84]

Cortices were then mechanically homogenized by pipetting up and down with a serological pipette and a syringe with a 25G needle. Afterwards, the homogenenate was enzymatically treated by adding 0,1mg/ml DNAse I (Sigma) and 0,0025% trypsin (Gibco, Life Technologies) to HBSS for 15 minutes at 37ºC. Serum-containing medium was added to stop trypsinization. Cells were centrifuged (Heraeus Megafuge 1.0) at 500g for 10 minutes, the supernatant was discarded and the pellet was resuspended in complete DMEM medium (**Table IV**) and then filtered into a clean 50 mL centrifuge tube through a 100 µm nylon cell strainer (BD Falcon). Cells were plated in T75 cm^2 poly-D-lysine-coated (Sigma) flasks at a density of 2 brains per flask in approximately 12 mL of medium. Cells were incubated in a tissue culture incubator with 5% CO_2 at 37ºC for approximately 10 days. Medium was changed every 2-3 days.

2.1.2. OPC isolation and culture

Rat OPCs were obtained from MGC cultures. When MGC cultures are confluent, approximately 10 days after plating, astrocytes form a thick layer and microglia and OPCs grow on top. Flasks were first pre-shaken on an INFORS AGCH-4103 BOTTMINGEN shaker for 2 hours at 200 r.p.m. at 37ºC, to remove microglia. The medium was collected and discarded, fresh media was added to each flask and the flasks were placed in the incubator with 5% CO_2 at 37ºC for 1 hour to equilibrate the pH. The flasks were shaked once again, this time at 220 r.p.m. overnight at 37ºC to detach OPC. The cell suspension from each flask was transferred to an untreated Petri dish (1 Petri dish per flask) and incubated at 37ºC for 2-3 hours to allow an efficient attachment of contaminant microglia and astrocytes to the Petri dish and ultimately isolate OPCs. As OPCs are not adherent in these untreated Petri dishes, these were collected from the Petri dishes in the cell suspensions and transferred to 50 mL centrifuge tube. Cells were centrifuged at 300g for 3 minutes, the supernatant was discarded and the pellet was resuspended in approximately 1 mL of OPC SATO 1× (**Table IV**). Live cells were finally observed under optical microscope and counted by Trypan Blue assay.

A summary of dissection, plating and culture of neonatal cortices (A) until OPC isolation and culture is displayed (B) in **Figure 9**.

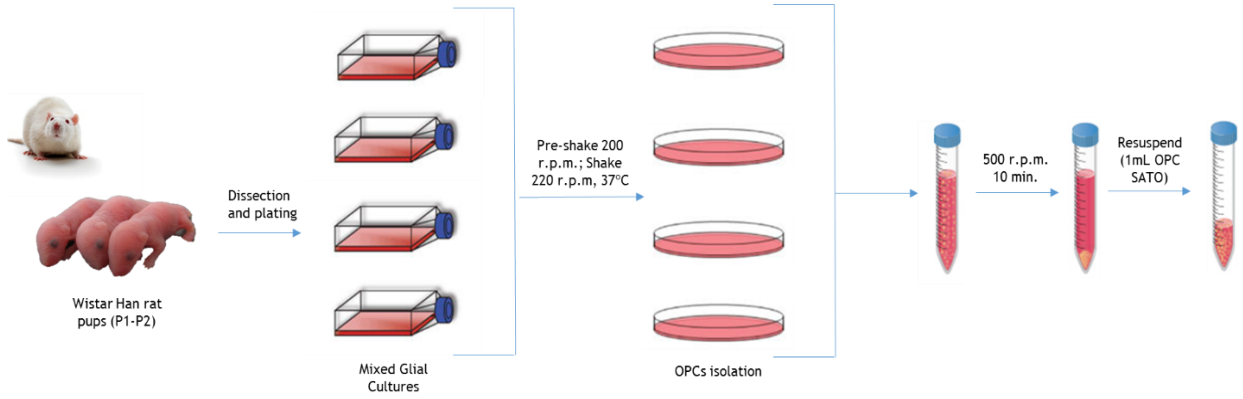


Figure 9. Summary of dissection, plating and culture of neonatal rat cortices until OPC isolation and culture.

Table IV. Summary of media compositions.

Media nomenclature	Base	Additional components
Complete DMEM	DMEM-(1x) Glutamax (Gibco)	10% FBS (F7524, Sigma-Aldrich) + 1% P/S
		100 mg Transferrin (T8158, Sigma-Aldrich) 100 mg BSA (Merck) 16 mg Putrescine (Sigma-Aldrich)

SATO 10× (150 mL)	DMEM-(1×) Glutamax (Gibco) (up to 150 mL)	60 µL Progesterone (1 mg/mL ethanol) (Sigma-Aldrich) 40 µL Na Selenite (1 mg/mL 0.1 NaOH) (Sigma-Aldrich) 40 µL Thyroxine (1mg/mL 0.1 NaOH) (Sigma-Aldrich) 30 µL Triiodo L-thyronine (1mg/mL 0.1 NaOH) (Sigma-Aldrich)
OPC SATO 1× (Proliferation medium)	DMEM-(1×) Glutamax (Gibco) + SATO 10×	10% P/S (10 mg/mL), Human insulin (final concentration 5 µg/mL) + human PDGFα(AA) (final concentration 10 ng/mL) + human FGF (basic) (final concentration 10 ng/mL)
OL SATO 1× (Differentiation medium)	DMEM-(1×) Glutamax (Gibco) + SATO 10×	10%Penicillin Streptomycin (10 mg/mL), Human insulin (final concentration 5 µg/mL) + FBS

2.2. OPC culture in fibrin hydrogels

2.2.1. Preparation of fibrin hydrogels

Prior to be used in the preparation of fibrin gels, fibrinogen was dissolved in ultra-pure water, dialysed against tris-buffered saline (TBS, pH 7.4), sterile-filtered, and diluted to 12 mg/mL with sterile TBS.

Polymerizing fibrin hydrogels, formed by applying equal volumes of a solution of plasminogen-free fibrinogen from pooled human plasma and a thrombin solution(all Sigma), containing $CaCl_2$ (Merck) and the bi-domain peptides HYD1, T1 or HYD1+T1 (GenScript), into the lower wells of a µ-Slide 8 Well ibiTreat(Ibidi®) (final concentration of fibrin components: 2, 4, 6, or 8 mg/mL fibrinogen; 2 NIH U/mL thrombin from human plasma; 2.5 mM $CaCl_2$; 5, 10 or 20 µM of the bi-domain peptides) (**Figure 10**).

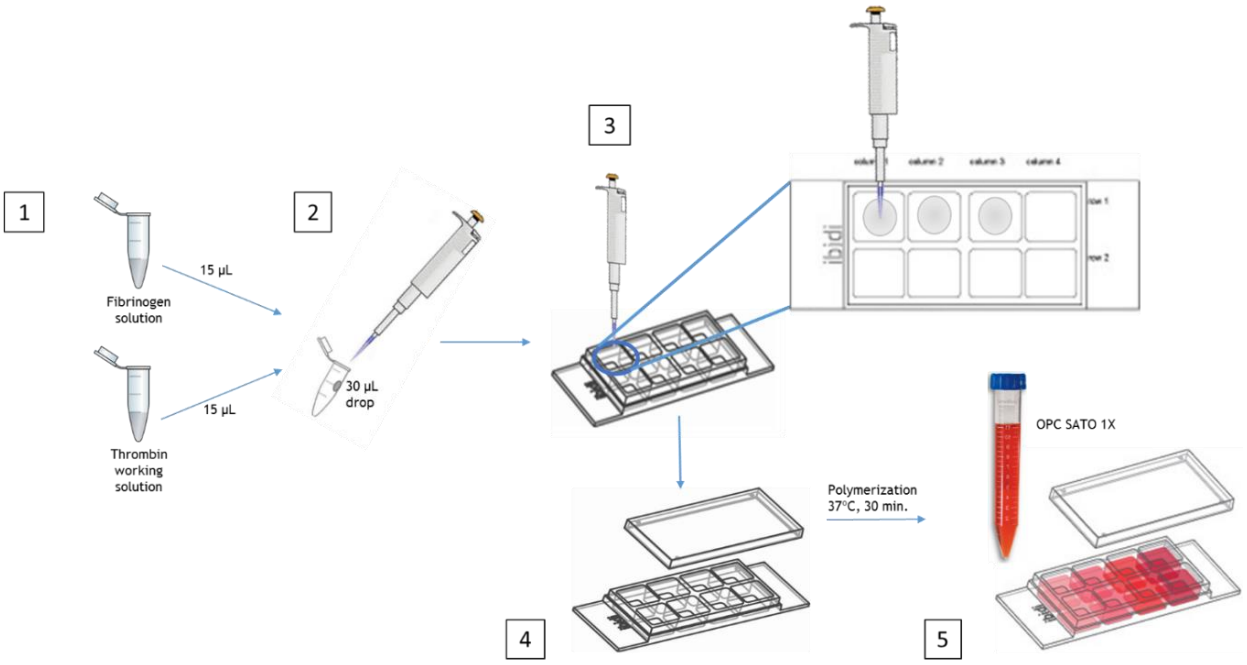


Figure 10. One-drop system for preparing 30µL Fibrin drops.

1 and 2. Take 15µL of the fibrinogen solution and place it on the lateral side of the Eppendorf; take 15µL of the thrombin working solution and add it to the fibrinogen drop;

3 Carefully transfer the 30 µL of fibrin polimerizing solution to a well of the µ-slide 8 Well ibiTreat;

4 Allow to polymerize for 30 minutes at 37ºC;

5 Add OPC SATO 1× supplemented with 5 µg/mL Aprotinin.

After being centrifuged at 200g for 3 minutes at 4ºC (eppendorf centrifuge 5417R) OPCs were resuspended in the correspondent fibrinogen solution prior polymerization. Further incubation at 37ºC with CO_2 for 30 min ensured full polymerization due to fibrinogen crosslinking by factor XIIIa (present in fibrinogen solution).

At the end of the polymerization period, 300 µL of OPC SATO 1× supplemented with 5 µg/mL Aprotinin from bovine lung (Sigma) were added to each well of the µ-Slide 8 well After 2 days under proliferation conditions, the proliferation medium was switched to a differentiation medium, OL SATO 1×. The differentiation medium supplemented with aprotinin was changed every 2 days until the end of the culture period.

2.2.1.1.Preparation of unmodified fibrin hydrogels

To evaluate the effect of the fibrinogen concentration on OPC behavior, fibrin gels with fibrinogen concentrations ranging from 2 – 8 mg/mL were prepared by poly

OPCs were embedded in fibrin hydrogels and cultured for 9 days, 2 days under proliferation conditions and 5 days under differentiation conditions.

At the end of the culture period, the effect of fibrinogen concentration on cell viability and process extension were assessed.

2.2.1.2. Preparation of fibrin hydrogels functionalized with T1, HYD1 and HYD1+T1

Fibrin gels were functionalized with HYD1 peptide, T1 peptide (GenScript) and a combination of both HYD1 and T1 peptides. Peptides were covalently bound to fibrin using the enzymatic cross-linking action of transglutaminase factor XIIIa. Fibrin gels with peptide concentrations of 5 μM, 10 μM and 20 μM were prepared.
OPCs were embedded in fibrin hydrogels functionalized with α6β1 ligands and cultured for 5 days, 2 days under proliferation conditions and 3 days under differentiation conditions. At the end of incubation period the effect of bi-domain peptides concentration on OPC process extension and myelin membrane formation was assessed.

2.3. Immunocytochemistry

2.3.1. F-actin/DNA fluorescent staining

Cells were fixed with 2% paraformaldehyde (Merck) in culture medium for 30 minutes in the incubator, washed and stored in PBS at 4ºC, until being immunostained. Cells were permeabilized with 0.2% (v/v) Triton X-100 (T-8787; Sigma) in PBS (P-3813; Sigma) for 45 minutes and washed with PBS. To minimize non-specific staining, the samples were incubated with 1% (w/v) Bovine Serum Albumin (BSA) solution (Merck) in PBS for 2-3 hours as a blocking solution, followed by the incubation with Alexa Fluor 594 phalloidin (Molecular Probes) diluted 1:40 in the 1% BSA solution overnight at 4ºC, in the dark and under stirring (50 r.p.m.). After washing with PBS, the samples were incubated with 0.1 μg/mL 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma). Finally, the samples were washed with PBS then again and kept in Fluor FluoromountTM Aqueous Mounting Medium (Sigma) at 4ºC in the dark until analysis in the high throughput fluorescence microscope InCell Analyzer 2000 (GE Healthcare).. Alexa Fluor® 594 phalloidin is a toxin with high-affinity for F-actin conjugated with red fluorescent Alexa Fluor® 594 dye (Excitation/Emission: 581/609 nm).

2.3.2. Immunostaining of a-tubulin and oligodendrocyte specific nuclear marker Olig2

Cells were fixed with 2% paraformaldehyde (Merck) in culture medium for 30 minutes in the incubator, washed and stored in PBS at 4ºC, until being immunostained.

Cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 45 minutes and washed with PBS. The samples were incubated with a blocking buffer of 5% Normal Goat Serum (NGS) (Sigma) and 3% Donkey Serum (DS) (Sigma) in PBS for 2-3 hours to minimize non-specific staining of the antibodies prior to the incubation with the primary antibodies: mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich) diluted 1:2000 and rabbit anti-Olig2 polyclonal antibody (Merck-Millipore) diluted 1:500 in PBS containing 5% NGS and 3%

DS, overnight at 4ºC, in the dark and under stirring (50 r.p.m.). After washing with PBS containing 5% NGS and 3% DS, the samples were incubated with the secondary antibodies: Alexa Fluor® 488 conjugate F(ab')2-Goat anti-Mouse IgG (H+L) Secondary Antibody, (Invitrogen) and Alexa Fluor® 647 conjugate Donkey anti-Rabbit IgG (H+L) Secondary Antibody (Invitrogen), both diluted 1:1000 in PBS containing 5% NGS and 3%DS for 6 hours at 4ºC. After washing with PBS, nuclei were stained with DAPI diluted 1:10000 in PBS. The samples were washed with PBS and finally kept in Fluoromount™ Aqueous Mounting Medium at 4ºC in the dark until CLSM observation.

2.3.3. Immunostaining of α-tubulin and oligodendrocyte specific Myelin Basic Protein (MBP)

Cells were fixed with 2% paraformaldehyde (Merck) in culture medium for 30 minutes in the incubator, washed and stored in PBS at 4ºC, until being immunostained.

Cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 45 minutes and washed with PBS. The samples were incubated with a blocking buffer of 5% NGS in PBS for 2-3 hours to minimize non-specific staining of the antibodies prior to the incubation with the primary antibodies: mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich) diluted 1:2000 and MBP chicken polyclonal anti-peptide antibody mixture (100 µg/mL) (MBP, Aves Labs Inc.) diluted 1:500 in PBS containing 5% NGS, overnight at 4ºC under stirring (50 r.p.m.). After washing with PBS containing 5% NGS, the samples were incubated with the secondary antibodies: Alexa Fluor® 594 conjugate Goat anti-Mouse IgG (H+L) Secondary Antibody, (Invitrogen) and Alexa Fluor® 488 conjugate Goat anti-Chicken IgY (H+L) Secondary Antibody, both diluted 1:1000 in PBS containing 5% NGS for 6 hours at 4ºC. After washing with PBS, nuclei were stained with DAPI diluted 1:10000 in PBS (final concentration: 0.1 µg/mL). The samples were washed with PBS and finally kept in Fluoromount™ Aqueous Mounting Medium at 4ºC in the dark until CLSM observation.

2.3.4. Cell viability assay

OPCs viability in 3D fibrin gels was assessed by incubating cells with Calcein AM and Propidium Iodide (PI) (Sigma) after 9 days of culture (2 days under proliferation and 7 days under differentiation. Hoechst 33342 Fluorescent Stain was used to stain DNA. Calcein AM freely diffuses into the cells, and is hydrolyzed by nonspecific esterases into fluorescent products that are retained by cells with intact plasma membranes. Propidium iodide (PI) is used as counterstain to identify nonviable cells. PI readily enters and stains cells with compromised membranes but cannot cross the membranes of viable cells.

A 2.8 µg/mL Hoechst 33342 (Molecular Probes) solution in PBS was initially prepared. Briefly, the cell/fibrin constructs were incubated with 2 µM of Calcein AM (Invitrogen Molecular Probes) diluted in the Hoechst 33342 solution, for 20 minutes at 37ºC in the dark. After removing this solution, cells were incubated with 2 µM of PI diluted in PBS for 10

minutes at 37ºC in the dark. Finally, pre-warmed OL SATO 1× supplemented with 5 µg/mL aprotinin was added and the samples were observed under CLSM as soon as possible (Calcein AM excitation/emission: 488/530 nm; PI excitation/emission: 535/617 nm).

2.3.5. Apoptosis assay

OPCs apoptosis and cell death was assessed using Annexin V-FITC Apoptosis Detection Kit (Biotool) after 9 days of culture. This kit uses recombinant annexin V conjugated to green-fluorescent fluorescein isothiocyante (FITC) dye to detect the externalization of phosphatidylserine in apoptotic cells and PI to detect necrotic cells by fluorescence microscopy. After incubation with these probes, apoptotic cells show green fluorescence and dead cells show red and green fluorescence, while live cells show little or none.

Cells were incubated with Annexin V-FITC and PI Staining solution both diluted 1:20 in 1x Binding Buffer for 20 minutes at room temperature. After the incubation period, cells were first washed with cold PBS and then kept in 1x Binding Buffer on ice until CLSM observation, which was performed as soon as possible.

2.4. Process outgrowth quantification

To assess if both fibrinogen concentration in the gels and functionalization with bi-domain peptides had significant effects in process outgrowth, IN Cell Developer Toolbox software was used for the acquisition of 3D images of the fibrin gels.

IN Cell Analyzer 2000 (GE Healthcare) is a widefield automated high throughput microscope. These systems are designed to provide the user with the throughput and performance necessary for both high content analysis and screening.

Since the purpose was to observe the OPC process extension in a 3D environment, the bottom of the gel, more specifically the interface where the gel meets the plastic surface of the well, was avoided in the acquisition whenever possible. The top and the bottom of the gels were found by performing sequential focus planes up and down in the DAPI channel until nuclei stopped being seen clearly. The mean between the top and the bottom of the gel was calculated. By considering an offset=0, a z position is obtained, which is subtracted to the mean. The value obtained is the offset to be considered in the acquisition. For each condition tested, 21 z-stacks of 64-72 fields were acquired, using a magnification of 20×0.75 Pan Apo.

The number of processes in OPCs was quantified using the Freehand Line function of ImageJ software, as shown in **Figure 11**. Only $Olig2^+$ cells were considered for the quantification.

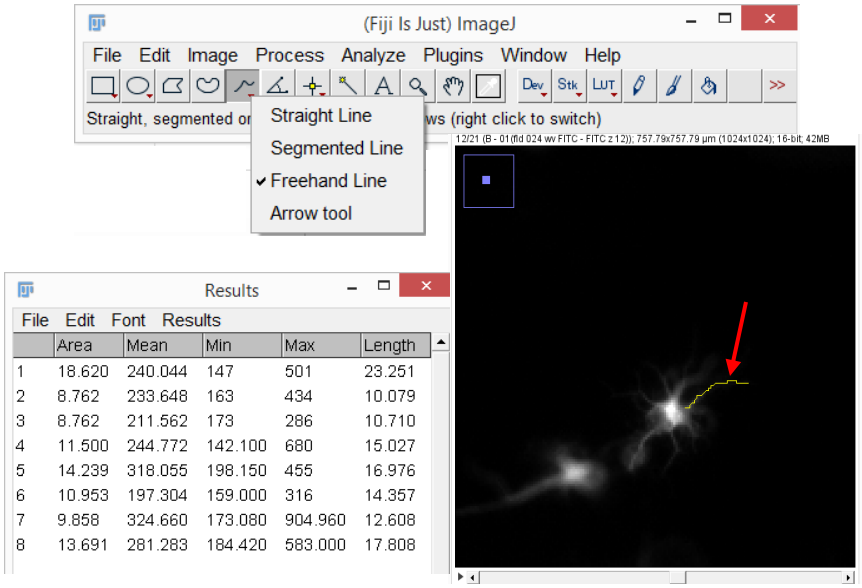


Figure 11. Manual OPC process counting and measurement using Freehand Line (in yellow) and Measure function from ImageJ software.

2.5. Apoptotic and dead cell quantification

To quantify the results correspondent to cell viability and apoptosis, the function Spot Detector from Icy Bio-Imaging Software was used. The Spot detector allows the user to count cells by detecting spots in noisy 3D images by defining parameters such as the input sequence, the type of detector, the scale and the type of filtering. A batch of files corresponding to CLSM z-stacks of the Hoechst 33342 channel, the PI channel or Annexin channel were loaded into the program as input sequences. After selecting the scale and the size of spots to be detected, the computation starts, displaying an image with the detection markers and a binary image for control as output. The output data is directly exported to Excel. With these data, the percentage of dead cells and apoptotic cells could be calculated. Cell viability (%) for each concentration was calculated by image analysis of the 2-D PI/DNA projected stacks, counting the total cell number (Hoechst 33342) and dead cells (PI) using the spot detector function from Icy Software and subtracting the obtained percentages to 100% to obtain the percentage of viable cells. A similar principle was used to calculate the percentage of apoptotic cells, using 2-D Annexin V/DNA projected stacks.

2.6. Statistical Analysis

Fibrin gels prepared with different fibrinogen concentrations were compared using *one-way ANOVA* followed by *the Bonferroni post-hoc test for multiple comparisons* (whenever homogeneity of variances was observed) or the *Kruskal-Wallis* non-parametric test (in case of homogeneity of variances was not observed) followed by the the *Mann-Whitney* U test for two sample comparisons.

Statistically significant differences between functionalized fibrin gels and unmodified gels (control) were assessed using the unpaired *t-test*. Functionalized fibrin gels were compared among them using *one-way ANOVA*.

Statistical treatment of data was performed using IBM® SPSS® Statistics Software (version 20). Results were considered significant for $p < 0.05$.

Chapter 3

Results

3.1. Optimization of OPC 3-D culture in fibrin hydrogels

For optimization of OPC 3-D cell culture in fibrin hydrogels, different cell seeding densities and aprotinin concentrations were initially investigated.

For this purpose, OPCs were seeded in fibrin hydrogels (prepared with fibrinogen concentrations ranging from 2 to 8 mg/mL) at 0.5, 0.75, 1.0, and 1.25 × 10^6 cells/mL of fibrin hydrogel and cultured for 7 days in the presence (1 or 5 µg/mL) or absence of aprotinin, a cell protease inhibitor. At the end of the culture period, the cell/fibrin constructs were processed for F-actin/DAPI staining, to qualitatively assess cell distribution and process extension within fibrin hydrogels. Results correspondent to OPCs entrapped in fibrin hydrogels prepared with 4 mg/mL of fibrinogen and cultured in the presence of 5 µg/mL of aprotinin are presented in **Figure 12**.

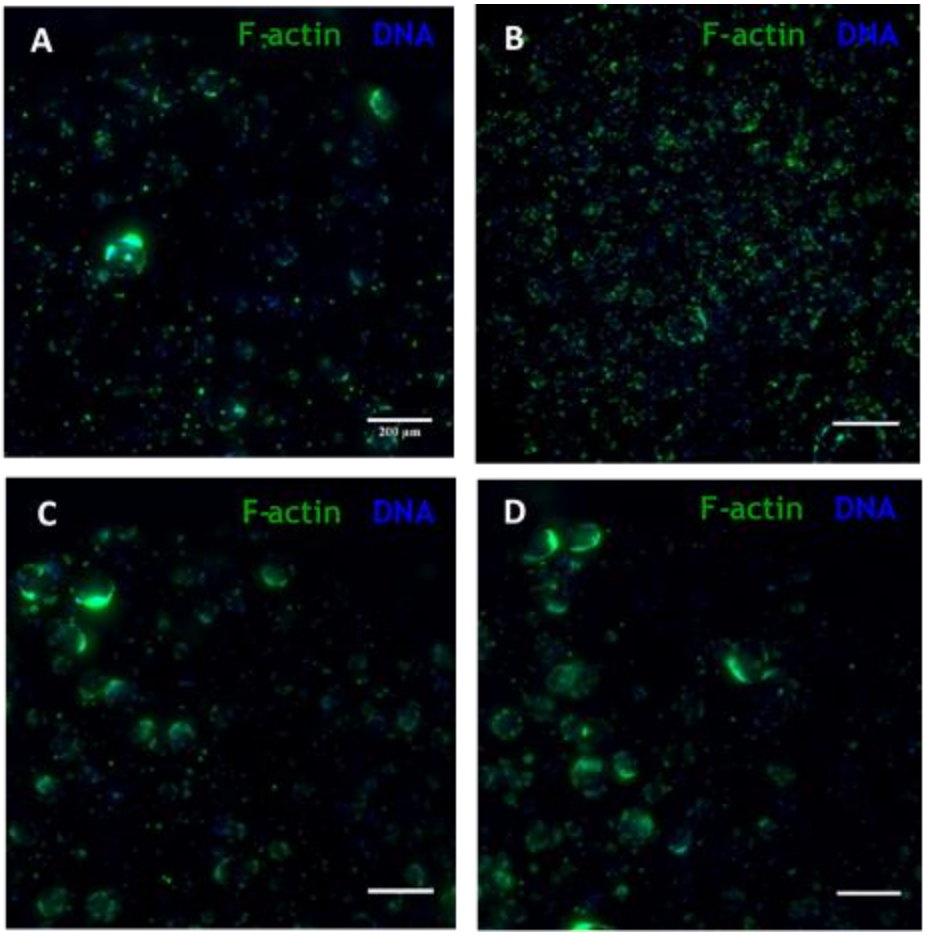


Figure 12. Distribution of OPCs within 3-D fibrin gels after 7 days of cell culture, as a function of the cell seeding density: (A) 0.5 × 10^6 cells/mL; (B) 0.75 × 10^6 cells/mL; (C) 1 × 10^6 cells/mL; (D) 1.25 × 10^6 cells/mL. Cell/fibrin constructs were cultured for 7 days, 2 days under proliferation conditions and 5 days under differentiation conditions in the presence of aprotinin (5 µg /mL), and subsequently processed for fluorescent labelling of and F-actin (in green) and DNA (in blue). Representative 2-D projections of stitched InCell Analyzer images of cell/fibrin constructs prepared with 4 mg/mL of fibrinogen and covering a depth of approximately~100 µm, are presented. Scale Bar: 200 µm.

Results revealed a trend for the presence of cell clusters with radially-migrating OPCs and fibrin degradation halos (the latter better observed under phase contrast microscopy) with increasing cell seeding densities (**Fig. 12**). Although the initial OPC suspension was mainly constituted by single cells, cell clusters could also be detected, suggesting that the observed cell clusters result of incomplete cell dissociation during the OPC isolation procedure. Increasing cell seeding densities led to the presence of more cell clusters. Since fibrin hydrogels seeded with 0.75×10^6 cells/mL presented a homogeneous distribution of cells and very few cell clusters as compared to hydrogels seeded with higher cell numbers, (**Fig. 12B**), this cell seeding density was selected for all subsequent assays. Aprotinin has shown to be required to avoid overall fibrin degradation. In its absence, fibrin hydrogels completely degraded overtime, independently from the initial cell seeding density and fibrinogen concentration tested. A 5 µg/mL aprotinin concentration was found to be sufficient to delay fibrin degradation while allowing fibrin degradation by migrating cells and process extension better observed at higher magnifications).

3.2. Effect of fibrinogen concentration on OPC behavior

Although for certain clinical applications such as in hemostatic plugs, fibrin hydrogels with high fibrinogen concentrations are desirable (due to their shorter gelation time at physiological conditions and higher resistance to enzymatic degradation), for use as a matrix for cell ingrowth and tissue engineering, fibrin gels with viscoelastic properties matching those of the native tissue are required, due to cell mechanosensing. Fibrin gels too stiff may hinder OPC survival, proliferation and differentiation. As among fibrin components, fibrinogen concentration is the one affecting fibrin structural and viscoelastic properties at a greater degree [68], the fibrinogen concentration leading to better retention of OPC viability and differentiation was first established. The selected concentration was be used for the functionalization studies.

3.2.1. Effect of fibrinogen concentration on OPC process outgrowth

OPC differentiation into myelinating OLs in a 3-D environment was evaluated by analyzing the process extension. To assess the effect of fibrinogen concentration on OPC process outgrowth in fibrin gels, OPCs were embedded in fibrin hydrogels prepared with increasing fibrinogen concentrations (2, 4, 6, and 8 mg/mL), and cultured for 9 days, 2 days with proliferation medium (OPC SATO 1×) and 7 days in differentiation medium (OL SATO 1×). At the end of this period, process extension was evaluated in cell/fibrin constructs previously processed for immunofluorescent labelling of α-tubulin and Olig2. An oligodendrocyte-specific nuclear marker (Olig2, a transcription factor promoting the formation of OPCs and their differentiation into OLs at later stages of development) was used to identify OLs, as primary cultures of OPCs might also contain astrocytes

and microglial cells as contaminants. Representative 2-D projections of CLSM stack images of cell/fibrin constructs are presented in **Figure 13**. Cellular processes containing α-tubulin (in green) were observed in all fibrin hydrogels, independently of the fibrinogen concentration, indicating that all hydrogels were permissive to OPC process extension. Still, for the lowest fibrinogen concentration tested (2 mg/mL), only a thin layer of fibrin matrix remained at the end of the cell culture period, regardless of medium supplementation with 5 μg/mL of aprotinin. This observation suggests that fibrin hydrogels prepared with 2 mg/mL of fibrinogen are not suitable for the 3-D culture of OPCs when considering a cell seeding density of 0.75×10^6 cells/mL, due to its fast degradation by cell-secreted proteases. Among the fibrin hydrogels tested, those correspondent to 4 and 6 mg/mL of fibrinogen revealed more frequently $Olig2^+$ cells extending processes, while those of 8 mg/mL revealed mostly cells with round morphology and extending few processes, as well as a tendency for cell aggregation.

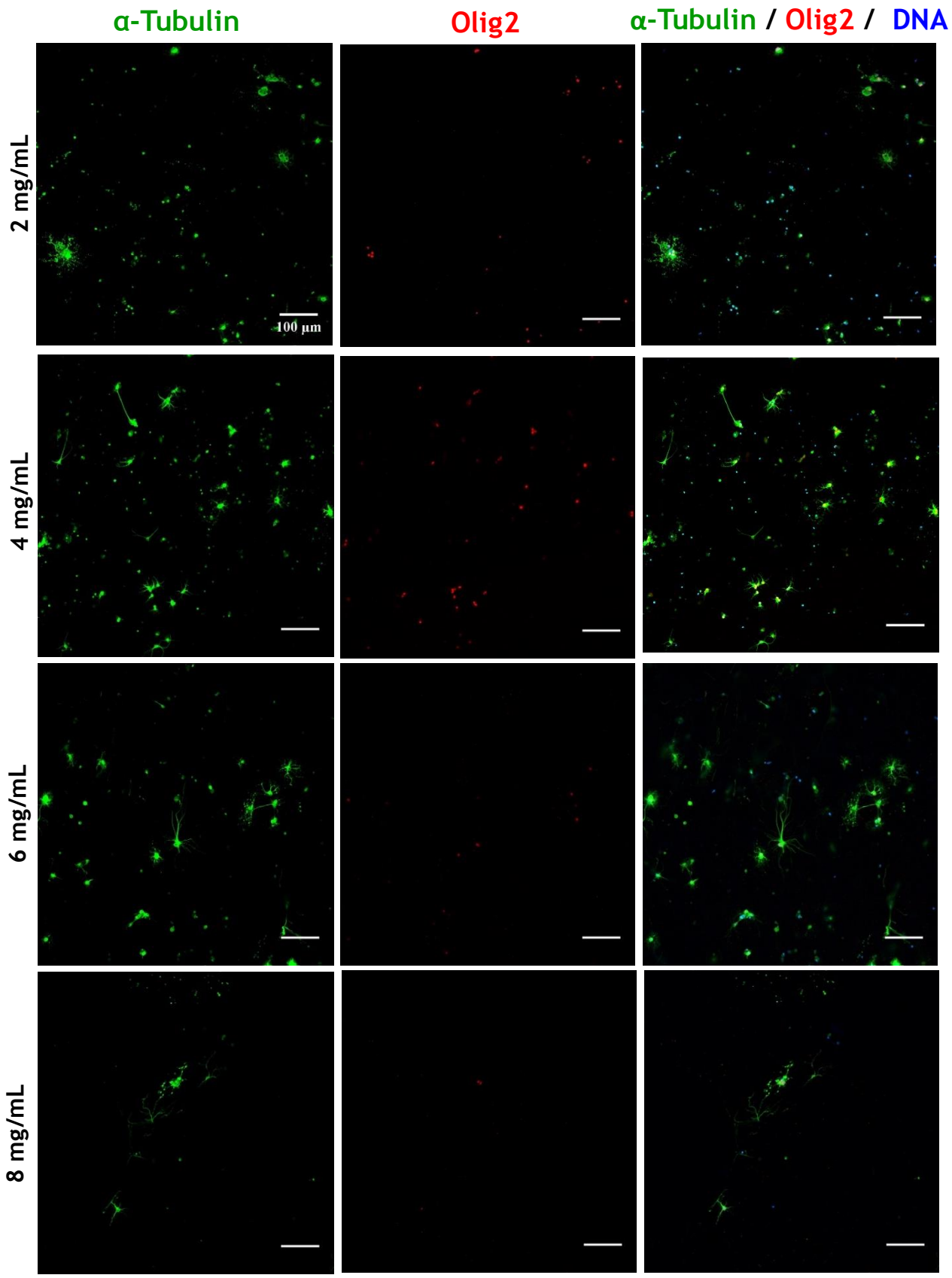


Figure 13. Immunocytochemistry analysis of OPC process extension in fibrin hydrogels as a function of fibrinogen concentration. Cell/fibrin constructs were cultured for 9 days (2 days under proliferation conditions and 7 days under differentiation conditions) and subsequently processed for immunofluorescent labelling of α-

tubulin/olig2. Representative 2-D projections of CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are presented. Scale Bar: 100 µm.

Following the qualitative analysis of 2-D CLSM projections suggesting that fibrin gels prepared with 4 mg/mL and 6 mg/mL of fibrinogen were more permissive to OPC process extension, quantitative image analysis was performed. Three parameters were evaluated to quantify process outgrowth: process number, maximum length and average length. The results obtained are presented in Figure 14.

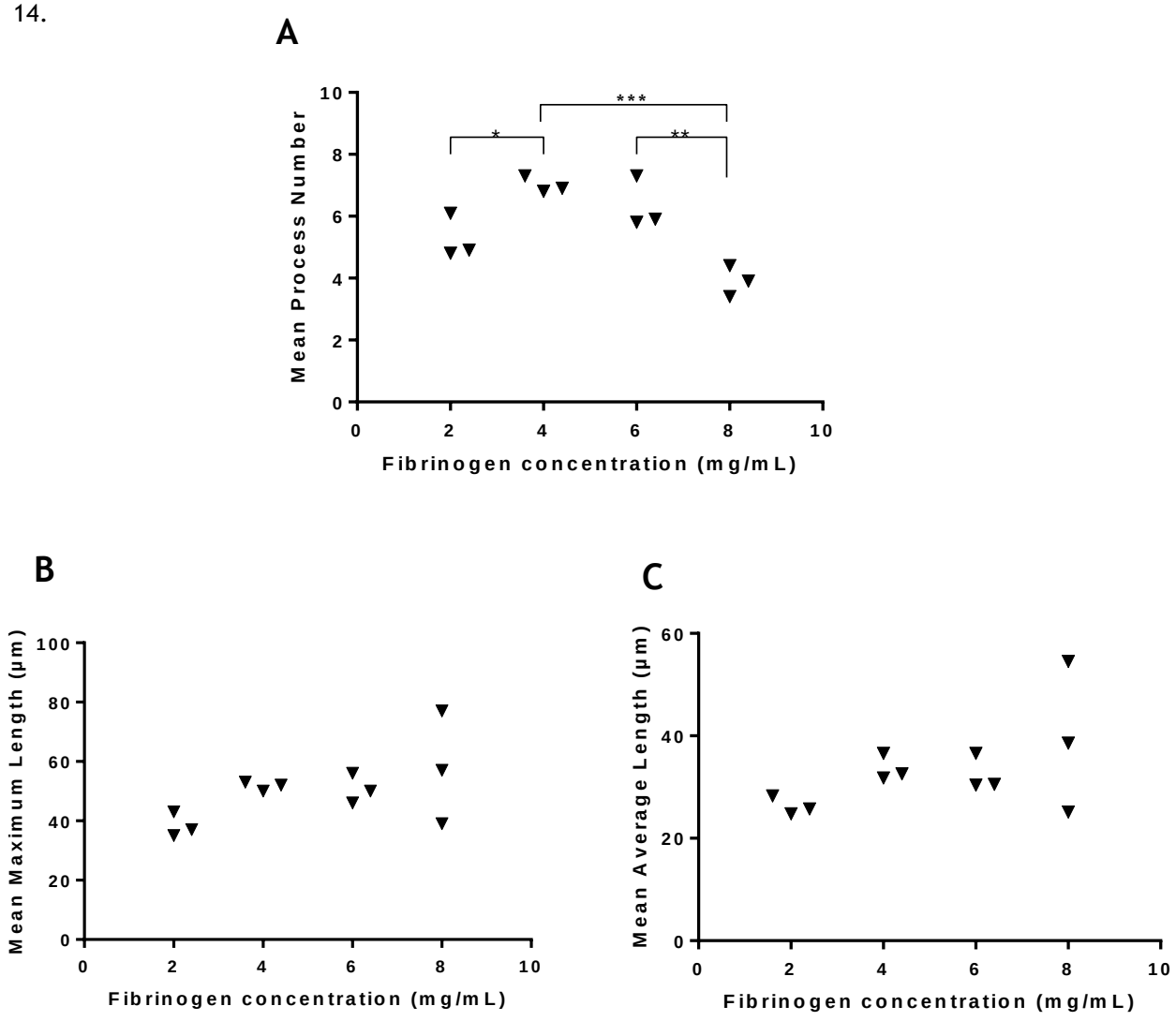


Figure 14. Quantitative analysis of process outgrowth of OPCs cultured in fibrin hydrogels, as a function of fibrinogen concentration. Cell/fibrin constructs were cultured for 9 days, 2 days under proliferation conditions and 7 days under differentiation conditions, and subsequently processed for immunofluorescent labelling of α-tubulin and olig2. Process outgrowth was determined in InCell Analyzer stack images of cell/fibrin constructs covering a depth of approximately~200 µm, by image analysis. Only cells expressing olig2 were considered for the analysis. To evaluate process extension three parameters were considered: (A) Mean process number; (B) Mean maximum length; (C) Mean average length. In each experiment, 45-60 cells were analyzed in two replicate cultures. Mean values from three independent experiments are shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$, as determined using the *one-way ANOVA* test followed by *Bonferroni post-hoc test for multiple comparisons*).

Fibrin hydrogels prepared with the lowest fibrinogen concentration tested (2 mg/mL) revealed a trend to have cells with lower number of processes per cell as well as with lower values of maximum length and average length, when compared to fibrin hydrogels prepared with 4 mg/mL of fibrinogen. The higher degradation rate observed in these gels may partially explain these differences, since cells presenting well developed processes had to be excluded from the analysis for being too close from the bottom of the wells.

Among the fibrin hydrogels tested, those prepared with 4 mg/mL and 6 mg/mL of fibrinogen showed the highest mean of number of processes (7.0 ± 0.3 and 6.3 ± 0.7, respectively; **Fig. 14A**), in line with the qualitative CLSM analysis of OPC sprouting in fibrin (**Fig. 13**). Although fibrin gels prepared with 4 mg/mL of fibrinogen presented a higher mean of number of cellular processes as compared to gels with 6 mg/mL of fibrinogen, this difference was not found to be statistically significant. Also when considering the mean of the maximum length and of the average length, higher values were found for fibrin gels prepared with 4 mg/mL of fibrinogen than for gels with 6 mg/mL of fibrinogen, although no significant differences were found (**Fig. 14B and C**). Increasing the fibrinogen concentration to 8 mg/mL led to a decrease of the mean of the number of processes (3.9 ± 0.5), a decrease which was found to be statistically significant when compared to fibrin gels with 4 or 6 mg/mL of fibrinogen. Nevertheless, the increase of fibrinogen concentration to 8 mg/mL did not significantly impact the mean of the maximum length or of the average process length (**Fig. 14B and C**).

Apart from comparing the mean values of the different process outgrowth parameters from each independent experiment, process outgrowth values pooled from the three independent experiments were also compared. Results are presented in **Figure A1 (Appendix A)**. In line with the previous analysis, increasing the fibrinogen concentration to 8 mg/mL resulted in a significant decrease of the number of cellular processes when comparing to fibrin gels with 4 or 6 mg/mL of fibrinogen (**Fig. A1.A**). Also in accordance with the previous analysis, the increase in fibrinogen concentration to 8 mg/mL did not significantly influence the two other process outgrowth parameters evaluated (maximum process length and average process length) (**Fig.A1.B and A1.C**).

3.2.2. Effect of fibrinogen concentration on OPC viability

Cell viability of OPCs embedded in fibrin hydrogels prepared with increasing fibrinogen concentrations (2, 4, 6, and 8 mg/mL) was assessed at the end of 9 days of cell culture (2 days under proliferation and 7 days under differentiation conditions), incubating the cell/fibrin constructs with Calcein AM/PI, and Hoescht 33342. Representative 2-D projections of CLSM stack images are shown in **Figure 15A**.

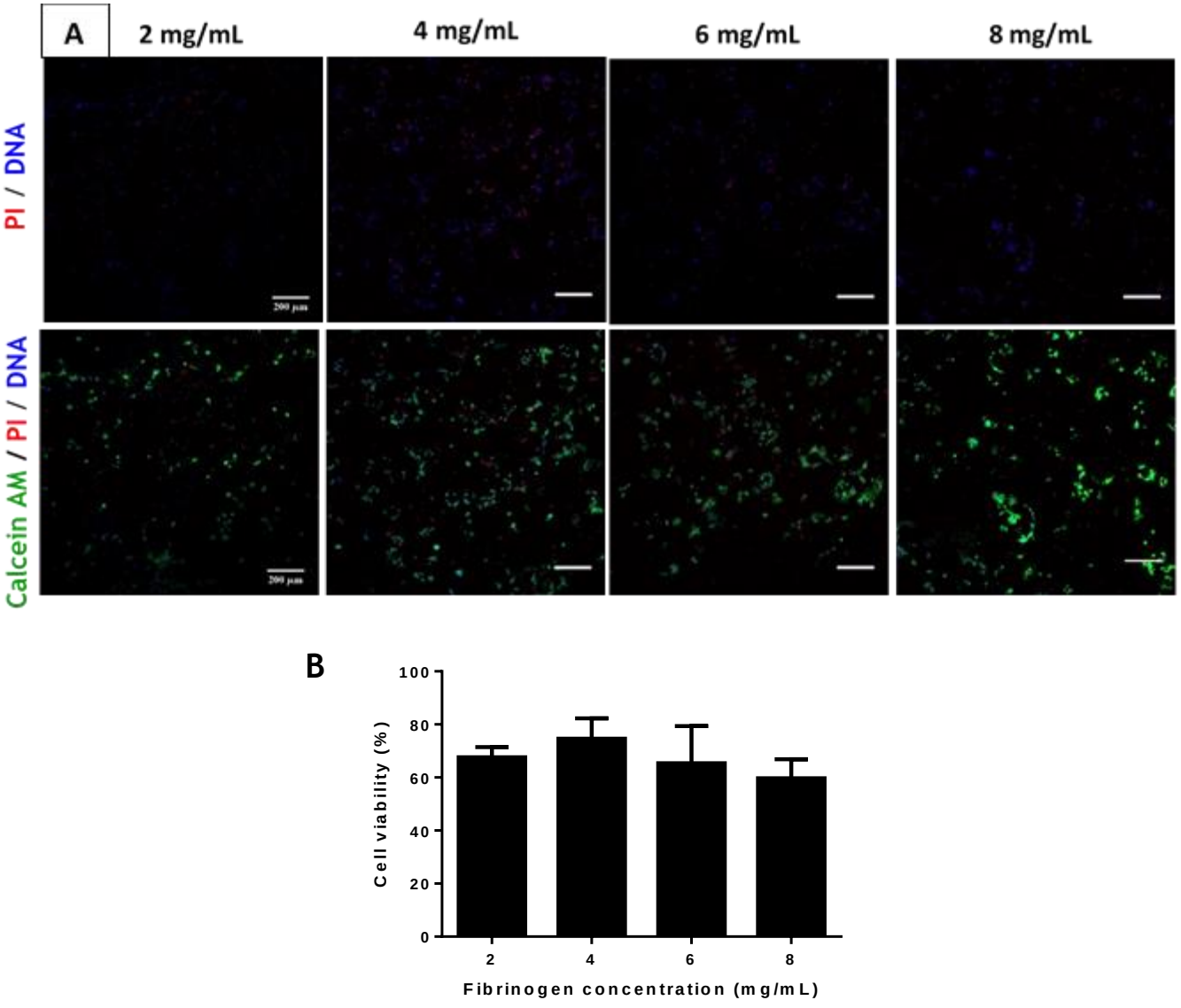


Figure 15. Cell viability of OPCs cultured in fibrin hydrogels, as a function of fibrinogen concentration. Cell/fibrin constructs were cultured for 9 days, 2 days under proliferation conditions and 7 days under differentiation conditions, and subsequently incubated with Calcein AM, PI and Hoescht 33342, for detection of live (in green), dead (in red), and DNA (in blue), respectively. (A) Representative 2-D projections of CLSM stack images of cell/fibrin constructs covering a depth of approximately 100 µm. Scale Bar: 200 µm. (B) Percentage of viable cells, as determined by image analysis of the 2-D projected CLSM stacks. Results from three independent experiments are shown (mean ± standard deviation). Results were not considered statistically significant (One-way ANOVA test).

The percentage of viable cells was estimate by image analysis, subtracting the number of PI+ cells to the total cell number. Image analysis of CLSM stack images revealed for fibrin gels prepared with 4 mg/mL of fibrinogen the highest percentage of viable cells (74 ± 7 %) while the lowest (60 ± 7 %) was observed for gels prepared with 8 mg/mL of fibrinogen, the highest fibrinogen concentration tested. Still, the fibrinogen concentration was not found to alter significantly OPC viability, within the range of fibrinogen concentration tested.

As OPCs cultured in 3-D fibrin gels prepared with 4 mg/mL of fibrinogen revealed better overall *in vitro* biological performance, this fibrinogen concentration was used in the functionalization studies.

3.3. Effect of immobilized α6β1 ligands on OPC behavior in 3-D fibrin hydrogels

Fibrin gels were functionalized with bi-domain peptides known to interact specifically with α6β1 integrin and the effect of immobilized α6β1 ligands on OPC process outgrowth, myelin membrane formation, and apoptosis, evaluated.

3.3.1. Effect of immobilized α6β1 ligands on OPC process outgrowth

To assess the effect of immobilized α6β1 ligands on OPC process outgrowth, OPCs were embedded in fibrin hydrogels (4 mg/mL of fibrinogen) functionalized with increasing molar concentrations (5, 10 and 20 μM) of bi-domain peptides (HYD1, T1, and the combination of HYD1 with T1), and cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions). Process outgrowth was evaluated at day 5 of cell culture (instead of day 9 of cell culture), to avoid overlapping of cell processes extending between neighboring cells, which could compromise image analysis of OPC projections.

OPC process outgrowth was evaluated in cell-fibrin constructs previously processed for immunofluorescent labelling of α-tubulin and Olig2. Representative 2-D projections of CLSM stack images of cell/constructs are presented in **Figure 16**. OPC process extension in fibrin hydrogels was not hindered by functionalization with bi-domain peptides, independently of the immobilized peptide and of the peptide concentration, since $Olig2^+$ cells with cellular processes containing α-tubulin (in green) were observed in all functionalized hydrogels.

The quantitative analysis of process outgrowth (mean process number per cell, mean maximum length and mean average length) is presented in **Figure 17**. Functionalization of fibrin with HYD1 bi-domain peptide resulted in a 1.4-fold increase in the mean number of cellular processes ($p < 0.05$), already for the lowest peptide input concentration tested (5 μM; **Fig. 17.A**). Still, increasing the concentration of bi-domain peptide from 5 μM to 10 or 20 μM did not result in a significant increase of the mean number of cellular processes, as assessed by one-way ANOVA. Immobilization of HYD1 also resulted in an increase in the mean of maximum length, although statistically significant

differences were not observed. Similarly, when considering the mean of the average length, no statistically significant differences were found between HYD1-functionalized fibrin hydrogels and unmodified gels, regardless of the input HYD1 bi-domain peptide concentration.

Functionalization of fibrin with T1 bi-domain peptide also revealed to be effective in increasing the mean number of cellular processes (1.3-fold increase, $p < 0.005$) and to higher values of maximum process length (1.2-fold increase), already for the lowest peptide input concentration tested (5 μM; **Fig. 17.B**), even though the differences in the mean of maximum length were not considered statistically different. Increasing the concentration of bi-domain peptide from 5 μM to 10 or 20 μM did not result in a significant increase either of the number of cellular processes or of the maximum process length. Following the trend observed for HYD1-functionalized gels, no statistically significant differences were found between T1-functionalized fibrin hydrogels and unmodified gels in terms of average process length, independently of the input T1 bi-domain peptide concentration.

Functionalization with both the bi-domain peptides (HYD1 and T1) led to an increase in the number of cellular processes (1.4-fold increase, $p < 0.05$) for the lowest input concentration tested (5 μM; **Fig. 17.C**). The functionalization with a combination of both peptides also led to higher values of maximum process length (1.13-fold increase) although the differences were not considered statistically different. Still, when considering this input concentration, no additive/synergetic effect of the two bi-domain peptides was found, since the values of the mean of the process number and of the maximum process length found in fibrin gels functionalized with a single bi-domain peptide do not differ significantly from those found in gels functionalized with both the bi-domain peptides (**Fig.18**). Once more, increasing the concentration of bi-domain peptides from 5 μM to 10 or 20 μM did not result in a significant increase either of the number of cellular processes or of the maximum process length (**Fig. 17.C**). Finally, no statistically significant differences were found between (HYD1+T1)-functionalized fibrin hydrogels and unmodified gels in terms of average process length, independently of the input bi-domain peptides concentration.

Apart from comparing the mean values of the different process outgrowth parameters from each independent experiment, process outgrowth values pooled from the three independent experiments were also compared. Results are presented in **Figure F1 (Appendix F)**. In line with the previous analysis, the functionalization with HYD1, T1 or the combination of both bi-domain peptides was successful in promoting an increase in the number of cellular processes, for the lowest peptide concentration 5 μM (**Fig. F1.A**). Also in accordance with the previous analysis, no additive/synergetic effect of combining the two bi-domain peptides was found (Supplementary Data – Figure F2).

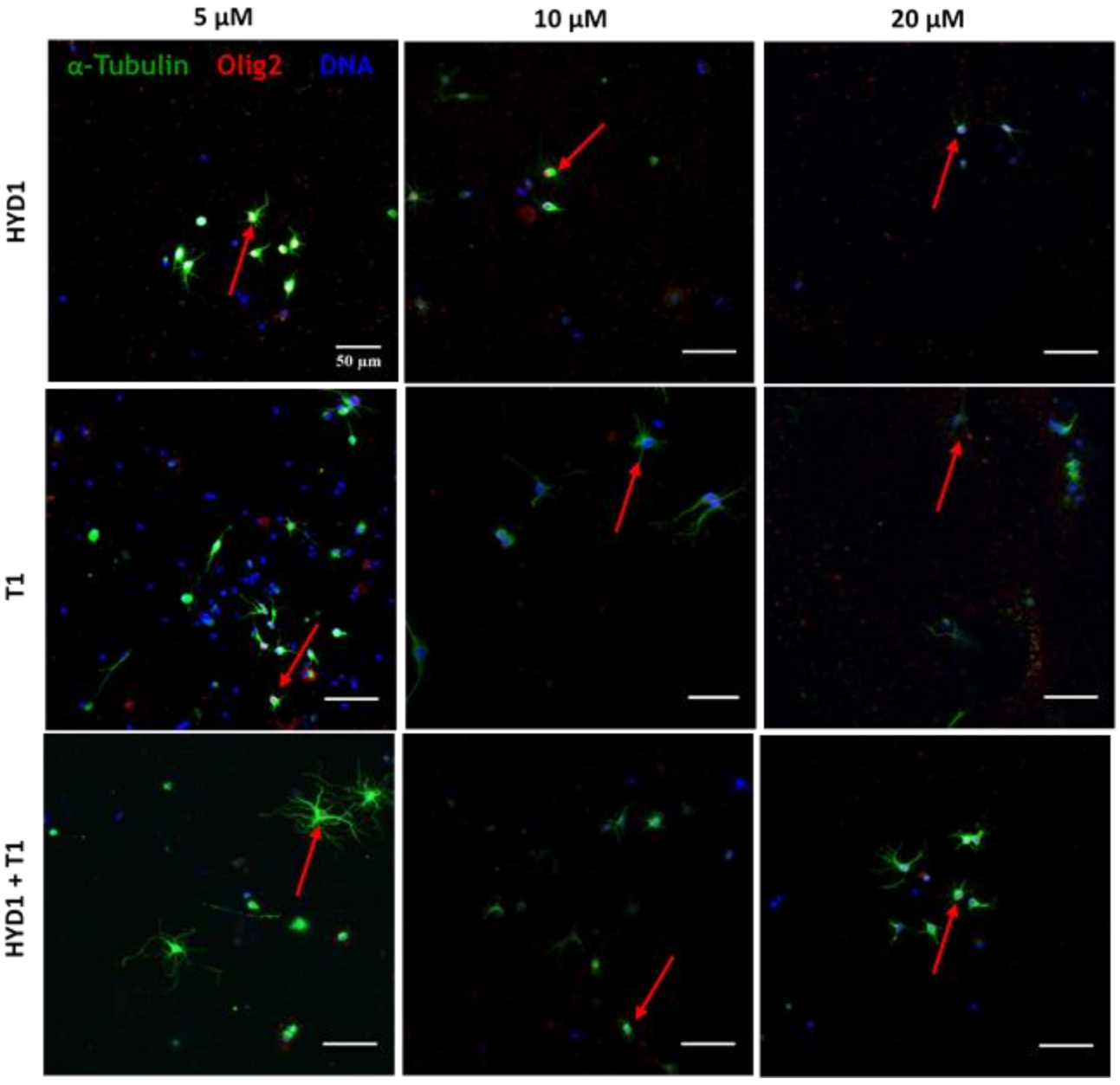


Figure 16. Immunocytochemistry analysis of OPC process extension in 4 mg/mL fibrin hydrogels functionalized with bi-domain peptides (HYD1, T1 and HYD1+T1), as a function of peptide input concentration in polymerizing gels. Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions) and subsequently processed for immunofluorescent labelling of α-tubulin (in green)/olig2 (in red). Representative 2-D projections of CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are presented. Red arrows indicate $Olig2^{+}$ cells. 2-D projections of split CLSM stack images are presented in **Supplementary Data – Appendix C, D and E.** Scale Bar: 50 µm.

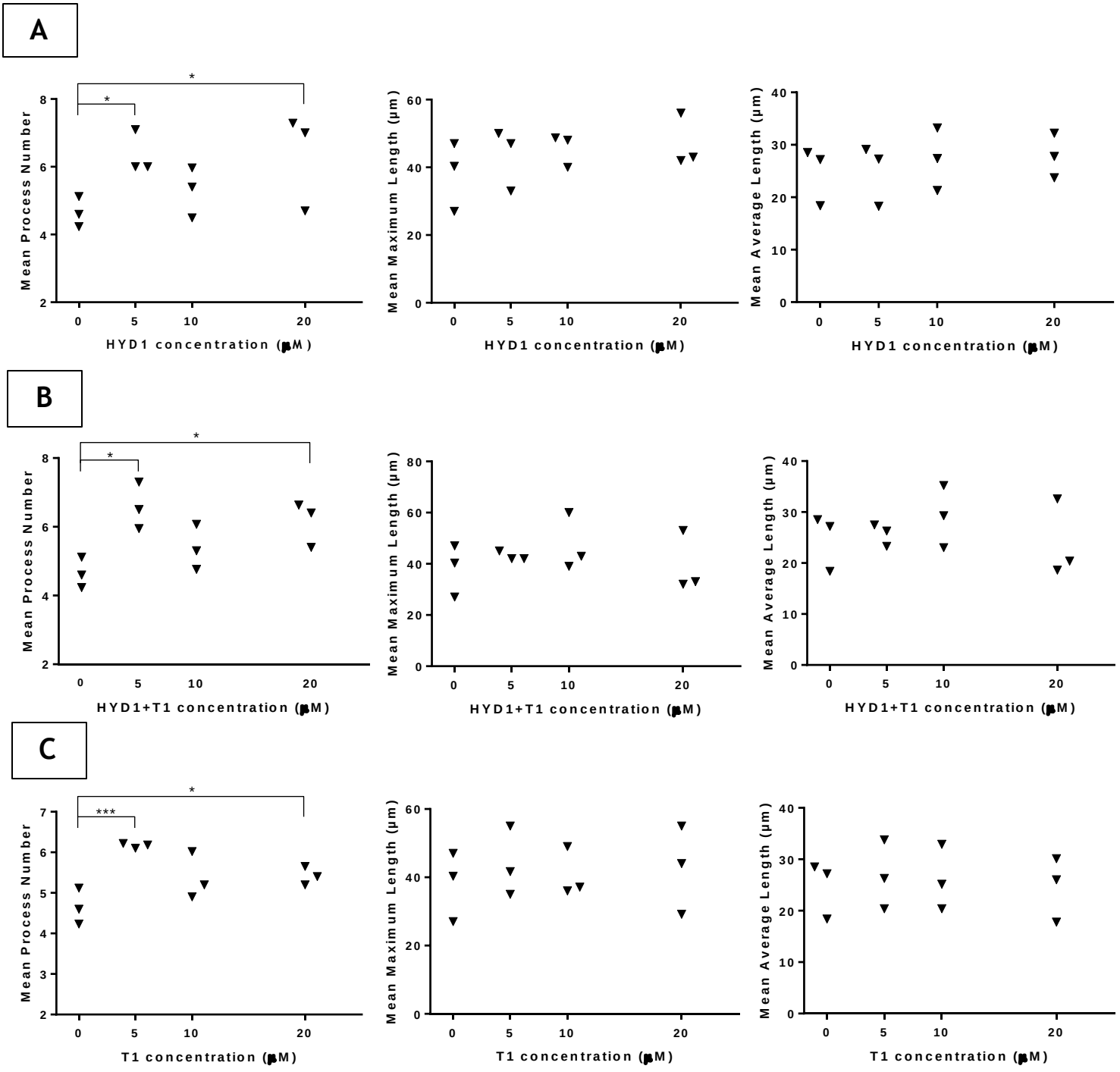


Figure 17. Process outgrowth quantitative analysis of OPCs cultured in fibrin hydrogels (4 mg/mL of fibrinogen) functionalized with bi-domain peptides, as a function of peptide input concentration in polymerizing gels: (A) HYD1; (B) T1 and (C) HYD1+T1. Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions), as assessed by image analysis of InCell Analyzer z-stacks covering a depth of approximately 200 μm. To evaluate process extension three parameters were considered: (A) Mean process number; (B) Mean maximum length; (C) Mean average length. In each experiment, 45-60 cells were analyzed in two replicate cultures. Mean values from three independent experiments are shown. Process outgrowth in functionalized fibrin hydrogels was compared to that in unmodified gels (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$). Functionalized gels were compared among them and no statistically significant differences were found (as assessed by *one-way ANOVA*).

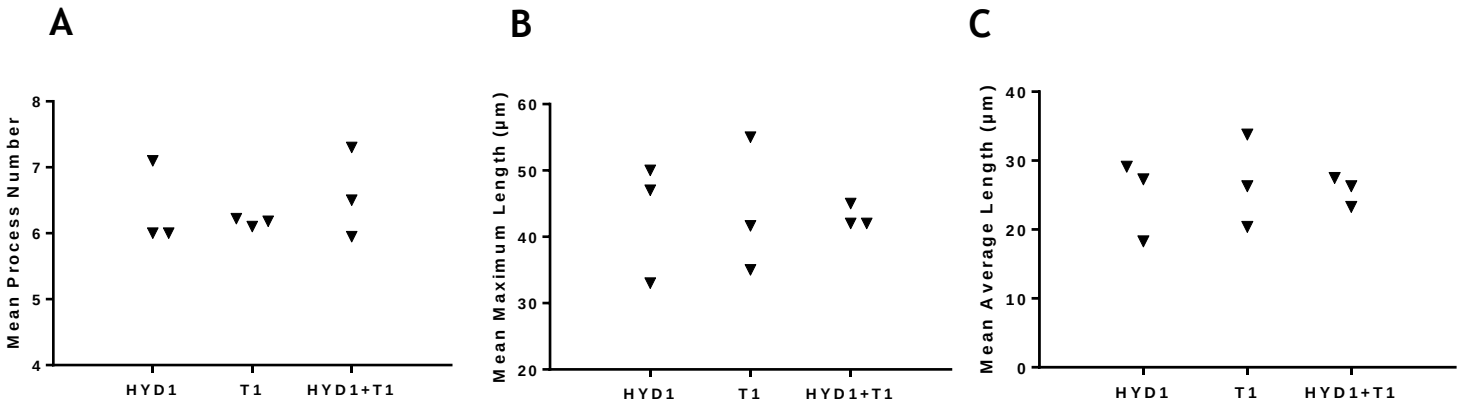


Figure 18. Process outgrowth quantification of OPCs cultured in 4 mg/mL fibrin hydrogels functionalized with 5 µM of bi-domain peptides (HYD1, T1 and HYD1+T1). Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions), as assessed by image analysis of InCell Analyzer z-stacks covering a depth of approximately 200 µm. Three parameters were considered to evaluate process extension: (A) Mean process number; (B) Mean maximum length; (C) Mean average Length. In each experiment, 45-60 cells were analyzed in two replicate cultures. Mean values from three independent experiments are shown. Results were not considered statistically different as assessed by *one-way ANOVA*.

3.3.2. Effect of immobilized α6ß1 ligands on myelin membrane formation

To assess the effect of immobilized α6ß1 ligands on OL myelin production, OPCs were embedded in fibrin hydrogels (4 mg/mL of fibrinogen) functionalized with increasing molar concentrations (5, 10 and 20 µM) of bi-domain peptides (HYD1, T1, and the combination of HYD1 with T1), and cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions). Myelin deposition was evaluated at day 5 of cell culture in cell/fibrin constructs previously processed for immunofluorescent labelling of α-tubulin and myelin basic protein (MBP), an important differentiation marker exclusively expressed by myelinating OLs in the CNS, localized on the cytoplasmic surface of the plasma membrane and myelin membrane. Representative 2-D projections of CLSM stack images of cell/fibrin constructs are presented in **Figure 19**.

Myelin membrane formation in fibrin hydrogels was not hindered by functionalization with bi-domain peptides, independently of the immobilized peptide and of the peptide concentration, since cells expressing MBP (in green) were observed in all functionalized hydrogels. This can be better observed in 2-D projections of split MBP channel (green) stack images presented in **Supplementary data - Appendix G**.

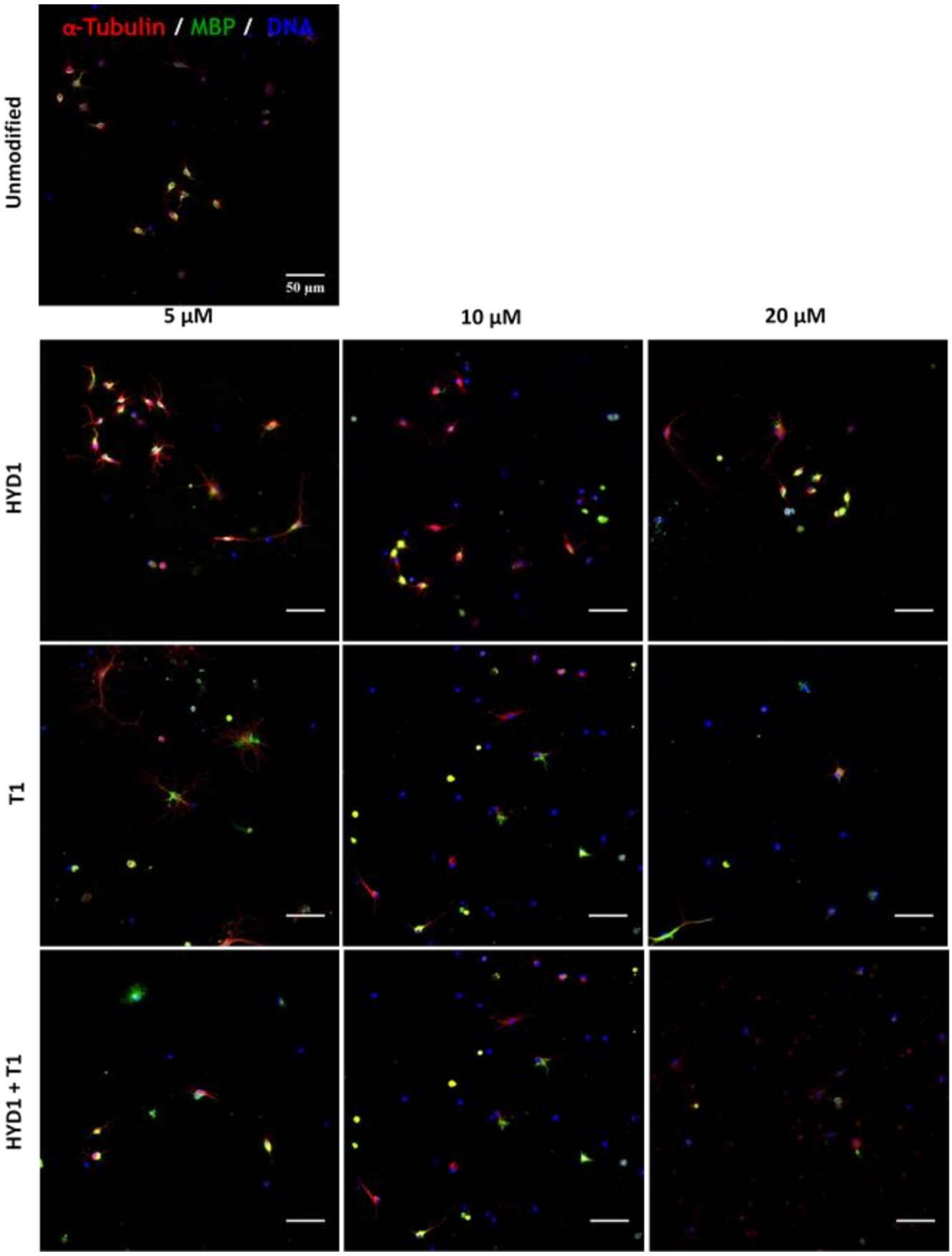
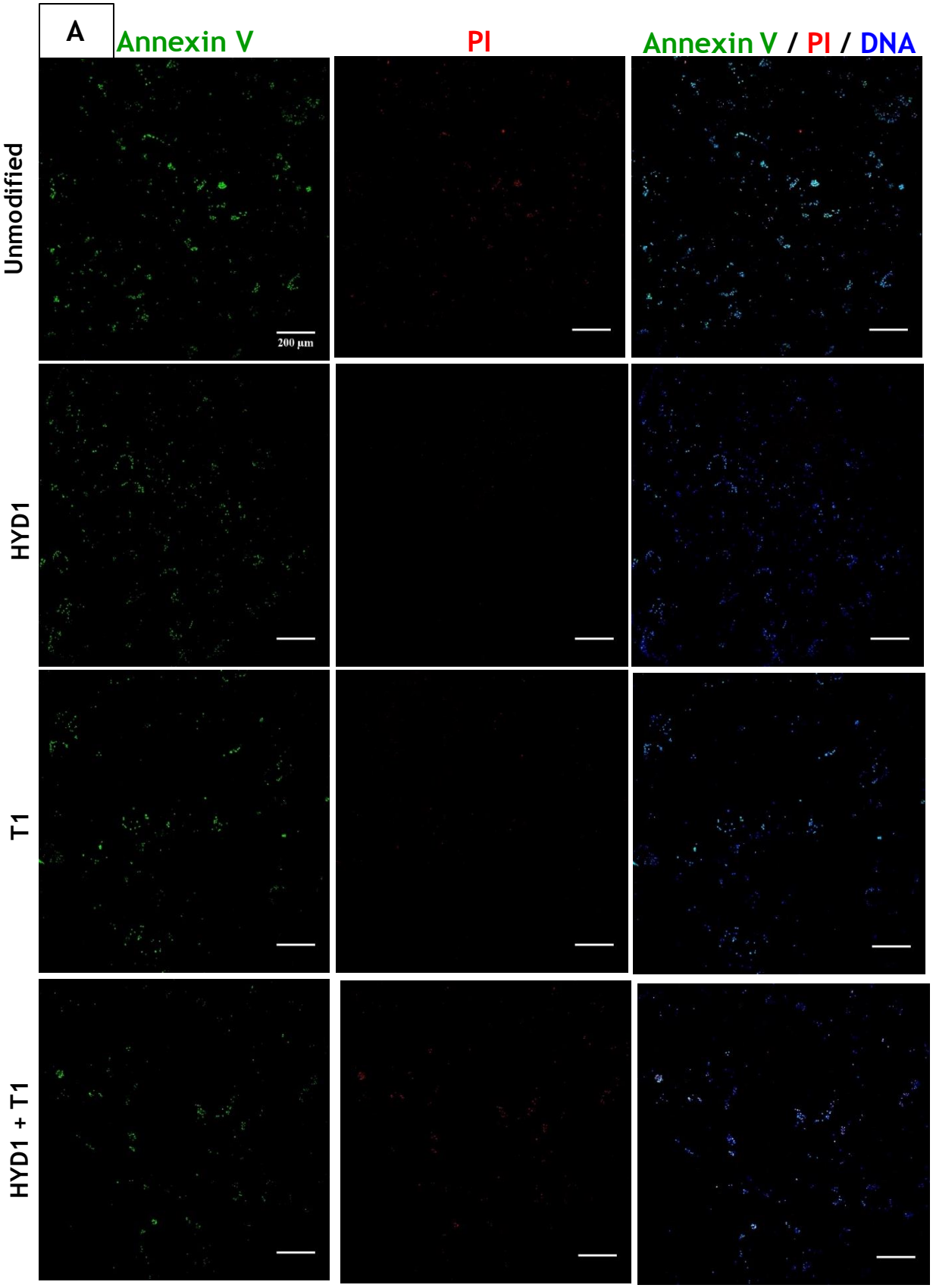


Figure 19. Immunocytochemistry analysis of OL myelin production in 4 mg/mL fibrin hydrogels functionalized with bi-domain peptides (HYD1, T1 and HYD1+T1), as a function of peptide input concentration in polymerizing gels. Cell/fibrin constructs were cultured for 5 days, 2 days under proliferation and 3 days under differentiation

conditions, and subsequently processed for immunofluorescent labelling of α-tubulin (in green) and anti-MBP (in red). Nuclei are shown in blue. Representative 2-D projections of CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are shown. 2-D projections of split MBP channel (green) stack images are presented in Supplementary Data – Appendix G. Scale Bar: 50 µm.

3.3.3. Effect of immobilized α6ß1 ligands on OPC apoptosis

An apoptosis assay was performed to address if functionalization of fibrin hydrogels with α6ß1 ligands could compromise OPC viability. In this assay, early apoptotic cells were stained with annexin V and dead cells with PI. Apoptosis was evaluated in OPCs cultured in fibrin hydrogels (4 mg/mL of fibrinogen) functionalized with 5 µM of bi-domain peptides (HYD1, T1 and the combination of HYD1 with T1), at the end of 9 days of cell culture (2 days under proliferation conditions and 7 days under differentiation conditions). Representative 2-D projections of CLSM stack images of cell/fibrin constructs incubated with annexin V and PI are shown in **Figure 20A**. Image analysis revealed for unmodified fibrin 46% of early apoptotic cells, and for functionalized fibrin hydrogels, percentages of apoptotic cells ranging from 30 to 47 %. Among functionalized fibrin hydrogels, those functionalized with HYD1 showed the lowest percentage of apoptotic cells (30%). The percentage of PI^+ cells in unmodified fibrin was 15%, and among functionalized hydrogels, those functionalized with HYD1 revealed the lowest percentage of dead cells (4%). Still, in gels functionalized with both HYD1 and T1, 38% of dead cells were found. Nevertheless, it should be noted that these results correspond to a single experiment and no obvious conclusions can be obtained from this data, as statistical analysis could not be performed.


A
Annexin V
PI
Annexin V / PI / DNA
Unmodified
HYD1
T1
HYD1 + T1
200 µm

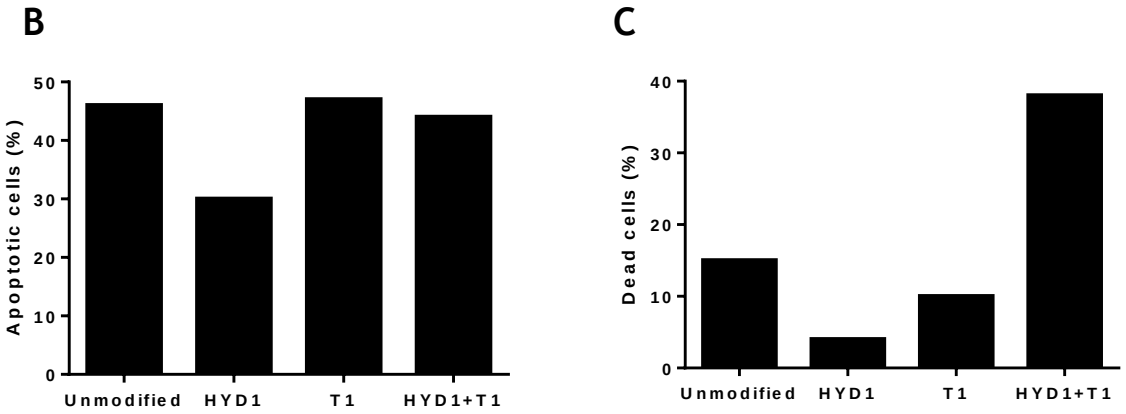


Figure 20. (A) Apoptotic analysis of OPCs cultured in unmodified 3-D fibrin hydrogels (4 mg/mL of fibrinogen) or fibrin hydrogel functionalized with 5 μM of HYD1, T1 and HYD1+T1. OPCs were cultured for 9 days (2 days under proliferation and 7 days under differentiation conditions). Cell/fibrin constructs were incubated with Annexin V (in green) and PI (in red), for detection of early apoptotic and dead cells, respectively. Nuclei were stained with Hoechst 33342 (in blue). Representative 2-D projections of CLSM stack images covering a depth of approximately 100 μm are presented. Scale Bar: 200 μm. (B and C) Percentage of apoptotic and dead cells, as assessed by image analysis of the 2-D projections of CLSM stacks. The results correspond to a single experiment (N=1).

Chapter 4

Discussion

Remyelination of CNS lesions associated with demyelinating disorders such as MS and SCI is still a challenging field. Natural remyelination mechanisms are not efficient in advanced stages of demyelination, eventually preventing neuronal function recovery.

As previously referred, transplantation of OPCs has emerged as a feasible therapy to promote remyelination of spared and regenerating axons after CNS damage. The transplantation of OPCs in the form of cell suspensions presents drawbacks such as low cell survival and poor proliferation and differentiation, which are related with the lack of an adequate mechanical and biochemical support. As an attempt to overcome these limitations, the combination of cell transplantation with biomaterial-based matrices stands up as a promising solution namely by providing a supportive environment for transplanted OPCs to survive and differentiate. [8, 42]

Although many hydrogels have been developed for cell delivery into the CNS, to date, only a few were explored for OPC delivery, among them, fibrin. [*Li et al. (2013); Asmani et al. (2013)*] In opposition to synthetic hydrogels, fibrin presents natural ligands for cell adhesion (two pairs of RGD sites and a pair of AGDV sites), proteolytic enzymes, growth factors, ECM proteins and protease inhibitors.

OPCs and OLs express a limited repertoire of integrins, including the laminin receptor α6β1 integrin, which has shown to enhance cell survival of myelinating oligodendrocytes and to promote myelination of axonal tracts *in vivo* [17,33,34]. Although fibrin contains binding sequences for integrins $\alpha_{IIB}\beta_3$, $\alpha_V\beta_3$, $\alpha_M\beta_2$ and $\alpha_X\beta_2$, it does not present natural ligands for integrin $\alpha_6\beta_1$ [68].

Therefore, the objective of this study was to assess OPC behavior in 3-D fibrin hydrogels containing covalently-bound α6β1 ligands. The functionalization of fibrin hydrogels with synthetic peptides with reported affinity for α6β1 integrin was expected to improve fibrin biospecificity towards OPCs, namely by promoting cell survival, process outgrowth and myelin membrane production *in vitro*, and ultimatly enhance cell survival of transplanted OPCs and theirability to remyelinate axons *in vivo*.

Initially, different cells seeding densities (0.5×10^6 cells/mL, 0.75×10^6 cells/mL, 1×10^6 cells/mL and 1.25×10^6 cells/mL) were tested for optimization of the cell seeding density in fibrin gels. A trend for the presence of cell clusters with radially-migrating OPCs and fibrin degradation halos was observed, with increasing cell seeding densities leading to the presence of more cell clusters. Besides presenting very few cell clusters when compared to hydrogels seeded with higher cell numbers, fibrin hydrogels seeded with 0.75×10^6 cells/mL also allowed a homogeneous cell distribution. Therefore, this cells seeding density was selected for further experiments. In addition, aprotinin (5 µg/mL) was shown to be required to avoid overall fibrin degradation.

As cells can sense and respond to the biophysical properties of the microenvironment through mechanotransductional mechanisms, we subsequently assessed the effect of fibrinogen concentration on OPC cell viability and differentiation in 3-D fibrin gels. Process extension greatly varies throughout stages of differentiation from OPCs towards myelinating OLs, since it follows a stepwise morphological transformation. At the first stages of differentiation, OPCs present very few and longer processes. As they start to differentiate into late progenitors called pro-OLs, an increasing in the number of processes is observed. As these immature OLs evolve to mature OLs, they acquire a more complex structure, with a higher number of sprouting processes that are shorter

in length and start to present several branching points, giving these cells a starry-like morphology [85, 86]. With this in mind, a higher process number indicates that OPCs have differentiated into mature OLs, whereas a higher maximum length and average process length mean OPCs still at more immature differentiation stages. Therefore, to assess the effect of fibrinogen concentration on OPC differentiation in 3-D fibrin gels, gels with increasing fibrinogen concentrations (ranging from 2 to 8 mg/mL) were prepared and the effect of fibrinogen concentration on cell viability and process extension evaluated.

Results showed that OPCs were able to extend processes in all fibrin gels, independently from the concentration. Fibrin hydrogels gels prepared with the lowest fibrinogen concentration, 2 mg/mL, revealed a higher degradation rate (even in the presence of 5 µg/mL aprotinin in the medium), which might explain the lower values obtained for the three parameters evaluated (process number, maximum length and average length) when compared to fibrin gels with 4 mg/mL of fibrinogen, since cells presenting well-developed processes had to be excluded from the analysis. Fibrin degradation rate is usually delayed by increasing the fibrinogen concentrations [65], which explains why fibrin gels prepared with 2 mg/mL of fibrinogen were more permissive to cell-secreted proteases.

When a CNS-compatible hydrogel is being developed to be used as a cell carrier in the adult CNS, one may not forget to guarantee the mechanical compliance between the biomaterial and the native tissue. As already mentioned, the properties of fibrin hydrogels can be controlled either by fibrinogen or thrombin concentration, which affects the fibrin network structure and mechanical properties of the hydrogels. Typically, when the fibrinogen concentration is increased, resultant fibrin gels are dense and turbid, composed of thicker fibers. In addition, gel stiffness is also affected by fibrinogen concentration, usually with greater fibrinogen concentrations giving rise to stiffer matrices.[68] The mechanical properties of the fibrin gels used in the present study was previously characterized by *Bento et al (2015, submitted)* (Supplementary data – Appendix B). Since the average pore area (μm^2) was higher for softer fibrin gels (4 mg/mL of fibrinogen) and diminished with the increase of fibrinogen concentration, it can be concluded that fibrin gels obtained from low fibrinogen concentrations are more permissive to cell infiltration. However, when fibrin gels are too soft, these may lack mechanical strength and robustness. In this sense, fibrin gels should be both permissive, allowing OPC infiltration and differentiation, but simultaneously ensure appropriate mechanical properties.

When compared with gels prepared with the highest fibrinogen concentration tested (8 mg/mL) fibrin gels prepared with 4 and 6 mg/mL of fibrinogen led to a 1.8-fold and a 1.6-fold increase in the mean of the process number, respectively. The lower number of cell processes in fibrin gels prepared with 8 mg/mL may be related to the higher stiffness of these gels, which present higher storage modulus (G') (G'= 1130.81 ± 46.10 Pa) when compared to fibrin gels with fibrinogen concentrations of 4 mg/mL (G'= 446.82 ± 50.51 Pa) and 6 mg/mL (G'= 786.86 ± 79.04 Pa), as reported by *Bento et al (2015, submitted)* (Supplementary data – Appendix B). *Asmani et al. (2013)* have already reported that cells respond to the mechanical properties of the environment. In this study, hydrogels with fibrinogen concentrations 2 mg/mL, 3 mg/mL and 4 mg/mL were compared regarding their mechanical properties. While fibrin gels prepared with 2 mg/mL and 3 mg/mL presented a storage modulus G' < 1000 Pa, being suitable for both glial and neuron survival and proliferation,

gels prepared with 4 mg/mL of fibrinogen presented a G' > 1000 Pa, being appropriate only for glial proliferation [66].

Even though the differences were not considered statistically significant, fibrin gels prepared with 4 mg/mL of fibrinogen presented, in average, a higher number of processes per cell as well as higher maximum process length and average process length, when compared to those prepared with 6 mg/mL of fibrinogen (7.0 ± 0.3 and 6.3 ± 0.7, respectively). Increasing the fibrinogen concentration to 8 mg/mL did not significantly impact the maximum process length or the average process length.

Regarding OPC culture in 3D hydrogels, it was previously reported by *Li X. et al. (2013)* [65] that OPCs may exhibit a biphasic change in morphology over increasing elastic modulus of hydrogels. When cultured in hydrogels considered too soft, OPCs displayed round morphology, extending very few processes. In hydrogels of medium stiffness, OPCs were able to spread, resembling the natural morphology and behavior of OPCs in the body. However, as the hydrogel stiffness continued to be increased, cell spreading seemed to decrease, with OPCs assuming once again round morphology and even forming cell aggregates. [65] Thus, considering 4 mg/mL and 6 mg/mL fibrin hydrogels as mid stiffness matrices, it was expected that these fibrinogen concentrations would improve OPC process extension, when compared to stiffer matrices. When the fibrinogen concentration was increased for 8 mg/mL, OPCs started to present more round morphologies with few sprouting processes and started to be more aggregated, which was an expected result, since these hydrogels might be too stiff to allow OPC process extension.

In addition to process outgrowth quantification, it was observed that the fibrinogen concentration did not significantly affect cell viability. Fibrin gels prepared with 4 mg/mL of fibrinogen presented the highest cell viability (74% of viable cells). In what concerns the lower values for cell viability compared to 2-D matrices, these can be explained by the fact that OPCs were cultured in 3-D fibrin hydrogels. When cultured in 2-D, dead cells eventually detach from the surface during medium replacement, whereas in 3-D hydrogels, cells, including non-viable cells, remain entrapped within the 3-Dfibrin network.

Once better overall *in vitro* biological performance of OPCs in terms of process extension and cell viability was observed for fibrin gels prepared with 4 mg/mL of fibrinogen, this fibrinogen concentration was used for further functionalization studies.

In order to enhance the biospecificity of fibrin hydrogels towards α6β1 integrin, these were functionalized with ligands specifically binding to this integrin: the T1 peptide of the angiogenic inducer CCN1, the HYD1 peptide and a combination of both peptides (HYD1+T1). Three peptide input concentration (5 μM, 10 μM and 20 μM), were investigated.

The functionalization of fibrin gels with bi-domain peptides did not hinder the extension of cell processes, as shown by the CLSM analysis of cell/fibrin constructs previously processed for immunofluorescent labelling of α-tubulin and Olig2.

Quantitative image analysis showed that the functionalization with HYD1, T1 or the combination of both bi-domain peptides was effective in promoting an increase in the number of cellular processes (1.4-fold increase *vs* unmodified gels for HYD1 and for the combination (HYD1+T1) and 1.3-fold increase vs unmodified gels for T1 bi-domain peptide), already for the lowest peptide concentration tested (5 μM). Furthermore, functionalization with 5 μM of bi-domain peptides also

led to higher values of maximum length, although statistically significant differences in maximum length were not found. Even though, no additive/synergetic effect resulted from the combination of both bi-domain peptides, in terms of the mean of the process number and of the maximum process length (Fig. 18). Increasing the concentration of bi-domain peptides from 5 μM to 10 or 20 μM did not result in a significant increase of either the number of cellular processes or maximum process length, either when added separately or in combination. In terms of average length, no statistically significant differences were observed between functionalized gels (HYD1, T1 and HYD1+T1) and unmodified ones, independently of the input bi-domain peptide concentration.

While 5 μM of bi-domain peptides were already sufficient to promote OPC process extension in fibrin gels (4 mg/ml of fibrinogen), in case of embryonic stem (ES)-derived neural stem/progenitors, maximal outward migration in fibrin hydrogels (6 mg/mL of fibrinogen) was observed for peptide input concentrations of 20 and 40 μM of HYD1 and T1, respectively. [75] This difference may be associated with the decrease of peptide binding efficiency with increasing fibrinogen concentrations. *Hall and Hubbell (2001)* reported that the amount of incorporation of L1Ig6 (a ligand for αvß3) depended on the fibrinogen concentration: in 2 mg/mL fibrin gels 72 ± 18% of the input iL1Ig6 was covalently incorporated, whereas in 4 mg/mL fibrin matrices the incorporation efficiency of iL1Ig6 decreased to 63 ± 8%. [87] The covalent binding of peptides to fibrin using the cross-linking action of factor XIIIa has been previously explored by *Schense and Hubbell* (1999), and is an effective approach for the binding of peptides with retention of biological activity. Based on this approach, as 4mg/mL of fibrinogen present a lankier and more porous structure, the ability of the bi-domain peptide to penetrate into the structure and bind to the polymerizing fibrin network through the factor XIIIa increases, thus allowing the attainment of higher amounts of immobilized peptides when compared with fibrin gels with 6 mg/mL of fibrinogen. The fact that an increasing trend in the process number was not observed when bi-domain peptide input concentrations were higher than 5 μM may be related with an excess of peptide, which can lead to the saturation of α6ß1 receptors in OLs, hindering the formation of new bindings. Consequently, the excess of bi-domain peptide would lose its ability to connect with OLs and the effect would no longer be felt by these cells. *Hall and Hubbell (2001)* have previously reported this effect by demonstrating that, among the iL1Ig6 (a fusion protein containing the transglutaminase Factor XIIIa-substrate sequence from α2-plasmin inhibitor at the N-terminus) concentration range tested (2.5, 1.2, 0.6, 0.3, and 0.15 μM), the covalent incorporation of iL1Ig6 was saturated at concentrations higher than 2.5 μM iL1Ig6. In fact, *Schense and Hubbell (1999)* have also reported maximal neurite extension (from E8 chicken dorsal root ganglia) for intermediate adhesion site densities and for higher densities neurite outgrowth inhibition, for fibrin gels functionalized with two adhesion domain bioactive sequences (RGD and DGEA. [88]

After evaluating the sprouting ability of OPCs in functionalized fibrin hydrogels, their ability to differentiate into myelinating OLs was assessed. As previously mentioned, MBP is an oligodendrocyte-specific protein, located in myelin membranes, essential for OL morphogenesis at late stages of differentiation. Confocal analysis of cell/fibrin constructs processed for α-tubulin/MBP staining showed that OPCs cultured in 3-D fibrin hydrogels functionalized with HYD1, T1, or with both peptides, apart from being capable of extending processes, were also able to differentiate into myelinating OLs, independently from the bi-domain peptide concentration. Image analysis of InCell

Analyzer stack images will be performed in the near future, to quantitatively assess the effect of immobilized α6β1 ligands on MBP expression.

Finally, since immobilized ligands should not compromise cell viability, OPC apoptosis was assessed. This assay was performed only in fibrin gels functionalized with 5 μM of bi-domain peptides, since higher peptide input concentrations did not result in significant improvement of OPC process outgrowth. Although results from a single experiment were reported, a low percentage of dead cells was observed in both functionalized and unmodified fibrin gels, except for (HYD1+T1)-functionalized gels, in which 38% of dead cells was found. The high percentage of early apoptotic cells observed for both functionalized and unmodified gels (from 30 to 47 %) was associated to the 3-D cell culture environment, which may have prevented the release apoptotic and dead cells from the gel along the cell culture period. Moreover, apoptotic phenomena are involved in differentiation transformations from OPCs to OLs, which may also explain the higher percentages of early apoptotic cells found. [89] Still, as only a single experiment was performed, further studies are necessary.

Chapter 5

Conclusion and Future Perspectives

5.1. Conclusion

The objective of this work was to develop and hydrogel for delivery of OPCs into the CNS. For this purpose, fibrin hydrogels were functionalized with bi-domain peptides with reported affinity for α6β1 integrin. The functionalization with these synthetic ligands was expected to improve biospecificity towards OPCs by promoting process outgrowth and myelin membrane formation *in vitro*.

The culture of OPCs in 3-D fibrin hydrogels was initially optimized in terms of cell seeding density and aprotinin concentration. A cell seeding density of 0.75×10^6 cells/mL allowed a homogenous distribution of OPCs in the hydrogel with few cell clusters, whereas 5 μg/mL of aprotinin were found to be sufficient to delay overall fibrin degradation while allowing fibrin local degradation by migrating cells and process extension. Varying the fibrinogen concentration did not significantly affect cell viability (in the range of concentrations tested, 2 - 8 mg/mL), although higher cell viability was attained for a 4 mg/mL fibrinogen concentration. Fibrinogen concentration was found to impact OPC differentiation, fibrin gels prepared with intermediate fibrinogen concentrations (4 mg/mL or 6 mg/mL) revealing the highest number of processes per cell, an indicator of differentiation. Since the fibrinogen concentration of 4 mg/mL showed a better overall biological performance, this was selected for use in functionalization studies.

Functionalization with HYD1, T1 or the combination of both bi-domain peptides was successful in promoting an increase in the number of cellular processes, already for the lowest peptide concentration 5 μM. In addition, functionalization with 5 μM of bi-domain peptides also led to higher values of maximum length, although statistically significant differences in maximum length were not found. However, increasing the bi-domain peptide concentration in fibrin gels from 5 μM to 10 μM or 20 μM did not result into an increase in process extension. In addition, no additive/synergetic effect was observed when fibrin gels were functionalized with a combination of HYD1 with T1 instead of HYD1 and T1 alone.

Functionalization with α6β1 ligands did not hinder OPC differentiation into myelin-producing OLs. Myelin production by OLs was observed for all HYD1-, T1-, and (HYD1+T1)-functionalized gels, independently of the bi-domain peptide concentration. Apoptosis has shown a high percentage of apoptotic cells for all the conditions tested, with HYD1 peptide showing the lowest percentage of apoptotic cells. Results from a single apoptotic assay performed after 9 days of cell culture showed similar percentages of early apoptotic cells for both functionalized and unmodified gels, as well as similar fractions of dead cells, except for fibrin gels functionalized with both the bi-domain peptides.

5.2. Future perspectives

Taking into consideration the results obtained in this study, future studies are needed not only to increase the robustness of the results obtained, but also to get insight into the effect of immobilized HYD1 and T1 bi-domain peptides on other parameters of OPC behavior in 3-D fibrin hydrogels.

Although significant differences were already obtained using 47-60 cells in the quantitative analysis of process outgrowth, this analysis will benefit from increasing the number of cells analyzed in each independent experiment to 100 cells, to increase the robustness of the results. In addition, other process outgrowth parameters should be assessed, namely primary and secondary branching points, as these can be associated to different stages of OL development.

The percentage of MBP^+ cells expressing Olig2 should also be quantitatively analyzed to assess the effect of the functionalization in OPC differentiation and myelin production.

Cell viability should be performed also in functionalized fibrin gels, to ensure these can be used as a safe platform for OPC survival and differentiation. In addition, cell viability could be assessed at other timepoints, for example at day 5 and day 7 of culture, since OPC viability was reported to decrease with time in 3D fibrin hydrogels by *Asmani et al (2013)*. Also, apoptosis should be assessed to accomplish a total of 3 independent experiments, to obtain more consistent results.

Finally, since significant increase in the number of processes per cell was already found for the lowest bi-domain peptide concentration, fibrin gels with input concentrations of bi-domain peptides lower than 5 μM should be prepared and their effect on OPC behavior evaluated.

Present results indicate that fibrin gels functionalized with α6ß1 ligands are interesting for OPC culture in a 3-D environment and potentially promising for OPC transplantation in an *in vivo* scenario.

References

[1] Nave Klaus-Armin, Myelination and support of axonal integrity by glia. Nature 2010, 468: 244-252.

[2] Miron V.E., Kuhlmann T. and Antel J.P, Cells of the oligodendroglial lineage, myelination, and remyelination. Biochimica et Biophysica Acta 2010, 1812: 184-193.

[3] Simons M. and Trotter J., Wrapping it up: the cell biology of myelination. Current Opinion in Neurobiology 2007, 17:533–540.

[4] Mekhail M., Almazan G. and Tabrizian M., Oligodendrocyte-protection and remyelination post-spinal cord injuries: A review. Progress in Neurobiology 96 (2012) 322–339.

[5] Jackman N., Ishii A. and Bansal R., Oligodendrocyte Development and Myelin Biogenesis: Parsing Out the Roles of Glycosphingolipids. Physiology 2009, 24: 290-297.

[6] Swiss V. et al., Identification of a Gene Regulatory Network Necessary for the Initiation of Oligodendrocyte Differentiation. PLoS ONE 2011, 6: 1-13.

[7] Boulanger J.J. and Messier C., From Precursors to Myelinating Oligodendrocytes: Contribution of Intrinsic and Extrinsic Factors to White Matter Plasticity in the Adult Brain. Neuroscience 2014, 269: 343-366.

[8] Franklin R.J. and ffrench-Constant C., Remyelination in the CNS: from biology to therapy. Nature Reviews 2008, 9: 839-855.

[9] Chang A. et al, Premyelinating oligodendrocytes in chronic lesions of Multiple Sclerosis. New England Journal of Medicine 2002, 346(3): 165-173.

[10] Ahrendsen J.T. and Macklin W., Signaling mechanisms regulating myelination in the central nervous system. Neuroscience Bulletin 2013, 29(2): 199-215.

[11] Cadotte D. and Fehlings M., Spinal Cord Injury: A Systematic Review of Current Treatment Options. Clin Orthop Relat Res 2011, 469:732–741.

[12] McDonald, J.W. and Sadowsky, Spinal-cord injury. Lancet 2002, 359: 417–425.

[13] Macaya D. and Spector M., Injectable hydrogel materials for spinal cord regeneration: a review. Biomedical materials 2012, 7(1): doi:10.1088/1748-6041/7/1/012001.

[14] Perale G. et al., Multiple drug delivery hydrogel system for spinal cord injury repair strategies. Journal of Controlled Release 2012, 159: 271–280.

[15] Totoiu M. and Keirstead H., Spinal Cord Injury is Accompanied by Chronic Progressive Demyelination. THE JOURNAL OF COMPARATIVE NEUROLOGY 2005, 486:373–383.

[16] Silver J. and Miller H., Regeneration beyond the glial scar. Nature Reviews Neuroscience 2004, 5: 146-156.

[17] Keirstead H. et al., Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Remyalinate and Restore Locomotion after Spinal Cord Injury. The Journal of Neuroscience 2005, 25(19): 4694-4705.

[18] Fitch M.T. and Silver J., CNS injury, glial scars, and inflammation: Inhibitory extracellular matricesand regeneration failure. Experimental Neurology 2008, 209: 294–301.
[19] Raff et al, Reconstitution of a Developmental Clock In Vitro: A Critical Role for Astrocytes in the Timing of Oligodendrocyte Differentiation. Cell 1985, 42: 61-69.
[20] Richardson, W.D., Kessaris and Pringle, Oligodendrocyte wars. Nature Reviews Neuroscience 2006, 7: 11-18.
[21] Buttery P. and ffrench-Constant, Laminin-2/Integrin Interactions Enhance Myelin membrane Formation by Oligodendrocytes. Molecular and Cellular Neuroscience 14: 199-212 (1999).
[22] Emery B., Regulation of Oligodendrocyte Differentiation and Myelination. Science 2010: 330, 779-782.
[23] Rivera, F.J. et al., Deciphering the Oligodendrogenic Program of Neural Progenitors: cell Intrinsic and Extrinsic Regulators. Stem Cells and Development 2010, 19: 595-606.
[24] Kremer D. et al., The Complex World of Oligodendroglial Differentiation Inhibitors. ANNALS of Neurology 2011, 69:602–618.
[25] Patel J. and Klein R., Mediators of oligodendrocyte differentiation during remyelination. FEBS Letters 2011, 585: 3730–3737.
[26] Archer D.R. et al. Myelination of the canine central nervous system by glial cell transplantation: a model for repair of human myelin disease. Nature Medicine 1997, 3(1): 54-59.
[27] Windrem M. et al., Neonatal chimerization with human glial progenitor cells can both remyelinate and rescue the otherwise lethally hypomyelinated shiverer mouse. Cell Stem Cell 2008, 2(6): 553–565.
[28] Colognato H. and Tzvetanova I., Glia Unglued: How Signals from the Extracellular Matrix Regulate the Development of Myelinating Glia. Developmental Neurobiology 2011, 924-955.
[29] Ma W. et al., Cell-Extracellular Matrix Interactions Regulate Neural Differentiation of Human Embryonic Stem Cells. BMC Developmental Biology 2008, 8:90.
[30] Alberts Bruce, Jonhson Alexander, Lewis Julian, Raff Martin, Roberts Keith and Water Pete, in Molecular Biology of the Cell. Fifth Edition. Ch. 19: pp. 1176-1177. Garland Science 2002.
[31] Barros C.S. et al., ß1 integrins are required for normal CNS myelinization and promote AKT-dependent myelin outgrowth. Development 2009, 136: 2717-2724.
[32] ffrench-Constant and Colognato H., Integrins: versatile integrators of extracellular signals. Trends in Cell Biology 2004, 14: 678-686.
[33] Relvas J. et al, Expression of dominant-negative and chimeric subunits reveals an essential role for ß1 integrin during myelination. Current Biology 2001, 11: 1039-1043.
[34] Donaghue I. et al, Cell and biomolecule delivery for tissue repair and regeneration in the central nervous system. Journal of Controlled Release 2014, 190:219–227.

[35] Colognato H. et al., CNS integrins switch growth factor signaling to promote target dependent survival. Nature Cell Biology 2002.

[36] Ozaki I. et al., Regulation of TGF-Β1-induced pro-apoptotic signaling by growth factor receptors and extracellular matrix receptor integrins in the liver. Frontiers in Physiology 2011, 2(78): 1-8.

[37] Lauren L., Chan W. and ffrench-Constant, An Integrin–Contactin Complex Regulates CNS Myelinationby Differential Fyn Phosphorylation. The Journal of Neuroscience 2009, 29 (29): 9174 –9185.

[38] Colognato et al., Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. The Journal of Cell Biology 2004, 164(2): 365–375. doi/10.1083/jcb.200404076.

[39] Liang X., Draghi N. and Resh M., Signaling from Integrins to Fyn to Rho Family GTPases Regulates Morphologic Differentiation of Oligodendrocytes. The Journal of Neuroscience 2004, 24(32): 7140-7149.

[40] Hamill K.J. et al., Laminin deposition in the extracellular matrix: a complex picture emerges. Journal of Cell Science 2009, 122: 4409-4417. doi:10.1242/jcs.041095.

[41] Huveneers S. and Danen E. H., Adhesion signaling – crosstalk between integrins, Src and Rho. Journal of Cell Science 2009, 122: 1059-1069.

[42] Taveggia C., Feltri M.L. and Wrabetz L., Signals to promote myelin formation and repair. Nature Reviews Neurology 2010, 6: 276-287.

[44] Richter-Landsberg C., The Cytoskeleton in Oligodendrocytes. Journal of Molecular Neuroscience 2008, 35: 55-63.

[45] Galiano M. R., Andrieux A., Deloulme J. C., Bosc C., Schweitzer A., Job D. et al, Myelin basic protein functions as a microtubule stabilizing protein in differentiated oligodendrocytes. Journal of Neuroscience Research 2006, 84: 534–541.

[46] Lee J., Gravel M., Zhang R., Thibault P., and Braun P. E., Process outgrowth in oligodendrocytes is mediated by CNP, a novel microtubule assembly myelin protein. Journal of Cell Biology 2005, 170: 661–673.

[47] Pakulska MM, Ballios BG and Shoichet MS, Injectable hydrogels for central nervous system therapy. Biomed Mater. 2012, 7(2):024101. doi: 10.1088/1748-6041/7/2/024101.

[48] Li X. et al., Engineering neural stem cell fates with hydrogel design for central nervous system regeneration. Progress in Polymer Science 2012, 37: 1105– 1129.

[49] Woerly S. Restorative surgery of the central nervous system by means of tissue engineering using NeuroGel implants. Neurosurg Rev 2000, 23: 59–77.

[50] Bjugstad K.B., Lampe K., Kern D.S., Mahoney M.J., Biocompatibility of poly(ethylene glycol)-based hydrogels in the brain: an analysis of the glial response across space and time. J Biomed Mater Res A 2010, 95A:79–91.

[51] Mahoney M.J., Anseth K.S., Three-dimensional growth and function of neural tissue in degradable polyethylene glycol hydrogels. Biomaterials 2006, 27:2265–74.

[52] Ma W., Fitzgerald W., Liu Q.Y., O'Shaughnessy T.J., Maric D., Lin H.J., Alkon D.L., Barker J.L., CNS stem and progenitor cell differentiation into functional neuronal circuits in three-dimensional collagen gels. Exp Neurol 2004, 190:276–88.

[53] Schense J.C., Bloch J., Aebischer P., Hubbell J.A., Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension. Nat Biotechnol 2000, 18:415–9.

[54] Tian W, Hou S, Ma J, Zhang C, Xu Q, Lee I, Li H, Spector M, Cui F. Hyaluronic acid poly-d-lysine-based three-dimensional hydrogel for traumatic brain injury. Tissue Eng 2005, 11: 513–25.

[55] Martin B.C., Minner E., Wiseman S., Klank R.L., Gilbert R.J., Agarose and methylcellulose hydrogel blends for nerve regeneration applications. J Neural Eng 2008, 5: 221–31.

[56] Liudmila N.N. et al., Alginate hydrogel and matrigel as potential cell carriers for neurotransplantation. J Biomed Mater Res A 2006, 77A: 242–52.

[57]Chenite A., Chaput C., Wang D., Combes C., Buschmann M.D., Hoemann C.D., Leroux J.C., Atkinson B.L., Binette F., Selmani A., Novel injectable neutral solutions of chitosan form biodegradable gels in situ. Biomaterials 2000, 21:2155–61.

[58] Tate M.C., Shear D.A., Hoffman S.W., Stein D.G., LaPlaca M.C., Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury. Biomaterials 2001, 22:1113–23.

[59] Irons H.R. et al., Three-dimensional neural constructs: a novel platform for neurophysiological investigation. J Neural Eng 2008; 5:333–41.

[60] Haile Y., Haastert K., Cesnulevicius K., Stummeyer K., Timmer M., Berski S., Dräger G., Gerardy-Schahn R., Grothe C., Culturing of glial and neuronal cells on polysialic acid. Biomaterials 2007, 28: 1163–1173.

[61] Nisbet D.R., Rodda A.E., Horne M.K., Forsythe J.S., Finkelstein D.I., Implantation of functionalized thermally gelling xyloglucan hydrogel within the brain: associated neurite infiltration and inflammatory response. Tissue Eng Part A 2010, 16:2833–42.

[62] Cheng T.Y. et al., Neural stem cells encapsulated in a functionalized self-assembling peptide hydrogel for brain tissue engineering. Biomaterials 2013, 34: 2005-2016.

[63] Tysseling-Mattiace et al., Self-Assembling Nanofibers Inhibit Glial Scar Formation and Promote Axon Elongation after Spinal Cord Injury. The Journal of Neuroscience 2008, 28(14):3814 –3823.

[64] Tysseling-Mattiace et al., Self-Assembling Peptide Amphiphile Promotes Plasticity of Serotonergic Fibers Following Spinal Cord Injury. Journal of Neuroscience Research 2010, 88:3161–3170.

[65] Li X. et al., Engineering an *in situ* crosslinkable hydrogel for enhanced remyelination. FASEB Journal 2013, 27: 1127–1136.

[66] Asmani M. et al., Three dimensional culture of differentiated endometrial stromal cells to oligodendrocyte progenitor cells (OPCs) in fibrin hydrogel. Cell Biology International 2013, 37: 1340-1349.

[67] J. R. Soc. Interface 2009 **6**, 1-10 Fibrin gels and their clinical and bioengineering applications, Paul A Janmey, Jessamine P Winer and John W Weisel. J. R. Soc. Interface 2009, 6: 1-10.

[68] Brown A.C. and Barker T.H., Fibrin-based biomaterials: Modulation of macroscopic properties through rational design at the molecular level. Acta Biomaterialia 2014, 10: 1502-1514.

[69] Stefanelli V.L. and Barker T.H., The Evolution of Fibrin-Specific Targeting Strategies. Journal of Materials Chemistry B 2015, DOI: 10.1039/C4TB01769B.

[70] Montgomery A. et al., Combining protein-based biomaterials with stem cells for spinal cord injury repair. OA Stem Cells 2014, 2(1):1-7.

[71] DeVolder R. and Kong H., Hydrogels for in vivo-like three-dimensional cellular studies. WIREs Systems Biology and Medicine 2012, 4:351–365.

[72] Janmey P.A., Winer J.P. and Weisel J.W., Fibrin gels and their clinical and bioengineering applications. J. R. Soc. Interface2009, 6: 1–10.

[73] Martino M.M. et al., Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix. PNAS 2013, 110(12): 4563–4568.

[74] Schense J.C., Bloch J., Aebischer P. and Hubbel J.A., Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension. NATURE BIOTECHNOLOGY 2000, 18: 415-419.

[75] Taylor S.J., McDonald III J.W. and Sakiyama-Elbert S.E., Controlled release of neurotrophin-3 from fibrin gels for spinal cord injury. Journal of Controlled Release 2004, 98: 281– 294.

[76] Sakiyama-Elbert S.E., Hubbell J.A., Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix, J. Control. Release 2000a, 69: 149– 158.

[77] Sakiyama-Elbert S.E., Hubbell J.A., Development of fibrin derivatives for controlled release of heparin-binding growth factors, J. Control. Release (2000b), 65:389–402.

[78] J.N. Silva, T. Laundos, P. Quelhas, A.P. Pêgo, and I.F. Amaral, Development of fibrin hydrogels functionalized with covalently bound alpha6beta1 ligands for neural stem cell (NSC) based transplantation therapies. Journal of Tissue Engineering and Regenerative Medicine 2014, 8(supplement S1), 39-40.

[79] Leu et al., Identification of a Novel Integrin α6ß1 Binding site in the Angiogenic Inducer CCN1 (CYR61). The Journal of Biological Chemistry 2003, 36: 33801-33808.

[80] Stroka T.C. et al., The minimum element of a synthetic peptide required to block prostate tumor cell migration. Cancer Biology &Therapy 2006, 5(11): 1556-1562.

[81] Sroka et al., Synthetic D-amino acid peptide inhibits tumor cell motility in laminin-5. Carcinogenesis 2006, 27: 1748-1757.
[82] Ichinose, A., et al. (1983) Elsevier Biomedical Press.
[83] McCarthy K.D. and deVellis J., Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. Journal of Cell Biology 1980, 85: 890 – 902.
[84] Chen Y. et al., Isolation and culture of rat and mouse oligodendrocyte precursor cells. Nature Protocols 2007, 2(5): 10441051.
[85] Su-Chun Zhang, Defining glial cells during CNS development. Nature Reviews 2001, 2: 840-843.
[86] Nicole Jackman, Akihiro Ishii, and Rashmi Bansal, Oligodendrocyte Development and Myelin Biogenesis: Parsing Out the Roles of Glycosphingolipids. Physiology 2009, 24: 290-297.
[87] Hall H., Baechi T.,and Hubbell, Molecular Properties of Fibrin-Based Matrices for Promotion of Angiogenesis in Vitro. Microvascular Research 2001, 62: 315-326.
[88]Jason C. Schense and Jeffrey A. Hubbell, Cross-Linking Exogenous Bifunctional Peptides into Fibrin Gels with Factor XIIIa. Bioconjugate Chemistry 1999, 10: 75-81.
[89] Gu C. et al., Oligodendrocyte Apoptosis Mediated by Caspase Activation. The Journal of Neuroscience 1999, 19(8): 3043-3049.]

Supplementary data

Appendix A

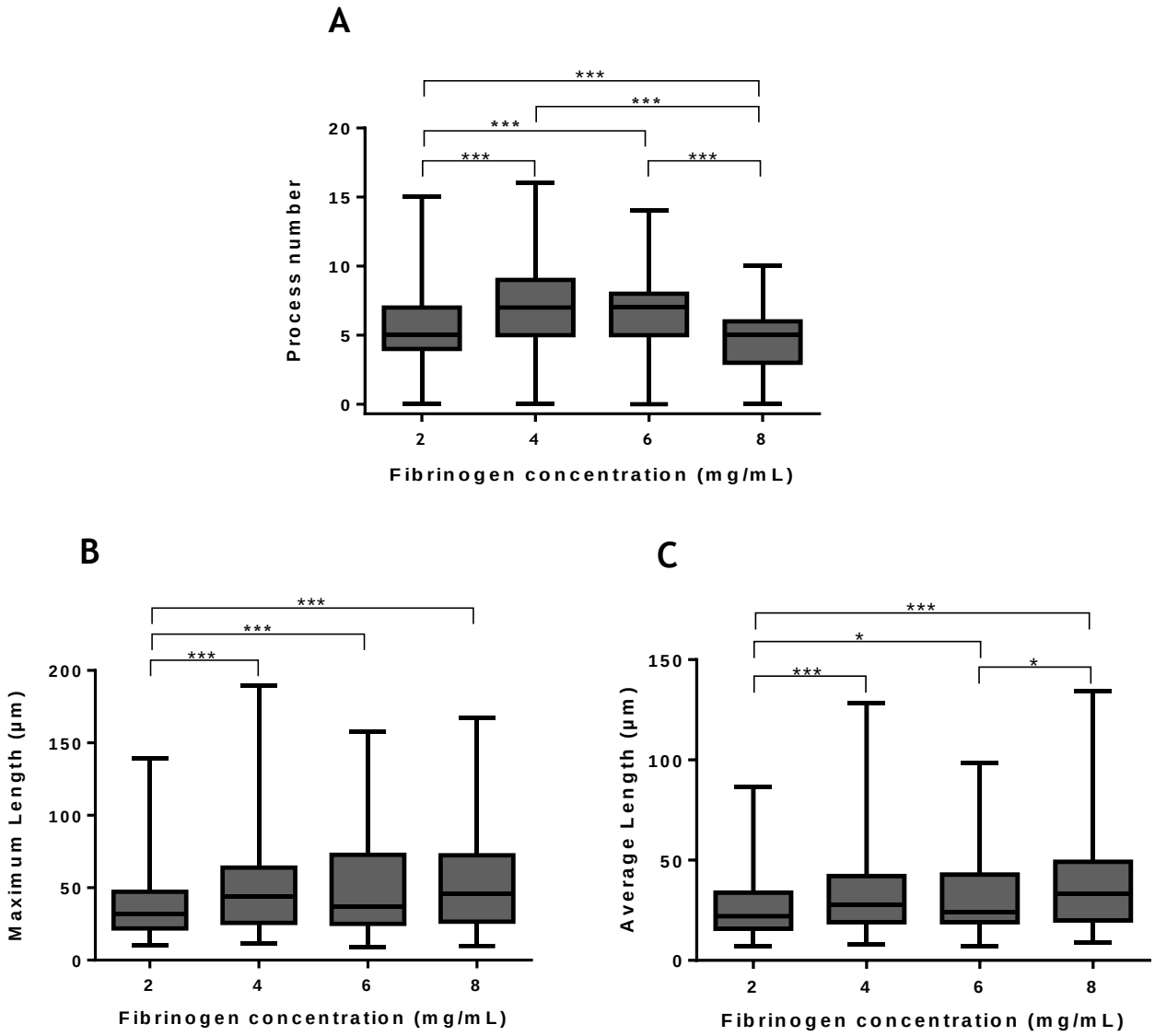


Figure A1. Quantitative analysis of process outgrowth of OPCs cultured in fibrin hydrogels, as a function of fibrinogen concentration. Cell/fibrin constructs were cultured for 9 days (2 days under proliferation conditions and 7 days under differentiation conditions), and subsequently processed for immunofluorescent labelling of α-tubulin and olig2. Process outgrowth was determined in InCell Analyzer stack images of cell/fibrin constructs covering a depth of approximately 200 µm, by image analysis. Only cells expressing olig2 were considered for the analysis. To evaluate process extension three parameters were considered: (A) Process number per cell; (B) Maximum length; (C) Average Length. In each experiment, 45-60 cells were analysed in two replicate cultures. Results pooled from three independent experiments are shown (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$, as assessed using the *Kruskal-Wallis non-parametric test* followed by *Mann-Whitney U* test).

Appendix B

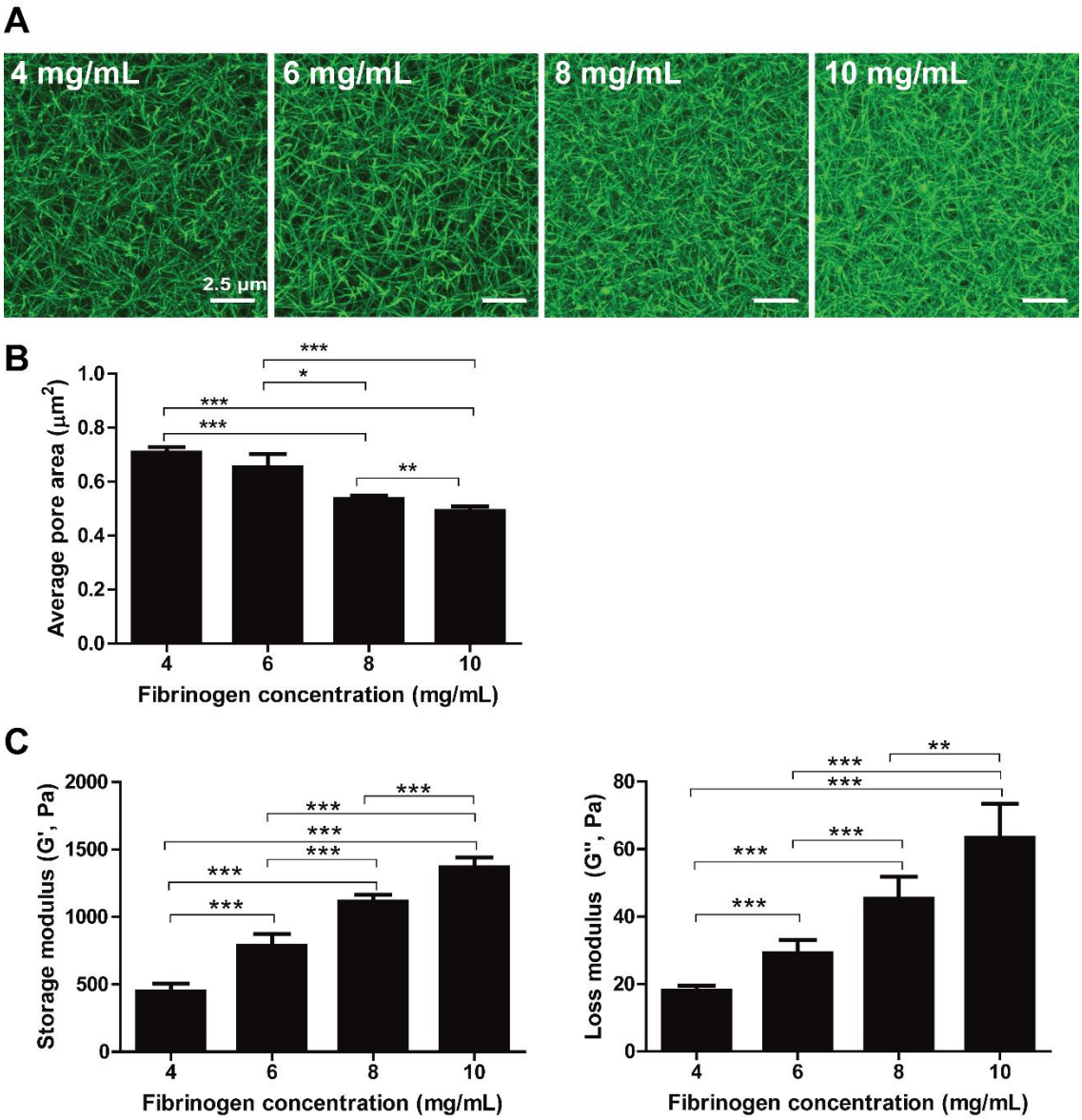


Figure B1. Effect of fibrinogen concentration on Fb hydrogels microstructure and stiffness. (A) Microstructure of Fb gels prepared with 4, 6, 8 or 10 mg/mL of fibrinogen spiked with Alexa Fluor 488-labeled fibrinogen. Representative 2-D projections of CLSM stack images covering a depth of 10 µm are shown. (B) Average pore area of Fb hydrogels, as determined by image analysis of the 2-D projected CLSM stacks (N = 4; *, $p < 0.05$). (C) Storage modulus (G') and loss modulus (G'') of Fb hydrogels, as assessed by rheological analysis (N = 6 independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$). (*Bento et al., 2015, submitted*)

Appendix C

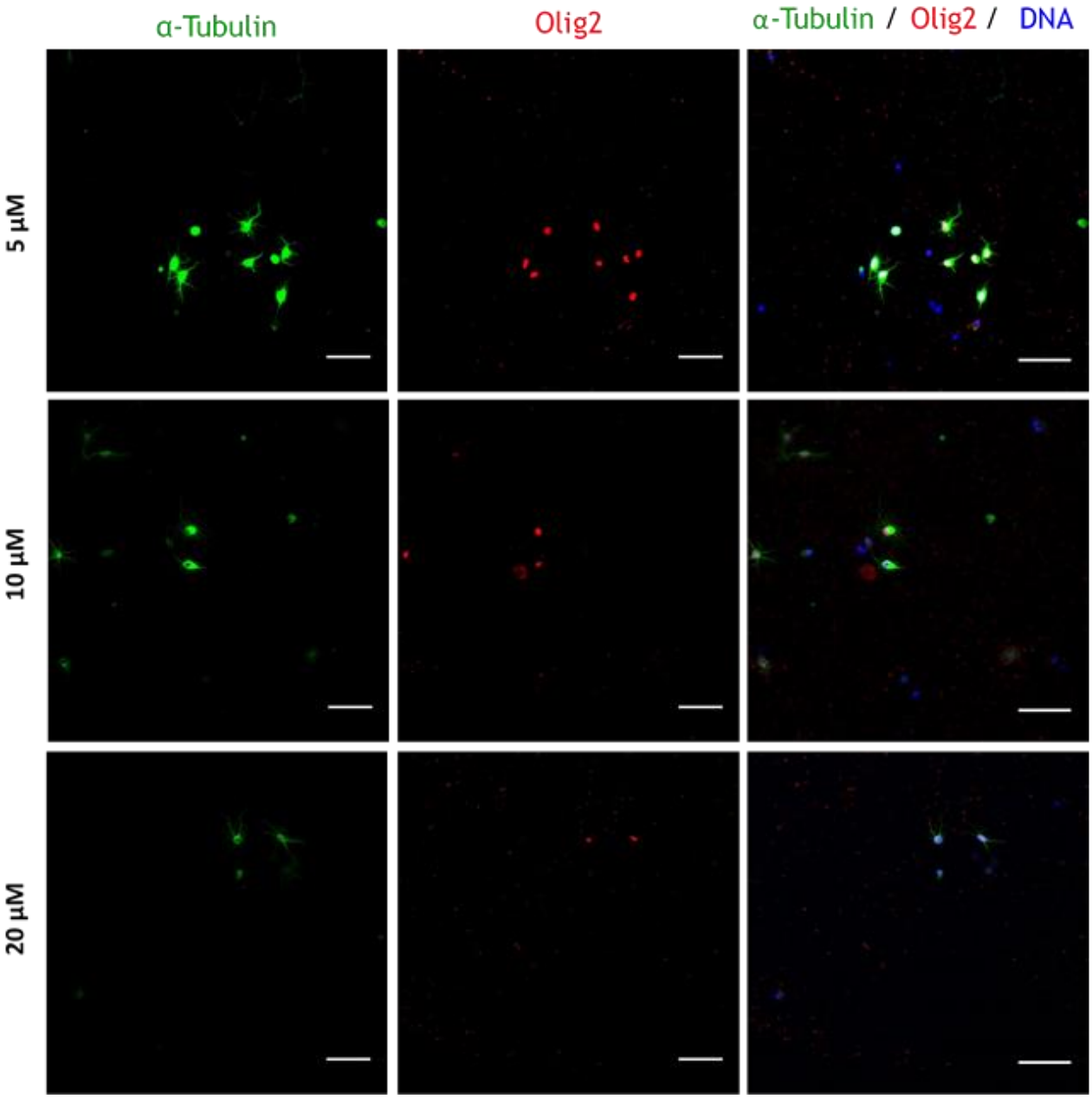


Figure C1. Immunocytochemistry analysis of OPC process extension in 4 mg/mL fibrin hydrogels functionalized with HYD1 bi-domain peptide as a function of peptide input concentration in polymerizing gels. Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions) and subsequently processed for immunofluorescent labelling of α-tubulin (in green)/olig2 (in red). Representative 2-D projections of split CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are presented. Scale Bar: 50 µm.

Appendix D

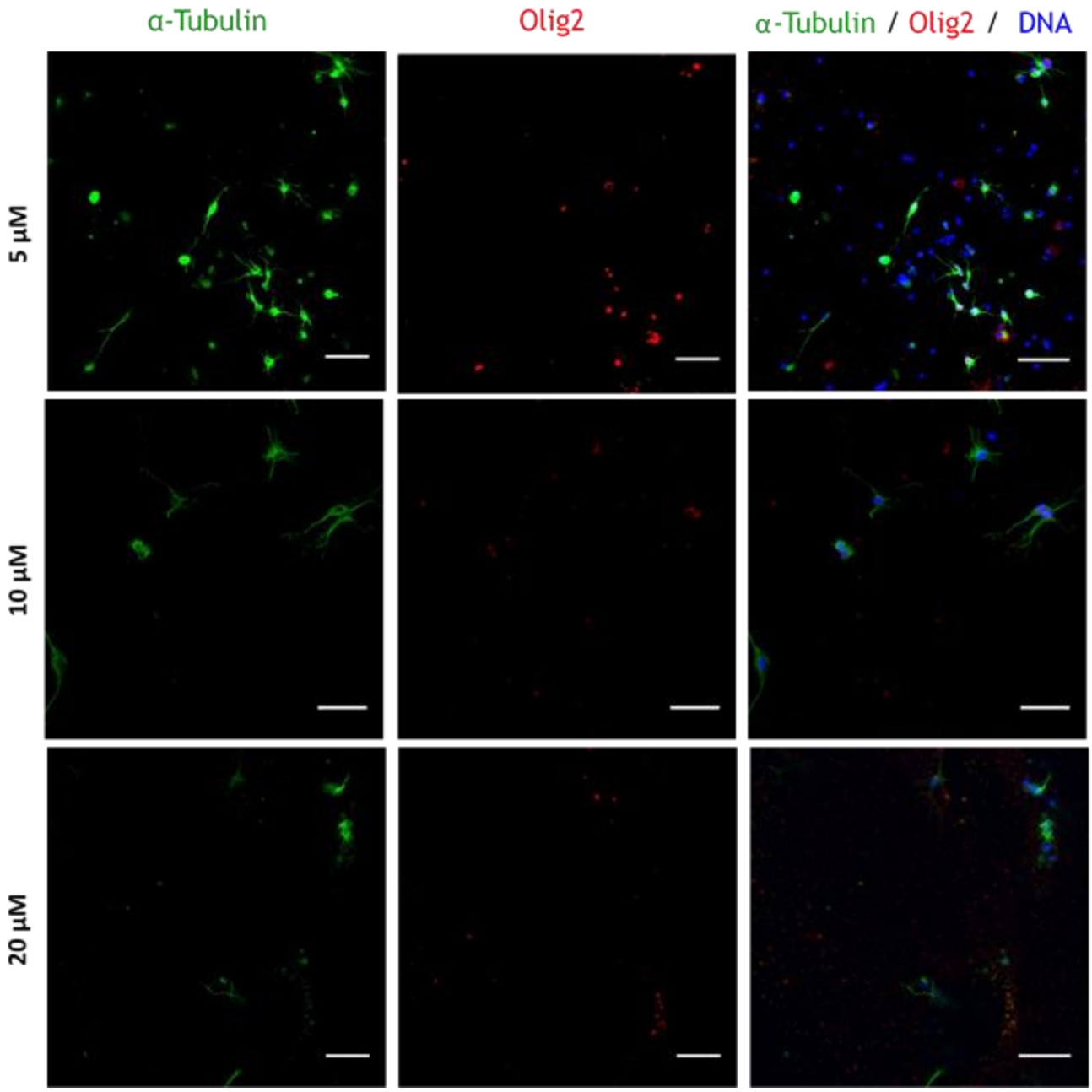


Figure D1. Immunocytochemistry analysis of OPC process extension in 4 mg/mL fibrin hydrogels functionalized with T1 peptide as a function of peptide input concentration in polymerizing gels. Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions) and subsequently processed for immunofluorescent labelling of α-tubulin (in green)/olig2 (in red). Representative 2-D projections of split CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are presented. Scale Bar: 50 µm.

Appendix E

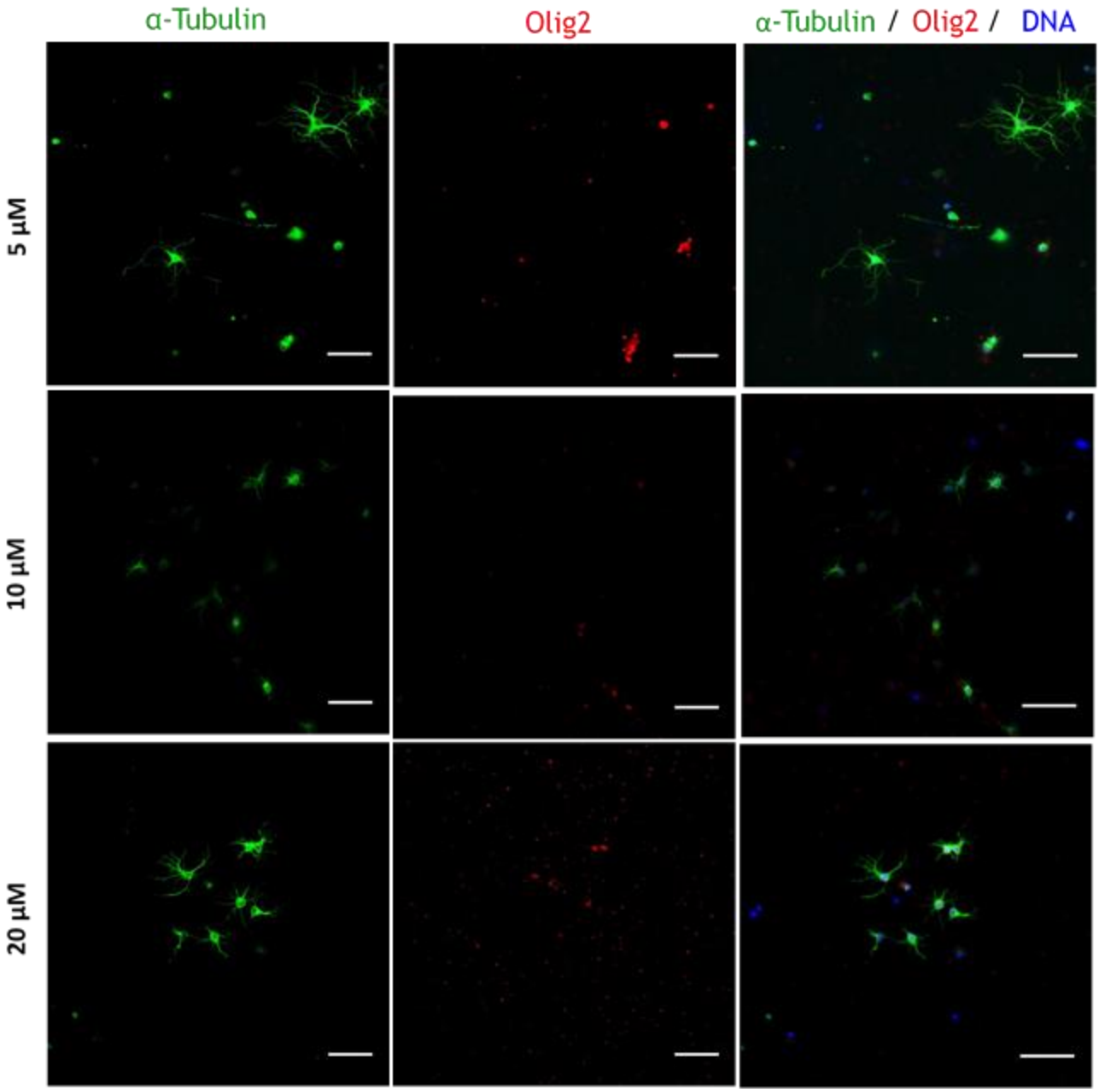


Figure E1. Immunocytochemistry analysis of OPC process extension in 4 mg/mL fibrin hydrogels functionalized with HYD1+T1 peptides as a function of peptide input concentration in polymerizing gels. Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions) and subsequently processed for immunofluorescent labelling of α-tubulin (in green)/olig2 (in red). Representative 2-D projections of split CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are presented. Scale Bar: 50 µm.

Appendix F

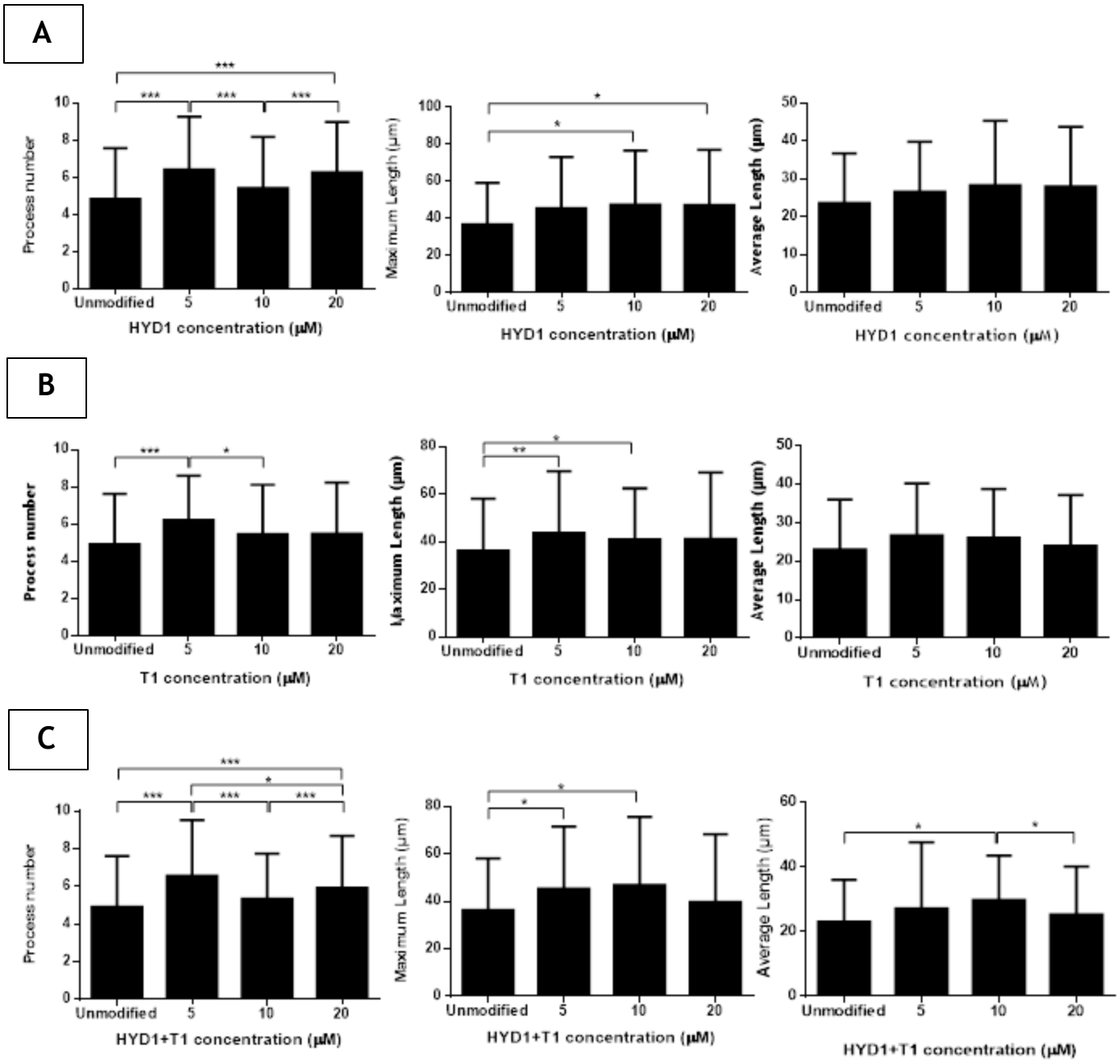


Figure F1. Process outgrowth quantitative analysis of OPCs cultured in fibrin hydrogels (4 mg/mL of fibrinogen) functionalized with bi-domain peptides, as a function of peptide input concentration in polymerizing gels: (A) HYD1; (B) T1 and (C) HYD1+T1. Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions), as assessed by image analysis of InCell Analyzer z-stacks covering a depth of approximately 200 μm. To evaluate process extension three parameters were considered: (A) Mean process number; (B) Mean maximum length; (C) Mean average length. In each experiment, 45-60 cells were analyzed in two replicate cultures. Results from three independent experiments are shown. Process outgrowth in functionalized fibrin hydrogels was compared to that in unmodified gels (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

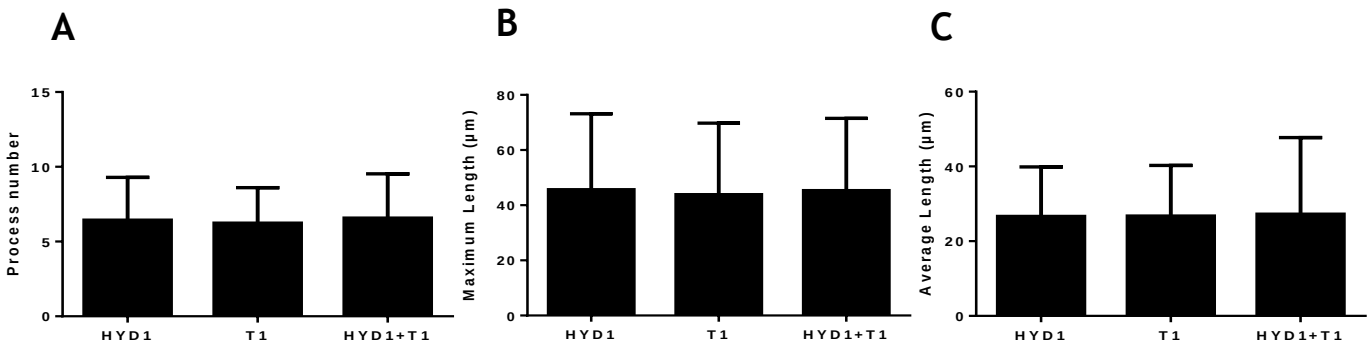


Figure F2. Process outgrowth quantification of OPCs cultured in 4 mg/mL fibrin hydrogels functionalized with 5 µM of bi-domain peptides (HYD1, T1 and HYD1+T1). Cell/fibrin constructs were cultured for 5 days (2 days under proliferation conditions and 3 days under differentiation conditions), as assessed by image analysis of InCell Analyzer z-stacks covering a depth of approximately 200 µm. Three parameters were considered to evaluate process extension: (A) Process number; (B) Maximum length; (C) Average Length. Results from three independent experiments are shown. In each experiment, 45-60 cells were analyzed in two replicate cultures. Results were not considered statistically different as assessed by *Kruskal-Wallis non-parametric* test.

Appendix G

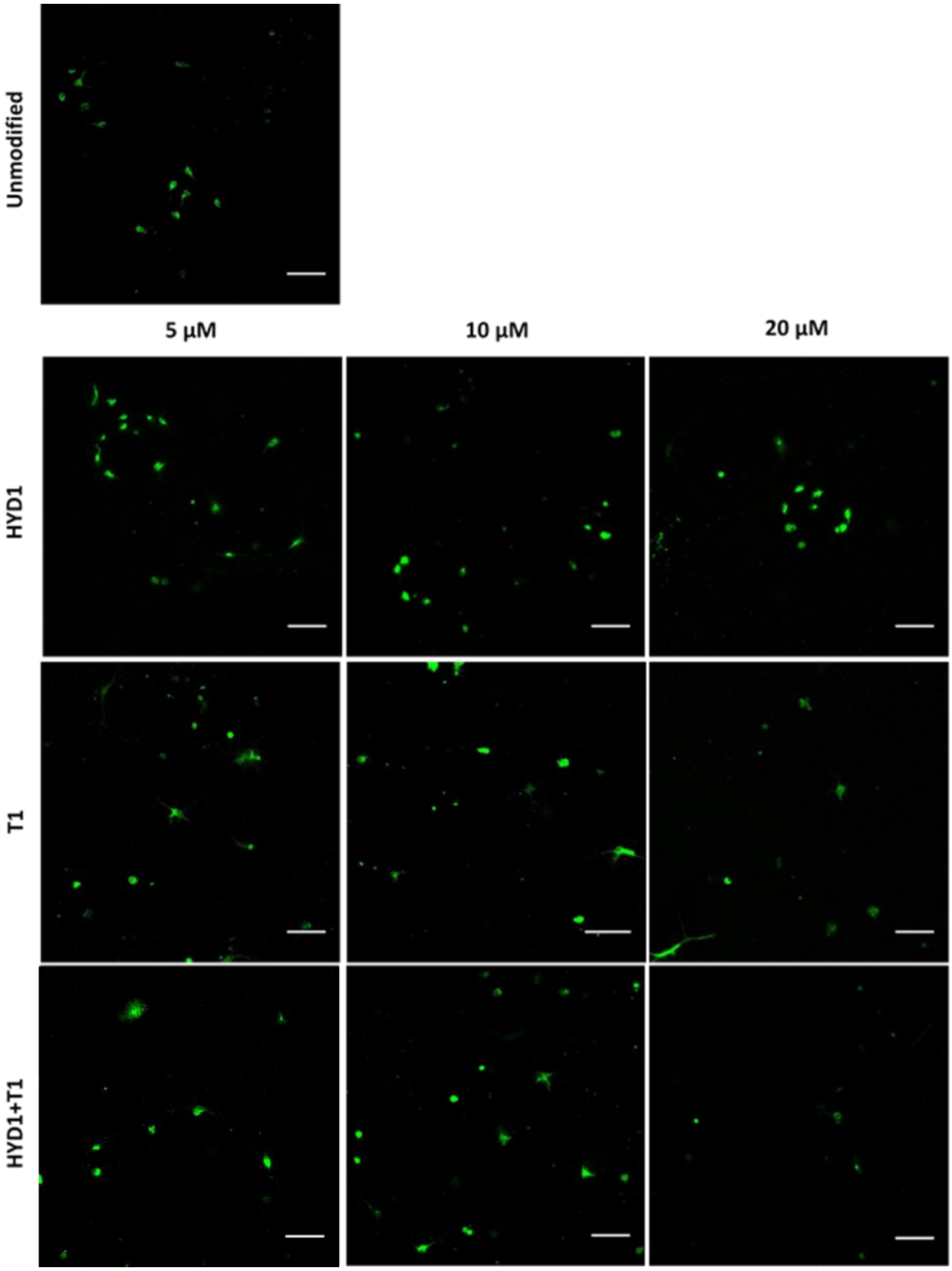


Figure G1. Immunocytochemistry analysis of OL myelin production in 4 mg/mL fibrin hydrogels functionalized with bi-domain peptides (HYD1, T1 and HYD1+T1), as a function of peptide input concentration in polymerizing gels. Cell/fibrin constructs were cultured for 5 days, 2 days under proliferation and 3 days under differentiation conditions. Representative 2-D projections of MBP channel (green) of CLSM stack images of cell/fibrin constructs covering a depth of approximately 30 µm are shown. Scale Bar: 50 µm.