MASTER THESIS

UNIVERSITY OF CRETE

School of Sciences and Engineering
Biology Department
Graduate Program: Molecular Biology & Biomedicine

Evaluation of embryonic neural stem cells (NSCs) growth and differentiation within scaffolds composed of graphene-based materials (GBM) combined with adipose decellularized extracellular matrix (adECM)

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October 2020

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Acknowledgments

First of all, I would like to express my appreciation to Prof. Ioannis Charalampopoulos, Prof. Achileas Gravanis and Dr. Emmanouel Stratakis for giving me the opportunity to work on such an interesting project. Specifically, I would like to thank Prof. I. Charalampopoulos for constantly providing advice and further insight regarding my career.

Special thanks to my supervisor Dr. Kanelina Karali whose unlimited help, constant guidance, support and patience, were valuable for the fulfilment of this thesis.

Additionally, I would like to thank all the members of the group for the cooperation and the friendly working environment that they offered. I really enjoyed my time working with you!

Finally, I would like to thank my family and friends for their endless love, continuous support and encouragement. I owe them more than I can describe.

Abstract

Spinal cord injury is a severely debilitating condition leading to neurological dysfunction, loss of independence, respiratory failure, psychological morbidities, and an increased lifelong disability. The injury triggers a complex cascade of pathological processes, culminating in formation of a scar. A bridging biomaterial construct that allows the axons to grow through has been studied for the repair of injured spinal cord. In the present study, two different compositions of adECM/GO scaffolds, seeded with neural stem cells were used. The results showed no cytotoxicity, and upregulated proliferation. Both compositions seem to favor NSCs differentiation towards OPCs, whereas one of them significantly increased differentiation into neurons. Collectively, these results suggest that adECM/GO scaffolds comprise a promising innovative treatment strategy for the injured spinal cord.

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List of Abbreviations

AdECM	adipose decellularized Extracellular Matrix
AD-MSCs	Adipose Derived Mesenchymal Stem Cells
BM-MSCs	Bone-Marrow Mesenchymal Stem Cells
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CSPGs	Chondroitin Sulfate Proteoglycans
DAM	Decellularized Adipose Matrix
DIV	Days in Vitro
ECM	Extracellular Matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ESCs	Embryonic Stem Cells
GAGs	Glycosaminoglycans
GO	Graphene Oxide
GQDs	Graphene Quantum Dots
GRMs	Graphene Related Materials
HBSS	Hanks' Balanced Salt Solution
iPSCs	induced Pluripotent Stem Cells
min	minutes
MSCs	Mesenchymal Stem Cells
NSCs	Neural Stem Cells
NT3	Neurotrophin-3
PBS	Phosphate Buffer Saline
PDGFAA	Platelet-derived growth factor A
PDL	Poly-D-Lysine
PFA	paraformaldehyde
PI	Propidium Iodide
PLA	Polylactic Acid
PLGA	Poly Lactic-co-glycolic Acid
P/S	Penicillin/Streptomycin
PSCs	Pluripotent Stem Cells
PVA	Polyvinyl Alcohol
rGO	reduced Graphene Oxide
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Scanning Electron Microscopy
SCB	sodium cacodylate buffer
SCI	Spinal Cord Injury
T3	triiodo-l-thyronine
U-MSCs	Umbilical cord Mesenchymal Stem Cells

1. Introduction

1.1 Spinal Cord Injury

The central nervous system (CNS) consists of the brain, spinal cord and retina. The main function of the spinal cord is transmitting information between the brain and the body, which allows us to direct our body's voluntary muscle movements, monitor sensations of touch, pressure, temperature, pain, and regulate autonomic functions such as digestion. It is limited in terms of its spontaneous regenerative capacity, limiting the possible treatment strategies. Spinal cord injury (SCI) is a severely debilitating condition leading to neurological dysfunction, loss of independence, respiratory failure, psychological morbidities, and an increased lifelong mortality rate (Marion et al. 2017), (Satkunendrarajah et al. 2018), (Marion et al. 2017), (Y. Wang, Xie, and Zhao 2018). Causes include vehicle accidents, accidental falls, violent acts and other traumatic events (Mackay-Sim et al. 2008; Fehlings et al. 2014). The etiologies of CNS injuries are apoptotic and necrotic death of neurons, astrocytes and oligodendrocytes, blood–brain barrier dysfunction, local inflammation, demyelination, disrupted nerve pathways (Silva et al. 2014), axonal injury, excitotoxicity, ischemia and oxidative damage (Tam et al. 2014). Neurological deficits and impairment include motor, sensory and autonomic (sexual, urinary, cardiovascular and intestinal) dysfunction, resulting in paraplegia or tetraplegia the severity of which depends on the degree of damage and the spinal level at which the injury occurs. To date, there is no effective cure for SCI and the only therapeutic option is physical rehabilitation (Kadoya et al. 2016; Behrman and Harkema 2007; Harvey 2016). To reverse outcomes caused by SCI, it is necessary to repair the damaged neural circuits. However, once transected, CNS axons form dystrophic endbulbs at their proximal tips, termed retraction bulbs, which render neurons unable to regenerate (Bradke, Fawcett, and Spira 2012). Despite decades of research and numerous regenerative approaches that demonstrated promising results in animal models, (Krueger et al. 2013; Badhiwala, Ahuja, and Fehlings 2018), the scientific community has yet to provide SCI patients with a viable option to prevent the devastating outcome of traumatic SCI or to reverse the neurological impairment brought about by the condition.

1.1.2 Barriers to Regeneration

There is an inhibitory microenvironment that impedes nerve regeneration, formed by a lack of nutritional factors and myelin proteins, inflammatory responses, blood flow disruption, and other adverse elements in the lesion (Fujita and Yamashita 2014), (K. Liu et al. 2011), (Seifalian et al., 2015).

On the one hand, at the acute/subacute stages of injury, the glial scar isolates the lesion area preserving the healthy tissue and limiting disruption and amplification of the injury (Yuan and He 2013). On the other hand, the glial scar shows a detrimental effect, constituting a mechanical, physical and chemical barrier to axonal regrowth and nerve fiber regeneration as well as in primordial cell regeneration (Yuan and He 2013), (Silver and Miller 2004). To re-establish connectivity of neural circuits, neurons need to be reorganized into existing or newly formed neural pathways and oligodendrocytes must myelinate the axons to facilitate electrical transmission. The glial scar potently restricts axon regeneration and anatomical plasticity by inhibiting neurite outgrowth (McKeon et al. 1991; Ahuja, Martin, and Fehlings 2016). This dense boundary structure is formed by CNS glial cells, which include astrocytes, oligodendrocytes, their progenitors, and microglia. Reactive astrocytes and other glial cells secrete chondroitin sulfate proteoglycans (CSPGs), which acts as a physical and chemical barrier that impedes endogenous tissue repair processes such as axonal sprouting and synaptic reorganization.

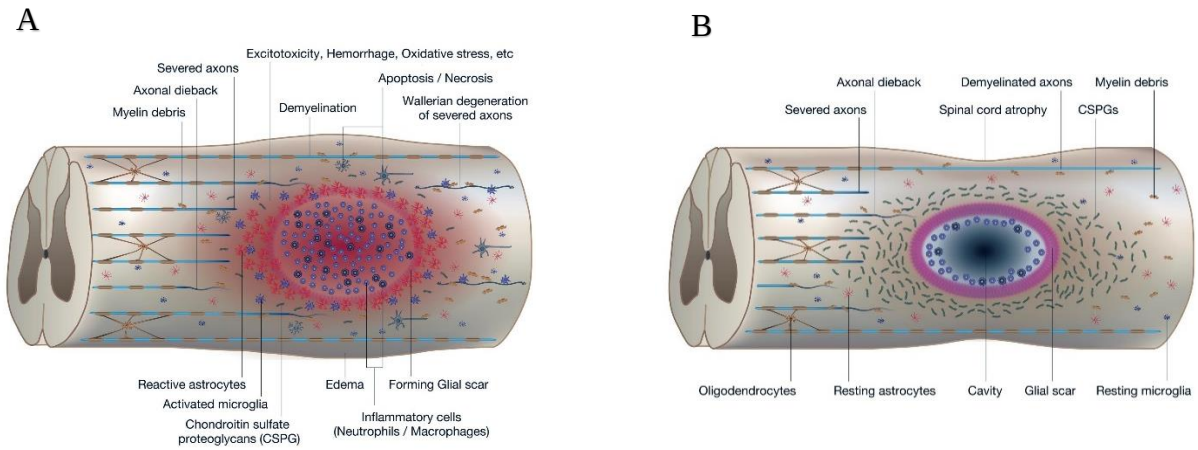


Figure 1: Pathophysiological events in SCI. The diagram shows the pathophysiological events occurring around the lesion site during (A) the acute to subacute phase of SCI. Resident neurons, oligodendrocytes, and astrocytes near the lesion are forced into apoptosis or necrosis, resulting in axonal degeneration. (B)During the chronic phase, the phenotype of reactive astrocytes has changed into scar-forming astrocytes that impede regenerating axons from crossing the lesion. Some inflammatory immune cells remain around the lesion. This image was taken from *Katoh, H., et al. (2019). Regeneration of Spinal Cord Connectivity through Stem Cell Transplantation and Biomaterial Scaffolds.*

1.1.3 Therapeutic approach

Treatment strategies combining cell transplantation, molecule delivery and biomaterial scaffold constructions are considered the greatest hope for possible regeneration and functional recovery in SCIs (Niu and Zeng 2015; Straley et al., 2010). Regenerative strategies have also aimed to increase synthesis and transport of materials required for growth, and to modulate axonal cytoskeletal dynamics to promote elongation or branching (Puttagunta et al. 2014; Hilton and Bradke 2017), (Cheah et al. 2016; Hellal et al. 2011), (Koseki et al. 2017).

1.1.3.1 Animal models

In rodent models, therapeutic interventions are applied usually early or immediately after the spinal cord lesion. However, differences in size, molecular signaling, anatomy and the recovery potential following SCI have made direct translation challenging. The ideal animal model should anatomically and pathophysiologically resemble human SCI, and require minimal training. However, larger animal models such as non-human primates can form an important intermediary model to confirm results from rodents by providing relevant safety, biodistribution and technical feasibility data (Nout et al. 2012; Kwon et al. 2015) but unique housing requirements make their use less common.

1.1.3.2 Cellular implants

Cell-based regenerative therapies for SCI are a promising approach as transplanted cells are capable of filling many roles including providing trophic support, regulating inflammatory response, modulating the regenerating lost neural circuits, and remyelinating denuded axons (Arriola et al. 2010; Lei Wang et al. 2009; Okamura et al. 2007). The use of cellular implants to bridge the site of the lesion is a popular technique that has been tried over many years with variable success. Currently, there are three approaches to rewire the spinal cord after injury: axonal regeneration (direct endogenous reconnection), axonal sprouting (indirect endogenous reconnection), and neural stem cell transplantation (indirect exogenous reconnection).

1.1.3.2.1 Axon Regeneration

Axon regeneration is the re-growth of transected axons across a lesion site towards their original synaptic targets, whereas other forms of axonal sprouting and synaptic remodeling result in circuit reorganization. These processes can all lead to restoration of function.

1.1.3.2.2 Myelin Regeneration

Demyelination in white matter has been observed in experimental and human SCI (Waxman, et al.1994). Preserved myelin seems to be related to the ability to improve motor function (Plemel et al. 2014), and some transplanted cells can potentially improve myelination. However, it is difficult to experimentally differentiate new myelinated axons from spared myelinated neurites. Thus, debates remain concerning the potential of remyelination after cell transplantation (Plemel et al. 2014).

1.1.3.2.3 Indirect exogenous reconnection

The multitherapeutic ability of stem cells that secrete growth factors, attenuate the glial scar and restrain the production of inhibitory proteoglycan is being evaluated as one of the most promising strategies for SCI (Hill et al. 2004; Ketschek et al. 2012). Several types of cells including embryonic stem cells (ESCs), neural stem cells (NSCs) (P. Lu et al. 2012), induced pluripotent stem cells (iPSCs), have been used for the transplantation therapies (Tetzlaff et al. 2011) and can contribute to the replacement of spinal cord architecture and functionality, thus overcoming the inhibitory glial scar environment (P. Lu et al. 2012; Bonner and Steward 2015). Other transplantations including non-neuronal cell sources such as olfactory ensheathing cells (Cao et al. 2007), (Ramón-Cueto et al. 1998), Schwann cells (Xu et al. 1995), mesenchymal stromal cells (Himes et al. 2006), (Bonner et al. 2011) or nerves have been performed into injured spinal cords allowing some regeneration of severed fibers (Houle et al. 2006), (Tom et al. 2009). However, non-neuronal cells can only provide structural and trophic support to the injury site and no regeneration for damaged axons.

1.1.3.3 Limits of Stem Cell Treatment in SCI

Different methods have been tested to release therapeutic cells into the injured spinal cord. The direct injection of cells into the injury site is the most widely used approach. However, injection of cells could have contraindications for treatment of the spinal cord, such as an unequal distribution of cells in the target tissue and greater risk of potential side effects.

1.2 Stem cells

A stem cell is defined by two criteria: self-renewal and multipotentiality. Self-renewal is the ability to proliferate in an undifferentiated state, whereas multipotency is the ability to differentiate towards several cell types. These abilities of stem cells expand the possibilities of applications in cell-based therapies such as tissue recomposition in regenerative medicine, drug screening, and treatment of neurodegenerative diseases. In addition to stem cells found in the embryo, various adult organs and tissues have niches of stem cells in an undifferentiated state among differentiated cells in the whole body after development. Scientists have found that these tissue-specific "adult" stem cells that are capable of both self-renewal and can generate various cell types from the originating organ. Adult stem cells are localized in the epidermis, brain, liver, bone marrow, skeletal muscle, blood and blood vessels.

1.2.1 Mesenchymal Stem Cells (MSCs)

MSCs are present in many tissues including bone marrow and adipose tissue. In particular, studies have explored the therapeutic potential of bone marrow-derived (BM-MSCs), adipose-derived (AD-MSCs), and umbilical cord (U-MSCs) (Charbord 2010). Also, the opportunity to harvest and transplant cells autologously greatly reduces concerns regarding immunogenicity and graft rejection. However, MSCs bring distinct limitations, including their inherent multipotency that restricts the repertoire of available cell fates.

1.2.2 Pluripotent Stem Cells (PSCs)

PSCs have the potential to differentiate into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system), but not into extra-embryonic tissues. PSCs include ESCs and iPSCs. ESCs are derived from the blastocyst of the embryo and can be indefinitely maintained and expanded in the pluripotent state *in vitro*. Human iPSCs are created from adult somatic stem cells through the overexpression of four transcription factors *in vitro*,

rendering them immortal (Takahashi et al. 2007; Thomson et al. 1998; Takahashi and Yamanaka2006).

1.2.2.1 iPSCs

During the last decade, human iPSCs have been the basis of autologous cell therapies, drug discovery, and new models of human disease. Since iPSCs can be derived directly from adult tissues, they not only bypass the need for ESCs but can be made in a patient-matched manner, which means that each individual could have their own pluripotent stem cell line. Considering that the supplies of autologous cells are unlimited, these cells could be used to generate transplants without the risk of immune rejection. Also, iPSCs-derived neural progenitor cells have been shown to exhibit ESC-like neural differentiation potentials both in vitro and in vivo (Tsuji et al.,2010).

1.2.2.2 ESCs

ESCs are regarded as the archetypal stem cell, with the capacity to endlessly self-renew and the ability to differentiate into any cell lineage (Keller 1995). One of the major strategies for treating the injured spinal cord is to induce ESCs to differentiate towards specific phenotypes to replace the desired cell (neurons or glia) or to produce factors that could limit the damage and sustain regeneration of the tissue. Acquisition of ESCs involves the isolation of embryonic cells from the inner cell mass of the developing blastocyst, resulting in its destruction (Vazin and Freed 2010). Thereby, ESCs raise legal, ethical and immunogenic concerns, limiting the application of ESCs in human SCI (Nussbaum et al. 2007; Oh et al. 2016). Additionally, the formation of teratomas, tumors masses composed of structurally and compositionally heterogeneous aggregates of differentiated somatic tissue, has been observed in numerous models of ESC-derived cell therapy (Nussbaum et al. 2007), (Thinyane et al. 2005).

1.2.3 NSCs

NSCs are self-renewing, multipotent cells that can give rise to neurons, astrocytes, and oligodendrocytes. In the adult brain, NSCs reside in the subventricular zone and the subgranular zone of the hippocampal dentate gyrus (Bond, Ming, and Song 2015). These adult NSCs continuously generate functional neurons throughout life, and this generation is suggested to be critical for biological functions such as olfaction, learning and memory (Ming and Song 2011). Neural stem cells are also present in the spinal cord, but they are unable to

generate neurons (Weiss et al. 1996; Sabelström, Stenudd, and Frisén 2014), (Alfaro-Cervello et al. 2014). Based on their functional multipotency, these cells are used for regenerative medicine, and their therapeutic effects result from the secretion of trophic tissue factors, as well as from interactions with infiltrating cells of the immune system through soluble molecules and exosomes (Luarte et al. 2016), (Teng et al. 2011). Albeit the high proliferative capacity is a hallmark of stemness, a unique characteristic of neural stem cells is their capability to quiescent for very long periods, providing a reserve pool of cells available for tissue regeneration and cell replacement throughout life (L. Li and Clevers 2010; Naik, Birbrair, and Bhutia 2019). Recent developments in stem cell research and regenerative therapy using NSC transplantation indicate this therapeutic strategy as the most promising to re-establish destroyed neural circuits (Assinck et al. 2017), (Abbaszadeh et al. 2018). Therefore, researchers aim at the production of an unlimited number of NSCs in vitro from other stem cells sources such as ESCs (Elkabetz et al. 2008), (S. Shin et al. 2006), PSCs (S. Shin et al. 2006; H. W. Choi et al. 2014) and MSCs (Fu et al. 2008), (Hermann 2004). The use of NSCs as a treatment strategy in CNS disease and injury has been tested for decades. Parkinson's disease specifically has gained the most momentum for potential therapeutic benefits (Studer 2017), however, similar work has been performed in Huntington's disease, stroke, and following spinal cord injury (Studer 2017; Vishwakarma et al. 2014).

1.3 Tissue engineering in SCI

The CNS has a limited capacity to spontaneously regenerate following traumatic injury or disease, requiring innovative strategies to promote tissue and functional repair. The development of tissue engineering technology has opened up new avenues for treating SCI (Dietz and Curt 2006), (Furlan et al. 2016), (X. Chen et al. 2018), (Yao et al. 2018). Tissue engineering is an interdisciplinary field that strives to develop biological substitutes to restore, maintain or improve function of a tissue or whole organ (Langer and Vacanti 1993). Recent advances in materials science have led to biomaterials that aim to promote functional tissue repair following SCI (Shroff 2016; Rossi et al. 2013). This approach could ameliorate repair in two ways: biomaterials can act a) as carriers that can maintain and release their payload (e.g., stem cells and their biofactors) and, from a structural point of view, can act b) as supporting materials for tissue regeneration (scaffolds) (Shoichet 2010). Biomaterials have emerged as an exciting strategy to fill cavitation defects and reproduce the complex structural architecture of the extracellular matrix (ECM) (Caicco et al. 2013; Mothe et al. 2013; Tam, Cooke, and Shoichet 2012; Ansorena et al. 2013; Itosaka et al. 2009). Many of these materials can be

engineered to biodegrade over time, release growth factors, and can even be seeded with stem cells to enhance engraftment (Bregman et al. 1995; Hamid and Hayek 2008).
The philosophy of developing regenerative biomaterials is to mimic the physiological extracellular matrix of the spinal cord and reconstruct a favorable regenerative niche for SCI. Although some progress has been made in SCI therapy by tissue engineering techniques, there are still many problems to be resolved. It is still a challenge to build an ideal regenerative microenvironment at the lesion site, because of the numerous cellular mechanisms that mediate the response to neuronal injury, (Fitch and Silver 2008).
Several strategies to promote tissue regeneration after injury are currently being pursued including cell-based therapies and delivery of bioactive molecules such as small molecules, growth factors, and antibodies (Pakulska, Ballios, and Shoichet 2012). Given the structureless nature of the cavity, scaffold-based strategies have established an alternative for neuroregeneration after SCI (Purushothaman, Sugahara, and Faissner 2012; Orive et al. 2009). Their aim is to provide structural and active growth support to the damaged axons (Brock et al. 2010), (J. Park et al. 2009),(P. Lu et al. 2012), (L. He and Richard Lu 2013). The implantation of a scaffold not only aims at the mechanical and trophic support of the spinal cord, or at the seeding of stem cells to facilitate nerve regeneration, but it also inhibits the glial scar formation, antagonizes myelin inhibitory signals, and combines cells, drugs or other substrates.
To improve the efficacy of SCI repair, combining natural and synthetic biomaterials can improve the performance of scaffolds. The biomaterials used for scaffold fabrication can be natural or synthetic polymers (degradable or non-degradable). Each of those has advantages and disadvantages (Kubinová and Syková 2012), (T. Liu et al. 2012).
To aid tissue growth and increase physiological relevance, scaffolds should aid cell adhesion, proliferation and differentiation. An ideal nerve conduit should be thin, flexible, porous, biocompatible, biodegradable, compliant, neuroinductive, and with appropriate surface and mechanical properties (Verreck et al. 2005). Scaffolds provide contact-mediated guidance for aligned axon growth across the lesion site and act as a vehicle to deliver drugs and biomolecules that favorably modify the environment as well as stem cells that repopulate the lost neural cells.

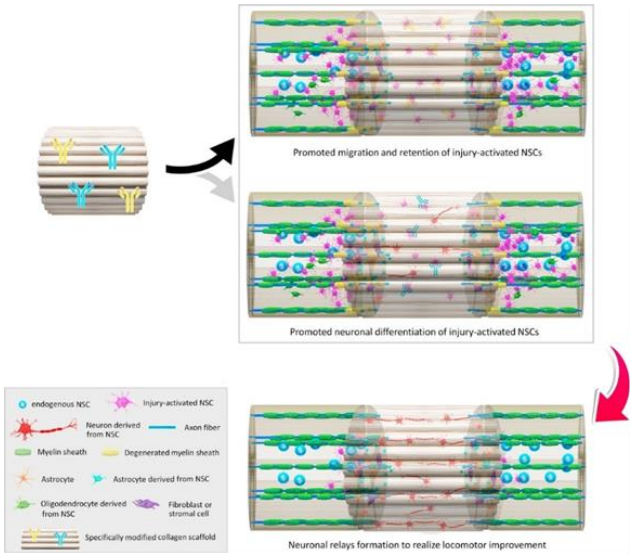


Figure (2) :Scaffold implantation in complete SCI Implantation of a scaffold into the lesion gap could modulate the directed migration and neuronal differentiation of NSCs. Eventually, they could facilitate functional reconnection and integration of the neurons derived from injury-activated NSCs with the transected neuronal axons to restore functional locomotion. This image was taken from *Li et al. (2019) Scaffold-facilitated locomotor improvement post complete spinal cord injury: Motor axon regeneration versus endogenous neuronal relay formation.*

1.3.1 Mechanical properties

As extracellular matrix substitutes for SCI, biomaterial scaffolds ought to meet certain requirements. Tissue engineering products are designed to mimic tissue architecture and responses. Firstly, they need to keep a balance of softness and mechanical strength, to avoid crushing the surrounding residual tissues and to maintain local structure (Rooney et al. 2008). The biomaterials should have the appropriate mechanical properties to mimic the ECM. That means appropriate apposite porosity, biodegradability, permeability, surface topography, and good biocompatibility for cells (J. Wang et al. 2015), (Perale et al. 2011), (Wen and Tresco 2006), (Verreck et al. 2005; Amado et al. 2008). Thus, scaffolds are processed to produce 3D structures, with appropriate shape, size, architecture, and physical properties tailored to fulfill specific functions.

1.3.2 Biocompatibility

Biocompatibility is a property of prime importance as it facilitates cell adhesion, proper functionality of cells and migration and proliferation of cells on the scaffold (O'Brien 2011). Surface modification of the scaffold can be done using bioactive molecules to make biomimetic materials. Bioactive molecules like long chains of ECM proteins including fibronectin, laminin, vitronectin and short peptide sequences are coated on the biomaterials.

1.3.3 Biodegradability

One of the major advantages of synthesizing biodegradable scaffolds is that they eliminate the need for surgical removal of the scaffold and they are absorbed by the surrounding tissues in the body. In the case of neural tissue engineering, controlled biodegradable scaffolds are preferred as the scaffold is meant to support the growth of nerve cells and then be degraded by the body as subsequent repair takes place (Subramanian, Krishnan, and Sethuraman 2009).

1.3.4. Porosity and pore size

An ideal scaffold should possess the appropriate shape and porosity required to mimic its natural tissue. High porosity and a pore size sufficient to aid in cell seeding, vascularization and diffusion of growth factors and nutrients into the scaffold and surrounding tissues is necessary. It is crucial to scaffolds that they have interconnected pores to facilitate cell penetration and diffusion of nutrients to cells and ECM present in the scaffold (O'Brien 2011).

1.3.5 Morphology-Topography

Topographical cues such as grooves, ridges, pores and nodes can influence cell adhesion, migration, proliferation and differentiation (Crapo, Gilbert, and Badylak 2011; Subramanian, Krishnan, and Sethuraman 2009). By altering surface morphology and topography it has been demonstrated that it is possible to guide the growth and migration of neurites. Especially, ridged/grooved surfaces are intended to guide axonal outgrowth of neurons for spinal cord regeneration.

The differentiation process of stem cells varies based on the scaffold components, soluble growth factors, physiological conditions, external stimuli, etc (Barrilleaux et al. 2006). Differentiation response to different stimuli and thus the availability of proper scaffolds and toxicity concerns of scaffold materials were the critical factors which limit the stem cell-based tissue engineering. Some scaffold formats include polymer gels, solid porous scaffolds, fibrous scaffolds, and acellular scaffolds (Lluch et al. 2015; Dhandayuthapani et al. 2011).

1.3.6 Composition

Many different types of scaffolds have been developed for the treatment of SCI (Wong et al. 2008; J. Liu, Cui, and Losic 2013), but based on composition they can be classified as natural polymers, synthetic biodegradable polymers, or synthetic non-degradable polymers).

1.3.6.1 Synthetic

Synthetic materials have a long list of advantages for use as scaffolds in regenerative medicine, including low inflammatory response, well-controlled biodegradability, low or non-toxicity, controllable porosity, and customized physicochemical and mechanical properties (Subramanian, Krishnan, and Sethuraman 2009). Different kinds of synthetic polymers can be mixed to form a new type of biomaterial with unique characteristics (Subramanian, Krishnan, and Sethuraman 2009). Thus, synthetic material scaffolds can be applied for adjuvant treatment of injuries in the central nervous system and produce good therapeutic outcomes. Various polymeric regenerative biomaterials have been used in the repair and treatment of SCI. To date, synthetic polymers such as polylactic acid (PLA), polyvinyl alcohol (PVA), polyhydroxyalkanoate , and poly (lactic-co-glycolic acid) (PLGA) were also utilized in the preparation of 3D scaffold due to their easily adapting porosity, degradation time, and mechanical characteristics and found to be effective in influencing the fate of stem cells (Dhandayuthapani et al. 2011; Carletti, Motta, and Migliaresi 2011). Most of the synthetic biodegradable materials are hydrophobic which limits their use as tissue engineering scaffolds. Hybrids of synthetic and natural biomaterials can combine desirable properties of both types of materials.

1.3.6.2 Natural

Owing to their biocompatibility, natural polymers are commended as biomaterials for preparing 3D scaffolds. They are easily obtained from natural sources and they have predictable physical, mechanical and biologic properties since they undergo highly controlled synthesis, resulting in regular structures. They are biodegradable and contain signals for cell adhesion, but they are also hard to be sterilized. Among those commonly used are:

Hyaluronic acid has a high concentration in the nervous system, especially in the central nervous system, so it is particularly beneficial for designing scaffolds for SCI repair.

Chitosan is a copolymer composed of N-glucosamine and N-acetyl-glucosamine,and has been demonstrated to be effective for nerve tissue regeneration in traumatic SCI due to its neuroprotective effect (Cho, Shi, and Borgens 2010),(Gnavi et al. 2013),(W. Wang and Peng 2017).

Gelatin is the hydrolysate of collagen in connective tissue (Pearlman and Sheppard 1996) . This form of biomaterial is easily combined with growth factors and cells for SCI.

Fibrin is mainly derived from plasma proteins and has significant blood or tissue compatibility, without toxic side effects or other adverse reactions to the recipient

*Collagen i*s the natural ECM component found in most of the cellular niches. Collagen is an abundant protein of the extracellular matrix in body tissue and is highly conserved between species. Thus, collagen is very easy to obtain and has low immunological rejection response after transplantation. Importantly, collagen can provide binding sites to support adhesion, migration, proliferation, and even differentiation of cells (Guan et al. 2013),(Murphy et al. 2017) . Because of the above advantages, collagen has become one of the most popular natural biological materials for treating SCI (Yoshii et al. 2004), (Kourgiantaki et al. 2020). Collagen materials are fabricated into multiple forms of scaffolds, such as sponge, hydrogel, and guidance conduit, to deliver cells, drugs, and proteins into the injured site.

1.3.7 ECM based scaffolds

The extracellular matrix in biological systems holds the cells together and provides a medium for the cells to interact and migrate (Dillon et al. 1998; L. M. Y. Yu, Leipzig, and Shoichet 2008). It creates a biologically active microenvironment and surface architecture that is favorable for a variety of cells to grow, differentiate and proliferate by providing the optimal required conditions (Wei and Ma 2009; Mandal and Kundu 2009).
ECM influences many cellular functions through three modes: (a) mechanical stimulation from substrates with different stiffness, (b) regulation of soluble factor availability and activity, and (c) intracellular signaling activated by cell adhesion molecules. Thus the synthetic scaffold should mimic the ECM in promoting cell adhesion, proliferation, and differentiation in vitro and in vivo (J. Lee, Cuddihy, and Kotov 2008), (Ma and Zhang 1999).
In addition, ECM proteins collectively represent a class of naturally derived biomaterials purified from harvested organs and tissues with increasing scientific focus and utility in tissue engineering and repair. Components such as collagens, elastins, trace cell-engaging proteins (fibronectin, vitronectin, osteopontin, glycosaminoglycans (GAGs), are assembled to form a complex structure, the ECM (Hynes 2009).
Decellularized ECM is useful *in vitro* model for studying the comprehensive roles of ECM because it retains a native-like structure and composition. Acellular scaffolds have been increasingly applied in tissue engineering, and are fabricated by removing the cellular components from a tissue or a whole organ (namely decellularization), leaving its 3D structure and the remnant ECM (Nishio 2009). Thus, this form of acellular scaffold can provide a

skeleton structure for the adhesion, proliferation, migration, and differentiation of seeding cells. Acellular scaffolds exhibit advantages over other tissue engineering scaffolds: they have similar if not the same chemical and biological composition as natural ECM, they retain native ECM architecture and mechanical properties and considerably reduce immunological complications.

1.3.7.1Adipose derived ECM

With the rapid development of adipose tissue engineering, decellularized adipose tissue (Rao Pattabhi, Martinez, and Keller 2014, Flynn 2010), (Mohiuddin et al. 2020) (C. F. C. Brown et al. 2015, C. Yu et al. 2017, Morissette Martin et al. 2018) has attracted much attention due to its wide range of sources and good regeneration capacity. Decellularized adipose matrix (DAM) is widely used in soft tissue regeneration because of its unique biological and physical properties. A large amount of adipose tissue can be obtained by using the developed method of degreasing and decellularization (Rao Pattabhi, Martinez, and Keller 2014, Flynn 2010). Thus, DAM, provides a natural microenvironment for the growth and differentiation of stem cells. ECM is composed of collagens I (B. N. Brown et al. 2011,Y. He et al. 2018, Young et al. 2011), IV(Rao Pattabhi, Martinez, and Keller 2014, Flynn 2010)(Y. He et al. 2018)(Q. Lu et al. 2014), (Giatsidis, Succar, Haddad, et al. 2019), and VI (Thomas-Porch et al. 2018), laminin (B. N. Brown et al. 2011),(Y. He et al. 2018),(Y. He et al. 2018; Young et al. 2011),(Thomas-Porch et al. 2018; S. Zhang et al. 2016),(Thomas-Porch et al. 2018; S. Zhang et al. 2016; Song, Liu, and Hui 2018), fibronectin(Giatsidis, Succar, Waters, et al. 2019), (Zhao, Fan, and Bai 2019), elastin(Y. C. Choi et al. 2012), GAGs (B. N. Brown et al. 2011), (Y. C. Choi et al. 2012), (Y. He et al. 2018; Young et al. 2011), (Q. Lu et al. 2014),(Lina Wang et al. 2013), and other biologically active macromolecules.

1.3.8 Graphene

1.3.8.1 Structure

The main critical element in graphene and its derivatives is carbon. Carbon is an abundant element that has important applications in the fields of science and technology. Many various carbon allotropes can be synthesized by altering the combinations of sp, sp2, and sp3 hybridization (Rinaldi 2010), (Orlita et al. 2008), (C. Lee et al. 2008), and a variety of carbon structures and nanostructures have been introduced to date. Each carbon atom has four valence electrons that could be shared through covalent bonds with other elements. Graphene is mainly

composed of two-dimensional sheets less than 10 nm thick. These sheets are made up of sp2 hybridized carbon atoms that are bonded in a honeycomb-like lattice.

1.3.8.2 Derivatives

Graphene and its derivatives have very similar structures that form 2D materials. Graphene family incorporates several derivatives with contrasts within the structure and properties such as graphene oxide (GO), reduced graphene oxide (rGO), graphene quantum dots (GQDs), graphene nanosheets, monolayer graphene, and few layer graphene. G-related materials (GRMs) are classified based on either number of layers in the sheet or their chemical modification. Each member of GRMs differs from the other in terms of number of layers, surface chemistry, purity, lateral dimensions, defect density and composition. GO is a highly oxidized form of chemically modified graphene that consists of single atom thick layer of graphene sheets with carboxylic acid, epoxide and hydroxyl groups in the plane. GO has outstanding aqueous processability, amphiphilicity, ease of surface functionalization. rGO is mainly produced to restore the electrical conductivity, optical absorbance in GO while reducing the oxygen content, surface charge, and hydrophilicity (Bagri et al. 2010). Having such different composition and structures, these compounds possess very diverse properties that have to be taken into consideration when planning biomedical applications, as they elicit completely different biological responses. Physicochemical properties like the unique planar 2D structure, high specific surface area and availability of free π electrons make graphene a good candidate for interaction with organic molecules and has been explored widely in drug delivery.

1.3.8.3 Properties

Due to its unique structure and geometry, graphene exhibits remarkable physical and chemical properties, that allow its chemical and biological functionalization (Jiang 2011), (S. Guo and Dong 2011). Graphene displays properties such as, biodegradability, electrical conductivity (Neto et al. 2009), transparency, thermal conductivity (Balandin et al. 2008), optical transmittance and mechanical strength (Balandin et al. 2008; C. Lee et al. 2008), that encourage further utilization for 3D cultures (S. R. Shin et al. 2016; Akhavan 2016). Also the porous morphology, great surface area and selective permeability of gases, enable graphene materials to be the best component for scaffold engineering. These properties offer an excellent capability to immobilize a large number of substances, including metals, drugs, biomolecules, fluorescent probes, and cells (Wray et al. 2012; Reina et al. 2014). In the field of tissue engineering,

graphene scaffolds can provide an environment for neural tissue regeneration: (1) Directing differentiation. It was reported that the unique surface properties of 2D graphene can induce stem cell to preferentially differentiate into specific lineage (Nayak et al. 2011; S. Y. Park et al. 2011) (2) 3D porous structure. Different from 2D stem cell culture system, the porous graphene foam could provide 3D microenvironments in which cells can resemble their in vivo counterparts. Interestingly, the features of the G scaffolds (i.e., stiff vs. soft) differentially affected cell adhesion and proliferation and could drive NSC differentiation toward the astrocyte and neuronal lineages, respectively.

The biocompatibility of the material must be excellent to eliminate the possibility of creating an adverse effect within the living tissues (Manavi-Tehrani et al. 2010; Y. B. Wang et al. 2013) and making sure at the same time that the material can lead to successful tissue engineering techniques is a key factor as well (Rasoulianboroujeni et al. 2019), (Eslami et al. 2018). Graphene shows improved biocompatibility compared to other classes of carbon nanostructures due to its unique surface physical and chemical characteristics, including the number and surface area of layers, chemical functional groups, surface charge density. Graphene nanomaterials with small size ranging are reported to induce cytotoxicity possibly because of the internalization by cells following their interactions (B. Zhang et al. 2016), (Mendes et al. 2015). Sufficiently large graphene flakes are reportedly more biocompatible when they are utilized as substrates to grow cells (Nayak et al. 2011),(Kenry et al. 2016),(Kalbacova et al. 2010). Additionally, adverse cytotoxic effect is not expected if the cells are not exposed to graphene-based nanommaterials in high concentrations for a long period (Pelin et al. 2017). In previous evaluations of graphene-based nanomaterials in vitro, the shape and concentration of graphene proved to be the most critical components in measuring the degree of its cytotoxicity and how the nanomaterials interact with biological systems and cells (Guazzo et al. 2018).

Published data suggest that GO is less toxic than bare G, rGO and hydrogenated-G (Bianco 2013), (Ou et al. 2016), (Akhavan, Ghaderi, and Akhavan 2012; Bramini et al. 2018). Indeed, it was shown that graphene induces high oxidative stress due to the generation of reactive oxygen species (Y. Zhang et al. 2010). Mechanisms of cytotoxicity of G nanosheets have been reported in literature on different cell types, and include the physical interaction with cell membranes (Seabra et al. 2014), disruption of cell cytoskeleton (Tian et al. 2017) oxidative stress due to production of reactive oxygen species (ROS) (J. Chen et al. 2016),(Mittal et al. 2016), DNA damage, such as chromosomal fragmentation, DNA strand breakages, point

mutations and oxidative DNA alterations (Akhavan, Ghaderi, and Akhavan 2012), (Fahmi et al. 2017), autophagy (G.-Y. Chen et al. 2014), apoptosis and/or necrosis (Lim et al. 2016).

1.3.8.4 Graphene-based materials in tissue engineering

Graphene may represent a promising scaffold to bridge nerve defects, favoring nerve regeneration, support neuronal growth and the development of synaptic activity (Martín et al. 2017). Most importantly, as conductive substrate graphene may provide cues to reinforce the formation of interconnected neural networks and electrical connections among cells (S. Y. Park et al. 2011; Serrano et al. 2014) . Carbon materials of different dimensions such as fullerenes, carbon nanotubes, and graphite were successfully employed in many tissue engineering investigations due to their mechanical stability (Rifai, Pirogova, and Fox 2019; Martinelli et al. 2018; Minami et al. 2015). These carbon nanomaterials provide unique features that are compatible with the ECM components such as collagen fibers due to their similar dimensions (Rifai, Pirogova, and Fox 2019; Martinelli et al. 2018; Minami et al. 2015), (Cheng, Rutledge, and Jabbarzadeh 2013; Starý et al. 2003). Owing to this, advances in stem cell-based tissue engineering have a huge reliance on graphene-based scaffolds, specifically in terms of inducing signals for cell differentiation and proliferation (S. K. Lee, Kim, and Shim 2013; Ding, Liu, and Fan 2015).

Graphene nanomaterials-based scaffolds have been employed in various medical applications including tissue engineering for the past few decades. Interestingly, most of the recent in vitro studies indicate that graphene-based nanomaterials (i.e. mainly graphene, graphene oxide and carbon nanotubes) promote stem cell adhesion, growth, expansion and differentiation. Hence, it can be potentially used as a reinforcement material in hydrogels, biodegradable films, electrospun fibers and other tissue engineering scaffolds. Various three-dimensional G and GO foams were shown to be compatible substrates for stem cells (Crowder et al. 2013),(N. Li et al. 2013), (W. Guo et al. 2016),(Sayyar et al. 2016). Given these premises, a large amount of research on G focuses on medical applications, and particularly in the field of neurology, where its mechanical and electronic features make it a strong candidate for replacing current devices (Kostarelos et al. 2017),(Reina et al. 2017). Another appealing aspect of GRM-based medical devices lies in the increasing evidences of G biocompatibility, an extremely important issue to take into consideration for any new biomaterial brought to the market.

1.4 Combinatorial methods

Concerns have been raised that NSCs might migrate into undesired locations within or outside the neuroaxis or remain undifferentiated for an extended period before becoming mutagenic and giving rise to cancer (Assinck et al. 2017). The transplanted stem cells ultimately die largely during the first 3 weeks after transplantation due to the deleterious microenvironment caused mainly by low oxygen levels (hypoxic) high levels of ROS, inflammatory cytokines, and cell-mediated immune response (Hill et al. 2007). For these reasons, there is currently no effective treatment for spinal cord injury. Accordingly, overcoming these multi-factorial conditions requires a combinatorial approach (Bunge 2008). A combinatorial approach using cells, growth factors and regenerative biomaterials should be applied to attain ideal scaffolds for spinal cord regeneration. Therefore, it is becoming increasingly more frequent to combine stem cell transplantation with other strategies that would enhance the effect of the transplanted cells (Ruff, Wilcox, and Fehlings 2012). Cell transplants can provide trophic support, neuroprotection and anti-inflammatory effects, as well as forming permissive tissue bridges across the spinal lesion. Furthermore, the utilization of undifferentiated stem cells cultured with suitable signals in the 3D culture could be developed into specific organ-based tissue, which supports the field of regenerative medicine by replacing the damaged organs. NSCs grown on 3D-Graphene foams were able to differentiate into neurons and astrocytes. Additionally, 3D-Graphene foams act as a platform for electrical stimulation of NSCs to enhance their differentiation. Similar results have been obtained more recently with rGO microfibers, which could support NSC viability and drive them toward a neuronal phenotype (W. Guo et al. 2017). In addition, interfacing G with neural cells was also proposed to be extremely advantageous for exploring their electrical behavior or facilitating neuronal regeneration by promoting controlled elongation of neuronal processes (N. Li et al. 2011), (Tu et al. 2014), (Fabbro et al. 2016). Combining mechanism-based biological strategies with targeted technological interventions could augment neuroplasticity, followed by rehabilitation to direct circuit reorganization, facilitating clinically meaningful recovery after SCI.

2. Aim

The aim of this present work is the evaluation of embryonic neural stem cell growth and differentiation within scaffolds composed of porcrine adipose derived decellularized extracelluar matrix (adECM) combined with GBM . NSCs harvested from E13.5 cortices of mouse embryos and maintained in 2D and 3D cultures will be evaluated based on their structure and differentiation in different time points and will be monitored both with and without the induction of the differentiation pathways. The proliferative and survival potentials of NSCs and/or neural lineages will be examined as well. The immunostained cells will be imaged using fluorescent and confocal laser scanning microscope. Further morphological information for the NSCs and the adECM scaffolds will be acquired using SEM.

3. Materials and Methods

3.1 Animals

C57/BL6 mice were housed in a temperature-controlled facility on a twelve hour light/dark cycle, fed by standard chow diet and water ad libitum in the Animal Facility of the Institute of Molecular Biology and Biotechnology (IMBB-FORTH, Heraklion, Greece). Animals were handled according to the international and national bioethical rules and conformed to the bioethics regulations of the Institution. Animal experimentation received the approval of Veterinary Directorate of Prefecture of Heraklion, Crete and was carried out in compliance with Greek Government guidelines and the guidelines of our ethics committee.

3.2 Embryonic NSCs harvest and culture

3.2.1 Cortical NSCs Isolation

Pregnant mice (gestational day 13.5) were sacrificed via cervical dislocation and amniotic sacs were removed from the belly into a petri dish with cold 1X Hanks' Balanced Salt solution (HBSS)/5% penicillin/streptomycin (P/S) medium. All embryos were removed from the amniotic sacs and placed under a stereoscope in a petri dish with 1X HBSS/5% P/S medium. The embryo was immobilised with forceps, in order to isolate the cortex. After the brain was exposed, it was dissected from the skull, then the midbrain was removed to isolate the forebrain and then the two hemispheres. Finally, the meninges were removed and the cortex was dissected from the ganglionic eminences and placed in a 15mL tube with cold HBSS/5% P/S medium.

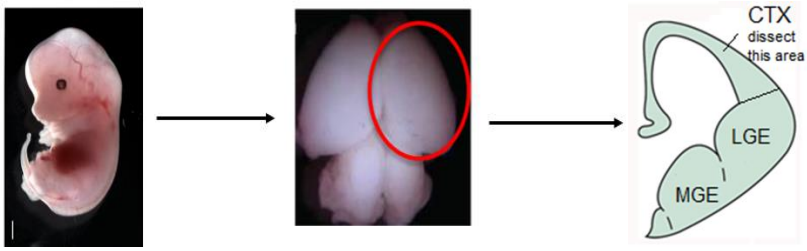


Figure (3): Dissection of mouse Cortical NSCs

3.2.1.1 Tissue processing for NSC

The excess of the 1x HBSS/5% P/S medium was removed and warm (37˚C) complete embryonic medium was added. The cells were dissociated with the use of a 1 mL tip and complete embryonic medium was added, followed by a 5 min spin at 100 g. The supernatant was discarded and the pellet was reconstituted in a complete embryonic medium. The concentration of the cells was measured with a haemocytometer. 5x104 cells/mL were plated in a T25 flask (Nunc™ EasYFlask™ TC 25cm2 Filter). Complete-embryonic medium was added to the cultures every other day. Primary neurospheres started to form at day 3. The neurosphere formation and morphology was checked every day. Averagely, on the fifth-seventh day neurospheres were ready to be passaged as they reached a diameter of around 200μm.

3.2.1.2 Passaging of NSC culture

Every three to five days, neural stem cells were ready for sub-culture. The contents of the T25 flask were put in a 50 mL tube and centrifuged for 5 min at 100 g at Room Temperature (RT). The supernatant was discarded and 200 uL accutase was added to the cell pellet for each 5 mL of initial culture, followed by incubation at 37˚C for 5-7 min with intermediate ups-downs using 1000 uL and 200uL pipette. When neurospheres were completely dissociated DMEM/F12 medium was added in a 10x volume of accutase in order to dilute the enzyme and refresh the cells. Similarly, cell suspension was centrifuged once again in the same way and finally cells are reconstituted in 1 mL complete- embryonic medium and measured with a haemocytometer. $5x10^4$ cells/mL were plated in T25 flasks for passaging or used for experiments. Neurospheres between passages 3 to 8 were collected after dissociation into single cell suspension and used for experiments. For the experiments in 2D cultures, dissociated NSCs were plated in a concentration of $5x10^4$ cells/mL in Poly-D-Lysine / laminin coated coverslips and treated as in 3D cultures depending on the experimental protocol.

Table (1): Table of used media for cell cultures

Embryonic NSC culture medium	Dissection medium
1.32% D-glucose (40% stock) (SIGMA G8769-100ML)	5% Penicillin/Streptomycin (10,000U/mL Gibco catalogue #15140122)
0.2% Primocin (50 mg/mL stock) (Invivogen ant-pm-1)	10% 10x HBSS medium (gibco catalogue #14185-045)
2% B27 supplement without Vitamin A (gibco catalogue #12587-010)	filtered sterile dH_2O
0.5 % FGF (20 ug/mL stock) (R&D systems catalogue: 233-FB-025)	
0.5% EGF (20 ug/mL stock) (R&D systems catalogue: 236-EG)	
DMEM/F12 (Thermo cat: 11330-032))	

3.2.2. Cell plating

Coverslips were covered with PDL(SIGMA, P6407) at 100 ug/mL (in dH_2O sterile filtered) and incubated for at least 2 hours in a humidified 37°C, 5% CO_2 incubator. PDL was removed and plates were washed with dH_2O. Then, coverslips were coated with 15 ug/mL of Laminin (Laminin stock 1mg/mL. L2020 SIGMA), and incubated for 2 hours (or more) in a humidified 37°C, 5% CO_2 incubator. Laminin was removed and coverslips were washed with dH_2O. Consequently, warm complete embryonic NSC medium was added and NSCs were plated in $5x10^4$ cells/mL for experiments.

3.3 AdECM-GO scaffolds

3.3.1 Scaffold Fabrication

Adipose extracellular matrix material (adECM) based foams were produced by solid-liquid phase separation followed by freeze-drying varying the content of adECM (30 and 50 wt.%). Compressive Modulus was measured at 35.83 ± 4.02 Pa. Pore size was variable with high interconnectivity. The cross-linker EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) was used for the covalent conjugation of rGO and adECM. Cross-linking with EDC:NHS (1:1 molar ratio) was performed using 0.0033mM of EDC per mg of adECM. adECM from porcine adipose tissue using organic solvent decellularization, was acquired from TECNALIA-(San Sebastián Spain). Briefly, cleaned and creamed porcine adipose tissue was homogenized on ice using a POLYTRON (PT3100) with two different rods at 12000rpm for 5min. Afterwards, the homogenized tissue was centrifuged at 5000 rpm for 5 min using ultrapure water. Phase separation produced and lipids were discarded obtaining a protein pellet which was treated with triton-x-100 and isopropanol. Decellularized tissue was thoroughly cleaned with phosphate buffer saline (PBS) and ultrapure water, frozen at -20 °C and lyophilized (Appendix A). Commercially available rGO was acquired from Graphenea (San Sebastián, Spain). Optimization of the methodologies for the adECM/GBM mixtures, preparation and characterization of adECM/GBM scaffolds were performed from University of Aveiro (UAVR).

3.3.2 Scaffold manipulation

Scaffolds were received dehydrated and non-sterile. Each scaffold was carefully sliced in 1 x 1 mm pieces with the use of surgical scalpel. Scaffolds were hydrated on the first day with 500uL of dH_2O. Scaffolds were rinsed with dH_2O every 30 min for 1,5 h at RT on shaker and stored in dH_2O at 4 ^oC. The same procedure was repeated the next day. On the third day, sterilization was achieved by washing scaffolds with 70 % ethanol for 30 min and then with dH_2O to remove and ethanol residues and rinsed with DMEM/F12. NSCs plating to scaffolds was succeeded by pipetting 3 uL containing $5x10^4$ NSCs on top of scaffolds, incubating at 37^oC for 15 min, and finally adding 500 uL NSC culture medium.

3.4 Time course experiments of E13.5 NSCs in 2D and 3D cultures.

Survival and proliferation were assessed at days 3 and 5 for the 2D cultures. Dissociated neurospheres were cultured in the presence of growth factors with medium change every other

day. Cultures were fixed at indicated days and processed for immunocytochemistry. For the spontaneous differentiation experiments, dissociated neurospheres were seeded in 2D conditions in the presence of growth factors for 3 days. On the 3rd day the medium was removed and replaced with growth medium without growth factors. In the case of induced differentiation experiments the 3rd day the medium was switched to the differentiation medium (see *Table 2*). On day 8 cultures are fixed and further processed for immunocytochemistry and imaging. For each experimental treatment, at least three samples were quantified.

Table (2): Table of used differentiation media

Embryonic NSC medium without growth factors (Spontaneous Differentiation medium)	Embryonic NSC medium for differentiation towards astrocytes	Embryonic NSC medium for differentiation towards neurons	Embryonic NSC medium for differentiation towards Oligodendrocyte Precursor Cells
2% B27 supplement without Vitamin A (gibco catalogue #12587-010)	2% supplement without Vitamin A (gibco catalogue #12587-010)	2% B27 supplement without Vitamin A (gibco catalogue #12587-010)	2% B27 supplement without Vitamin A (gibco catalogue #12587-010)
1.32 % D-glucose (40% stock) (SIGMAG8769-100ML)	1.32% D-glucose (40% stock) (SIGMA G8769-100ML)	1.32% D-glucose (40% stock) (SIGMA G8769-100ML)	1% N2 Supplement (SIGMA 17502048)
0.2% Primocin (50 mg/mL stock) (Invivogen ant-pm-1)	0.2% Primocin (50 mg/mL stock) (Invivogen ant-pm-1)	0.2% Primocin (50 mg/mL stock) (Invivogen ant-pm-1)	1% Fetal Bovine Serum (Gibco™ # 10270-106 Fetal Bovine Serum, qualified, E.U. approved, South America origin
DMEM/F12 (Thermo cat: 11330-032))	1% FBS (Gibco™ # 10270-106 Fetal Bovine Serum, qualified, E.U.-approved, South America origin)	Retinoic acid 0.1uM	60ng/mL triiodo-l-thyronine (T3) (Sigma T6397-250MG)
	DMEM/F12 (Thermo cat: 11330-032)	DMEM/F12 (Thermo cat: 11330-032)	10ng/mL Neurotrophin-3 (NT3) (Peprotech #450-03)
			10ng/mL Platelet-derived-Growth –Factor A (PDGFAA) (Peprotech 100-13A-2UG)
			DMEM/F12 (Thermo cat: 11330-032)

3.4.1 Viability assay

Viability was assessed through the live/dead assay using calcein-AM and propidium iodide (PI) solution. Calcein-AM, acetoxymethyl ester of calcein, is highly lipophilic and cell membrane permeable. Though calcein-AM itself is not a fluorescent molecule, the calcein

generated from Calcein-AM by esterase in a viable cell emits a strong green fluorescence (λex 490 nm, λem 515 nm). Therefore, calcein-AM only stains viable cells. Alternatively, the nuclei staining dye PI cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membrane, and intercalates with the DNA double helix of the cell to emit red fluorescence (λex 535 nm, λem 617 nm). In order to assess the viability in the culture, medium was removed and replaced with 5 uM calcein AM (live dye; Thermo # C3100MP) and 3uM PI (dead dye) and Hoechst (Thermo # H3570) 1:10000 diluted in warm 1x PBS.

3.4.2. Proliferation assay

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a synthetic nucleoside that is an analog of thymidine. BrdU is commonly used in the detection of proliferating cells and can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle during which DNA is replicated), substituting for thymidine during DNA replication. Antibodies specific for BrdU were used to detect the incorporated chemical, thus indicating cells that were actively replicating their DNA. Six hours prior completion of the experiment, 1 uM of BrdU analogue, was added to the cultures, in order to assess the proliferation of the cells. (BD Pharmingen # 51-2420 KC).

3.4.3 Immunocytochemistry

Samples were fixed by 4% paraformaldehyde (PFA) and washed PBS for 5 min at RT. In the case of BrdU staining, cells were incubated in 2N HCl at 37 ^{o}C for 30 min, then rinsed in 0.1 M sodium tetraborate pH 8.5 at RT for 10 min and washed twice with 1x PBS for 10 min, at RT. The rest of the procedure is identical for all immunocytochemistry assays followed. Samples were washed in 0.1%PBSTx (1x PBS 0.1% Triton X-100), and blocked in 10% Normal (goat or donkey) Serum (specific to the species the secondary antibodies are raised) 0.1% bovine serum albumin (BSA) in 0.3% PBSTx at RT for 1 h (2 h for 3D cultures). Samples were incubated in primary antibodies (see *Table 3*) diluted in 0.1% PBSTx, 1% Normal Serum overnight at 4 ^{o}C. The next day samples were washed twice in 0.1% PBSTx for 10 min at RT, incubated in fluorophoreconjugated secondary antibodies (Thermo) diluted 1:1000 in 0.1% PBSTx for 1 h (2 h for 3D cultures) at RT, washed twice in 0.1% PBSTx and PBS, and counterstained with nucleus stain (1:10000 for HOESCHT) in 1x PBS for 10 min (30 min for 3D cultures),at RT. For 2D experiments, samples were washed twice with 1x PBS for 5min

and the coverslips were carefully placed in superfrost plus slides (Thermo) with VECTASHIELD® Antifade Mounting Medium (VECTOR H-1000). For 3D experiments, scaffolds were washed with 1xPBS and stored in 1xPB 1% P/S.

3.5 Imaging, Image analysis

2.5.1 Imaging

Samples were imaged at Leica TCS SP8 inverted confocal microscope using a ×40 oil-immersion objective lens. For 2D experiments ten randomly selected planes per technical replicate (in total 30 planes) per experiment were required. For 3D experiments five to six randomly selected planes per experiment were required.

2.5.2 Image analysis

Images were processed by Fiji Image J software in process that consisted of two steps as follows: (1) identification of cell nuclei at each plane counted manually based on HOECHST staining. (2) Manually counted cells based on markers of interest using the "Cell Counter" plug in ($GFAP^+$, $Tuj1^+$, and $PDGFRa^+$, BrdU etc.) as well as based on confocal z-stacks due to staining pattern complexity. Tuj^+ neurons, $GFAP^+$ astrocytes or $PDGFRa^+$ OPCs were counted manually based on Hoechst nuclei surrounded by Tuj1, GFAP or PDGFRa staining. The results represent the mean values of three different experiments.

Experimental data are expressed as mean ± SEM. Statistical analysis was performed using the Prism software (Graphpad Prism 6). Statistical significance was assessed by unpaired two-tailed Student's t tests (two-group comparisons) or analysis of variance (ANOVA) assuming a statistical significance level of 0.05.

3.6 Scanning electron microscopy (SEM)

Cell-seeded scaffolds were washed twice in 0.1 M sodium cacodylate buffer (SCB) for 15 min, fixed in 2% glutaraldehyde, 2% formaldehyde in 1% SCB for 30min, washed twice in 1% SCB for 15 min, and dehydrated in serial ethanol solutions. Samples were sputter coated by a 10 nm-thick gold layer (BAL-TEC SCD 050 Sample Sputter Coater) and imaged in a JEOL 7000 scanning electron microscope (JEOL, Tokyo, Japan) at 15 kV voltage.

Antibody	Host animal	Dilution for immonocytochemistry	Distributor-Cat. num
Anti-Glial Fibrillary Acidic Protein (GFAP)	chicken	1:1000	Millipore #AB 5541
Nestin	chicken	1:1000	NOVUS #NB100-1604
PDGFR-a	goat	1:200	R&D #AF 1062
Neuron-Specific ClassIII Beta Tubulin (Tuj1)	mouse	1:1000	Biologend #801201
BrdU	mouse	1:200	Invitrogen MoBU-1 # B3512,
Sox2	rabbit	1:200	Abcam #AB 15830
Synaptophysin	rabbit	1:100	Abcam#AB32127

Table (3): Table of used antibodies

4.Results

4.1 Scanning Electron Microscopy

To study the scaffold - NSC interactions and in order to investigate the infiltration of NSCs within the scaffold, cells were seeded and imaged inside the adECM/GO scaffold using SEM. NSCs were seeded in a single-cell suspension and allowed to grow over three days. It was observed that NSCS easily infiltrated inside scaffold pores in both the adECM/GO 70/30 [Fig. 4 A-D] adECM/GO 50/50 [Fig. E-H] scaffolds, where they adhered as single cells or formed neurosphere-like aggregates conquering the pore cavities.

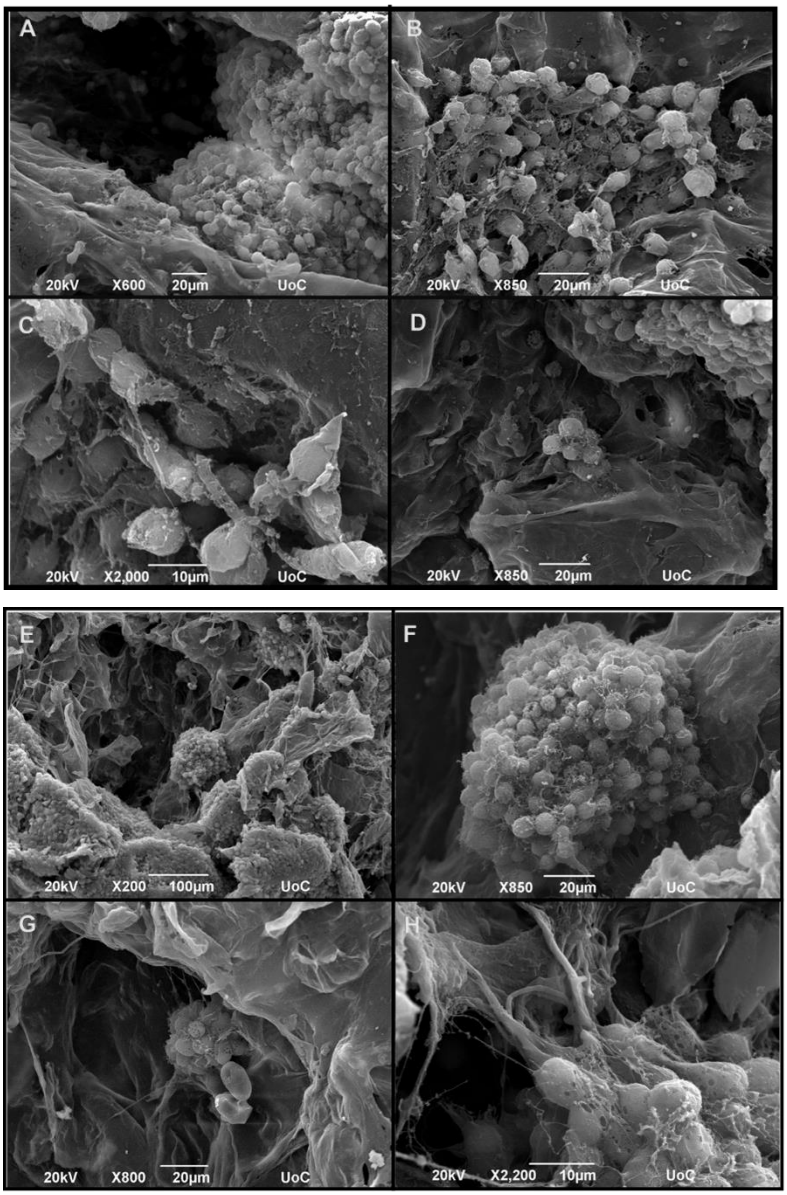


Figure (4): SEM images. SEM images from adECM/GO 70/30 [A-D] adECM/GO50/50 [E-H] scaffolds highlighting their structure. High-magnification image in B, C, F, G, showing neurospheres grown inside scaffolds at 3 DIV.

4.2 Evaluating the viability of E13.5 NSCs

4.2.1 Cell viability in 2D cultures

NSCs in single cell suspension were cultured in the presence of growth factors (EGF/FGF) in 2D conditions. After 3 and 5 days in vitro (DIV) the viability of the NSCs was evaluated using the live/dead assay (Fig. 5 A-H). The number of calcein, PI and HOECHST positive cells was assessed in three independent experiments (n=3). The percentage of dead cells (PI^+) in respect to the total number of cells ($HOESCHT^+$) was calculated at 3DIV at 11,48 ± 0,6030 % and at 5DIV at 13.31 ±2.160 %. There was no significant ($P_{student's\ t\text{-}test} > 0.05$) difference in the viability of NSCs between 3DIV and 5DIV (Fig. 5)

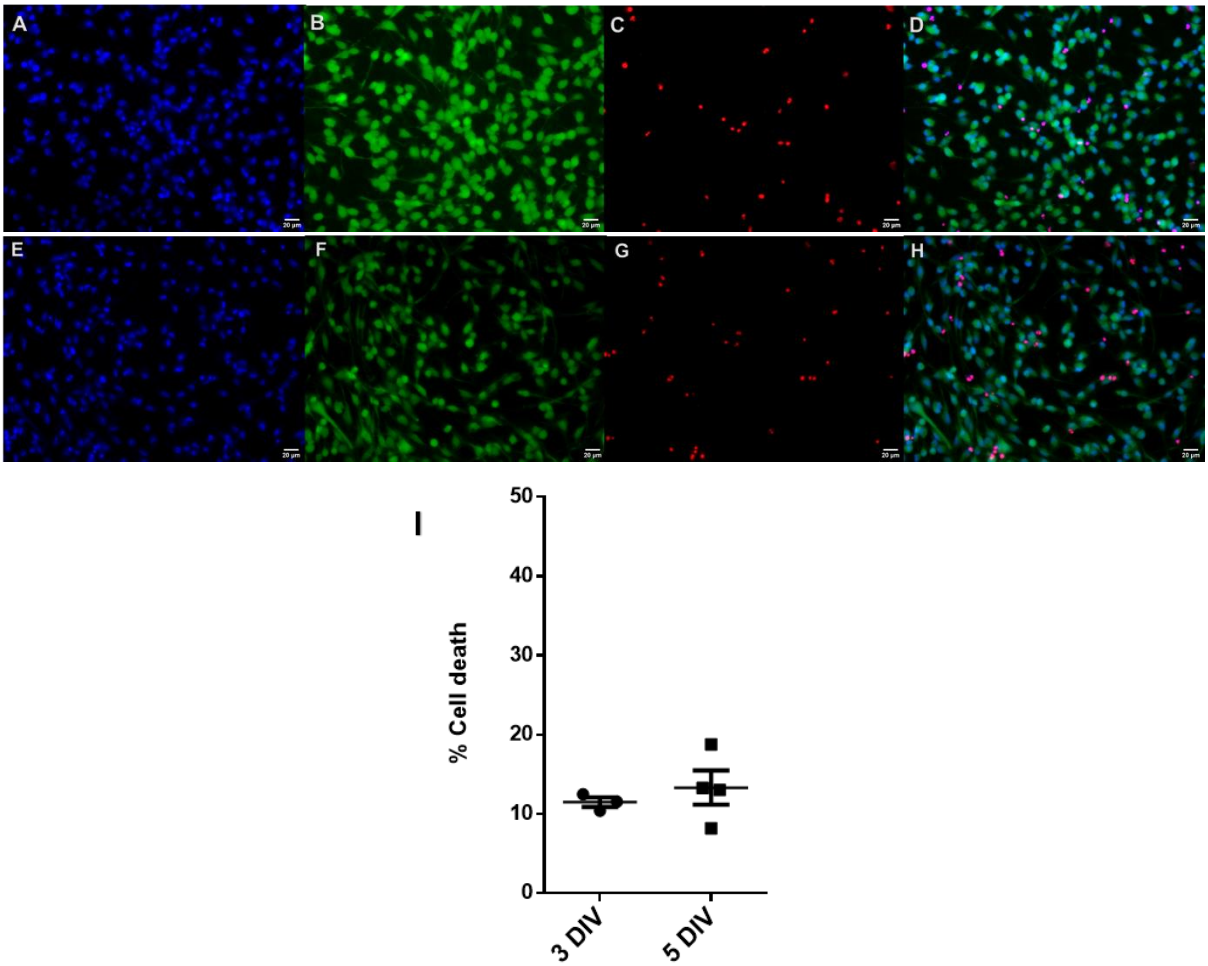
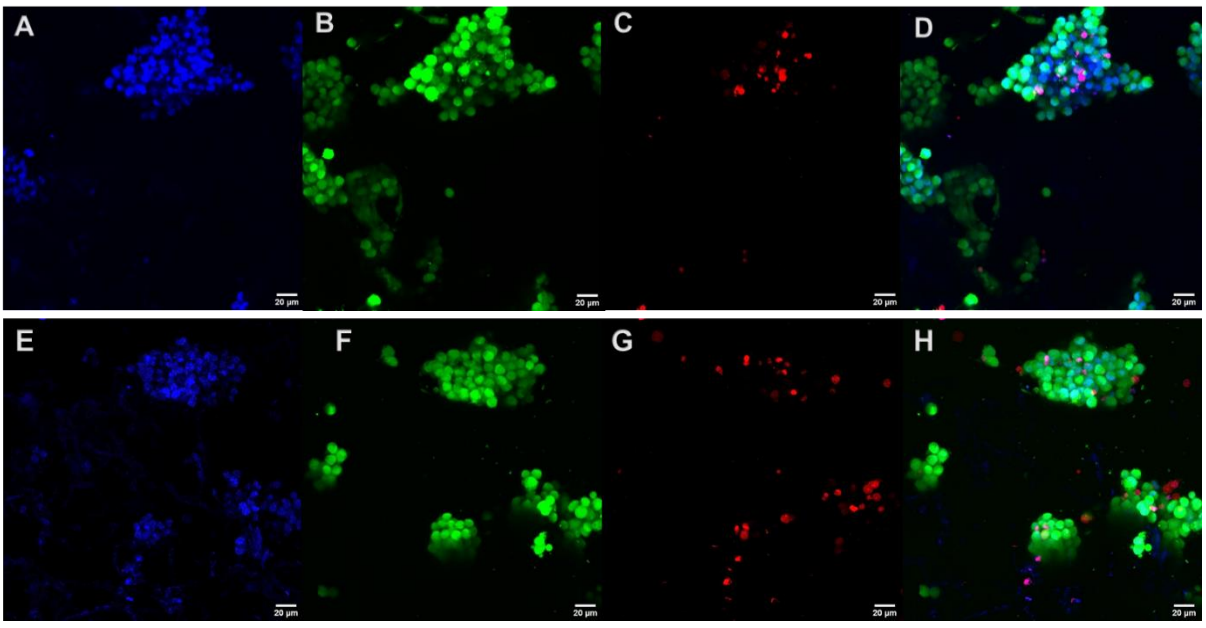


Figure (5): 3 DIV compared to 5 DIV Viability of E13.5 NSCs in 2D cultures. A representative image of Live-Dead staining. [A,E] Hoechst staining (blue) showing the nucleus. [B,F] Calcein (green) showing living cells [C,G] Propidium Iodide (red) showing the dead cells and [D,H] merged image. Nominal magnification 32x. Scale bar 20µm. Dot plot of cell death in 2D cultures in 3DIV compared to 5DIV. Fault rates are presented as mean ± SD. [5-I]

4.2.2 Cell viability in 3D cultures

NSC viability was assessed in 3D cultures within the adECM/GO 70/30 (Fig. 6,7) and adECM/GO 50/50 (Fig. 6,7) scaffold to assess possible detrimental effects of the scaffold composition to the NSCs. The cells were cultured in the presence of growth factors (EGF/FGF). After 3 and 5 DIV the viability of the NSCs was evaluated using the live/dead assay. The number of calcein, PI, and HOECHST positive cells was assessed in three independent experiments (n=3). The percentage of dead cells (PI^+) in respect to the total number of cells ($HOESCHT^+$) was calculated at 3DIV adECM/GO 70/30 at 16,20 ± 3,425%, adECM/GO 50/50 at. 20,01 ± 2,554. It was demonstrated that there is no significant ($P_{\text{one-way ANOVA}} > 0.05$) difference in NSC viability in either within the different scaffold compositions of with the 2D control condition. The percentage of dead cells (PI^+) in respect to the total number of cells ($HOESCHT^+$) was also calculated at 5DIV adECM/GO 70/30 at 21,71 ± 2,563%, adECM/GO 50/50 at. 29,84±3,248%. It was shown that at the adECM/GO 50/50 scaffolds there is a significant increase ($P_{\text{one-way ANOVA}} < 0.01$) in the number of dead cells compared to the 2D conditions.



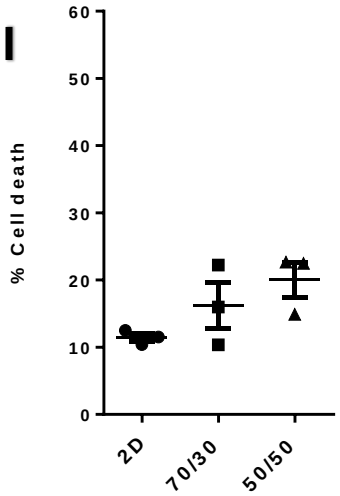


Figure (7): 3**DIV Viability of E13.5 NSCs in 3D cultures**. A representative image of Live-Dead staining. *AdECM/GO 70/30* [A] Calcein (green) showing living cells, [B] Hoechst staining (blue) showing the nucleus), [C] Propidium Iodide (red) showing the dead cells and [D] merged image. *AdECM/GO 50/50* [E] Calcein (green) showing living cells, [F] Hoechst staining (blue) showing the nucleus), [G] Propidium Iodide (red) showing the dead cells and [H] merged image. Nominal magnification 40x. Scale bar 20µm

Dot plot of cell death in 2D cultures compared to 3D in 3DIV. Fault rates are presented as mean ± SD [6-I]

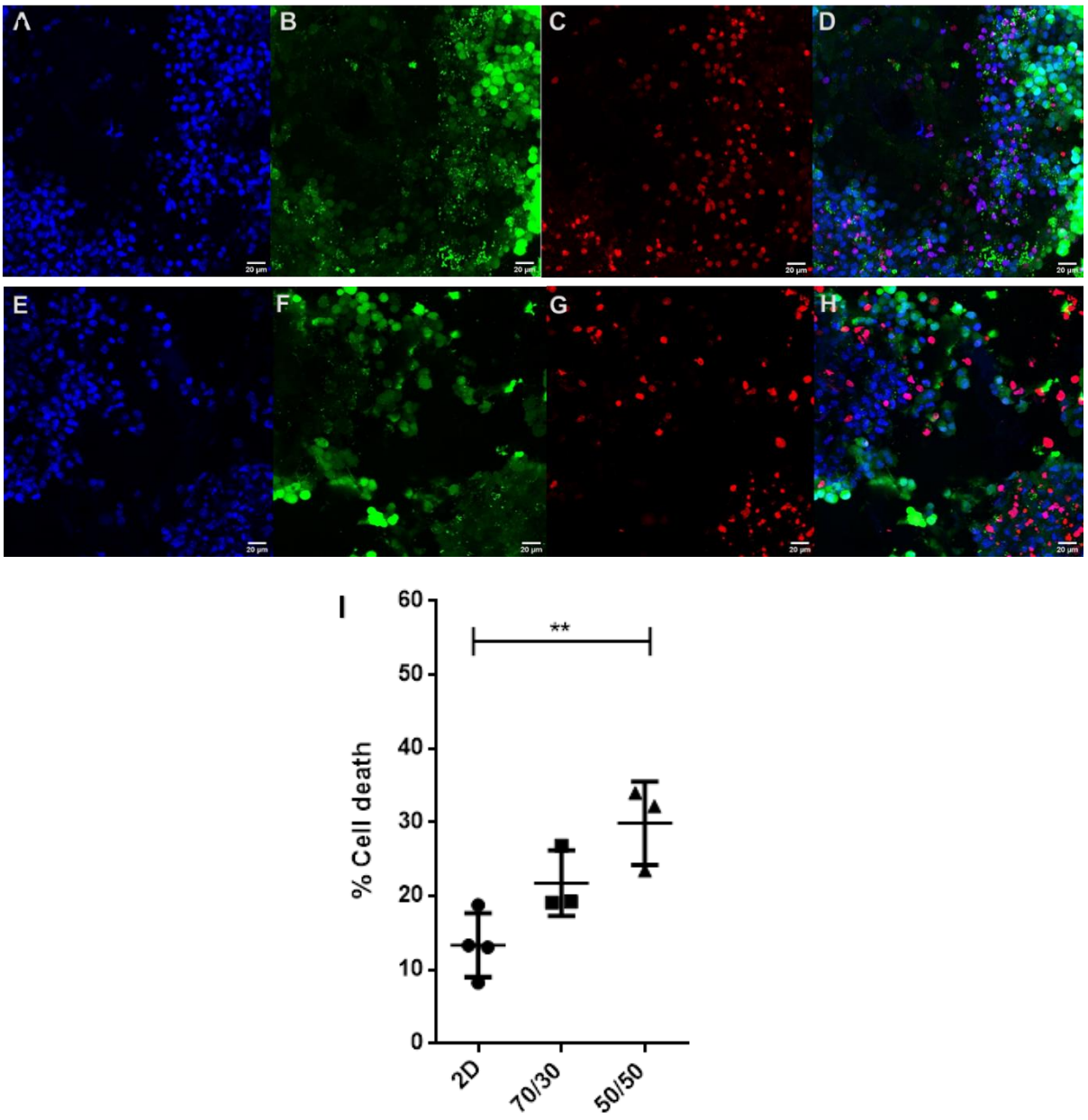


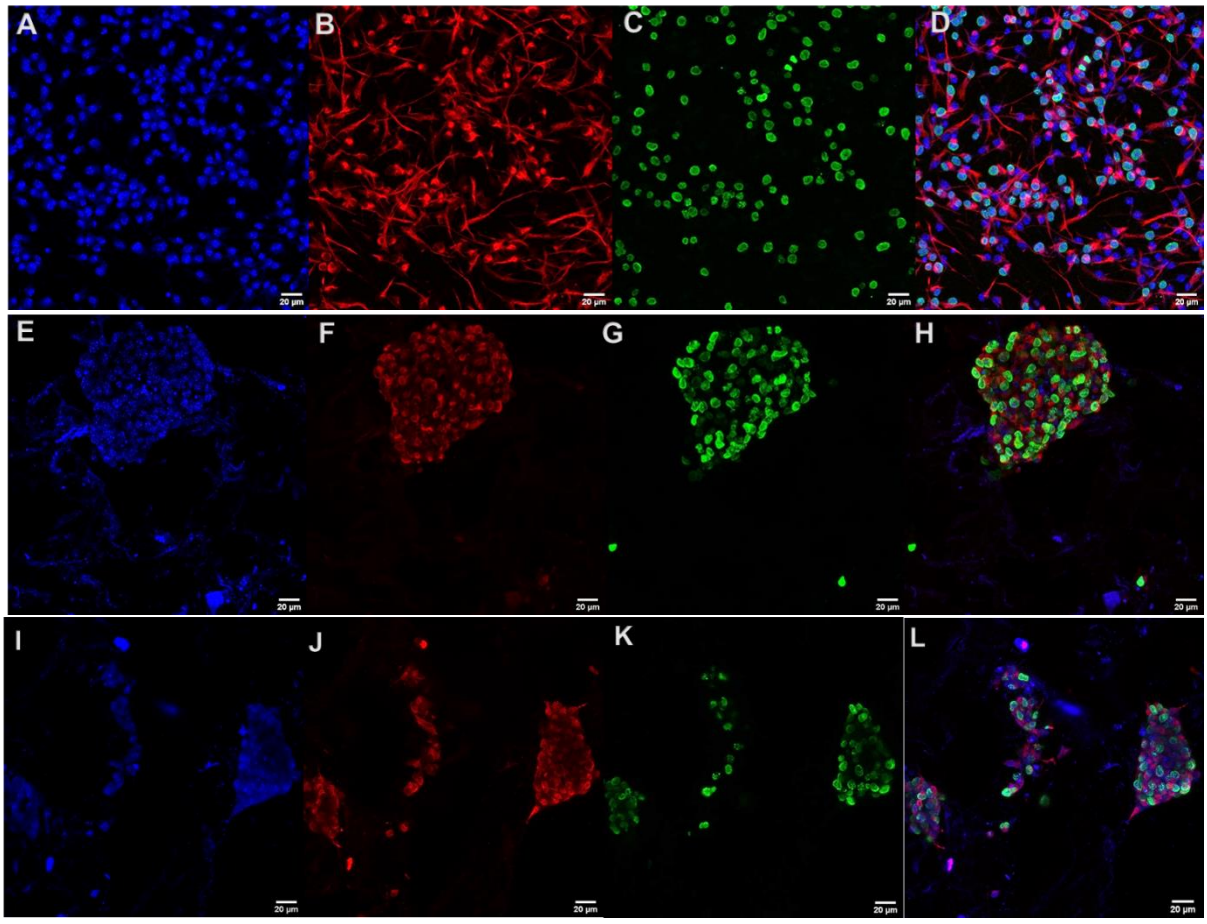
Figure (9): 5**DIV Viability of E13.5 NSCs in 3D cultures**. A representative image of Live-Dead staining. *AdECM/GO 70/30* [A] Calcein (green) showing living cells, [B] Hoechst staining (blue) showing the nucleus), [C] Propidium Iodide (red) showing the dead cells and [D] merged image. *AdECM/GO 50/50* [E] Calcein (green) showing living cells, [F] Hoechst staining (blue) showing the nucleus), [G] Propidium Iodide (red) showing the dead cells and [H] merged image. Nominal magnification 40x. Scale bar 20µm

Dot plot of cell death in 2D cultures compared to 3D in 5DIV. Fault rates are presented as mean ± SD. **P < 0.01 [7-I]

4.3 Evaluating the proliferation of E13.5 NSCs

4.3.1 Cell proliferation in 2D cultures and 3D cultures 3DIV

A key feature of the NSCs, their ability to proliferate, was then evaluated. NSCs in single cell suspension were cultured in the presence of growth factors (EGF/FGF). After 3 DIV the proliferation of the NSCs was assessed by evaluating the levels of BrdU incorporation within cells (Fig.8 A-L), in three independent experiments (n=3). The percentage of proliferative cells ($BrdU^+$) in respect to the $Nestin^+/HOECHST^+$ was calculated in 3DIV 2D at 62,99 ± 2,390%, adECM/GO 70/30 at 77,77± 1,268%, adECM/GO 50/50 at 84,36± 3,355%. It was demonstrated that the 3D conditions both in the adECM/GO 70/30 ($P_{one\text{-}way\ ANOVA} < 0.05$) and the adECM/GO 50/50 ($P_{one\text{-}way\ ANOVA} < 0.01$) significantly boosted proliferation in comparison with the 2D (Fig 8M).



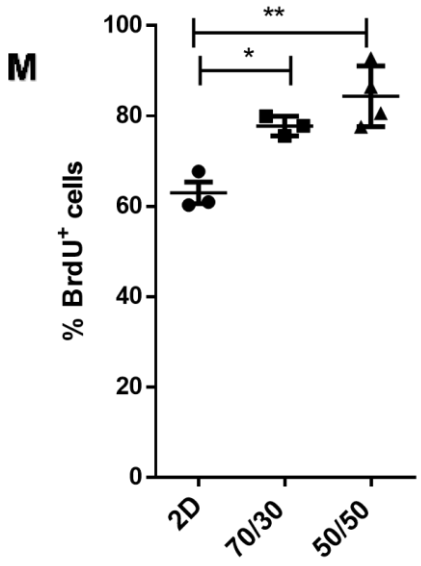
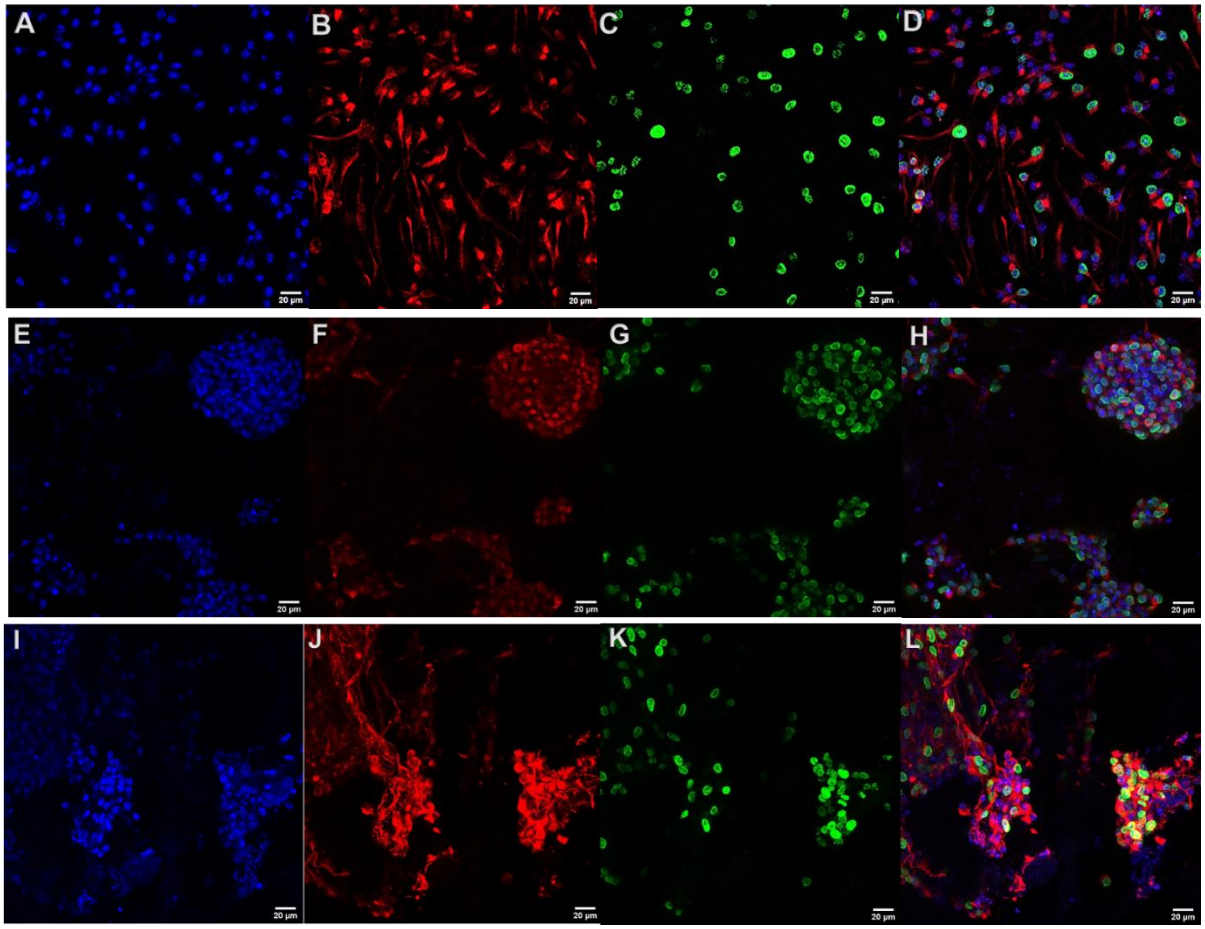


Figure (10): 3DIV Proliferation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of proliferated NSCs in 2D cultures [A-D] in 3D cultures inside *AdECM/GO 70/30* scaffolds [E-H] ,in 3D cultures inside AdECM/GO 50/50 scaffolds [I-L] [A,E,I] Hoechst staining (blue) showing the nucleus, [B,F,J] Nestin staining (red) showing neural stem cells, [C,G,K] BrdU staining (green) showing proliferated cells [D,H,L] merged image. Nominal magnification 40x. Scale bar 20µm. Dot plot of cell proliferation in 3DIV of 2D cultures compared to 3D. Fault rates are presented as mean ± SD. *P < 0.05, **P < 0.01, [8-M]

4.3.2 Cell proliferation in 2D cultures and 3D cultures 5DIV

The proliferating capacity of the NSCs was also evaluated at a later timepoint. NSCs in single cell suspension were cultured in the presence of growth factors (EGF/FGF). After 5 DIV the proliferation of the NSCs was assessed by evaluating the levels of BrdU incorporation within cells (Fig. 9 A-L), in three independent experiments (n=3). The percentage of proliferative cells ($BrdU^+$) in respect to the $Nestin^+/HOECHST^+$ was calculated 2D at 57,38± 3,794%, AdECM/GO 70/30 at 72,14± 3,818%, adECM/GO 50/50 at 63,22 ± 3,591 %. No significant difference ($P_{one\text{-}way\ ANOVA} > 0.05$) was observed between the scaffolds and the 2D conditions.



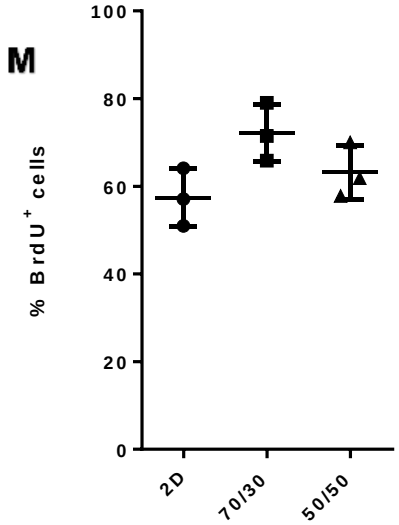


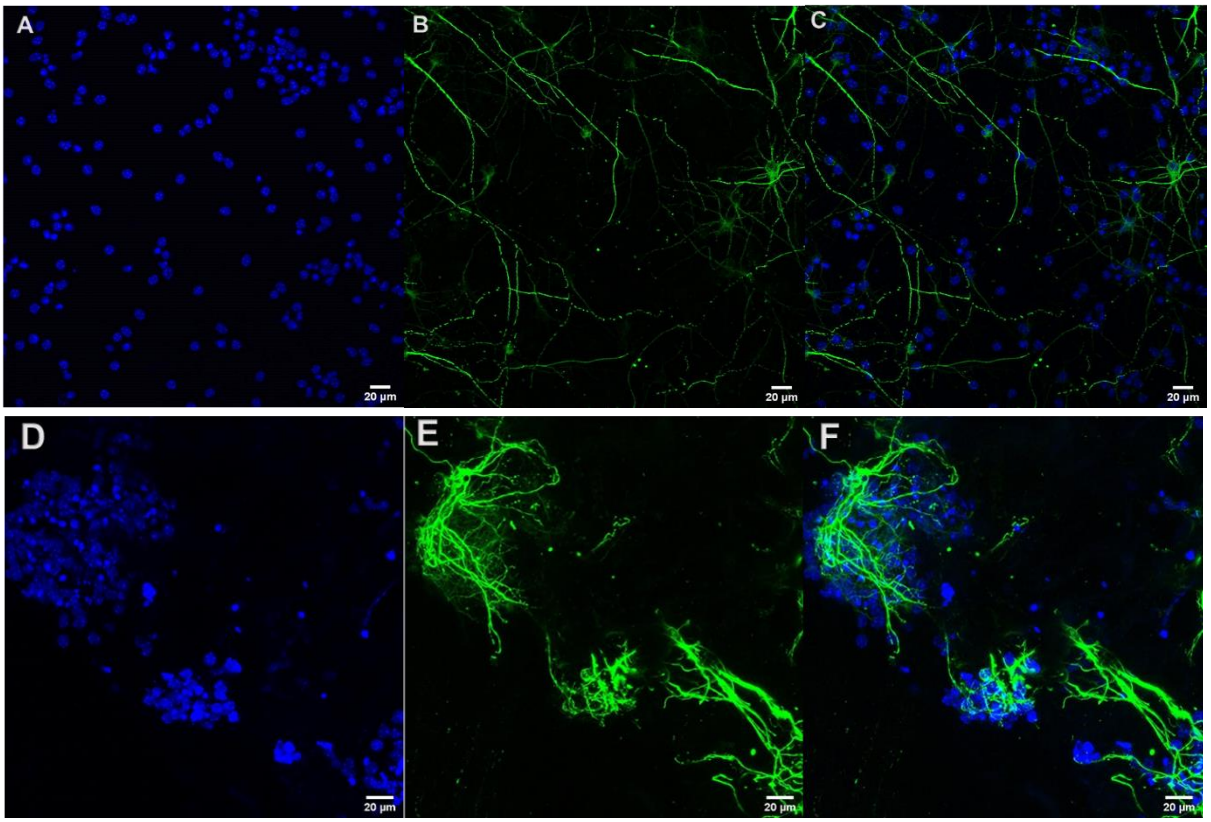
Figure (13): 5**DIV Proliferation of E13.5 NSCs in 2D and 3D cultures**. Representative confocal images of proliferated NSCs in 2D cultures [A-D] in 3D cultures inside *AdECM/GO 70/30* scaffolds [E-H] ,in 3D cultures inside AdECM/GO 50/50 scaffolds [I-L] [A,E,I] Hoechst staining (blue) showing the nucleus, [B,F,J] Nestin staining (red) showing neural stem cells, [C,G,K] BrdU staining (green) showing proliferated cells [D,H,L] merged image. Nominal magnification 40x. Scale bar 20µm.

Dot plot of cell proliferation in 5DIV of 2D cultures compared to 3D. Fault rates are presented as mean ± SD.[9-M]

4.4. Evaluating spontaneous differentiation

4.4.1 Spontaneous neuronal differentiation of E13.5 NSCs

One of the key features of the NSCs is their ability to differentiate in the three main cell types of the CNS, namely neurons, astrocytes and oligodendrocytes. For the spontaneous differentiation experiments, NSCs in single cell suspension were cultured in the presence of growth factors (EGF/FGF) for three days. After 3 DIV the medium is switched to the EGF/FGF free medium in order to investigate whether the 3D of the adECM/GO scaffolds direct the fate of NSC differentiation. On day 8 cultures are fixed and further processed for immunocytochemistry. The number of $Tuj1^{+}$ and HOECHST positive cells was assessed (Fig 10 A-I) in three independent experiments (n=3). The percentage of $Tuj1^{+}$ in respect to the total number of cells ($HOESCHT^{+}$) was calculated 2D at 11,54± 0,5062 %, AdECM/GO 70/30 at 12,99 ± 0,8933%, adECM/GO 50/50 at 20,20± 3,034%. It was discovered that the adECM/GO 50/50 significantly ($P_{\text{one-way ANOVA}} < 0.05$) promotes the differentiation towards neurons when compared with the 2D condition (Fig 10 J).



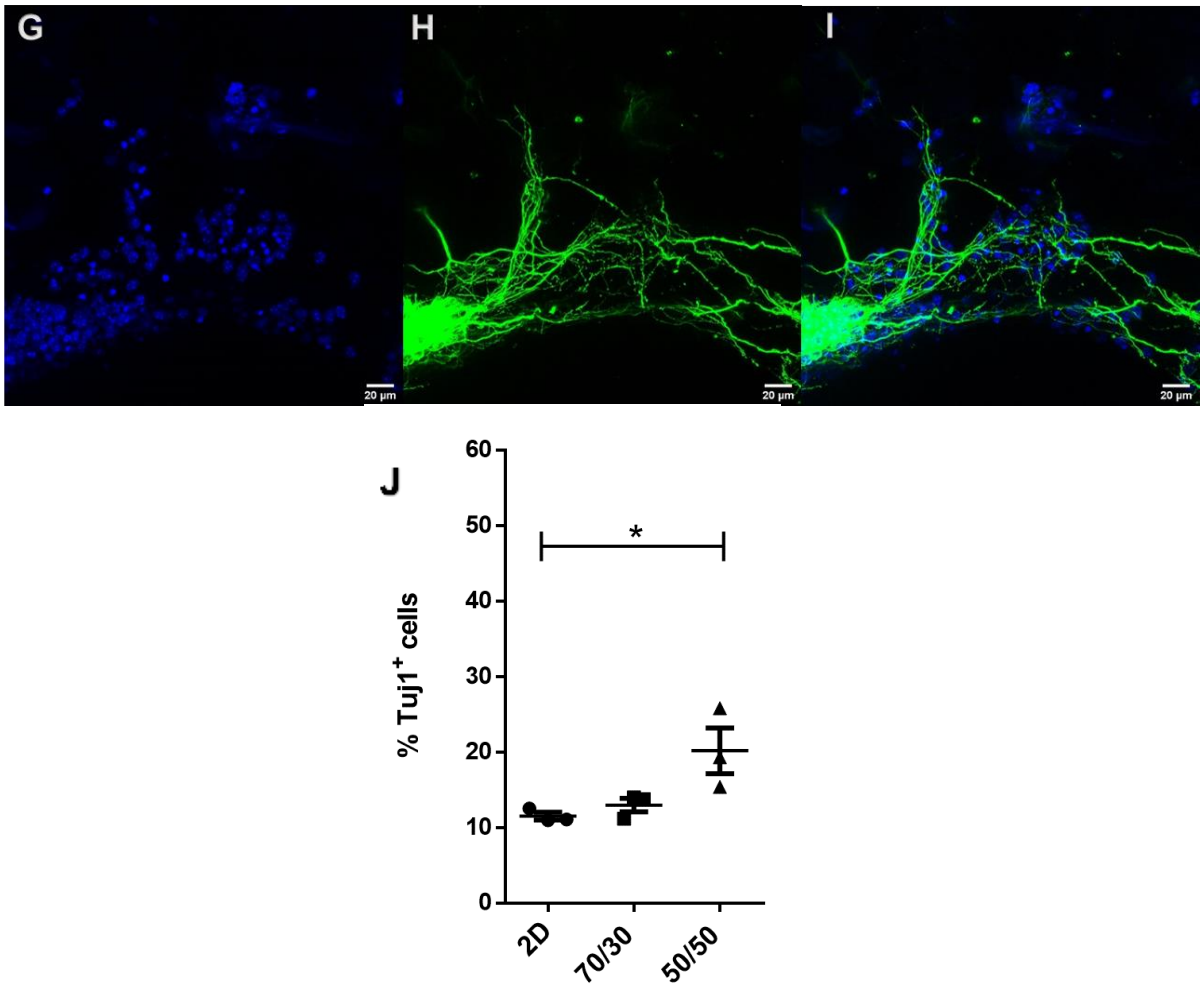
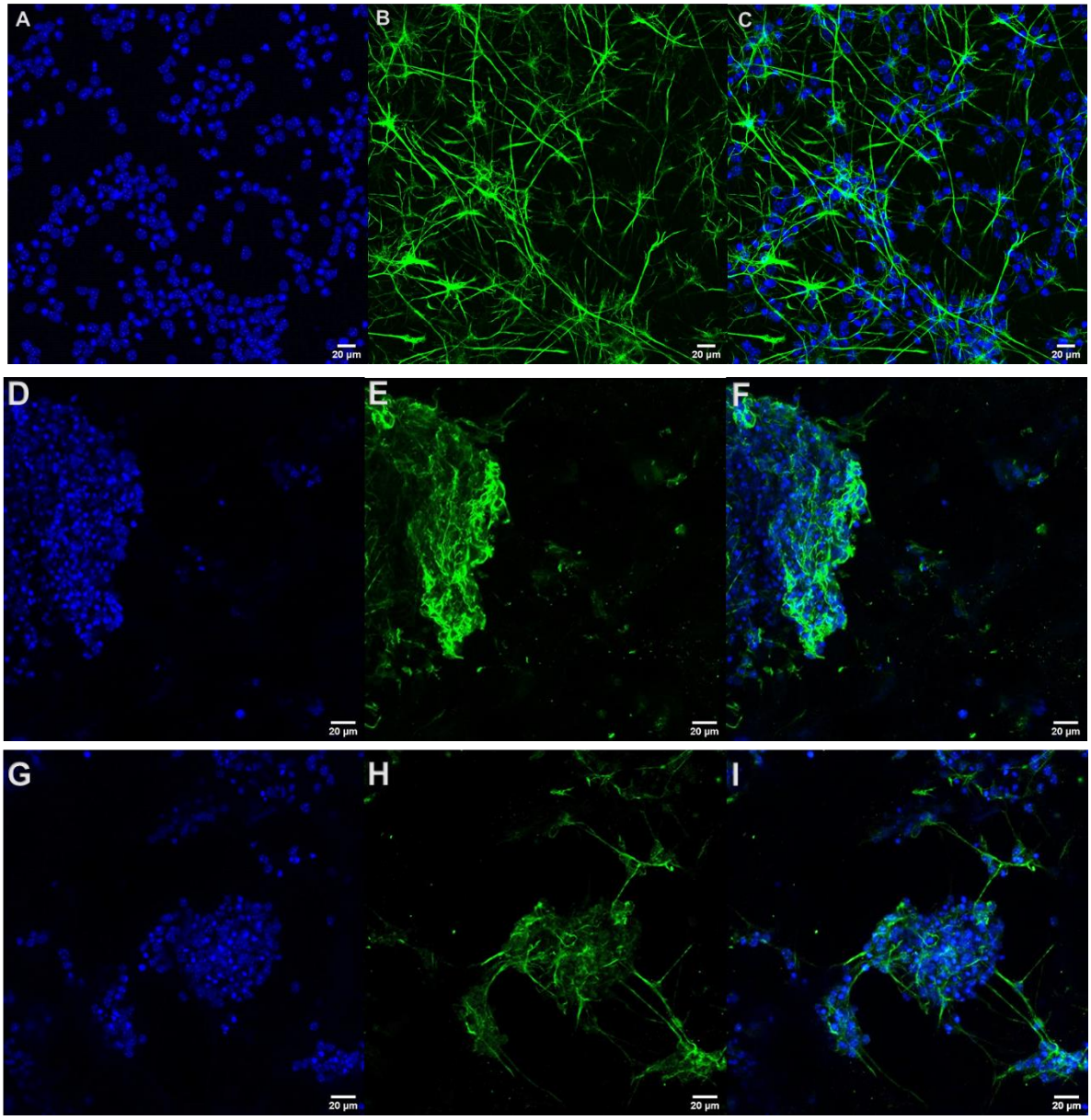


Figure (14): Spontaneous neuronal differentiation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of NSCs stained for Tuj1$^+$ (neurons) in 2D cultures [A-C] in 3D cultures inside *AdECM/GO 70/30* scaffolds [D-F] ,in 3D culturesinside AdECM/GO 50/50 scaffolds [G-I] [A,D,G] Hoechst staining (blue) showing the nucleus, [B,D,H] Tuj1 staining (green) showing neurons, [C,F,G] merged image. Nominal magnification 40x. Scale bar 20µm.

4.4.2 Spontaneous astrocyte differentiation of E13.5 NSCs

The number of GFAP$^+$ and HOECHST positive cells was also assessed (Fig. 11) in three independent experiments (n=3). The percentage of GFAP$^+$ in respect to the total number of cells (HOESCHT$^+$) was calculated 2D at 24,86± 1,416 %, AdECM/GO 70/30 at 23,92± 4,037%, adECM/GO 50/50 at 21,03± 2,950%. No significant difference ($P_{\text{one-way ANOVA}} > 0.05$) was observed between the scaffolds and the 2D conditions in the number of astrocytes (Fig 11J)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20 µm

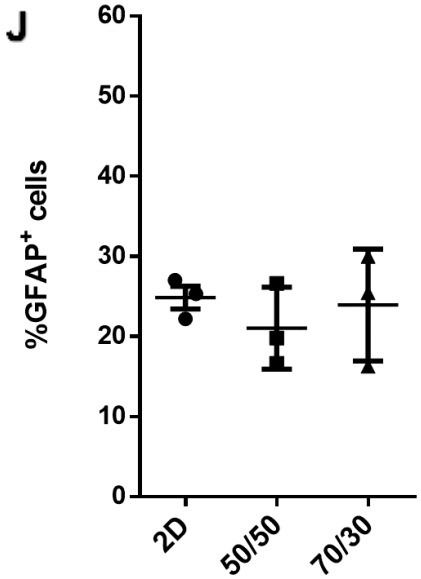
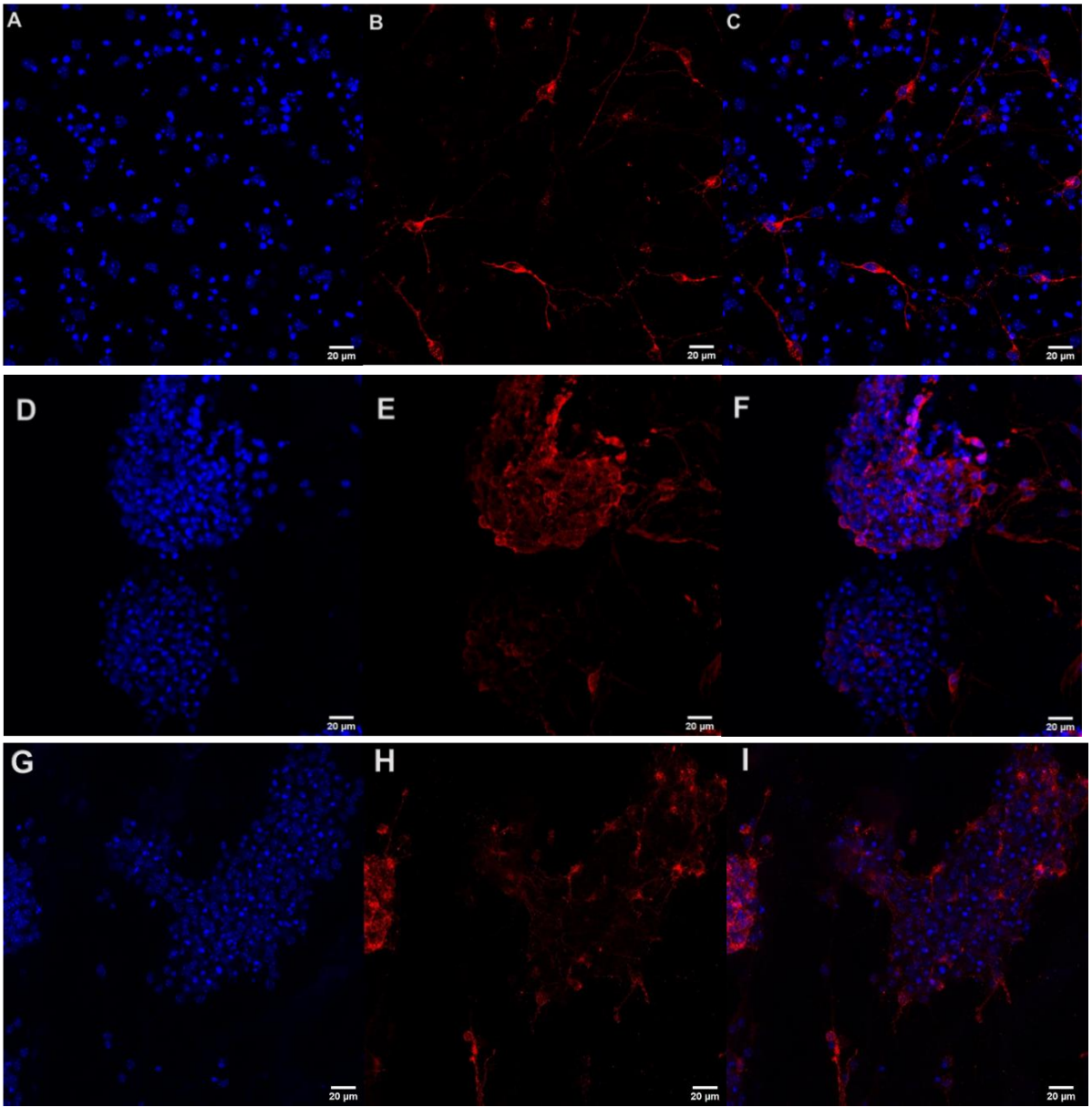

J
%GFAP+ cells
60
50
40
30
20
10
0
2D
50/50
70/30

Figure (15): Spontaneous astrocyte differentiation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of NSCs stained for GFAP$^+$ (astrocytes) in 2D cultures [A-C] in 3D cultures inside *AdECM/GO 70/30* scaffolds [D-F] ,in 3D cultures inside AdECM/GO 50/50 scaffolds [G-I] [A,D,G] Hoechst staining (blue) showing the nucleus, [B,D,H] GFAP staining (green) showing astrocytes, [C,F,G] merged image. Nominal magnification 40x. Scale bar 20μm.
Dot plot of GFAP$^+$ cells in 2D cultures compared to 3D. Fault rates are presented as mean ± SD. [11-J]

4.4.3 Spontaneous OPCs differentiation of E13.5 NSCs

The number of PDGFRa$^+$ and HOECHST positive cells was assessed (Fig 9) in three independent experiments (n=3). The percentage of PDGFRa$^+$ in respect to the total number of cells (HOESCHT$^+$) was calculated at 2D at 8,286 ± 1,834%, AdECM/GO 70/30 at 28,43 ± 2,783 %, adECM/GO 50/50 at 31,58 ± 2,972%. It was demonstrated that the 3D conditions both in the adECM/GO 70/30 ($P_{\text{one-way ANOVA}} < 0.01$) and the adECM/GO 50/50 ($P_{\text{one-way ANOVA}} < 0.01$) significantly promoted oligodendrogenesis in comparison with the 2D (Fig 12J).



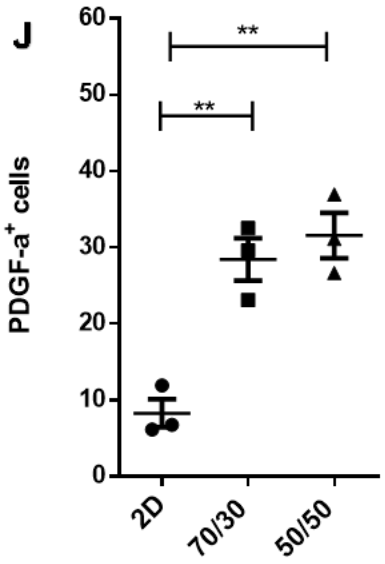
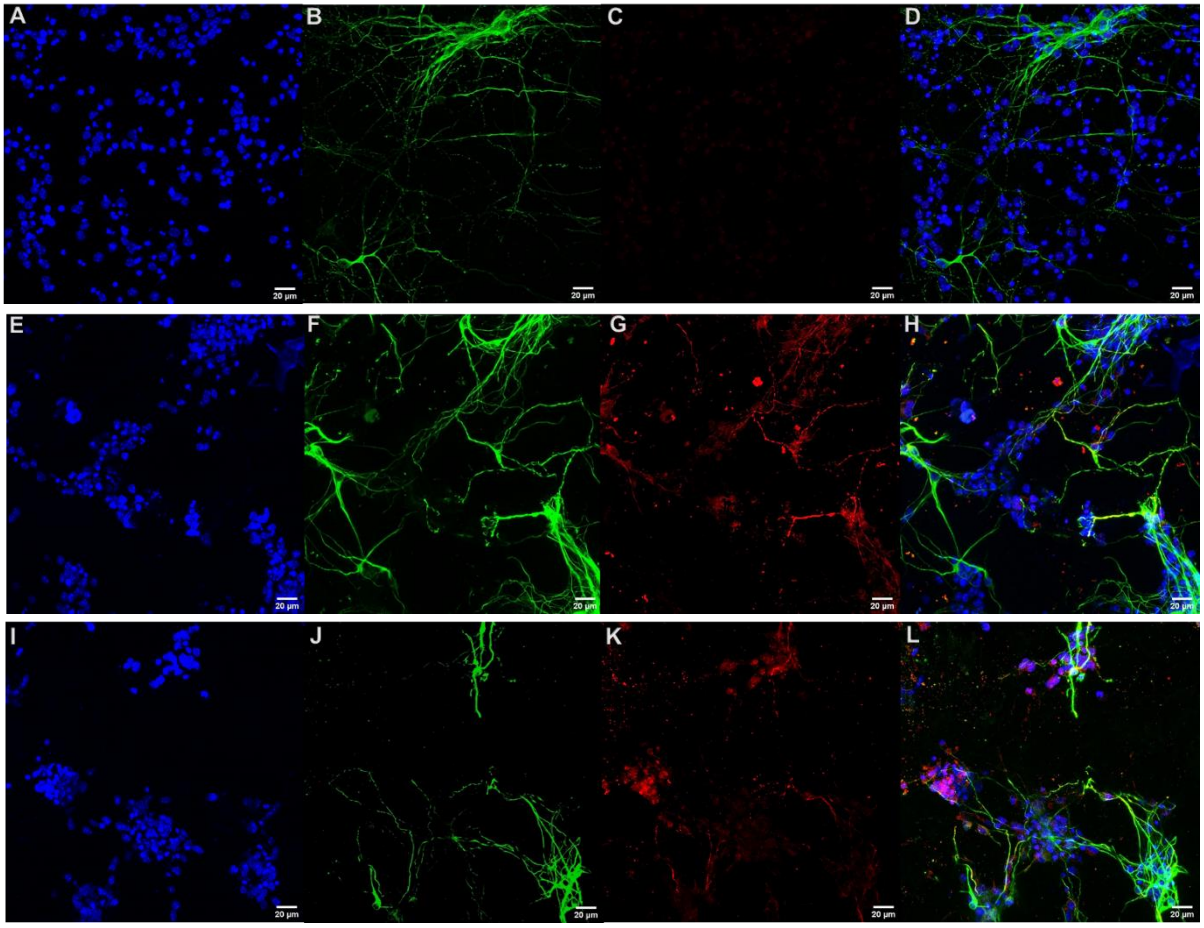


Figure (18): **Spontaneous OPCs differentiation of E13.5 NSCs in 2D and 3D cultures**. Representative confocal images of NSCs stained for PDGFRa$^+$ (OPCs) in 2D cultures [A-C] in 3D cultures inside *AdECM/GO 70/30* scaffolds [D-F] ,in 3D cultures inside AdECM/GO 50/50 scaffolds [G-I] [A,D,G] Hoechst staining (blue) showing the nucleus, [B,D,H] PDGFRa staining (red) showing OPCs, [C,F,G] merged image. Nominal magnification 40x. Scale bar 20μm.
Dot plot of PDGFRa$^+$ cells in 2D cultures compared to 3D. Fault rates are presented as mean ± SD. **P < 0.01 [12-J]

4.5 Evaluating induced differentiation

4.5.1 Induced neuronal differentiation of E13.5 NSCs

After showing the adECM/GO scaffolds on their own may favor the commitment of the NSCs toward neurons and OPCs the focus was turned towards which effect the combination of the soluble signals within the differentiation medium and the microenvironment created by the adECM/GO scaffolds may have. The number of Tuj1$^+$ and HOECHST positive cells was assessed after inducing differentiation (Fig.10) in three independent experiments (n=3). The percentage of Tuj1$^+$ in respect to the total number of cells (HOESCHT$^+$) was calculated 2D at 20,55± 1,408%, AdECM/GO 70/30 at 16,10± 2,135%, adECM/GO 50/50 at 21,66 ± 3,580%. No significant difference ($P_{\text{one-way ANOVA}} > 0.05$) was observed between the scaffolds and the 2D conditions in the number of neurons (Fig 13-J).


A
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20 µm

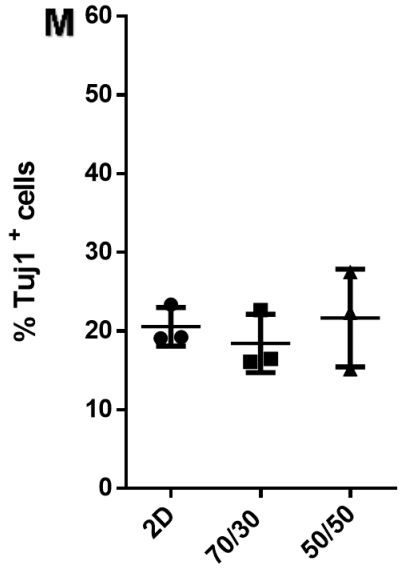

M
% Tuj1 + cells
60
50
40
30
20
10
0
2D
70/30
50/50

Figure (19): Induced neuronal differentiation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of NSCs stained for Tuj1^{+} (neurons) in 2D cultures [A-D] in 3D cultures inside *AdECM/GO 70/30* scaffolds [E-H] ,in 3D cultures inside AdECM/GO 50/50 scaffolds [I-L] [A,E,I] Hoechst staining (blue) showing the nucleus, [B,F,J] Tuj1 staining (green) showing neurons, [C,G,K], Synaptophysin 1 (red) shown synapses ,[D,H,L] merged image. Nominal magnification 40x. Scale bar 20µm.
Dot plot of Tuj1^{+} cells in 2D cultures compared to 3D. Fault rates are presented as mean ± SD [13-M]

Although neuronal differentiation was not further boosted in the adECM/GO scaffolds. The differentiated neurons was shown to express synaptophysin (Fig. 13G K, Fig 14) which is a an integral membrane glycoprotein that occurs in presynaptic vesicles of neurons . This finding is indicative that synaptogenesis has occurred and there are possibly functional neurons within the adECM/GO scaffolds whilst synaptophysin expression was absent at 2D (Fig 13 C)

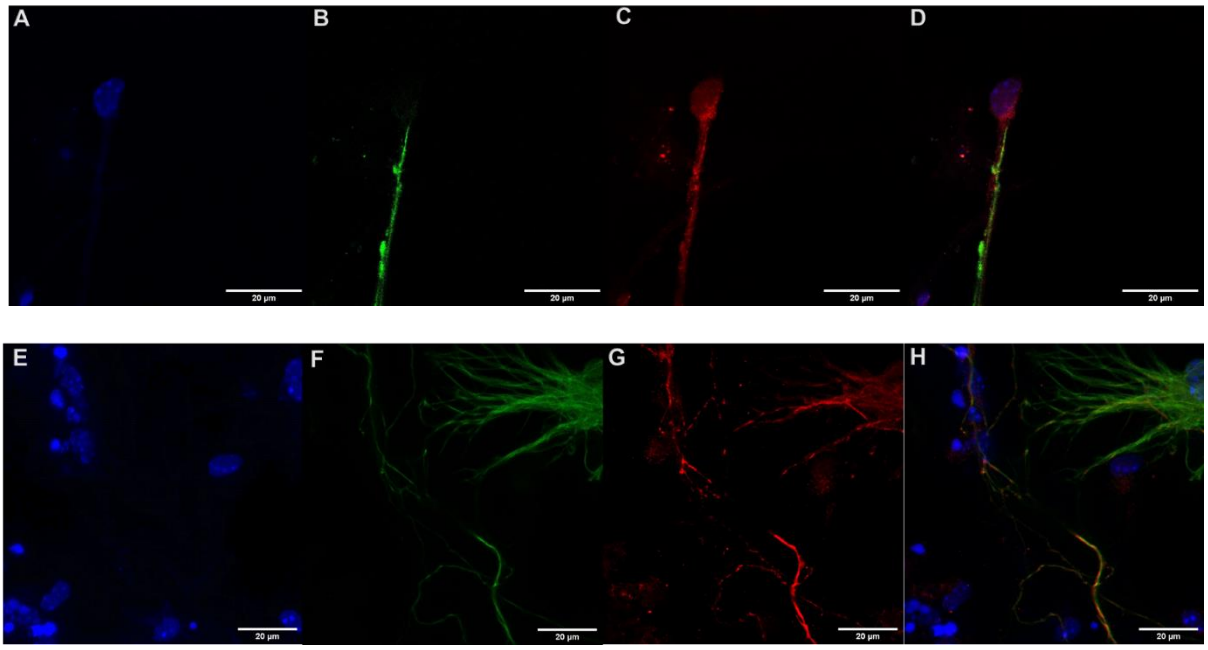
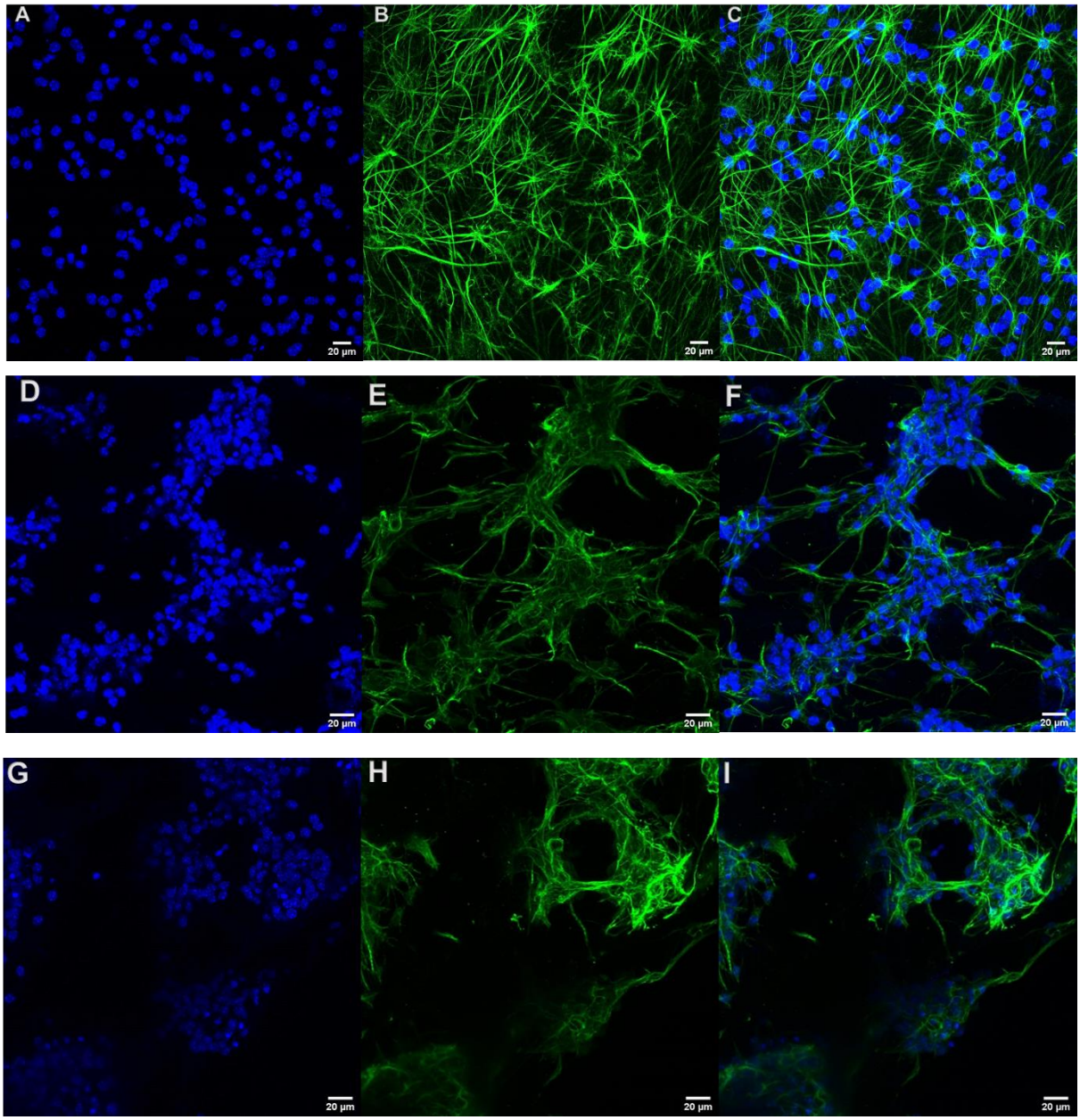


Figure (20): Induced neuronal differentiation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of NSCs stained for Tuj1+ (neurons in 3D cultures inside *AdECM/GO 70/30* scaffolds [A-D] in 3D cultures inside AdECM/GO 50/50 scaffolds [E-H] [A,E] Hoechst staining (blue) showing the nucleus, [B,F] Tuj1 staining (green) showing neurons, [C,G], Synaptophysin 1 (red) shown synapses ,[D,H] merged image. Nominal magnification 63x. Scale bar 20µm.

4.5.2 Induced astrocyte differentiation of E13.5 NSCs

The number of GFAP+ and HOECHST positive cells was assessed after the induction of NSCs differentiation towards astrocytes (Fig15) in three independent experiments (n=3). The percentage of GFAP+ in respect to the total number of cells (HOESCHT+) was calculated 2D at 43,67 ± 2,208 %, adECM/GO 70/30 at 38,04 ± 4,105%, adECM/GO 50/50 at 35,87± 3,064%. No significant difference (Pone-way ANOVA >0.05) was observed between the scaffolds and the 2D conditions (Fig 15-J).



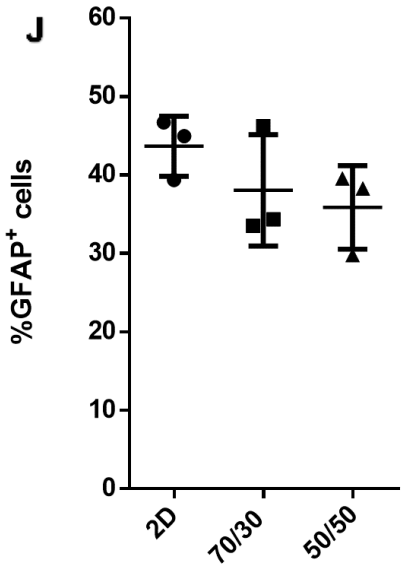


Figure (21): Induced astrocyte differentiation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of NSCs stained for GFAP$^+$ (astrocytes) in 2D cultures [A-C] in 3D cultures inside *AdECM/GO 70/30* scaffolds [D-F] ,in 3D cultures inside AdECM/GO 50/50 scaffolds [G-I] [A,D,G] Hoechst staining (blue) showing the nucleus, [B,D,H] GFAP staining (red) showing astrocytes, [C,F,G] merged image. Nominal magnification 40x. Scale bar 20μm.
Dot plot of GFAP$^+$ cells in 2D cultures compared to 3D. Fault rates are presented as mean ± SD.[15-J]

4.5.3 Induced OPCs differentiation of E13.5 NSCs

The number of PDGFRa$^+$ and HOECHST positive cells was assessed after induction of differentiation (Fig 16) in three independent experiments (n=3). The percentage of PDGFRa$^+$ in respect to the total number of cells (HOESCHT$^+$) was calculated 2D at 23,91 ± 1,302%, adECM/GO 70/30 at 42,85 ± 1,769%, adECM/GO 50/50 at 40,79 ± 2,812 %. It was demonstrated that the 3D conditions both in the adECM/GO 70/30 ($P_{\text{one-way ANOVA}} < 0.01$) and the adECM/GO 50/50 ($P_{\text{one-way ANOVA}} < 0.01$) significantly promoted oligodendrogenesis in comparison with the 2D (Fig16 J).

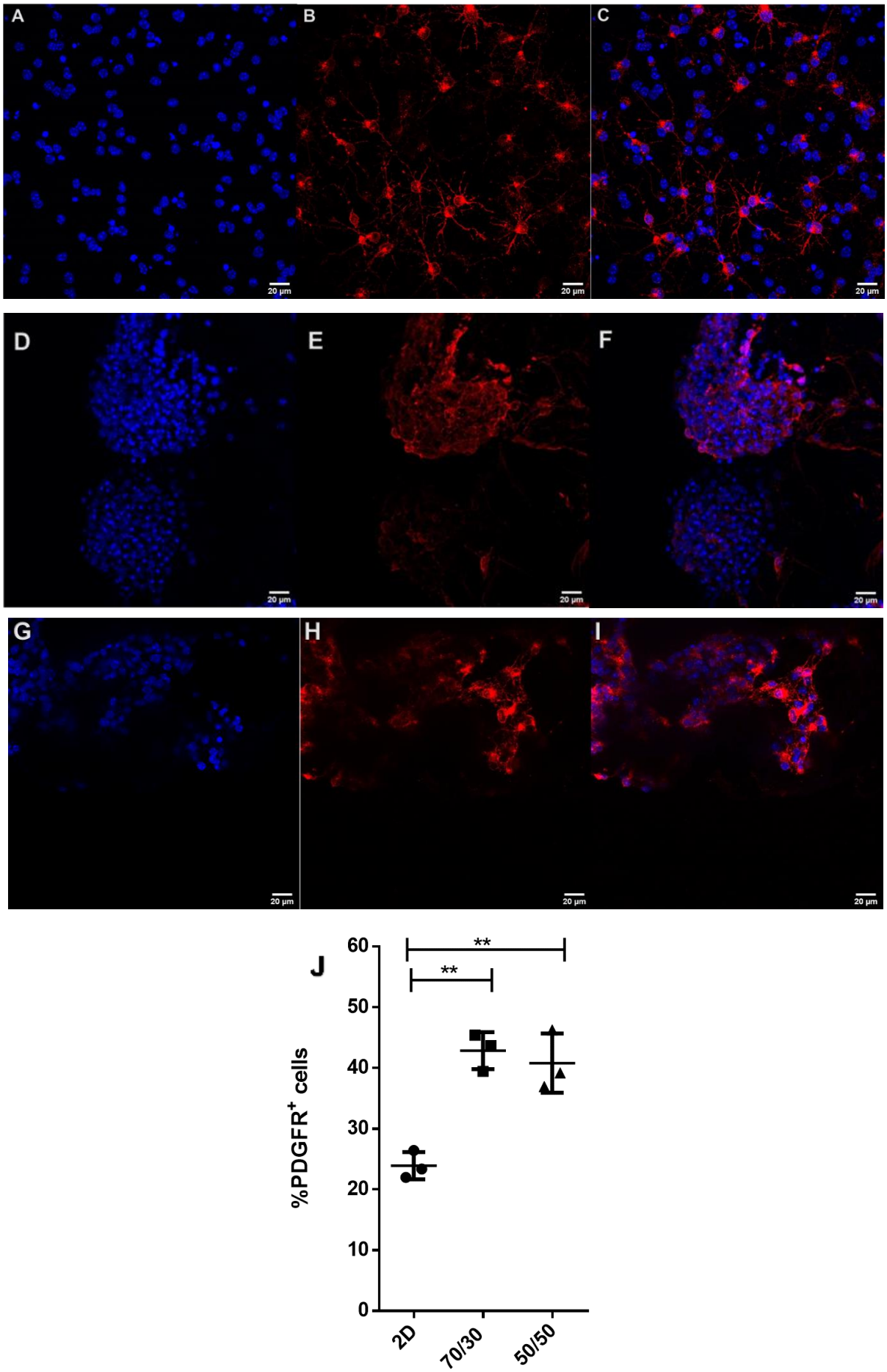


Figure (22): Induced OPCs differentiation of E13.5 NSCs in 2D and 3D cultures. Representative confocal images of NSCs stained for PDGFRa$^+$ (OPCs) in 2D cultures [A-C] in 3D cultures inside *AdECM/GO 70/30* scaffolds [D-F] in 3D cultures inside AdECM/GO 50/50 scaffolds [G-I] [A, D, G] Hoechst staining (blue) showing the nucleus, [B, D, H] PDGFRa staining (red) showing OPCs, [C, F, G] merged image. Nominal magnification 40x. Scale bar 20µm.

Dot plot of PDGFRa$^+$ cells in 2D cultures compared to 3D. Fault rates are presented as mean ± SD. **P < 0.01 [16-J]

5.Discussion

Spinal cord injury is a severely debilitating condition leading to neurological dysfunction, loss of independence, respiratory failure, psychological morbidities, and an increased lifelong disability (Marion et al. 2017), (Satkunendrarajah et al. 2018), (Marion et al. 2017), (Y. Wang, Xie, and Zhao 2018). To date, there is no effective cure for SCI and the existing clinical treatments (small molecules and surgical procedures) have limited efficacy and short-term effects. One of the main obstacles is the formation of the glial scar at the site of the injury, creating an inhibitory microenvironment at the lesion area, constituting a mechanical, physical and chemical barrier to axonal regrowth and nerve fiber regeneration (Yuan and He 2013), (Silver and Miller 2004).

SCI research has aimed to repair the disrupted neural network, reestablish functional neuronal connectivity as well as the support of remyelination facilitating electrical transmission (Li et al. 2017), (Abematsu et al. 2010). Thus, tissue engineering appears to be a promising alternative for providing SCI patients valuable functional and sustainable recovery. There is evidence that transplanted stem cells may offer significant possibilities, such as tissue recomposition (Vieira et al. 2018), (Shastri and Martin 2002). However, significant development is required to safely deliver NSCs at SCI lesions. Given the structureless nature of the cavity, scaffold-based strategies have been proposed as an alternative to promote neuroregeneration at the site of SCI by concomitantly bridging the lesion site while acting as a drug and/or cell carrier. Several types of biomaterials (synthetic and natural) are regularly used in tissue engineering. Among them, extracellular matrix and graphene exhibit distinct mechanical and chemical properties making them appropriate candidates for scaffold manufacturing.

Before any in vivo application, a new scaffold formulation is required to be examined in detail *in vitro* conditions first. Within the scope of the NEUROSTIMSPINAL project which has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 829060, an international consortium aims to fabricate an array of scaffold composed of graphene-based materials in combination with adipose tissue decellularized extracellular matrix. The present study focuses on the effects of two different scaffold compositions on NSCs by investigating their proliferation, viability, and differentiation *in vitro*.

The present study demonstrates that seeded NSCs adhered robustly on adECM/GO scaffolds, forming neurosphere-like aggregates inside the pore cavities indicating that the microenvironment within the adECM/GO scaffolds supports the growth of NSCs. This initial observation was further confirmed by the evaluation of NSC viability in the scaffolds. It was shown that there was no significant difference in cell viability at 3DIV in adECM/GO scaffolds compared to 2 D cultures indicating a biocompatible and non-toxic scaffold composition (Fig 6). This is congruent with previous studies demonstrating that G and GO foams are appeared to be compatible substrates for stem cells. Crowder et al. (2013) showed that three-dimensional graphene foams used as culture substrates for human mesenchymal stem cells can maintain stem cell viability and promote osteogenic differentiation. In another study provided by Li et al. (2013) it is indicated that 3D-Graphene Foams could offer a platform for NSC research, neural tissue engineering, and neural prostheses. Results also demonstrate that both scaffold compositions induce NSC proliferation (Fig 8M), indicating a beneficial effect of the 3-D microenvironment. Several studies support these findings. For instance, a study provided by Waele et al (2016) showed that in decellularized brain ECM, NSCs can attach, proliferate, and retain their stemness. When matrices from porcine optic nerve, spinal cord, brain, and urinary bladder were compared, it has appeared that all four matrices promoted neural differentiation while the central nervous system materials also promoted cell migration (Crapo et al. 2012). Thus, 3D scaffolds provide support, and topological features that regulate stem cell adhesion, proliferation and behavior as it is in natural tissues. Owing to this, advances in stem cell-based tissue engineering have a huge reliance on graphene-based scaffolds, specifically in terms of inducing signals for cell differentiation and proliferation (Lee et al. 2013; Ding et al. 2015). In agreement with previous studies, the present research shows that adECM/GO scaffold 50/50 significantly promotes neuronal differentiation compared to both 2D cultures and adECM/GO 70/30 scaffold (Fig 10-J). The ability of adECM/GO 50/50 scaffold to support the neuronal maturation was further supported by the synaptophysin positive staining (Fig 14), indicating the formation of functional neuronal networks. Accordingly, Serrano et al (2014)showed that graphene may provide cues to reinforce the formation of interconnected neural networks and electrical connections among cells. There is evidence that graphene due to it unique surface property can promote the differentiation of hNSCs toward neurons rather than glia, offering tremendous opportunities in stem cell research, neuroscience, and regenerative medicine(Park et al. 2011). In the present study, it has appeared that both compositions seem to favor NSCs differentiation towards OPCs compared to 2D cultures (Fig 12). Considering that OPCs can self-renew and generate mature oligodendrocytes, consisting the main myelinating cell

population in CNS throughout adulthood (Boulanger and Messier 2014), these results further support a promising ability of the scaffolds, to support axonal growth and myelination, thus, bypassing the inhibitory effects of the glial scar. Additional studies and electrophysiology experiments will provide information to test this hypothesis. The multitherapeutic potential of stem cells and their ability to release potential beneficial factors at the damaged site along with the beneficial effects of biomaterial fabricated scaffolds could facilitate clinically meaningful recovery after SCI.

6.References

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