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Towards 3D bio-printed spinal cord organoids

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Abstract

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The development of 3D bioprinting technology has provided a new direction for the replacement of organs or tissues and the development of drug testing models. Testing cell adhesion, proliferation, and differentiation in different printed scaffolds for creating functional 3D bio-printed structures provides the possibility of establishing a patient-specific in vitro model for neurodegenerative diseases. This thesis aims to establish a 3D bio-printed spinal cord model for drug research of ALS by exploring the factors affecting cell adhesion, growth, and differentiation in different hydrogels, and the suitable printing conditions.

In Paper I, we compared the adhesion and cell survival rates of BCs on the surfaces of the scaffolds with different stiffness and different chemical covering substracts and found the effects of physical and chemical factors for cell adhesion, proliferation, and differentiation through comparison, which can be used as a reference for exploring the conditions for further 3D printing mixing with cells inside.

In Paper II, gelatin-based hydrogel was selected as the main material for printing the scaffold. By testing the survival rate of BCs in the different concentrations of gelatin with different concentrations of crosslinker, we selected a protocol that is suitable for cell viability, cell differentiation, and bioprintability. Unfortunately, when this protocol is applied to hiPSCs, it can obtain the viability of cells after printing, but cell differentiation was only observed on the surface of the scaffolds since cells in the middle of the printed structure lack contact with the surrounding culture medium.

Paper III showed that BCs attracted endothelial cells sprouting from aortic rings in their co-cultured 3D-printed scaffolds and guided the migration direction of endothelial cells. Also, after implantation at the injury DRTZ, they helped with vascularization by increasing the blood vessel volume and vessel diameters.

In Paper IV, we improved the protocol from Paper II for hiPSCs-derived MNs by reducing the concentration of gelatin and adding MSP loaded with cintrofin and gliafin. Two printable methods that could keep the printed structures during culturing were tested, and one was chosen for further printing based on cell viability during bio-ink preparation. A lower concentration of gelatin helped with getting better access to the surrounding culture medium and achieving motor neuron differentiation inside the scaffolds.

Keywords: 3D bioscaffold, gelatin, iPSC, differentiation

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Dedication

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Han, Y**., Baltriukien, D., Kozlova, EN. (2020) Effect of scaffold properties on adhesion and maintenance of boundary cap neural crest stem cells in vitro. *Journal of Biomedical Materials Research Part A*, 108(6):1274–1280

II. **Han, Y**., King, M., Tikhomirov, E., Barasa, P., Souza, C., Lindh, J., Baltriukiene, D., Ferraiuolo, L., Azzouz, M., Gullo, M., Kozlova, EN. (2022) Towards 3D Bioprinted Spinal Cord Organoids. *International Journal of Molecular Sciences*, *23*(10):5788

III. Trolle, C., **Han, Y**., Mutt, SJ., Christoffersson, G., Kozlova, EN. (2024) Boundary cap neural crest stem cells promote angiogenesis after transplantation to avulsed dorsal roots in mice and induce migration of endothelial cells in 3D printed scaffolds. *Neuroscience Letters*, 826:137724

IV. **Han, Y**., King, M., Fjerdingstad, HB., Gullo, M., Zeger, L., Kádár, R., Ivert, P., Glover, J., Ferraiuolo, L., Azzouz, M., Kozlova, EN. (2024) Differentiation of human motoneurons in a 3D-printed scaffold - a step towards standardized and personalized human spinal cord tissue for modeling motoneuron diseases. *Manuscript Submitted*

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Abbreviations

ALS	Amyotrophic Lateral Sclerosis
BCs	Boundary Cap Neural Crest Stem Cells
C9orf72	Chromosome 9 Open Reading Frame 72
CNS	Central Nervous System
DREZ	Dorsal Root Entry Zone
DRTZ	Dorsal Root Transitional Zone
hiPSCs	Human Induced Pluripotent Stem Cells
HUVECs	Human Umbilical Vein Endothelial Cells
MNPs	Motor Neural Progenitors
MNs	Motor Neurons
MS	Multiple Sclerosis
MSP	Mesoporous Silica Particles
mTG	Micro-transglutaminase
NHLF	Normal Human Lung Fibroblasts
NPCs	Neural Progenitor Cells
PAA	Polyacrylamide
PBS	Phosphate-buffered Saline
PEO	Polyethylene Oxide
PNS	Peripheral Nervous System
PPO	Polypropylene Oxide
RA	Retinoic Acid
SCI	Spinal Cord Injury
SHH	Sonic Hedgehog
SMA	Spinal Muscular Atrophy
SNs	Sensory Neurons
SOD1	Superoxide Dismutase 1
sulfo-SANPAH	N-sulfosuccinyimidyl-6-(40-azido-20-nitrophenylamino) hexanoate
TARDBP	TAR DNA-binding Protein
TEMED	N, N, N’, N’-tetramethylethylenediamine
VEGF	Vascular Endothelial Growth Factors
VZ	Ventricular Zone

Introduction

Spinal Cord

The spinal cord is a long cylindrical structure of nerve tissue covered with three layers of protective membranes located within the vertebral canal, extending from the brainstem, passing through the foramen magnum, then the conus medullaris near the second lumbar vertebra, and ending at the filum terminale. (Purves and Williams, 2004)

During early development, the neural plate which is located in the middle of the dorsal ectoderm starts to differentiate, the neural folds are formed by folding it ventrally in the center and raising the edges. Then the neural folds curve upwards and fuse in both the head and tail direction to form the neural tube. The formed neural tube starts to vascularize when the neuropores closed. (Purves and Williams, 2004) The tail direction of the neural tube thickens as the neural canal gets thinner. Neuroepithelium surrounding this thin central canal develops into neurons and macroglia (including astrocytes and oligodendrocytes) within the spinal cord. (Ashwell, 2009) The border of the neural plate becomes the neural crest which will migrate along the dorsal neural tube and give rise to the entire peripheral nervous system (PNS) later. (Greene and Copp, 2014; Purves and Williams, 2004)

The cross-section of the spinal cord (Fig. 1) shows that it is divided into gray matter and white matter. The gray matter is composed of three parts. The wider part facing the ventral side is called the anterior horn, the thinner part on the dorsal side is the posterior horn, and the triangular part between them is the lateral horn. A large number of neuron cell bodies, synapses, and dendrites are concentrated in the butterfly-shaped gray matter in the center of the spinal cord. The surrounding white matter consists of axon tracts to transfer information to and from the brain. The axons of those sensory neurons (SNs) and motor neurons (MNs) are wrapped by a white lipid called myelin, which causes the color of the white matter. (Purves and Williams, 2004)

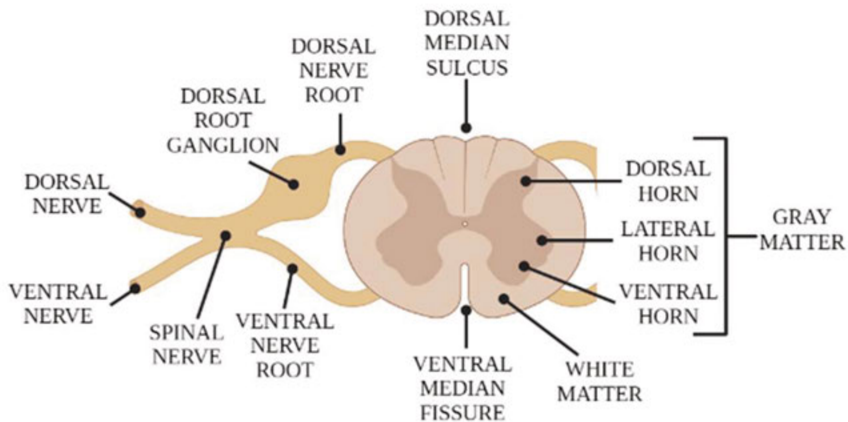

Figure 1. Spinal Cord cross section (Maugeri et al., 2023).

Cell functions in the spinal cord

Neurons and glial cells are the two cell types that make up the nervous system. Neurons are in charge of detecting stimuli from changes in the surrounding environment and conducting electrochemical signals, while glial cells provide support, nutrition, and insulation to neurons, as well as remove metabolic waste. (Merkle and Alvarez-Buylla, 2006)

Glial Cells

Radial glial cells, which originate from early neural tube epithelial cells, are neural progenitor cells (NPCs) that play an important role in the early stages of development. (Ever and Gaiano, 2005; Götz and Barde, 2005) During development, the bipolar structures of radial glial cells connect the surface of the ventricular zone, where newly generated neurons reside, to the outermost layer of the developing brain, the pia surface, thus providing a scaffold for those neurons to migrate to their corresponding locations in the cerebral cortex. (Sild and Ruthazer, 2011), They migrate to the surface of the cortex, where most of them become astrocytes, at the end of cortical development. Müller glial cells are a type of radial glial cell that appears in the retina after the first round of neurogenesis, (Bhattacharjee and Sanyal, 1975) whose main role is to maintain the stability of retinal cells. In addition, in the zebrafish model, Müller glial cells can proliferate after injury and have the possibility to generate retinal neurons. (Bernardos et al., 2007) Bergmann cells in the cerebellum also come from radial glial cells, but unlike bipolar radial glial cells, their apical processes are retracted, leaving only basal processes, which are called

monopolar glial cells. (Leung and Li, 2018) Their neurites are vertical assisting granule neurons to migrate along their radial direction during development and also retaining the synaptic connections in the mature cerebellum. (Buffo and Rossi, 2013)

Astrocytes are distinctive star-shaped glial cells that arise from neural stem cells. During the development, the neural stem cells or radial glia generated spinal cord astrocytes in the ventricular zone (VZ). (Akdemir et al., 2020) The physical framework composed of astrocytes provides support for neurons and their connections in the spinal cord, maintaining the structure of neural circuits. In addition, the high permeability of astrocytes to potassium allows them to clear extra potassium ions, preventing neuron depolarization (Walz, 2000) to ensure that electrical signals can be properly transmitted. Furthermore, when neurotransmitters (such as glutamate) are excessive in the synaptic cleft, astrocytes can absorb glutamate to prevent neurons from excitotoxicity. (Agulhon et al., 2010; Volterra and Meldolesi, 2005)

Oligodendrocytes are glial cells mainly for producing and maintaining a fatty substance called myelin that covers axons to form segments of the protecting sheath as an insulator. (Baumann and Pham-Dinh, 2001; Bradl and Lassmann, 2009) Myelin reduces the leakage of ions and also increases the speed of transmitting action potentials energy-efficiently. (Fry, 2007) The oligodendrocyte myelination is adaptive, oligodendrocyte progenitor cells can differentiate and then produce new myelin sheaths depending on the activities of neurons. (Czopka et al., 2013; Mount and Monje, 2017; Xin and Chan, 2020) In addition, oligodendrocytes can also provide the nutrition support required for myelinated axons, by converting pyruvate into lactate (Lee et al., 2012) or assisting in glucose or lactate delivery with astrocytes (Philippot et al., 2021).

Microglia cells are glial cells as the primary active immune defense in the central nervous system (CNS). The immune surveillance function of microglia makes them play a key role in responding to infection and injury. (Ginhoux et al., 2013) In response to injury or infection, microglia cells are activated and then release chemokines and pro-inflammatory cytokines. (Aloisi, 2001) When pathogens appear in the CNS environment, microglia cells are responsible for phagocytizing foreign antigens after detecting them, as well as metabolic waste or dead cell debris elimination. (Gehrmann et al., 1995)

Ependymal cells are glial cells lining the ventricles of the brain and the central canal of the spinal cord. It has different morphologies, including cuboidal ependymal cells, elongated cells, and radial ependymal cells. (Deng et al., 2023) The cilia that face the cerebrospinal fluid on the ependymal cells generate local movement and drive the cerebrospinal fluid flow. Stem cell-like characteristics of ependymal cells were shown after spinal cord injury (SCI).

Stimulated ependymal cells in the central canal of the spinal cord proliferate, then migrate to the injured site and start the multilineage differentiation. (Meletis et al., 2008) Most of the migrated ependymal cells differentiate into astrocytes, forming neural scars at the injured site, and a small number differentiate into oligodendrocytes. (Ren et al., 2017)

Neurons

SNs in the spinal cord whose cell bodies are located in the ganglia are neural cells that receive stimuli and transmit impulses to the CNS about alterations from the internal and external environments. Axons, dendrites, and the cell body make up an SN. Since they only have one axon that is divided into two branches, the majority of SNs are pseudo-unipolar. The finger-like dendrites receive the input and send it to the cell body via the axon. (Purves and Williams, 2004) MNs whose cell bodies are located in the cerebral cortex or the brainstem, known as upper MNs, transfer signals down to the lower MNs whose cell bodies are located in the ventral horn of the spinal cord. The lower MNs transmit impulses from the spinal cord to muscles and control their movements. MNs are multi-polar, with one axon and several dendrites. (Purves and Williams, 2004) Interneurons, unlike SNs and MNs, are not myelinated and have shorter axons that are not used to transport signals over long distances. In the spinal cord, they are located between SNs and MNs in the grey column, transferring sensory information to MNs, and when a complicated reaction to a stimulus is needed, more interneurons become active. (Kepecs and Fishell, 2014; Purves and Williams, 2004)

Spinal cord injury and diseases

The SCI which usually is trauma directly or indirectly to the spinal cord or the surrounding tissue, causes permanent or temporary trouble in sending sensory signals and receiving information from the brain for MNs below the injury site. (Harrow-Mortelliti et al., 2024) The most common ways to cause SCI are traffic accidents, surgical complications, or falling (Sekhon and Fehlings, 2001), in addition, spinal inflammation or tumors could also lead to it (Celani et al., 2001; New et al., 2014). Depending on the site of the spinal cord that got damaged, the severity of the effects on the body will vary. (Furlan et al., 2016; Li et al., 2017; Moonen et al., 2016; Stroman et al., 2016) Usually, SCI can be classified as incomplete injury since the damaged spinal cord still has some functions left, and complete injury, in which the spinal cord below the injury site is completely blocked from nerve communication and loses sensation. With the development and application of 3D bio-printing technology, printed scaffolds mixed with nutrient factors, growth factors, and stem cells are transplanted into the injury site (Liu et al., 2013; Song et al., 2012; Yang

et al., 2015) to provide a suitable microenvironment for regeneration and promote axon growth, which has become one of the breakthrough points for repairing spinal cord injuries. For example, Li's research showed that when cetuximab, which can inhibit the differentiation of NPCs into glial cells, was mixed into a collagen scaffold with NPCs and transplanted into the site of SCI, the implanted NPCs could differentiate into neurons more in vivo and promote nerve regeneration at the injured site. (Li et al., 2013)

The dorsal root avulsion is one of the most serious spinal cord injuries, which could be caused by difficult births, where the baby's shoulder is stuck in the birth canal for a long time (Berman et al., 1998), bullet wounds, or motorcycle accidents. (Carlstedt, 2016a; Carlstedt and Havton, 2012) It leads to shoulder and arm muscle weakness, paralysis, loss of sensation, and severe nerve pain. Prompt surgery after the injury can restore motor functions to some proximal muscles by reimplanting the avulsed brachial plexus nerve roots. (Cullheim et al., 1989; Risling et al., 1983) Unfortunately, the damaged sensory nerve axons are blocked by the extracellular matrix at the injured dorsal root transitional zone (DRTZ), and cannot re-enter the spinal cord (Ramer et al., 2001), making it difficult to restore sensory functions. (Carlstedt, 2016b) In our previous research, we demonstrated that blood vessels at the dorsal root PNS-CNS interface provide the bridge for transplanted human SNs from the periphery to bypass the astroglia block (Hara et al., 2017) in the DRTZ. (Kozlova et al., 1997)

Amyotrophic lateral sclerosis (ALS) is a well-known neurodegenerative disorder, in which MNs are gradually lost, leading to progressive loss of muscle strength. As the disease becomes more and more serious over time, the upper and lower neurons gradually degenerate, cannot receive signals, and eventually die. The patients will gradually lose muscle functions of their upper and lower extremities and eventually suffer breathing and swallowing issues, leading to death. (Hardiman et al., 2017) Among the various mutations that cause ALS, the superoxide dismutase 1 (SOD1) mutation, the chromosome 9 open reading frame 72 (C9orf72) mutation, and the TAR DNA-binding protein (TARDBP) mutation are the most common ones. (Renton et al., 2013) We used the C9orf72 mutation for our 3D printing protocol from **Paper IV**. Currently, there is no cure for ALS, and the only way to prolong the patient's life is through medication such as Riluzole, and some assisted breathing devices. Because of the various genetic and sporadic forms of ALS, it is particularly important to establish a patient-specific in-vitro model for mechanistic and diagnostic purposes, as well as for drug testing.

Multiple sclerosis (MS) is a long-term CNS disease, caused by an autoimmune attack on oligodendrocytes. This leads to loss of myelin and action potential transmission failure. (Compston and Coles, 2002) Depending on the location

and degree of myelin damage, the symptoms displayed by patients are very diverse. Vision problems, loss of precise control, difficulty maintaining balance, urinary and bowel problems, and slow reactions, could be their symptoms. (Hartung et al., 2014) Also, the loss of myelin sheaths causes hard glial scars ("sclerae"). After diagnosis, the disease may get better and recur, but even if the symptoms disappear, permanent nerve damage remains and is irreversible. Even though there is no cure for MS, there are treatments that can effectively help in reducing the number of attacks and restoring some of the body mobilities. (McGinley et al., 2021)

Spinal muscular atrophy (SMA) is a genetic motor neuron disorder, which is characterized by the progressive death of MNs due to mutations in the SMN1 gene. (Brzustowicz et al., 1990) When the SMN gene is transcribed to SMN mRNA and translated to the SMN protein (motor neuron survival factor), the MNs that connect to the skeletal muscles are able to survive. (Lefebvre et al., 1995) Therefore, after the SMN1 gene mutates, the lack of SMN protein will further cause the progressive death of the MNs and the connected skeletal muscles will become smaller due to the lack of signals from the MNs. (Monani, 2005) The later the age of onset, the milder the symptoms, so the symptoms of SMA that begin in childhood are the most serious ones, causing weak crying, difficulty with sucking, and developmental delays. Fortunately, with the help of a spontaneous breathing system, the survival rate of babies with SMA has increased. (Bach et al., 2000)

Boundary cap neural crest stem cells

Boundary cap neural crest stem cells (BCs) are a self-renew transient group of cells, migrating from the dorsal neural tube during the development. They are located at the dorsal root entry zone (DREZ), (Aldskogius et al., 2009; Hjerling-Leffler et al., 2005) which can differentiate into SNs and Schwann cells in vitro and in vivo (Hjerling-Leffler et al., 2005), as well as into astrocytes in vitro (Zujovic et al., 2011) as a source of multipotent stem cells.

In previous research, BCs showed the ability to increase the proliferation of insulin-producing beta-cells in both co-culture and co-implantation with pancreatic islets. (Grouwels et al., 2012; Ngamjariyawat et al., 2013, 2012; Wang et al., 2016) The previous studies also showed that the vascularization and innervation of implanted pancreatic islets were promoted through angiogenic and neurotrophic factors that released by BCs. (Grapensparr et al., 2015; Lau et al., 2015) In addition, BCs demonstrated a high survival (Han et al., 2021) and proliferation rate (Han et al., 2024) in 3D-printed structures with extreme culturing conditions in space (under microgravity). Due to the ability to survive in tough conditions, differentiate into a variety of cell types in vitro, and

support other cells in co-culture, BCs became our initial cell type for exploring 3D printing conditions and as potential supporting cells for the establishment of microvasculature in 3D-printed scaffolds (**Paper III**).

Human iPSCs

Human induced pluripotent stem cells (hiPSCs) are human cells that have been genetically reprogrammed from adult somatic cells, typically skin cells, to an embryonic-like state, in which they can proliferate infinitely and have the potential to give rise to any type of cells. Due to this ability, hiPSCs have become a useful tool for disease modeling, drug development, stem cell differentiation, regenerative medicine, and tissue engineering. (Takahashi and Yamanaka, 2006) Shinya Yamanaka's discovery of iPSCs got the Nobel Prize in 2012. There is also a growing trend toward using hiPSCs in 3D bioprinting to test and screen bio-inks in pilot experiments, create patient-specific models for drug testing, and create implantable tissues and organs for replacement. (Gaetani et al., 2015; Ong et al., 2018) In **Paper II** and **Paper IV**, we used MNs and astrocytes derived from hiPSCs to explore suitable bio-inks and printed structures.

There are several protocols of hiPSCs-derived spinal motor neurons (sMNs) (Amoroso et al., 2013; Du et al., 2015a; Qu et al., 2014) for drug development against devastating neurodegenerative diseases such as ALS, SMA, etc. The most widely utilized way involves patterning with Sonic Hedgehog (SHH) and Retinoic Acid (RA) (Li et al., 2005; Qu et al., 2014), once the SMAD pathway is inhibited by small molecule inhibitors like LDN193189 or SB431542, which lead the cells towards neuroectodermal lineage for forming NPCs. The hiPSCs differentiation method that was improved by Maury's research (Maury et al., 2015) enables hiPSCs to rapidly differentiate without skipping the in-vivo development stage, producing effective sMNs and cMNs in about 14 days by early Wnt activation and γ-secretase inhibition. In **Paper II**, the hiPSCs-derived MNs we used followed the highly pure and quicker protocol from Su-Chun Zhang's group (Du et al., 2015b), which uses Wnt activator CHIR and NOTCH inhibitor. Motor neural progenitors (MNPs) could be expanded in 12 days, and young MNs could be used for 3D bioprinting at day 19. In **Paper IV**, we did not only use this hiPSCs-derived motor neuron but also used neurospheres generated from hiPSCs for the printing.

Bioprinting

3D Bioprinting is the process of creating functional layer-by-layer tissue-like structures with living cells, growth factors, and bio-inks which are materials

that support living cells to facilitate their adhesion, proliferation, and differentiation. (Moroni et al., 2018) Printed structures that are later used in drugs, potential treatments, and tissue engineering fields. Extrusion-based bioprinting, droplet bioprinting, and laser-assisted bioprinting (Khalil and Sun, 2009; Wüst et al., 2011) are the three primary 3D bioprinting modalities that can be broadly categorized, additionally, there are also some new modalities, such as stereolithography (Wang et al., 2015) (Fig. 2). And for extrusion-based bioprinting, it has three main ways: pneumatic-driven, piston-driven, and screw-driven bioprinting. (Murphy and Atala, 2014) In **Paper II**, an air-pressure printing head was used first, since soft materials were chosen as the bio-inks based on the requirements of cell survival, the air pressure could not provide the extrusion evenly and continuously. Therefore, in subsequent printing attempts (**Paper II, III, and IV**), the more stable piston-driven bioprinting was used.

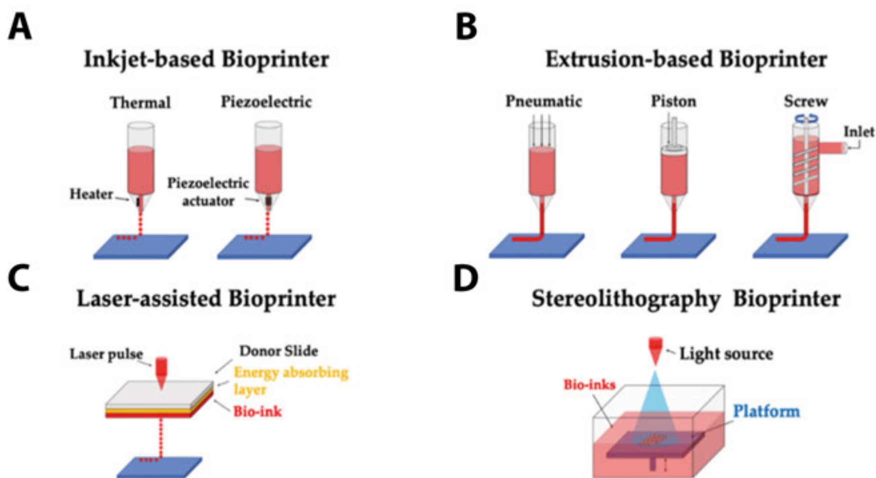

Figure 2. Different types of 3D bioprinters (Yu et al., 2020). (A) Inkjet-based printers (B) extrusion-based printers.(Malda et al., 2013) Copyright 2013 John Wiley and Sons. (C) laser-assisted printer (Keriquel et al., 2017) (D) stereolithography-based printer (Kačarević et al., 2018)

The selection of a suitable bio-ink needs to be considered from many aspects. First of all, biocompatibility and cell viability need to be considered. The bio-ink needs to be non-toxic and non-inflammatory for cells, also supporting cell adhesion, proliferation, and differentiation. Preliminary experiments are necessary before printing to screen out the range of bio-inks suitable for cell viability. (Lee et al., 2015) Therefore, within this appropriate range, the printability of the bio-ink needs to be tested. For example, too soft bio-inks that do not have enough strength and flexibility would not be able to maintain a layer-by-layer printing structure during the printing process, so it is necessary to

increase the stiffness of the bio-ink or print it in combination with other bio-inks that have stronger supportive properties to ensure a stable structure during the printing process and long-term culture. In some cases, biocompatibility and printability are not consistent, requiring a compromise in cell viability to find a balance between them. Also, to keep reproducibility, the potential for large-scale production with minimal batch-to-batch variability is required (Loo et al., 2015), which ensures the sustainable production of 3D bio-printed tissue-like structures for drug research or tissue engineering.

Based on the factors mentioned above, different types of cells require different bio-inks to support their growth in the printed scaffolds. The development of suitable bio-inks for MNs and astrocytes is a critical aspect of the 3D printing of spinal cord organoids.

Vascularization in bioprinting

One of the major challenges in 3D bioprinting is that the cells inside the thick printed scaffold lack a microvascular system that can circulate culture medium with nutrients to improve cell survival and vitality. (Jafarkhani et al., 2019) In vivo, the blood vessels are formed by the blood islands which are clusters of a special type of cells called hemangioblast. (Murray and Wilson, 1997) During the development, most of the central cells of the blood islands will die, and the cells in the left periphery become endothelium precursor cells, which will differentiate and then form the endothelium of the blood vessel. (Choi et al., 1998) After the blood islands coalesce together to create the vascular cords, mesenchymal cells will surround the vascular cords, and differentiate into vascular smooth muscles and the connection parts to form the mature blood vessels. (Sabin, 1920) Besides forming from embryonic mesenchyme, blood vessels can also form through angiogenesis. When the vascular endothelial growth factors (VEGF) are around the blood vessel, the endothelial cell on the blood vessel will change to a tip cell and migrate towards the VEGF. The cells next to the tip cell will proliferate and follow the tip cell to become new blood vessels. (Nieves et al., 2009)

In 3D bio-printing, setting up complex simulated vascular structures is still a big challenge. Using some special bio-inks that have reversible gelation properties affected by temperature, good fluidity, and low residue as sacrificial inks is the most common way to create tubal structures in printed soft tissues. In Lee's research (Lee et al., 2014), 10% gelatin was used as sacrificial ink for printing inside the collagen hydrogel, which will melt at 37 °C to create the channels. Once the printed gelatin temporary gelation was done, normal human lung fibroblasts (NHLF), human umbilical vein endothelial cells (HUVECs), Fibrinogen, and thrombin were mixed and placed between two gelatin

channels. After melting the gelatin, endothelial cells were seeded inside the channels. The microvascular network was formed between two channels, and also the endothelial cells from two large channels sprouted and eventually connected with the capillaries. A composite of triblock copolymer (polyethylene oxide (PEO)–polypropylene oxide (PPO)–PEO) was used as sacrificial ink, which will become liquid and be removed from the scaffolds at 4 °C, in Lewis's group (Kolesky et al., 2016). After removing the sacrificial ink, HUVECs were placed inside the channels, as well as the fibroblast cell-laden matrix was outside the channels. Lumen formation was detected in 6 weeks of perfusion on a chip. Besides using sacrificial inks, some laser-based methods are also used for creating tubal structures in harder tissues, especially for bone implants. (Matena et al., 2015) Each method has its advantages and limitations. Combining the functions of the printed tissue to find the appropriate simulated vascular structure is one of the keys to establishing complex 3D-printed models.

Methodology

Cell preparations before 3D-Printing

BCs were cultured in 6-well low attachment plates for 2 weeks, with the medium changing every other day, until form round neurospheres with clear edges. The free-floating neurospheres were collected and dissociated by trypLE for further material testing or printing in **Paper II, III, and IV**.

In **Paper II and IV**, iPSCs-derived NPCs were cultured on Matrigel-coated 6-well plates in the neural induction medium for 6 days with the medium changing every day. Then the medium was switched to basal medium with 3uM CHIR, 2uM DMH1, 2uM SB431542, and 0.1uM RA for 6 days. After that, basal medium with 0.5uM and 0.1uM purmorphamine was used for 6 days before the cells were dissociated by Accutase for further printing. In addition, **in Paper II**, directly induced NPCs were cultured in 6-well plates with fibronectin-coated surfaces in DMEM medium with 10% FBS, 0.05% N2, and penicillin-streptomycin to produce astrocytes. After 7 days in culture, the derived astrocytes were passaged by Accutase for further printing. In **Paper IV**, motor neuron progenitors were also cultured as neurospheres in 6-well low attachment plates before mixing with bio-ink for printing.

Materials selection for scaffold fabrication

Several biomaterials were tested for setting up the suitable 3D bio-printed structure. Polyacrylamide (PAA) was used in **Paper I** to test the effect of different stiffnesses on cell adhesion and maintenance. Following the protocol from Wang's research (Wang and Pelham, 1998), 8% acrylamide was mixed with 0.1%, 0.5%, and 0.7% bisacrylamide, respectively, and then polymerized by adding N, N, N', N'-tetramethylethylenediamine (TEMED) and ammonium persulfate. The PAA gel was then exposed to UV light for photoactivation with the cover of 10 mM HEPES (pH 8.5) containing N-sulfosuccinyimidyl-6-(40-azido-20-nitrophenylamino) hexanoate (sulfo-SANPAH) and used for cell seeding after washing 3 times with phosphate-buffered saline (PBS). Due to the complexity of the preparation process and the long photoactivation time, PAA gel can only be used to seed cells on the surface of the scaffold but cannot be used as a bio-ink that mixes with cells before printing.

Therefore, gelatin-based hydrogel was tried in different concentrations in **Paper II, III, and IV**. Gelatin-based bio-inks have temperature and enzyme gelation properties, which allow us to achieve a temporary gel state during the printing process by controlling the temperature, and then reach irreversible gelation under the action of enzymes to maintain the printed structure in subsequent cell culture at 37 °C in the incubator. 15% w/v gelatin powder was mixed in PBS while stirring at 70 °C on the hotplate, and 1M NaOH solution was added to adjust the pH to around 7 after the gelatin powder was completely dissolved. The flow point of gelatin was measured three times by thermic cycling rheometry (Anton-Parr) to get the range of suitable preparation temperatures for different concentration gelatin-based bio-inks. Micro-transglutaminase (mTG) enzyme was tested for suitable printing concentration and used for permanent gelation by adding into or covering the bio-ink. To improve the protocol from **Paper II**, the concentration of gelatin was reduced to get better access to the surrounding culture medium for cells inside the scaffolds.

To increase the printability of very soft bio-ink, we tried different approaches. Alginate, which uses $CaCl_2$ solution as the crosslinker was introduced into our test system. Alginate and gelatin were mixed (Fig. 3) in different ratios to make the bio-ink preparation process more concise and the printed 3D structure more stable. However, the disadvantage of alginate-gelatin-based hydrogel is that the $CaCl_2$ solution needs to be continuously added to the culture medium to maintain the concentration of calcium ions during long-term culture to keep the crosslinking. Moreover, the adhesion and differentiation of cells on the surface of the alginate-gelatin scaffolds were not ideal, compared with the low-concentration gelatin bio-ink. Eventually, 4% gelatin was selected as the final bio-ink in **Paper III and IV**.

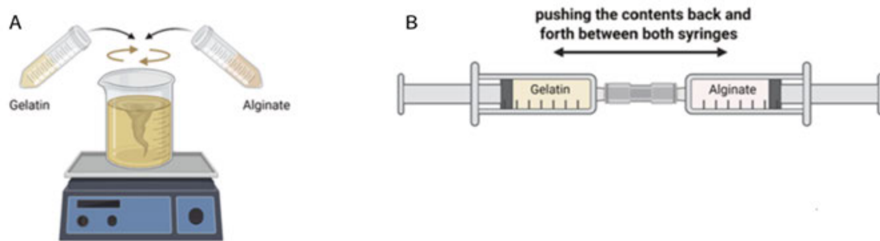

Figure 3. Two different methods of mixing gelatin with alginate for bio-ink.

Hydrogel ink preparation and 3D printing settings

Extrusion-based bioprinters were chosen in this thesis. BIO X syringe pump printhead from CELLINK (**Paper II**) and BioScaffolder 3.3 PRIME from

GeSiM (**Paper III and IV**) were used to print the 3D scaffolds with different concentrations of bio-inks. In **Paper II**, the original printing head with air pressure was used first, however, to extrude the soft cell-containing bio-ink continuously and evenly, the syringe pump had more stable performance. Cells mixed with bio-ink were loaded into a 3 ml Luer-lock syringe (BD Syringe) with a 0.41 mm inner diameter nozzle (Precision Tips, Nordson EFD), and printed on the 10 °C printing bed. Since the printing bed temperature of the BIO X printer is difficult to stabilize at its lowest temperature (10 °C) and cannot achieve the ideal low-temperature printing requirement (8-4°C), we switched to the GeSiM printer in **Paper III and IV**.

In **Paper III**, to achieve co-culture cell spheres and aortic rings inside the scaffolds, first, cell spheres were loaded with gelatin-based hydrogel for printing first, then when the three-layer structure was halfway through printing, the process was paused, the aortic ring was placed near the cell spheres, and then printing continued until the aortic ring was covered, completing the preset three-layer structure. The whole scaffolds were then crosslinked by mTG solution.

In **Paper IV**, similar methods were applied to iPSCs-derived MNs with slightly modified. Method 1 is only mixing 4% gelatin and mTG solution in the syringe first, and cells in another syringe were added by Luer-locker after the gelatin mixture became like Newtonian fluid. Method 2 is that cells were mixed with 4% gelatin and mTG solution, then after printing, the whole scaffolds were covered with mTG solution for 10 mins to allow the structures to reach permanent gelation. Cell viability was tested for both methods, and Method 2 was then used for the final prints.

Bio-printed scaffold sectioning

To visualize the differentiation of cells in 3D-printed structures, the printed structures were sectioned and stained (**Paper II and IV**). In **Paper II**, scaffolds were fixed in 4% paraformaldehyde (4% PFA) for 15 min, then covered with Tissue-Tek (Sakura) and frozen with carbon dioxide 2.8/4.5 for sliding into 10um serial sections. However, in **Paper IV**, we lowered the concentration of the gelatin bio-ink, therefore, to maintain a relatively complete section, the scaffolds were soaked in mTG solution for 5 minutes before fixation and the section thickness was increased to 12um as well.

Analytical methods for printed scaffolds and sections

The stained sections in **Paper II and IV** were imaged by Carl Zeiss LSM 700 Laser Scanning Microscope. Additionally, in **Paper III**, the printed scaffolds were not sectioned, which were imaged by a Zeiss 5 Live confocal microscope. The motor neuron differentiation inside the scaffolds in **Paper IV** was assessed by counting the intersections of neurite outgrowth and cell bodies. Z-stack merged images were placed in evenly distributed horizontal lines and the neurite outgrowth and cell bodies that crossed the line were counted to get the ratio for assessing the differentiation. (Rønn et al., 2000)

Present Investigations

Paper I

Effect of scaffold properties on adhesion and maintenance of boundary cap neural crest stem cells in vitro

Aims

To find suitable chemical and physical cues for BCs adhesion and maintenance in-vitro on scaffolds.

Background

Stem cell differentiation can be affected not only by chemical factors but also by physical cues. Neural stem cell differentiation depends on their preferred stiffness. Exploring the adhesion, proliferation, and differentiation of BCs under different stiffnesses of the material and the covering substrate can establish a preliminary foundation for the 3D bio-printed structure in the future.

Results and discussion

In our study, collagen- or laminin-PAA scaffolds of three different stiffness (0.5, 1, or ~7 kPa) were chosen for examining BCs adhesion, proliferation, and differentiation in the same culture medium.

Our findings indicate that the surface characteristics (collagen or laminin) of scaffolds with low stiffness are critical for cell adherence, however, scaffolds with higher stiffness show less dependence on the surface covering substrate.

The maintenance ability of BCs on the collagen-covered scaffold of 7kPa stiffness was better with a clear growth trend compared to 0.5 and 1kPa stiffness scaffolds. However, many cells attached to the surface with weak adhesion, therefore, they detached from the surface after washing as well as after longer culturing. For laminin-covered scaffolds, the 7kPa stiffness surface showed better maintenance ability of BCs, and also better support for BC proliferation compared to 1kPa and 0.5kPa scaffolds.

For cell differentiation, high-stiffness scaffolds demonstrated significant advantages for BC differentiation with both chemical coatings. Laminin-covered scaffolds of all three different stiffnesses supported cell differentiation well.

Especially on surfaces with 1kPa stiffness, the expression of the astrocyte marker GFAP was consistent with GFAP expression in early differentiated astrocytes.

The scaffold stiffness significantly affected the capacity of BCs to adhere and maintain a high cell density and differentiation with especially laminin surface. These results point to some crucial factors in achieving optimal stem cell properties for 3D bio-printed scaffolds.

Paper II

Towards 3D Bio-printed Spinal Cord Organoids

Aims
To develop a 3D printing protocol BCs and furtherly apply it with human iPSC-derived neural cells for spinal cord organoids.

Background
As organoid research develops, it becomes an increasingly attractive tool for in vitro organ modeling and new drug testing. Currently, 3D co-culturing of cells that partially represent the composition of the spinal cord shows that there is the possibility to achieve a 3D bio-printed structure similar to the mature human spinal cord using hiPSCs-derived neurons and glial cells with suitable bio-inks and under appropriate printing conditions. BCs as an attractive stem cell resource that could differentiate into different kinds of neural cells such as neurons and astrocytes were chosen as the testing cells for the initial printing protocol for spinal cord organoids.

Results and discussion
We examined if BCs could survive and differentiate in gelatin-based bio-ink with different concentrations of bio-ink and cross-linker and analyzed the survival of BCs on the surface with scaffolds and inside the scaffolds.
The survival of BCs after seeding on the surface of the scaffolds in different concentrations was tested. Several concentrations of gelatin-based bio-inks showed good support for BC survival and proliferation, especially, 12% gelatin. Additionally, 12% gelatin showed the capacity for BC differentiation from day 1 after seeding, earlier than other bio-inks with different stiffnesses. On the other hand, the survival tests for BCs mixed in different layers of scaffolds were proceeded by Alamar Blue staining, and the 3-layer scaffold showed the best survival compared to the 1-layer and 5-layer scaffolds.

According to the results above, the 12% gelatin with 25mg/ml mTG in the 3-layer structure was selected for BCs 3D Bio-printing. The cells in the scaffolds had a high survival rate after 5 weeks of culture, and cell differentiation was detected not only at the edge but also inside the scaffolds. Unfortunately, the cells at the edge and on the surface were showing advanced differentiation compared to the cells in the middle of the scaffolds.

Thereafter, the protocol developed for BCs was applied to human iPSCs-derived motor neuron precursors and astrocytes. It shows that neural differentiation was more advanced closer to the edge of the scaffolds than in the central parts of the scaffolds, presumably because it was easier for edge located cells to get access to the differentiation medium. The gelatin-based and enzymatically cross-linked hydrogel is suitable for BCs and human iPSCs survival, but to achieve uniform neural differentiation, it needs further modifications.

Paper III

BCs promote angiogenesis after transplantation to avulsed dorsal roots in mice and induce migration of endothelial cells in 3D-printed scaffolds

Aims

To determine whether BCs could help with increasing vascular volume after implantation in vivo and promote angiogenesis in vitro.

Background

The DRTZ is the interface between the CNS and the PNS. During the development, BCs help sensory axons to enter through the DRTZ into the spinal cord, and also prevent motor neuron migration from the spinal cord. Some severe traumatic dorsal root avulsions can lead to a loss of connection between the spinal roots and their spinal cord attachments, sensory loss, and compromised vascular integrity at the site of injury. According to previous studies, sensory axons of transplanted neurons can use blood vessel growth to reenter the spinal cord. Increasing vascularization at the site of injury may be beneficial for providing nutrition and support to sensory axons reenter. Based on the properties of BCs, such as its beneficial effects on increasing vascular density and producing neurotrophic factors that appear to be useful for dorsal root regeneration, we tested the pro-angiogenic effects of BCs in both in vivo and in vitro (the 3D-printed co-culture model), which provide the possibility of improving vascularization in 3D-printed scaffolds.

Results and discussion

When aortic rings and BCs were co-cultured, the sprouting of endothelial cells in aortic rings was much higher than that in the culture without BCs.

Endothelial cells sprouting around aortic rings without BCs had no specific sprouting direction but were entangled near the aortic rings, however, in the 3D structure of co-culture, endothelial cells were strongly attracted by BCs, extended in the direction of BCs, and appeared to make contact with them. These findings show that BCs are able to guide the migration and assembly of endothelial cells in the 3D-printed scaffolds.

We analyzed the effect of BCs on the injury area after implantation. Compared with mice that underwent dorsal root avulsion but did not receive BCs transplants, mice that received BCs transplants had a more orderly vasculature with more uniform vessel diameters and significantly increased blood vessel volume in the injured DRTZ area.

The results show that BCs promote vascularization in a model of SCI and a 3D co-culture system with aortic ring slices.

Paper IV (Manuscript)

Differentiation of motor neurons in the 3D printed scaffold – next step toward spinal cord organoid

Aims

To improve the protocol for 3D bioprinting of human IPSC-derived MNs.

Background

Our previous research (Paper II) showed that our gelatin-based scaffolds with human iPSCs-derived motor neuron precursors achieved neurite arborization on the surface but there was no neurite outgrowth extensive inside the printed scaffolds, which showed that the concentration of the previous bio-ink is not suitable for motor neuron differentiation. While gradually reducing the density of the bio-ink, rheology tests are used to explore the appropriate printing temperature and crosslinking time to adjust the suitable bio-ink and then achieve motor neuron differentiation.

Results and discussion

The concentration of gelatin-based bio-ink was reduced from 12% to 4% to facilitate access to the differentiation-promoting factors in the medium for MNs in the deep parts of the printed structure. Based on the soft properties of 4% gelatin, we set up two printing methods to ensure the printability of the bio-ink and the structural stability while culturing at 37 °C after printing. To optimize the printing temperature, crosslinking time, and the effects of adding mesoporous silica particles (MSP) loaded with the neurotrophic peptide mimetics cintrofin and gliafin in the bio-ink, rheology tests were done on 4%

gelatin with and without MSP. Rheology tests have shown that when the temperature drops from 37°C to 4 °C, 4% gelatin takes about 10 minutes to reach a gel state (G' > G"). It can also reach a gel state when the temperature drops to about 8 °C, however, the gel state cannot be stably maintained during the subsequent actual printing process. Also, added MSP does not show a significant effect on the viscoelastic properties of the bio-ink. After determining the gelation time of the bio-ink (non-permanent crosslinked) and the printing temperature, we also tested the stiffness of the final gelatin scaffold, which is 0.5 kPa, matching the neuronal differentiation conditions.

After comparing the cell loss of the two methods with the survival rate of BCs, one of the methods was selected as the final method for printing hiPSCs. In the scaffold containing MSP, NPCs differentiated into more advanced neurons, and the differentiation level of astrocytes in the scaffold was also higher. The monolayer culture of MN progenitor cells in the scaffold had a large number of neurites and astrocyte processes growing in the scaffold after 30 days of culturing. This improved printing protocol for generating 3D-printed scaffolds with human MNs will be very valuable for setting up the patient-matched spinal cord organoid.

Conclusions and Future Perspectives

According to our research in **Paper II and IV**, MNs can survive and differentiate in 3D-printed scaffolds by gradually optimizing printing conditions and printing materials, which shows the possibility of building an in vitro spinal cord organoid. Different nerve cells prefer different scaffold stiffness, therefore, finding chemical and physical conditions (**Paper I**) suitable for their adhesion, survival, and differentiation will be the basis for printing the scaffolds with multi-layer of cells and multi-bio-inks.

In **Paper III**, the BCs were shown to have an attractive effect on endothelial cell sprouting from aortic rings, making the sprouting direction more orderly, and could increase vascular volume and vessel diameter after transplantation into the injured spinal cord in mice. In the future, we can try to introduce HUVECs into 3D-printed scaffolds we got from **Paper IV** with hiPSCs-derived MNs to promote the generation of microvascular systems in that model and allow other neural cells in the printed structures to communicate with the surrounding culture medium. It could also be interesting to test if BCs have an attractive effect on human endothelial cell sprouting or not, therefore, BCs could be one of the support cells in the whole printing structures for vascularization. Additionally, microfluidic systems can also be tested to see if they facilitate angiogenesis, or directly create channels in printed structures using sacrificial bio-inks to deliver nutrients to cells that are located deep in the thick printed tissues.

To achieve a stable multi-material layered structure and also reduce costs, we need to test the adhesion, growth, and differentiation of MNs on other bio-inks as well, such as GelMA in further experiments. And hiPSCs-derived astrocytes were tested on gelatin and the current results show that there is no specific concentration of gelatin that can significantly support its differentiation. Therefore, in further studies, astrocytes, oligodendrocytes, and microglia could also be tested on different bio-inks with different concentrations, to select the optimal one for each cell type.

Ethical considerations

All procedures were approved by the Regional Ethical Committee for Research on Animals and carried out according to the Guide for the Care and Use of Laboratory Animals (the National Academy of Sciences, USA).

The hiPSCs used in **Paper II** were provided by the Cedars-Sinai Medical Center's David and Janet Polak Foundation Stem Cell Core Laboratory.

The work with hiPSCs in **Paper IV** was performed with the approval of the Southeastern Norway Regional Ethics Committee (approval REK 2017/110).

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