Biomaterials Science

REVIEW

View Article Online
View Journal | View Issue

Cite this: *Biomater. Sci.*, 2016, **4**, 768

Utilizing stem cells for three-dimensional neural tissue engineering

Stephanie Knowlton,[a] Yongku Cho,[b] Xue-Jun Li,[c] Ali Khademhosseini[d,e] and Savas Tasoglu*[a,f]

Three-dimensional neural tissue engineering has made great strides in developing neural disease models and replacement tissues for patients. However, the need for biomimetic tissue models and effective patient therapies remains unmet. The recent push to expand 2D neural tissue engineering into the third dimension shows great potential to advance the field. Another area which has much to offer to neural tissue engineering is stem cell research. Stem cells are well known for their self-renewal and differentiation potential and have been shown to give rise to tissues with structural and functional properties mimicking natural organs. Application of these capabilities to 3D neural tissue engineering may be highly useful for basic research on neural tissue structure and function, engineering disease models, designing tissues for drug development, and generating replacement tissues with a patient's genetic makeup. Here, we discuss the vast potential, as well as the current challenges, unique to integration of 3D fabrication strategies and stem cells into neural tissue engineering. We also present some of the most significant recent achievements, including nerve guidance conduits to facilitate better healing of nerve injuries, functional 3D biomimetic neural tissue models, physiologically relevant disease models for research purposes, and rapid and effective screening of potential drugs.

Received 18th August 2015,
Accepted 2nd February 2016

DOI: 10.1039/c5bm00324e

www.rsc.org/biomaterialsscience

1. Introduction

Recent progress in the field of neural biology and tissue engineering offers a deeper understanding of stroke, spinal cord injuries, traumatic brain injuries, and neurodegenerative disorders. However, much remains unknown and clinical treatment options remain limited for the 15 million stroke cases per year,[1] over 35 million people living with Alzheimer's disease,[2] the 1% of the world population with Autism Spectrum Disorder,[3] and others suffering from neurological injuries and diseases worldwide. The power of tissue engineering lies in the application of life science knowledge into engineering designs to give rise to cutting-edge biotechnology. A prime example of this intersection between biology and engineering research is neural tissue engineering, a field which stands to gain a great deal from implementing the recent progress in stem cell research and three-dimensional (3D) neural tissue engineering.

Here, we closely examine the intersection of three-dimensional (3D) tissue engineering, neuroscience, and stem cell biology. We discuss the vast potential, as well as the challenges, unique to the integration of these distinct areas of research (section 2). We examine a variety of tissue engineering strategies which take advantage of a wide range of fabrication techniques and combine these techniques with knowledge of stem cell biology and neuroscience to engineer neural tissues (section 3). Finally, we present several of the most innovative and important recent achievements which advance the field of neural tissue engineering: (i) engineering therapeutic nerve guidance conduits to facilitate better healing of nerve injuries (section 4), (ii) developing functional 3D tissue models to mimic nervous system tissues (section 5-A), (iii) designing physiologically relevant disease models for the purpose of better understanding neurological disorders (section 5-B), and (iv) screening potential drugs to treat disease and injury to the nervous system (section 5-C). Current research aims to engineer biomimetic neural tissues either as a replacement for

[a]*Department of Biomedical Engineering, University of Connecticut, 260 Glenbrook Road, Storrs, CT 06269, USA. E-mail: savas@engr.uconn.edu*
[b]*Department of Chemical & Biomolecular Engineering, University of Connecticut, 191 Auditorium Road, Storrs, CT 06269, USA*
[c]*Department of Neuroscience, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA*
[d]*Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital Harvard Medical School, Harvard-MIT Division of Health Sciences and Technology Massachusetts Institute of Technology, Cambridge, MA 02139, USA*
[e]*Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA 02115, USA*
[f]*Department of Mechanical Engineering, University of Connecticut, 191 Auditorium Road, Storrs, CT 06269, USA*

damaged or disease tissues or as platforms to study neural tissue structure and electrical function.

2. Motivation and approach to the challenge of neural tissue engineering

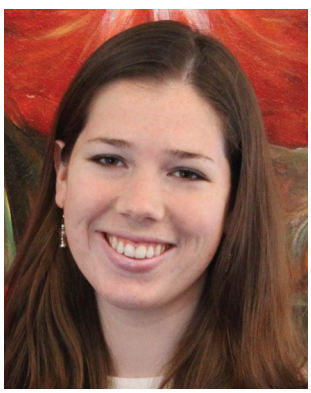
In vitro tissue models offer huge potential across many fields of medicine. Such artificial constructs which closely mimic natural tissues have the potential to serve as a replacement for tissues in the body to cure or treat a variety of medical issues.[4,5] However, in light of current challenges, alternative applications have been proposed for engineered tissues as models for biological research, drug screening, and preclinical testing of materials and therapies.[4] Even though animal models are widely used and essential in preclinical trials, translating results from animals to humans may be limited in certain cases.[6–8] Engineered tissues offer a low-cost, reproducible, and high-throughput alternative to current methods. Engineered tissues also afford the ability to use human cells, making engineered models as close to the clinical stage as possible without the use of human subjects. Use of stem cells enables patient-centered therapies while 3D approaches enable replication of 3D cellular structures, recreation of tissue functionality, and control over stem cell differentiation.

2.1 Engineering tissues for *in vitro* research

Tissue engineering strategies allow *in vitro* tissue formation in a way that mimics the natural developmental process to form 3D complex tissue structures, which can ultimately contribute to our basic understanding of developmental biology and tissue structure and function. There is also potential to create disease models using human stem cells to accurately recreate diseased conditions for the purpose of understanding the disease and developing effective therapies.[9] One application with the potential for significant economic impact is *in vitro* drug screening.[10] The cost associated with the discovery and development of each drug candidate is estimated to be between $800 million to $1 billion,[11] partly due to ineffectiveness of animal models at predicting the human response to a drug. In some cases, preclinical trials are successful in animal models but fail in clinical trials, partly due to ineffectiveness of animal models at predicting the human response to a drug.[7] In other cases, such as fibromyalgia syndrome, no animal models exist to replicate the attributes of human disease.[8] Therefore, *in vitro* disease models which use human cells from subjects carrying the disease may be developed to closely mimic the diseased tissues and offer a promising platform for rapid and efficient screening of clinical therapies (discussed further in section 5).

2.2 Stem cells as a source of other cell types

Stem cells have the ability to self-renew indefinitely, allowing researchers to obtain large numbers of cells with the same genotype as the patient for high-throughput testing.[9] Their differentiation potential makes it possible to obtain different cells types and create organoid models from a small number of isolated cells. Since all cells derived from the stem cells carry the donor's genome, it is possible to regenerate entire tissues which can be used to replace degenerated or damaged tissues with low risk of rejection or be used *in vitro* as a model of the *in vivo* tissue. Stem cells harvested from patients with a disease of interest can be used to create disease models as a viable alternative to current transgenic animals and transformed cell lines. Genetic modification of stem cells, such as knockout models where a particular gene is removed, can help to examine the role a specific gene plays in genetic disorders at the tissue level. Neurodegenerative disease research stands to



Stephanie Knowlton

Stephanie Knowlton earned her BS in 2015 and is now a graduate student in Biomedical Engineering at the University of Connecticut, Storrs, CT. Her current research interests include point-of-care diagnostics and microfluidics for tissue engineering applications with a focus on translational research. Her recent work includes the use of magnetic levitation for rapid, on-site diagnosis of sickle cell disease, which was featured in several news outlets including Medical Design Briefs, Labcritics, and UConn Today and was awarded an honorable mention in the 2015 Create the Future Design Contest. Stephanie is a member of the Biomedical Engineering Society, the Society of Women Engineers, and Phi Sigma Rho, a sorority for women in engineering.

Yongku Cho

Dr Yongku Cho is an assistant professor of Chemical and Biomolecular Engineering at the University of Connecticut. He obtained a B.S. degree from the Seoul National University, and a Ph.D. in Chemical Engineering from the University of Wisconsin-Madison. During his doctoral studies, he worked under Dr Eric Shusta to identify antibodies that target membrane receptors at the blood–brain barrier. He then conducted postdoctoral research with Dr Ed Boyden to engineer light-gated ion channel proteins for optical control of neural activity. His research group focuses on developing technologies to study gene function in the brain and other complex multicellular systems.

gain significantly from application of stem cells because differentiation protocols are well developed for neural cell types compared to others and because only a limited number of primary human neurons can be isolated directly from a patient.[9]

2.3 Replicating natural 3D cellular structure *in vitro*

It is well known that cells in the human body adhere strictly to a higher order of spatial distribution and often exhibit spatial polarity. This higher order characterizes tissues and organs and allows complex functions to take place – most notably, in the nervous system. Thus, replicating the 3D spatial distribution of different cell types and the relative orientation of individual cells is crucial to generating accurate models of these tissues. Complex biomimetic 3D structures cannot fully form in traditional 2D cell cultures, necessitating a shift of neural tissue engineering efforts into the third dimension in the interest of more accurately modeling the organization and structure of the tissues.[12]

Embryoid bodies formed from mouse embryonic stem cells (ESCs) differentiated into neural progenitors have been cultured on 3D fibrin scaffolds, revealing enhanced cell proliferation and differentiation compared to 2D culture (on top of the same scaffold).[13] It has been shown that in 3D microenvironments, neural stem cells (NSCs) migrate randomly, grow longer neurites, and retain their undifferentiated state more than in 2D cultures.[14]

An example of the importance of 3D culture in forming natural architecture is highlighted in a study aiming to regenerate brain cortical neurons.[15] In 2D *in vitro* cultures, these cells exhibit polarity and spatial patterns in the form of rosettes. But, in 3D, self-organization of cortical progenitors and neurons can take place to form cortical-like cytoarchitecture with time-dependent spatial patterning similar to that *in vivo* (Fig. 1). Other studies have observed rosette formation from human ESCs in 3D culture systems of degradable poly (α-hydroxy esters) as well as formation of 3D vascular structures in the presence of neurotrophins.[16]

Recent interest in creating organoid models borrows its approach from developmental biology in that it aims to reengineer the process of embryogenesis rather than simply engineering the end-stage tissue.[17] This approach calls for replication of the embryonic microenvironment and makes use of stem cells' unique, innate capability for self-organogenesis. The relatively recent advent of induced pluripotent stem cells (iPSCs) offers yet another viable source of stem cells for tissue engineering. Neural crest stem cells (NCSCs) have been derived from both ESCs and iPSCs and show great promise for cell therapies and neural tissue engineering.[18] NCSCs were shown to accelerate regeneration of sciatic nerves when seeded in nerve conduits, which served as a bridge for transected sciatic nerves, and to differentiate into Schwann

Xue-Jun Li

*Xue-Jun Li received her PhD in Neurobiology from Shanghai Medical College of Fudan University in 2000. She then worked as a lecturer at Fudan University for one and a half years. In 2002, she joined the Stem Cell Research Program at the University of Wisconsin-Madison, first as a postdoctoral associate (2002–2005) and then as an assistant scientist (2005–2007). In 2007, she joined the Neuroscience Department at the University of Connecticut Health Center as an assistant professor. She is also a PI of the University of Connecticut Stem Cell Institute. Dr Li published the seminal report on the specification of spinal motor neurons from hESCs in 2005 (*Nature Biotechnology, *2005). Her lab has recently successfully established human stem cell models for spinal muscular atrophy (*Cell Research, *2013;* Disease Models & Mechanisms, *2016) and hereditary spastic paraplegias (*Stem Cells, *2014;* Human Molecular Genetics, *2014). By combining cellular, molecular, bioengineering and system approaches, research in her lab focuses on specifying functional neuronal subtypes and modeling motor neuron diseases with the ultimate goal to develop therapeutics for the treatment of these debilitating diseases.*

Ali Khademhosseini

Prof. Ali Khademhosseini is a Professor of Medicine and Health Sciences and Technology at Harvard-MIT's Division of Health Sciences and Technology and the Harvard Medical School. His research is based on developing micro and nano-scale technologies to control cellular behavior with particular emphasis in developing microscale biomaterials and engineering systems for tissue engineering and drug delivery. He is an author on ~450 journal articles, ~60 book chapters/editorials, over 250 abstracts, and 20 patent/disclosure applications. He has been cited >22 500 times and has an H-index of >79. Dr Khademhosseini's interdisciplinary research has been recognized by over 30 major national and international awards, including the TR35 by the Technology Review Magazine and the Presidential Early Career Award for Scientists and Engineers. He is also a fellow of the American Institute of Medical and Biological Engineering (AIMBE), Royal Society of Chemistry (RSC), and Society and American Association for the Advancement of Science (AAAS). He received his PhD in bioengineering from MIT, and MASc and BASc degrees from University of Toronto, both in chemical engineering.

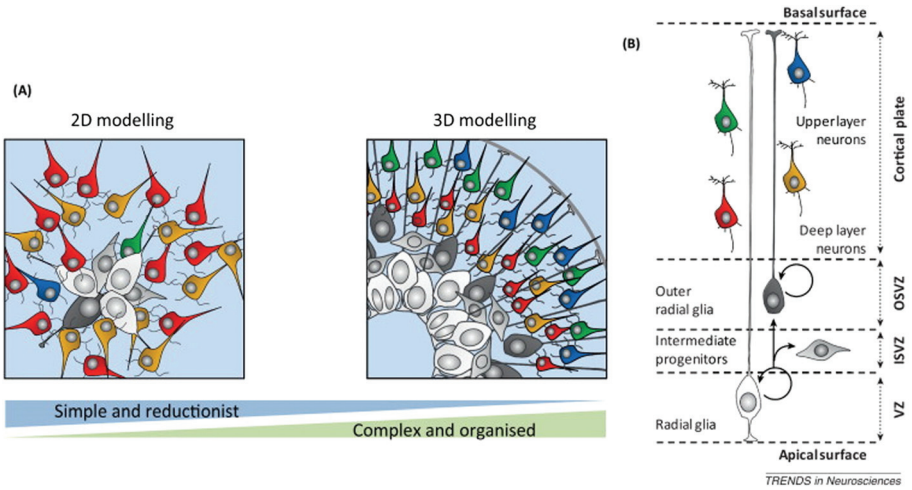


Fig. 1 Spatial patterning of cells in 2D and 3D. (A) Comparison of 2D and 3D models for neural tissue, demonstrating key aspects of corticogenesis in 2D, but more biomimetic spatial patterning and cytoarchitecture formation within 3D cultures. (B) Representation of the 3D spatial patterning of different cell types *in vivo*. SV: subventricular zone; ISVZ: inner subventricular zone; OSVZ: outer subventricular zone. Reprinted from *Trends in Neurosciences*, 37/6, J. van den Ameele, L. Tiberi, P. Vanderhaeghen, I. Espuny-Camacho, Thinking out of the dish: what to learn about cortical development using pluripotent stem cells, 334–332 2014, with permission from Elsevier.[51]

cells (SCs) and help form the myelin sheath around nerve axons.[18]

Using 3D scaffolds also provides opportunities to temporally control the tissue regeneration processes. The degradation rate of the scaffold may be tuned to temporally control neural regeneration. One study tuned the degradation rate of polyethylene glycol (PEG) hydrogels to control the rate at which embryonic neural cells extend processes in 3D culture, giving control over the time scale of neural process extension between 1 and 3 weeks.[19]

Savas Tasoglu

Dr Savas Tasoglu is an Assistant Professor in the Department of Mechanical Engineering at the University of Connecticut. He received his Ph.D. in 2011 from UC Berkeley, and held a post-doctoral appointment at Harvard Medical School and Harvard-MIT Division of Health Sciences and Technology until he joined UConn in 2014. Dr Tasoglu's achievements in research and teaching have been recognized by fellowships and awards including Chang-Lin Tien Fellowship in Mechanical Engineering, Allen D. Wilson Memorial Scholarship, UC Berkeley Institute Fellowship for Preparing Future Faculty, and AHA Scientist Development Award. Dr Tasoglu has published 30+ articles in journals such as: Nature Communications, Nature Materials, Advanced Materials, PNAS, Small, ACS Nano, Chemical Society Reviews, Trends in Biotechnology, Scientific Reports, Physics of Fluids, and Journal of Computational Neuroscience. His work has been featured as the cover of Advanced Materials, Small, Trends in Biotechnology, and Physics of Fluids and highlighted in Nature, Nature Physics, Nature Medicine, Boston Globe, Reuters Health, and Boston Magazine.

2.4 3D approaches enable recreation of tissue function

Cells are intimately connected to surrounding cells through intercellular signaling and closely regulated by interactions with the extracellular matrix (ECM). The great advantage of the recent shift from traditional 2D monolayer culture to biomimetic 3D microenvironments is that such conditions facilitate these complex interactions more so than 2D cultures. According to the organizing principle proposed by Bissell and colleagues, cell–cell and cell–ECM signaling defines a tissue's specificity and drives homeostatic regulation.[20] These interactions with the surrounding microenvironment are known to influence progression through a cell's life cycle, including proliferation, migration and apoptosis.[12,20] Cell–cell and cell–ECM interactions are also crucial for proper tissue formation in the developmental stages.[4] This necessitates use of 3D culture methods to allow accurate replication of the developmental process for studying the progression of nervous system development as well as proper adult structures.

The effects of 3D culture compared to 2D are evidenced by a recent study where differentiated human NSCs were cultured in an inert 3D scaffold (Alvetex scaffold circles) and compared to those cultured on a comparable 2D surface.[21] The NSCs in the 3D scaffold formed spontaneously functional 3D networks (unlike those in 2D conditions). The study also noted a significant difference in gene expression between the two conditions, specifically in genes coding for neuronal function, ECM, and cytoskeleton. In another scaffold, PuraMatrix™, human ESC-

derived neural cells grew into more branched structures when encapsulated in the matrix than when grown on top of the same material, even forming spontaneously functional 3D networks.[22]

3D culture conditions are of particular value in neural tissue models because they facilitate the formation of 3D neural networks characteristic of the cytoarchitecture found in cortical tissue. 2D studies fail to replicate the extension of neural processes in 3D. 3D scaffolds, however, have been shown to facilitate improved development of functional network connectivity and neural synapses.[23,24] In order to model network dynamics in the 3D space (compared to previous studies of 2D neural networks grown on rigid substrates), a recent study used 3D networks coupled to microelectrode-arrays to demonstrate the difference between 2D and 3D network dynamics.[25] Compared to 2D networks, 3D neural networks showed morphology, connectivity, and ECM more closely mimetic of the *in vivo* situation, spatial segregation of signals, and greater complexity. The 3D networks also exhibited a time delay between an external electrical stimulation and propagation of that signal to lower layers to evoke an electrical response signal. Observed variability in the time delay between stimulation and response in the presence of synaptic blockers demonstrates synaptic reverberation and amplification through the layers (as opposed to low synaptic connectivity or density), verifying that the 3D system generated functionally interconnected neuronal networks.

2.5 Effect of 3D environment on stem cell differentiation

A consideration which is specific to the use of stem cells is the influence of the microenvironment on differentiation. Thus, *in vitro* 3D scaffolds which contain these cells must be designed to mimic the *in vivo* conditions in order to regulate stem cell behavior as it is in natural tissues. It is known that in 2D cultures, substrate stiffness greatly influences neural stem cell differentiation.[26–28] Studies suggest that mechanical cues are sensed by the actin cytoskeleton and transduced by the RhoA pathway to influence differentiation.[29] Thus, in order to take full advantage of the range of cell types which a stem cell is capable of generating, it is necessary to pursue a range of 3D scaffolds with various mechanical properties which favor differentiation of these cell types.

One culture system used collagen to maintain high viability, allow migration, and induce differentiation into neurons, astrocytes, and oligodendrocytes by optimizing the concentration of the collagen peptide.[30] Compared to previous results regarding the effect of 2D substrate stiffness on stem cell behavior, similar results have been replicated with mesenchymal stem cells (MSCs) in 3D thixotropic gels.[31] A study of NSCs encapsulated in alginate hydrogels revealed that a low hydrogel modulus (around 183 Pa, which is similar to that in brain tissues) promoted greater proliferation as well as neuronal differentiation than stiffer hydrogels.[32] A controlled study used neural progenitor cells (NPCs) encapsulated in hyaluronic acid to characterize the effect of mechanical properties on differentiation.[33] The study reported that NPCs cultured in low-modulus scaffolds (1.5 kPa, similar to that measured in neonatal rat brains) differentiated into a neuronal lineage. In contrast, NPCs in scaffolds of greater stiffness, similar that of the adult brain, differentiated mostly into astrocytes. Another study used alginate to expand stem cell lines while retaining pluripotency and observed differentiation under a range of culture conditions.[34]

3. Applied 3D fabrication techniques

There exists an incredible amount of technology established for fabricating such 3D scaffolds for tissue engineering applications.[35–49] This work has strengthened our understanding of the effect of fabrication techniques and culture conditions on stem cell behavior, particularly for neural cell differentiation. One popular application of the 3D techniques described herein is to develop culture systems *in vitro*, generally for the purpose of controlling stem cell differentiation and organization and ultimately functional tissue formation. Another application which is gaining popularity is the nerve conduit to facilitate regeneration of peripheral nerves; this application will be discussed in detail in section 4.[50] Here, we review 3D fabrication techniques which have shown promise in using stem cells for neural tissue engineering.

3.1. Vascularization

Despite the recent successes reported in 3D neural tissue engineering, there remains a challenge associated with the shift from traditional 2D to 3D for long-term culture. Cells cultured in 3D scaffolds do not have immediate access to the oxygen and nutrients needed to survive, nor are they able to successfully expel wastes and CO_2 as in traditional 2D monolayer cell cultures. Thus, large 3D tissues cannot grow past a certain thickness in traditional culture conditions, making it impossible to replicate and study the later stages of development in vitro.[51] One study using adipose-derived stem cells (ASCs) transplanted into a nerve conduit were not viable in significant quantities after 14 days, limiting the success of the otherwise promising study, which indicated improved axonal growth and SC proliferation in the first few days.[52]

This problem has been addressed in one study, which achieved long-term viability in 3D culture beyond 13 weeks by optimizing the culture of ESC-derived cortical neuroepithelium cells to improve oxygen delivery throughout the culture.[15] This approach involved formation of aggregates with a transforming growth factor beta (TGFβ) inhibitor and a Wnt inhibitor for 18 days to cause telencephalic differentiation. This was followed by suspension culture under 40% O_2 conditions in the presence of a chemically defined lipid concentrate. This protocol also included addition of fetal bovine serum, heparin, and a low concentration of Matrigel (1%) on Day 35, use of a high O_2 penetration dish after Day 56, and an increase in Matrigel concentration to 2% with addition of a B27 supplement on Day 70 to achieve long-term maintenance of ventricular zone progenitor cells. Another study[53] used a similar but much simplified system[54] in which the aggregates were attached and cultured in Neurobasal medium sup-

plemented with N2, B27 and L-glutamine under normal culture conditions. Cultures were examined at day 50 and 70 after differentiation; results revealed the formation of synapses in these long-term cultures, suggesting that some cells could mature under basic 3D aggregation cultures.[53] In another approach, methacrylamide chitosan (MAC) has been conjugated with perfluorocarbons (PFCs), which take up and release oxygen at optimal levels to facilitate proliferation and enhanced neuronal differentiation of neural stem/progenitor cells (NSPCs).[55]

A more recent study which is discussed in more detail in section 5.2 revealed the formation of cerebral organoids in cultures using a spinning bioreactor to improve the absorption of nutrients in the 3D aggregation system.[56]

Future work in this area, such as strategies for introducing vasculature, will help improve long-term viability in 3D tissues.[57,58] These strategies involve facilitating vasculogenesis and angiogenesis, or the formation of new blood vessels, from existing vessels or endothelial cells by using optimized scaffolds, patterning cells, and delivering growth factors and signaling molecules.[59,60] Other novel techniques for vascular tissue engineering include assembly of microscale cell-laden hydrogel constructs,[61] use of microfluidic platforms,[62] electrospinning fibrous meshes,[63] as well as scaffold-based techniques to either pattern vessels into synthetic scaffolds (prevascularization) or take advantage of existing architecture in natural scaffolds.[64] A recurring theme in many of the studies discussed in this section is an effort to encourage mass transport for oxygen and nutrient delivery in the interest of achieving long-term viability in 3D cultures.

3.2. Porous scaffolds

Porous scaffolds offer a significant advantage for 3D tissue engineering because they create a higher surface area on which cells can attach and grow. Interconnected porous structures allow for mass transport of nutrients, waste, and biofactors and also facilitate cell infiltration and migration. Pore size is also a factor to consider: several studies have reported that a smaller pore size leads to greater differentiation while larger pore sizes favor cell proliferation.[65] One hypothesis is that the cells reach confluency earlier in scaffolds with smaller pores, and thus lower surface area, which inhibits proliferation and initiates differentiation.[65]

Human bone marrow MSCs were shown to differentiate more effectively in 3D terpolyesters of 3-hydroxyalkanoates scaffolds than in 2D; pore sizes in the range of 30–60 μm were tested and smaller pores were found to increase differentiation.[66] 3D macroporous cellulosic hydrogels have been used to promote human MSC growth, with a 14-fold demonstrated increase in cell proliferation over a period of 7 days.[67] The same study demonstrated that nearly all of the MSCs differentiated into neurons and glial cells on this scaffold after 14 days induction, as supported by PCR.

To increase pore size in photo-crosslinkable MAC scaffolds, D-mannitol crystals have been used as a porogen, a material admixed into a hydrogel and subsequently dissolved from the cured hydrogel to leave pores in the scaffold.[68] Using 20% D-mannitol crystals increased the pore size over 2-fold (attaining 7600 ± 1550 μm^2 pores) compared to control scaffolds (which had 3150 ± 220 μm^2 pores), increasing the oxygen diffusion in the scaffold. NSPCs were cultured on this scaffold and found to differentiate to neural cell types, with the most significant differentiation occurring on the more porous (20% D-mannitol) scaffolds.

In the cryogelation method, a polymer or monomer is frozen such that ice crystals are formed and act as a porogen.[69] After complete gelation, the cryogel is thawed at room temperature to melt the ice crystals and washed to remove unpolymerized monomers, leaving an interconnected pore structure. This technique has been applied to neural tissue engineering by using dextran or gelatin scaffolds linked to laminin with an optimal pore size of 80–100 μm, providing mechanical support to developing nerve tissue with 3D complexity. The scaffold allowed infiltration of seeded cells and delivery of morphogens to cause stem cell differentiation and served as a platform for development of 3D neural-like tissues.[70] Stem cells from human umbilical cord blood were seeded onto the scaffold and exposed to medium for neural commitment for 7–10 days followed by neural differentiation medium for 7–14 days, resulting in differentiated neuron-like and glial lineage cells. When implanted into rat brain tissue, the facilitated infiltration of host neuroblasts into the scaffolds.

Another method of pore generation is freeze-drying in which a scaffold is dissolved and dispersed in a solvent, which is then frozen and sublimated under a vacuum to remove the solvent and leave behind a porous scaffold. This method gives control over pore size by controlling the pH and freezing rate. This method has been applied to chitosan/gelatin scaffolds to observe the effect of pore size on NSPCs.[71] Chitosan/gelatin scaffolds which were hybridized with hyaluronic acid and heparan sulfate were shown to have a 96% porosity with 90–140 μm interconnected pores. Addition of hyaluronic acid and heparan sulfate to the scaffold improved cell adhesion, facilitated long-term growth, and enhanced neuronal differentiation compared to control chitosan/gelatin scaffolds. Another porous chitosan scaffold, also fabricated using the freeze-drying technique, exhibited a similar highly porous, interconnected structure.[72] The scaffold was found to facilitate growth and survival of dental pulp stem cells and enhance differentiation into nerve cells when exposed to differentiation media.

In thermally induced phase separation, a polymer is dissolved in a solvent and the sample is cooled to decrease the solubility of the polymer and induce phase separation into a polymer-rich and a polymer-poor phase. The sample is then freeze-dried to cause the solvent to sublime, forming a porous polymer scaffold. A highly porous poly (D,L-lactic acid) scaffold, formed *via* thermally induced phase separation, has been used to culture mouse ESCs and was shown to enhance differentiation into neural cells compared to in 2D cultures .[73]

Conductive scaffolds present a promising trend, as electrical stimulation has been shown to affect the migration, differentiation, and proliferation of NSCs.[74,75] A 3D porous

graphene foam was developed as a conductive scaffold which promoted differentiation into predominantly neurons as well as astrocytes and maintained the active proliferation state (as measured by upregulation of Ki67) compared to 2D graphene films.[76] The conductive properties of the graphene were capable of stimulating the cells *via* capacitive charge injection with voltage in the range of −0.2 to +0.8 V.

3.3. Fibrous scaffolds

Fibrous scaffolds show promise for neural tissue engineering because, based on 2D tests, they have the ability to orient and align neurites and encourage directed growth.[77] 3D nanofibrillar scaffolds, which closely mimic the geometry and porosity of natural basement membranes, have been shown to enhance proliferation and self-renewal of mouse ESCs.[78] Nanofibers may be formed through self-assembly, which occurs spontaneously without external guidance; in some cases, this material may be injected into a tissue cavity and will form a 3D hydrogel under physiological conditions.

A range of functional motifs were conjugated to a self-assembling peptide, RADA16, to generate "designer" peptide nanofiber scaffolds and observe their effect on mouse NSCs.[79] Functionalization with certain bone marrow homing motifs was shown to enhance survival and proliferation and to promote neuronal differentiation. Later, RADA16 self-assembling peptides were optimized for NSC culture at a concentration of 0.5%, which has an optimal stiffness for NSC culture of 10–1000 Pa.[80,81] RADA16 functionalized with different functional motifs: bone marrow homing motifs was shown to promote NSC proliferation while RGD was optimal for NSC differentiation, resulting in neurons, astrocytes, and oligodendrocytes after 5 days of culture on the scaffold. The motifs used can be easily varied by dissolving the desired peptides in aqueous solution followed by self-assembly into nanofiber scaffolds; different scaffolds can elicit a desired response from stem cells within this scaffold. Another study functionalized RADA16 with an IKVAV motif, which offered guidance to direct adhesion of encapsulated NSCs as well as neuronal differentiation.[82] When injected into rat brain defects, the hydrogel supported survival and differentiation of the encapsulated NSCs. The NSCs also reduced glial astrocyte formation, which can be a barrier to neural regeneration.

Nanofibrillar scaffolds may be formed using a process called electrospinning, in which a liquid material, often a polymer solution, is electrically charged, causing a charged jet of solution to eject toward a receiving substrate. As the solvent evaporates, nanofibers are deposited on the substrate in a non-woven mat with controllable fiber diameter and porosity.[83] Nanofiber diameter plays an important role in stem cell behavior. Rat NSPCs demonstrated a 20% increase in neuronal differentiation when cultured on 749 nm laminin-coated electrospun Polyethersulfone fibers compared to cultures on standard tissue culture polystyrene.[84]

Another technique is microfluidic spinning, in which a microfluidic chip is used to engineer continuous fibers with tunable control over complex morphological, structural, and chemical features.[85] By digitally coding microfluidic flow rates of different components, hydrogel microfibers were generated with varying chemical composition, spindle knots, gas bubbles, and tapering with grooved surfaces. Rat embryonic neurons were found to grow and extend along the axis of smooth and grooved fibers. A significant improvement in neuronal alignment and extension was seen on nano-grooved fibers compared to smooth fibers.

3.4. Decellularized scaffolds

Decellularized scaffolds, or scaffolds derived from removing the cellular components from tissue samples using detergents, retain the natural ECM proteins and biomolecules as well as their 3D architecture. This offers a notable advantage over the natural and synthetic scaffolds described previously, where these features need to be artificially reproduced through various fabrication and culture techniques, but also introduces complications with biocompatibility and sample-to-sample variability. Proliferation (as reported by proliferation marker Ki67) was enhanced in 3D scaffolds compared to 2D. Decellularized mouse brain sections (1.5 mm thick) were shown to be conducive to murine NSC survival and growth for up to 7 weeks and formation of a 3D maze-like interconnected cellular structure.[86] Cells were observed to retain their stem cell properties when stimulated with mitogens, epidermal growth factor (EGF), and human FGF-2 to trigger cell division.

3.5. Immobilization of growth factors

Use of biomolecules and growth factors is a well-established approach to deliver signals to cells either *in vitro*, through addition to the culture medium, or *in vivo*, through any one of many proposed drug delivery strategies. Immobilization of such growth factors within the 3D scaffold allows the molecules to be delivered more specifically to the targeted cells and remain localized over time to continue delivering the biomolecular signal. In one study, brain-derived neurotrophic factor (BDNF) was immobilized on electrospun poly-ε-caprolactone nanofiber scaffolds.[87] Results of this study indicate that NSC proliferation and differentiation into neurons and oligodendrocytes was enhanced by BDNF when it was immobilized onto the scaffold compared to when it was dissolved in the culture media. Another study used a layer-by-layer self-assembly method to pattern BDNF into heparin/poly-L-lysine (PLL) polyelectrolyte multilayers electrospun poly-ε-caprolatone nanofibers.[88] This layer-by-layer spatial distribution of the growth factor resulted in higher cell number and longer neurite outgrowth of neural progenitor cells compared to scaffolds without BDNF and scaffolds where BDNF was physically adsorbed.

Another study immobilized biotin-rat interferon-γ (IFN-γ), a cytokine shown to influence differentiation into neurons, on photocrosslinkable MAC modified with streptavidin *via* biotin-streptavidin binding.[89] Neuronal differentiation was enhanced in the presence of IFN-γ both immobilized on the scaffold and dissolved in the medium compared to control scaffolds without the growth factor. Further, growth factor-immobilized

scaffolds were shown to give rise to a more neuronal-committed cell population as evidenced by elevated expression of βIII tubulin and nestin compared to culture with soluble growth factor (where cells expressed βIII tubulin and nestin as well as an oligodendrocyte marker, RIP) and control scaffolds (where little differentiation was observed).

An extension of this work assessed differentiation *in vivo* using IFN-γ in addition to platelet derived growth factor-AA (PDGF-AA) and bone morphogenic protein-2 (BMP-2) to signal differentiation into neurons, oligodendrocytes, and astrocytes, respectively.[90] The scaffolds were implanted subcutaneously in mice and after 28 days, growth-factor immobilized scaffolds induced greater differentiation than adsorbed and control scaffolds. Further, cells in IFN-γ-immobilized scaffolds formed neural rosette-like arrangements and neural tube-like structures.

Proteins and growth factors may also be patterned in 3D scaffolds to direct cell attachment, growth, and differentiation with spatial control. One such method used a photoactive agarose hydrogel and photochemically patterned fibroblast growth factor-2 (FGF-2) *via* either disulfide bonding of cysteine groups in FGF-2 to photoexposed agarose thiols or human serum albumin bound to these photoexposed thiols followed by stable conjugation of FGF-2 to the albumin binding domain of the protein.[91] This method can be applied to a broad range of proteins and growth factors which express this albumin binding domain. This method and others which enable spatial patterning of proteins have potential to give rise to powerful neural tissue engineering strategies for producing neural tissues with spatial complexity.

3.6. 3D bioprinting

3D bioprinting techniques for neural tissue engineering are particularly promising because this method provides ultimate control over the 3D architecture and spatial arrangement of cells. Direct cell printing is one method which has been used to pattern rat embryonic neural cells in collagen hydrogel.[92] In this technique, a collagen precursor was printed in a layer, then embryonic neurons and astrocytes are printed in the same manner, and the process was repeated to construct a 3D construct in a layer-by-layer manner. Another study printed murine NSCs in collagen and vascular endothelial growth factor (VEGF)-releasing fibrin hydrogels with high viability (92.89+/−2.32%).[93] The results showed that cells printed within 1 mm of the VEGF-releasing areas of the construct migrated toward those areas over a distance of 100 μm in 3 days in contrast to control gels with no VEGF, in which cells did not proliferate or migrate. This indicates sustained release of growth factor from the fibrin gel over the 3-day period.

3.7. Magnetic levitation

Magnetic levitation is a unique approach to attaining a desired spatial arrangement of cells for 3D tissue engineering. In this method, cells were suspended in a hydrogel with bacteriophage, magnetic iron oxide, and gold nanoparticles.[94] Murine NSCs were mixed into the hydrogel in a media solution, causing the components to either enter the cell or bind to the membrane. When an external magnetic field was applied, the cells and hydrogel levitated to the air-medium interface. After incubation for 12 hours, the cells were shown to form multicellular assemblies showing characteristic branching morphogenesis, a developmental process implicated in tissue formation.[95]

3.8 Microfluidic flow

Microfluidic approaches offer precise and repeatable spatiotemporal control over the cell culture microenvironment, which is critical to understanding and controlling neural stem cell behavior.[96] One microfluidic device was designed to apply fluid flow to align 3D encapsulated mouse NSCs cultured in the device.[97] Consistent fluid flow was applied as Matrigel was undergoing gelation within the microfluidic channel, producing an ECM with 70% of the structures aligned. This enabled growth of primary rat cortical neurons and mouse NSCs with a high degree of orientation in the neuronal processes. Other useful applications of microfluidic devices to stem cells include maintenance of steady-state culture conditions over time as well as generation of gradients of growth factors as they are delivered to the cells.[96] Considering the rapid advancements in biological micro-electro-mechanical systems (BioMEMS) and the broad range of capabilities being developed, it is likely that this approach, combined with further application of stem cells, will serve as a platform for future innovation in the field of 3D neural tissue engineering.

4. Nerve conduits for *in vivo* nerve regeneration

Since there are limited nerve regenerative therapies available clinically to treat lesions and injuries to peripheral nerves, nerve guidance conduits (tubes) present a potential treatment option to bridge large gaps between severed nerve ends, facilitating peripheral nerve regeneration while preventing infiltration by surrounding cell types.[50] These conduits provide a cylindrical tubular vessel in which these 3D scaffolds and fabrication strategies developed *in vitro* (described in the previous section) can be patterned to affect neurite regeneration and extension through the defect and ultimately reconnection of the severed neurite ends *in vivo*. Recent research in the interest of improving the effectiveness of this therapy has made use of stem cells seeded within the tube as well as several of the various 3D fabrication techniques described previously (Fig. 2). Future work taking advantage of these two powerful approaches will be able to bridge nerve defects more quickly and restore more sensory and motor function than possible with current therapies.

4.1. Stem cells seeded in nerve conduits

Bone marrow stem cells (BMSCs) are widely used for regenerative therapies because they are easily harvested from the patient, reduce the risk of rejection, and are able to differentiate into multiple cell lineages. BMSCs may be seeded in

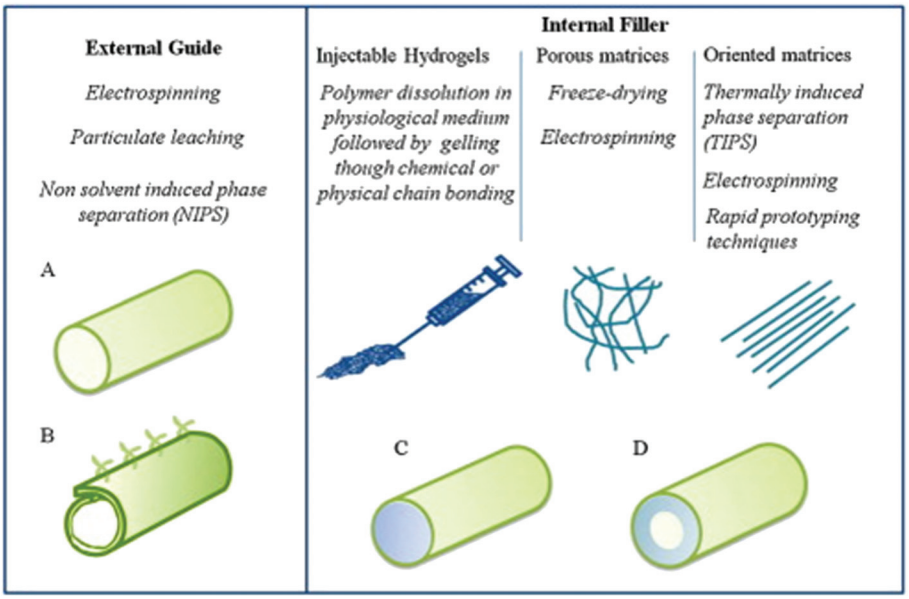


Fig. 2 Engineering strategies for nerve guidance conduits. External guides for nerve guidance conduits are fabricated into (A) hollow tubes or (B) flat membranes which are rolled into tubes and secured. Internal fillers for nerve guidance conduits include injectable hydrogels, porous matrices, and oriented matrices used to (C) fill the tube cavity or (D) create two or more layers of different fillers. Reprinted from *Progress in Neurobiology*, Chiono and Tonda-Turo, Trends in the design of nerve guidance channels in peripheral nerve tissue engineering, 2015, with permission from Elsevier.[127]

nerve guidance conduits to enhance regeneration and, in one study, were found to survive and maintain their phenotype for 6 weeks when seeded in polyglycolic acid tubes and implanted *in vivo* for facial nerve regeneration in a rat model.[98] BMSCs seeded in chitosan nerve guidance tubes have been shown to survive and proliferate on the scaffolds for 8–16 weeks *in vivo* in a rat model.[99] This study concluded that BMSCs enhance peripheral nerve regeneration across an 8 mm gap. A later study by the same group found that nerve functionality (as quantified by the sciatic nerve function index in rat models) and the number and size of fibers in the defect were improved in chitosan tubes containing BMSCs, approaching the regenerative success observed in rats which received autografts[100] (which serve as the current gold standard for peripheral nerve regeneration). Addition of a laminin coating to the chitosan tubes improved BMSC adhesion, thus enhancing the ability of the cells to suppress the inflammation and fibrotic response to chitosan over long-term *in vivo* transplantation in rats, ultimately facilitating peripheral nerve regeneration over a 10 mm defect.[101]

BMSCs have also been seeded in polycaprolactone nerve guidance conduits. MSCs in these conduits were shown to prevent decrease in denervation in muscle when implanted into mice *in vivo* as evidenced by relatively high levels of creatine phosphokinase in MSC-treated conduits compared to conduits without cells.[102] These results indicate that the cells help to maintain tissue activity, likely through the release of various trophic factors, ultimately improving functional recovery in mice. A later study by the same group revealed a significant increase in myelinated fibers when BMSCs were seeded in polycaprolactone conduits and implanted into mice,[103] consistent with the findings for BMSC-seeded chitosan conduits.

Bone marrow-derived cells (BMDCs) also show promise for facilitating nerve regeneration when seeded inside nerve guidance tubes. One study seeded resorbable collagen guidance tubes with BMDCs and found that motor function of mice which received sciatic nerve grafts with BMDC-seeded tubes recovered motor function faster than mice which received guidance tubes without cells.[104] The number of myelinated fibers and the total myelinated area was found to be higher in the group which received BMDC-seeded grafts. Another study found that when BMDCs were co-seeded with SCs in a collagen nerve guidance tube implanted *in vivo* in mice, the BMDCs transdifferentiated into SCs.[105] Consistent with the previous study, the BMDCs were found to enhance sciatic nerve regeneration.

ASCs have also been used as a stem cell source. One study found that autologous ASCs improved axon growth, nerve reinnervation, and functional recovery when seeded in a decellularized artery conduit compared to use of the artery conduit alone when implanted in an 8 mm facial nerve defect in a rat model.[106] Another recent study tested different adult stem cell types, including ASCs and dental pulp stem cells (DPSCs), co-cultured with SCs within microporous nerve conduits.[107] *In vitro* studies revealed synergistic nerve growth factor (NGF) production in co-cultured conduits with ASCs and SCs. When implanted *in vivo* in rat models to bridge a 15 mm sciatic nerve defect, conduits with ASCs and SCs resulted in the greatest functional recovery. Further, conduits seeded with DPSCs and SCs promoted better functional recovery than those

seeded with DPSCs alone, highlighting the positive effects of co-culturing multiple cell types.

Another recent approach to improve peripheral nerve regeneration is to harvest donor MSCs and differentiate them into neural cells prior to seeding into nerve conduits. This approach may be used to obtain an alternative source for SCs, which are known to play an important role in nerve regeneration, as it is difficult to obtain large quantities of cells due to donor site morbidity and low proliferation rate.[108] SC-differentiated MSCs have been seeded in a decellularized human vein/muscle conduit and implanted *in vivo* in mouse models.[108] In this study, MSCs were differentiated into SC-like cells. *In vivo* results indicate that the muscle-stuffed vein was biocompatible and biodegradable, fully degrading after 8 weeks post-implantation. The scaffold was found to be completely replaced by the differentiated MSCs seeded on it after these 8 weeks. A later study by these authors seeded neural-differentiated MSCs into a collagen-coated polylactic-glycolic acid conduit.[109] This conduit was also shown to facilitate cell attachment, survival, and proliferation of cells when implanted sub-muscularly in mice. ASCs have also been used with this approach. ASCs, after being differentiated into SC-like cells and seeded in an allogenic artery conduit, were shown to enhance facial nerve regeneration in rat models better than undifferentiated cells and nearly as well as SCs.[110] ASCs have also been differentiated into neurosphere-like cells on a chitosan-coated plate prior to seeding into a chitosan-coated silicone tube.[111] This approach also resulted in an improvement in the number of myelinated axons formed in the tube as well as myelin thickness and functional recovery when used *in vivo* in rat models to bridge a 10 mm sciatic nerve defect.

Stem cells may also be transfected to induce production of certain neurotrophic factors known to enhance peripheral nerve regeneration. One study used this method to transfect NSCs with BDNF or glial cell line-derived neurotrophic factor (GDNF).[112] When implanted *in vivo* into a sciatic nerve defect in rat models, GDNF-transfected NSCs improved the degree of myelination, regenerated tissue mass, number of blood vessels in the defect area, as well as functional recovery compared to the same non-transfected cells.

4.2. 3D scaffolds for nerve conduits

3D scaffolds prove particularly useful for encouraging alignment and directional growth of neurites within a nerve conduit in order to bridge the defect more quickly. One study used fabricated poly (D,L-lactide) conduits with microlithography to etch 3 μm grooves in a silicon wafer, soft lithography to create polydimethylsiloxane (PDMS) submasters, and solvent casting to create micropatterned grooves.[113] NSCs were seeded on the scaffold and 84.7% of NSCs were aligned within 72 hours of seeding. Alignment of NSCs resulted in upregulation of NGF and BDNF expression on the micropatterned conduits, which is possibly regulated by the cytoskeletal rearrangement associated with alignment. This altered gene expression may have contributed to nerve regeneration, resulting in functional recovery of a 10 mm sciatic nerve gap in 6 weeks. Another study also seeded NSCs on a micropatterned aligned scaffold formed from two layers.[114] The outer layer was a porous micropatterned film fabricated by casting a co-polymer with PEG as a porogen on an etched silicon wafer with square grooves and perpendicular walls. The inner layer was an aligned fibrous mat fabricated by electrospinning of a co-polymer. NSCs were seeded on the films and mats and allowed to proliferate, then the tubular constructs were formed by rolling the micropatterned film around the aligned fibrous mat. NSCs were shown to align and elongate in the direction of fiber alignment, wrapping around the fibers in bundles, in contrast to unoriented clusters formed on randomly oriented fibers. Astrocytes were also seeded and observed to align in the same orientation while supporting NSC alignment.

Another approach to obtaining 3D scaffolds for nerve conduits is deriving decellularized scaffolds from harvested tissues. An allogenic artery was used in two studies, obtained by treating the artery tissue with trypsin-EDTA and Triton X-100 detergent, then crosslinking with ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC) to reduce degradation rate.[106,110] The decellularized allogeneic artery conduits were implanted with ASCs which were either undifferentiated[106] or differentiated into neural-like cells.[110] These conduits supported attachment and proliferation of seeded cells, degraded at a moderate rate, and did not cause an inflammatory response. Another study used human vein and muscle tissue decellularized *via* liquid nitrogen immersion and hydrolysis with hydrochloric acid.[108] The acellular tissue provided a stable living nerve conduit with high biocompatibility and biodegradability over 8 weeks when implanted *in vivo*.

Bioactive conduits may also be produced by conjugating biomolecules to the scaffold. For example, one group immobilized acidic fibroblast growth factor 1 (FGF-1) on microporous/micropatterned poly (D,L-lactic acid) conduits *via* open air plasma treatment.[115] Conduits were grafted with chitosan, which has antimicrobial qualities but is mechanically weak, and gold nanoparticles (nano Au), which served to reinforce the chitosan scaffold. This nanocomposite provided an ECM-like environment to support nerve regeneration while also promoting sustained release of the FGF-1, which has been shown to promote growth and have neuroprotective properties. Immobilization of FGF-1 using this approach preserved the bioactivity of the molecule compared to tubes without the nanocomposite, ultimately improving regeneration and functional recovery when seeded with NSCs and implanted in a 15 mm defect.

5. Recent progress in functional 3D tissue formation using stem cells

5.1. Modeling human cortical tissue development, structure, and functionality

Combining multiple cell and 3D scaffolding techniques has proven to be effective in modeling human cortical tissue. One approach uses a 3D culture system to screen potential cells for

their therapeutic effects for chronic neurological injury and disease.[116–118] The 3D culture system proposed was shown to maintain astrocytes in a state which more closely mimics their behavior *in vivo* than 2D cultures.[116,117] Astrocytes we co-cultured with a range of stem cell types to examine astrocyte reactivity, which is associated with glial scarring and is known to limit neural regeneration.[118] Of three stem cell therapies evaluated using the platform developed here, NCSCs from hair follicles, differentiated SC-like ASCs, and bone marrow MSCs, only the bone marrow MSCs caused significant astrocyte activation.

Another approach to engineering human cortical tissue is to replicate the developmental process over time *in vitro.* Development occurs "inside out;" that is, neurons formed in the proliferative zone migrate radially outward to form layer 6 first, then continuing in this manner until layer 2 is formed (with layer 1 originating from the cortical plate and having very few neurons). The cortex is ultimately composed of a 2–3 mm thick layer of gray matter with a folded, convoluted structure and neurons comprising excitatory (pyramidal) and inhibitory neurons. Neurons form a highly organized and interconnected network, with the layered structure reflecting on the radial organization to facilitate input and output signaling, a theme common across all of the diverse areas within the cortex.[51,119]

One study replicated the developmental process of the telencephalon by culturing human iPSCs in suspension with rostral neutralizing factors.[54] Though previous studies had shown differentiation into anterior forebrain-like tissue, this study aimed to mimic the transcriptional activity which gives rise to the telencephalon. Results showed that the transcriptional activity of these cells during differentiation closely mimics that in the early stages of dorsal pallium development in humans and the approach produced a self-organized multilayered 3D structure in 8–10 weeks *in vitro*. Further presence of synaptic vesicles, neurotransmitter receptors, transporters, and astroglial differentiation suggested early synapse formation.

A later study also observed self-organization of cortical-like higher order 3D structures formed from human ESCs cultured in a 3D suspension culture containing Matrigel.[15] The structure exhibited axial polarity and inside-out layering reminiscent of cortical development (Fig. 3). Outer radial glia (oRG)-like progenitors were developed on day 91 of culture (13 weeks, or the equivalent of the second trimester), implying that the development rate was similar to that of a fetus.

5.2. Application to disease modeling

A recent study has developed cerebral organoids from human stem cells in a 3D culture system developed using Matrigel, growth factors to improve growth conditions, and a spinning bioreactor which enhances absorption of nutrients.[56] These organoids were used to model microcephaly, a neurological disease caused by abnormal brain development which is associated with a significantly smaller head than normal (hypoplasia).[120] iPSCs were derived from skin fibroblasts from a patient with microcephaly and used to form organoids.[56] In the early stages of development (day 22), the area of neuroepithelial regions was smaller, with an increased number of differentiated neurons compared to controls derived from "normal" iPS cells (obtained commercially), indicative of premature neural differentiation in microcephaly patients. Compared to the control organoids, the radial glia from the microcephaly patient-derived stem cells were fewer in number and showed little horizontal orientation. The horizontal orientation of the spindle as observed in control organoids is crucial for normal symmetric expansion of NSCs during development, so the lack of horizontal orientation in microcephaly samples gives insight regarding the disease phenotype. The overall size of the organoids formed was smaller than those formed with control cells, reminiscent of the hypoplasia characteristic of microcephaly. The study also attempted to rescue the microcephaly phenotype by adding cyclin-dependent kinase-5 Regulatory Subunit Associated Protein-2 (CDK5RAP2) protein *via* electroporation on day 12. The result was formation of organoid regions with larger neuroepithelium compared to the tissues electroporated with GFP only.

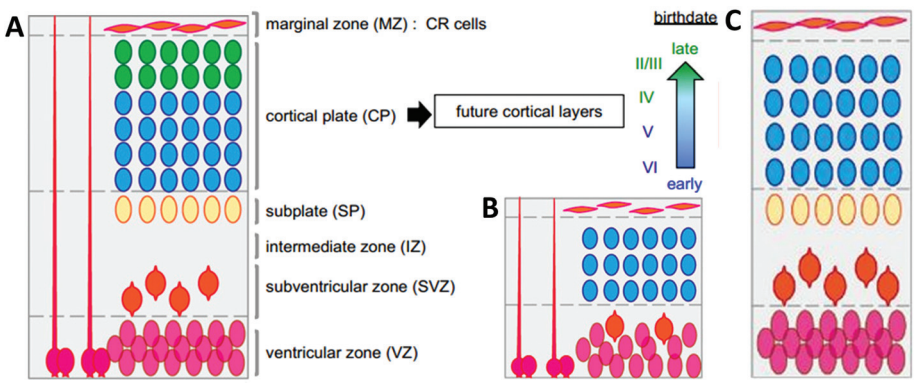


Fig. 3 Comparison of natural development and experimental results obtained by Kadoshima, *et al.* (A) Cortical Lamination at mid-gestation, (B) Self-assembled laminar cortical structure formed using previous culture techniques. (B) The experimental results seen after long-term culture. Reprinted with permission from *PNAS*.[15]

Models with RNAi knockdown of CDK5RAP2 also indicated premature neural differentiation, consistent with observations in the microcephaly model. Both of these experiments support the conclusion that the microcephaly phenotype is specific to loss of the CDK5RAP2 protein, demonstrating the value of using tissue engineered disease models to study diseases *in vitro*. Future studies may aim to generate disease models from both microcephaly patients as well as age-matched controls to further study the disease *in vitro*.

Alzheimer's disease, the most common form of dementia, is characterized by memory loss, disorientation, and memory changes, progressively worsening over time.[121] The molecular hallmarks of the disease include amyloid-β plaques and neurofibrillary tangles (aggregates of hyper-phosphorylated tau protein), which were replicated in a novel 3D Matrigel-based Alzheimer's disease model which expresses both of these features.[122] Human NSCs were transfected with lentivirus vectors to overexpress human β-amyloid precursor protein (APP) or APP and presenilin 1 (PSEN1) with familial Alzheimer's disease mutations; increased levels of amyloid-β isoforms and accumulations of insoluble amyloid-β aggregates were found in these cells after 6 weeks. Treatment with the γ-secretase modulator SGSM41, however, decreased the level of amyloid-β aggregates. In addition to higher neuronal and glial differentiation in 3D culture compared to 2D, this study also reported elevated levels of 4-repeat adult tau isoforms using 3D culture conditions. Tau levels were found to be increased in cells with familial Alzheimer's disease mutations, which, like amyloid-β, were decreased in the presence of the γ-secretase inhibitor DAPT. These results imply that the presence of aggregated phosphorylated tau is driven by amyloid-β aggregation, which is consistent with the amyloid hypothesis of Alzheimer's disease. These results validate this Alzheimer's disease model as a platform for further studies in this area and offers valuable insight into disease pathology.

5.3. Tissue models for drug screening

The drug discovery and development process involves a comprehensive process of screening around 10 000 potential compounds, honing in on the most promising compounds, extensive *in vitro and in vivo* testing, and ultimately United States Food and Drug Administration approval, ultimately narrowing the study down to a single safe and effective drug (Fig. 4).[123] However, many drugs which show promise through the preclinical stage fail at the clinical trial stage; only about 8% of compounds from the preclinical stages successfully pass the clinical trial phases and are approved for marketing.[124] In light of the large time investment (10–15 years[11]) and high costs associated with clinical trials (an estimated $15.2 million for Phase I, $23.4 million for Phase II, and $86.5 million for phase III),[124] more accurately assessing safety and efficacy at the preclinical stages can significantly reduce the cost of drug development due to failed clinical trials.

One recent study has addressed this need by creating a microarray platform which enables high-throughput toxicology assays which offer the capability to assess the toxicity of chemical compounds, including new drug candidates, to stem cells as well as their differentiated progeny.[125] This group seeded human NSCs and differentiated glial cells into the 3D microarray platform by embedding the cells in alginate and spotting on a glass slide with a microcontact microarray spotter in a 6 × 8 × 8 array of 60 nl spots over previously patterned 60 nl alginate-only spots. To demonstrate the ability to test toxicity of different compounds using this platform, human NSCs and differentiated glial cells were challenged with one of 5 compounds with a range of doses for 24 hours: neurotoxicants (cadmium chloride, retinoic acid and dexamethasone), an anti-proliferative anti-cancer agent (5-fluorouracil), or a non-toxic control compound (acetaminophen). Acetaminophen was verified to have a high IC_{50} value (low toxicity) and the toxicity of the neurotoxicants and anitproliferative agents was found to be consistent with previous studies using human NSCs. Further, a significant difference was observed in the IC_{50} values of both dexamethasone and acetaminophen between stem cells and differentiated cells. This study demonstrates that cell type-specific toxicity assays have potential to determine the effect of potential new drugs and other chemical compounds on neural cells at different stages of development in a high-throughput manner.

6. Conclusion and future directions

Here, we have reviewed relevant 3D fabrication techniques which have proven to be useful for the generation of 3D neural tissue models with stem cells. These scaffolds include porous scaffolds, nanopatterned structures, scaffolds designed specifically to facilitate proper structure and function of neural tissue, and several unique fabrication strategies which offer greater control over the 3D engineered tissue construct. It is likely that a combination of several of these approaches combined with the application of stem cells will generate significant innovation in the area of 3D neural tissue engineering. There is also promise in tunable scaffolds, such as elastin-like protein hydrogels which may be designed with a range of elastic moduli and ligand densities,[126] as well as "designer" scaffolds which can be functionalized with a variety of signaling molecules.[79] We have discussed an application of such techniques which has seen recent *in vitro* success: nerve guidance conduits, which are used to clinically bridge the gap between critical defects in the peripheral nervous system and promote axonal regeneration. These conduits have been shown to be greatly improved by seeding of stem cells as well as incorporation of 3D scaffolding within the conduit. Further application and optimization of these powerful 3D fabrication techniques and use of stem cells, particularly strategic combinations of two or more of these strategies, will likely advance the field of neural tissue engineering one step closer to clinical applications.

We have also discussed recent novel applications of stem cells to 3D neural tissue engineering. Self-organizing

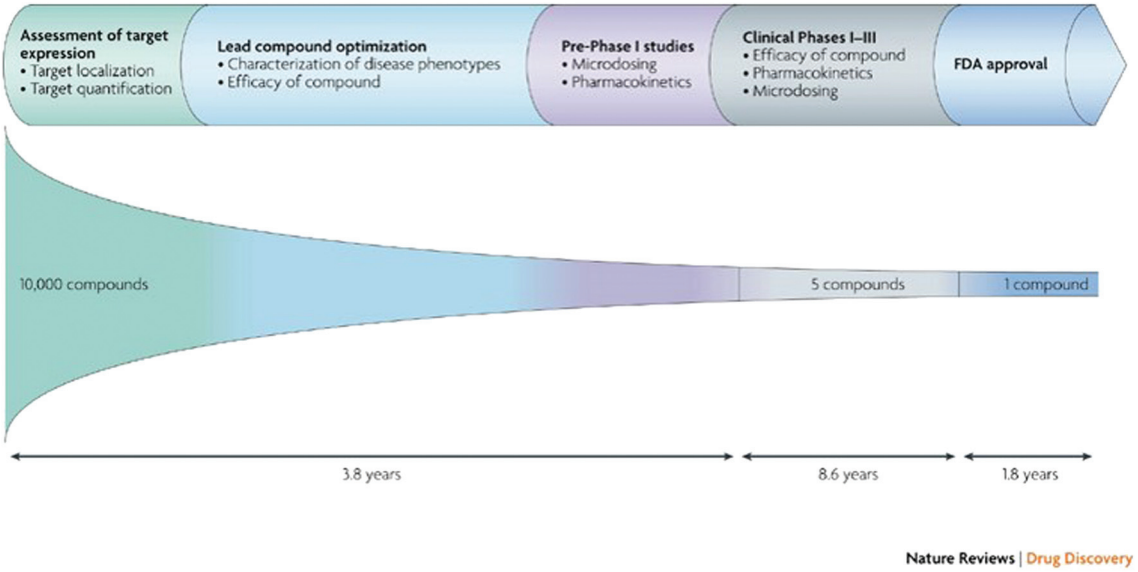


Fig. 4 Drug development process. The drug development process begins with assessing around 10 000 compounds and identifying lead compounds for use in pre-clinical trials. Compounds that succeed in this phase proceed to clinical trials with increasing numbers of participants in each phase. Of the approximately 10 000 compounds tested, only one will obtain Food and Drug Administration approval. Reprinted with permission from: reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery*, copyright 2008.[123]

cortical tissue models give insight into the developmental processes of cortical tissue. These *in vitro* models offer a powerful tool to further understand neural tissue development with the ability to conduct monitoring, imaging, and sampling of living tissue and better control the culture environment. Use of stem cells from patients carrying diseases allows a similar study of abnormal development of diseased tissues as well as readily available, accurate models of abnormal tissues to test potential therapies. Further applications of these 3D neural tissue and disease models include screening potential drugs as well as improving the efficiency of the drug development process. These applications may soon replace animal models for neural research and disease therapies, as animal model studies can produce inaccurate results due to cross-species differences in tissue development and structure, disease pathogenesis, and response to drugs. Translation of current cutting-edge studies carried out using 2D models can be readily extended to 3D models to continue progress in these areas.

Great potential lies in translating current advancements in 2D tissue models to 3D cultures, which create a more biomimetic environment and thus produce more physiologically relevant results. Future work in modeling of human neural tissue will focus on optimizing 3D culture methods, like those described here, in order to improve the biomimicry of both development and ultimate structure. The ability to closely study the self-organization process will give better insight into the developmental process of the human brain *in vitro* and shed light on the processes and pathways within the brain which remain a mystery. In addition, improving culture methods to extend the culturing period beyond 13 weeks will give information about the late stages of more complex cortical development.

The conclusions drawn from recent studies using disease models have given insight into the underlying mechanisms associated with microcephaly and Alzheimer's disease. However, these only hint at the potential knowledge that may be gained by further studies using accurate models of these and other diseases. Further advancements in 3D neural tissue models using stem cells will allow use of cells from patients carrying a particular disease to generate a relevant disease model and further understand the developmental and structural abnormalities associated with the disease. These models may also be used to develop and test therapies specific to such diseases. Some abnormalities may be due to patient-specific mutations, so the use of a patient's stem cells to generate a neural tissue model creates a vehicle for the advent of personalized medicine. In this way, physicians will ultimately be able to understand even the rarest disorders, and therefore treat these disorders with therapies targeted toward a specific patient. Further potential for disease modeling of developmental disorders lies in use of stem cells from diseased patients in previously described models which replicate the developmental process of human cortical tissue.[15,54] For example, the human iPSCs used previously[54] may be derived from patients with genetic diseases in order to closely study the developmental process leading to such diseases as Down syndrome and Fragile X syndrome. This approach may aid in hope of pinpointing therapies to intervene in diseased developmental processes.

Future work in drug screening will focus on comparison of 3D engineered tissues to subjects in order to validate the correlation between *in vitro* and *in vivo* models. It is also possible to determine the efficacy of disease models by comparison with

healthy disease models. One critical feature of drug screening tissue models will be adaptability to high-throughput testing. It will also be important to develop models of other tissues, such as liver and kidney, to watch for adverse side effects of a particular drug on the body and thus predict *in vivo* safety.

A long-term goal of using stem cells for 3D tissue engineering is application to personalized regenerative medicine. Using a patient's own stem cells to regenerate a damaged or diseased tissue will decrease the rate of transplant reduction and reduce issues associated with limited donor supply and donor site morbidity. Furthermore, use of 3D fabrication techniques will facilitate formation of biomimetic structures and, thus, proper organ function, ultimately improving the quality of life for those with developmental diseases, neurodegenerative disorders, and injuries to the nervous system.

Acknowledgements

ST acknowledges the UConn Research Excellence Program Award.

References

1 The Internet Stroke Center, Stroke Statistics, http://www.strokecenter.org/patients/about-stroke/stroke-statistics/, (accessed 15 June, 2015).
2 C. P. Ferri, R. Sousa, E. Albanese, W. S. Ribeiro and M. Honyashiki, World Alzheimer Report: 2009 Executive Summary, Alzheimer's Disease International, 2009.
3 Centers for Disease Control and Prevention, Autism Spectrum Disorder (ASD): Data & Statistics, (http://www.cdc.gov/ncbddd/autism/data.html).
4 M. C. Gibbons, M. A. Foley and K. O. H. Cardinal, *Tissue Eng., Part B*, 2013, **19**, 14–30.
5 O. Lindvall and Z. Kokaia, *Nature*, 2006, **441**, 1094–1096.
6 H. B. van der Worp, D. W. Howells, E. S. Sena, M. J. Porritt, S. Rewell, V. O'Collins and M. R. Macleod, *PLoS Med.*, 2010, **7**, e1000245.
7 A. M. Hopkins, E. DeSimone, K. Chwalek and D. L. Kaplan, *Prog. Neurobiol.*, 2015, **125**, 1–25.
8 G. W. Jay, R. B. Demattos, E. J. Weinstein, M. A. Philbert, I. D. Pardo and T. P. Brown, *Toxicol. Pathol.*, 2011, **39**, 167–169.
9 J. L. Sterneckert, P. Reinhardt and H. R. Schöler, *Nat. Rev. Genet.*, 2014, **15**, 625–639.
10 L. Kimlin, J. Kassis and V. Virador, *Expert Opin. Drug Discovery*, 2013, **8**, 1455–1466.
11 innovation.org, Drug Discovery and Development, http://www.phrma.org/sites/default/files/pdf/rd_brochure_022307.pdf, (accessed 20 March, 2015).
12 F. Pampaloni, E. G. Reynaud and E. H. K. Stelzer, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 839–845.
13 S. M. Willerth, K. J. Arendas, D. I. Gottlieb and S. E. Sakiyama-Elbert, *Biomaterials*, 2006, **27**, 5990–6003.
14 A. Ribeiro, E. Powell and J. Leach, *IFMBE Proc.*, 2015, **32**, 422–425.
15 T. Kadoshima, H. Sakaguchi, T. Nakano, M. Soen, S. Ando, M. Eiraku and Y. Sasai, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **11**, 20284–20289.
16 S. Levenberg, J. A. Burdick, T. Kraehenbuehl and R. Langer, *Tissue Eng.*, 2005, **11**, 506–512.
17 I. Martin, *Tissue Eng., Part A*, 2014, **20**, 1132–1133.
18 A. Wang, Z. Tang, I. Park, Y. Zhu, S. Patel, G. Daley and S. Li, *Biomaterials*, 2011, **32**, 5023–5032.
19 M. Mahoney and K. Anseth, *Biomaterials*, 2006, **27**, 2265–2274.
20 M. J. Bissell, D. C. Radisky, A. Rizki, V. M. Weaver and O. W. Petersen, *Differentiation*, 2002, **70**, 537–546.
21 I. Smith, V. Silveirinha, J. L. Stein, L. de la Torre-Ubieta, J. A. Farrimond, E. M. Williamson and B. J. Whalley, *J. Tissue Eng. Regener. Med.*, 2015, DOI: 10.1002/term.2001, [Epub ahead of print].
22 L. Yla-Outinen, T. Joki, M. Varjola, H. Skottman and S. Narkilahti, *J. Tissue Eng. Regener. Med.*, 2014, **8**, 186–194.
23 W. Ma, W. Fitzgerald, Q. Liu, T. O'Shaughnessy, D. Maric, H. Lin, D. Alkon and J. Barker, *Exp. Neurol.*, 2004, **190**, 276–288.
24 H. R. Irons, D. K. Cullen, N. P. Shapiro, N. A. Lambert, R. H. Lee and M. C. LaPlaca, *J. Neural Eng.*, 2008, **5**, 333–341.
25 M. Frega, M. Tedesco, P. Massobrio, M. Pesce and S. Martinoia, *Sci. Rep.*, 2014, **4**, 5489.
26 S. Bai, W. Zhang, Q. Lu, Q. Ma, D. L. Kaplan and H. Zhu, *J. Mater. Chem. B*, 2014, **2**, 6590–6600.
27 M. S. N. Leipsiz, *Biomaterials*, 2009, **30**, 6867–6878.
28 K. Saha, A. J. Keung, E. F. Irwin, Y. Li, L. Little, D. V. Schaffer and K. E. Healy, *Biophys. J.*, 2008, **95**, 4426–4438.
29 D. M. Cohen and C. S. Chen, in *StemBook*, ed. S. B. a. J. Polak, The Stem Cell Research Community, 2008, p. http://www.stembook.org.
30 K. Watanabe, M. Nakamura, H. Okano and Y. Toyama, *Restor. Neurol. Neurosci.*, 2007, **25**, 109–117.
31 Y. S. Pek, A. C. A. Wan and J. Y. Ying, *Biomaterials*, 2010, **31**, 385–391.
32 A. Banerjee, M. Arha, S. Choudhary, R. S. Ashton, S. R. Bhatia, D. V. Schaffer and R. S. Kane, *Biomaterials*, 2009, **30**, 4695–4699.
33 S. K. Seidlits, Z. Z. Khaing, R. R. Petersen, J. D. Nickels, J. E. Vanscoy, J. B. Shear and C. E. Schmidt, *Biomaterials*, 2010, **31**, 3930–3940.
34 T. G. Fernandes, S. J. Kwon, S. S. Bale, M. Y. Lee, M. M. Diogo, D. S. Clark, J. M. Cabral and J. S. Dordick, *Biotechnol. Bioeng.*, 2010, **106**, 106–118.
35 K. Y. Lee and D. J. Mooney, *Chem. Rev.*, 2001, **101**, 1869–1879.
36 A. Khademhosseini, R. Langer, J. Borenstein and J. P. Vacanti, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 2480–2487.

37 L. E. Freed, G. Vunjak-Novakovic, R. J. Biron, D. B. Eagles, D. C. Lesnoy, S. K. Barlow and R. Langer, *Biotechnology*, 1994, **12**, 689–693.
38 S. Tasoglu and U. Demirci, *Trends Biotechnol.*, 2013, **31**, 10–19.
39 S. Tasoglu, C. H. Yu, V. Liaudanskaya, S. Guven, C. Migliaresi and U. Demirci, *Adv. Healthcare Mater.*, 2015, **4**, 1469–1476.
40 S. Tasoglu, C. H. Yu, H. I. Gungordu, S. Guven, T. Vural and U. Demirci, *Nat. Commun.*, 2014, **5**, 4702.
41 P. Chen, Z. Luo, S. Guven, S. Tasoglu, A. V. Ganesan, A. Weng and U. Demirci, *Adv. Mater.*, 2014, **26**, 5936–5941.
42 S. Tasoglu, E. Diller, S. Guven, M. Sitti and U. Demirci, *Nat. Commun.*, 2014, **5**, 3124.
43 N. G. Durmus, S. Tasoglu and U. Demirci, *Nat. Mater.*, 2013, **12**, 478–479.
44 S. Tasoglu, D. Kavaz, U. A. Gurkan, S. Guven, P. Chen, R. Zheng and U. Demirci, *Adv. Mater.*, 2013, **25**, 1137–1143.
45 U. A. Gurkan, S. Tasoglu, D. Kavaz, M. C. Demirel and U. Demirci, *Adv. Healthcare Mater.*, 2012, **1**, 149–158.
46 S. Guven, P. Chen, F. Inci, S. Tasoglu, B. Erkmen and U. Demirci, *Trends Biotechnol.*, 2015, **33**, 269–279.
47 S. Tasoglu, U. A. Gurkan, S. Q. Wang and U. Demirci, *Chem. Soc. Rev.*, 2013, **42**, 5788–5808.
48 S. Knowlton, S. Onal, C. H. Yu, J. J. Zhao and S. Tasoglu, *Trends Biotechnol.*, 2015, **33**, 504–513.
49 I. Ozbolat and M. Hospodiuk, *Biomaterials*, 2016, **76**, 321–343.
50 D. Arslantunali, T. Dursun, D. Yucel, N. Hasirci and V. Hasirci, *Med. Devices (Auckl)*, 2014, **7**, 405–424.
51 J. van den Ameele, L. Tiberi, P. Vanderhaeghen and I. Espuny-Camacho, *Trends Neurosci.*, 2014, **37**, 334–342.
52 P. Erba, C. Mantovani, D. F. Kalbermatten, G. Pierer, G. Terenghi and P. J. Kingham, *J. Plast. Reconstr. Aesthet. Surg.*, 2010, **63**, e811–e817.
53 J. Mariani, M. V. Simonini, D. Palejev, L. Tomasini, G. Coppola, A. M. Szekely, T. L. Horvathm and F. M. Vaccarino, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 12770–12775.
54 M. Eiraku, K. Watanabe, M. Matsuo-Takasaki, M. Kawada, S. Yonemura, M. Matsumura, T. Wataya, A. Nishiyama, K. Muguruma and Y. Sasai, *Cell Stem Cell*, 2008, **3**, 519–532.
55 H. Li, A. Wijekoon and N. D. Leipzig, *Ann. Biomed. Eng.*, 2014, **42**, 1456–1469.
56 M. A. Lancaster, M. Renner, C.-A. Martin, D. Wenzel, L. S. Bicknell, M. E. Hurles, T. Homfray, J. M. Penninger, A. P. Jackson and J. A. Knoblich, *Nature*, 2013, **501**, 373–379.
57 C. L. E. Novosel and P. Kluger, *Adv. Drug Delivery Rev.*, 2011, **63**, 300–311.
58 H. Bae, A. S. Puranik, R. Gauvin, F. Edalat, B. Carrillo-Conde, N. A. Peppas and A. Khademhosseini, *Sci. Transl. Med.*, 2012, **4**, 160ps123.
59 M. Nikkhah, N. Eshak, P. Zorlutuna, N. Annabi, M. Castello, K. Kim, A. Dolatshahi-Pirouz, F. Edalat, H. Bae, Y. Yang and A. Khademhosseini, *Biomaterials*, 2012, **33**, 9009–9018.
60 R.-Z. L. Ying-Chieh Chen, H. Qi, Y. Yang, H. Bae, J. M. Melero-Martin and A. Khademhosseini, *Adv. Funct. Mater.*, 2012, **22**, 2027–2039.
61 Y. Du, M. Ghodousi, H. Qi, N. Haas, W. Xiao and A. Khademhosseini, *Biotechnol. Bioeng.*, 2011, **108**, 1693–1703.
62 A. Hasan, A. Paul, N. E. Vrana, X. Zhao, A. Memic, Y. S. Hwang, M. R. Dokmeci and A. Khademhosseini, *Biomaterials*, 2014, **35**, 7308–7325.
63 A. Hasan, A. Memic, N. Annabi, M. Hossain, A. Paul, M. R. Dokmeci, F. Dehghani and A. Khademhosseini, *Acta Biomater.*, 2014, **10**, 11–25.
64 G. H. Borschel, Y. C. Huang, S. Calve, E. M. Arruda, J. B. Lynch, D. E. Dow, W. M. Kuzon, R. G. Dennis and D. L. Brown, *Tissue Eng.*, 2005, **11**, 778–786.
65 T. Mygind, M. Stiehler, A. Baatrup, H. Li, X. Zou, A. Flyvbjerg, M. Kassem and C. Bunger, *Biomaterials*, 2007, **28**, 1036–1047.
66 L. Wang, Z. H. Wang, C. Y. Shen, M. L. You, J. F. Xiao and G. Q. Chen, *Biomaterials*, 2010, **31**, 1691–1698.
67 H. Gu, Z. Yue, W. S. Leong, B. Nugraha and L. P. Tan, *Regen. Med.*, 2010, **5**, 245–253.
68 H. Li, A. Wijekoon and N. D. Leipzig, *PLoS One*, 2012, **7**, e48824.
69 T. M. A. Henderson, K. Ladewig, D. N. Haylock, K. M. McLean and A. J. O'Connor, *J. Mater. Chem. B*, 2013, **1**, 2682–2695.
70 M. Jurga, M. B. Dainiak, A. Sarnowska, A. Jablonska, A. Tripathi, F. M. Plieva, I. N. Savina, L. Strojek, H. Jungvid, A. Kumar, B. Lukomska, K. Domanska-Janik, N. Forraz and C. P. McGuckin, *Biomaterials*, 2011, **32**, 3423–3434.
71 S. Guan, X.-L. Zhang, X.-M. Lin, T.-Q. Liu, X.-H. Ma and Z.-F. Cui, *J. Biomater. Sci., Polym. Ed.*, 2013, **24**, 999–1014.
72 X. Feng, X. Lu, D. Huang, J. Xing, G. Feng, G. Jin, X. Yi, L. Li, Y. Lu, D. Nie, X. Chen, L. Zhang, Z. Gu and X. Zhang, *Cell Mol. Neurobiol.*, 2014, **34**, 859–870.
73 N. Zare-Mehrjardi, M. T. Khorasani, K. Hemmesi, H. Mirzadeh, H. Azizi, B. Sadatnia, M. Hatami, S. Kiani, J. Barzin and H. Baharvand, *Int. J. Artif. Organs*, 2011, **34**, 1012–1023.
74 K. A. Chang, J. W. Kim, J. A. Kim, S. E. Lee, S. Kim, W. H. Suh, H. S. Kim, S. Kwon, S. J. Kim and Y. H. Suh, *PLoS One*, 2011, **6**, e18738.
75 Y. J. Huang, H. C. Wu, N. H. Tai and T. W. Wang, *Small*, 2012, **8**, 2869–2877.
76 N. Li, Q. Zhang, S. Gao, Q. Song, R. Huang, L. Wang, L. Liu, J. Dai, M. Tang and G. Cheng, *Sci. Rep.*, 2013, **3**, 1604.
77 L. Yao, N. O'Brien, A. Windebank and A. Pandit, *J. Biomed. Mater. Res., B*, 2009, **90**, 483–491.

78 A. Nur-E-Kamal, I. Ahmed, J. Kamal, M. Schindler and S. Meiners, *Stem Cells*, 2015, **24**, 426–433.
79 F. Gelain, D. Bottai, A. Vescovi and S. Zhang, *PLoS One*, 2006, **1**, e119.
80 C. Cunha, S. Panseri, O. Villa, D. Silva and F. Gelain, *Int. J. Nanomed.*, 2011, **6**, 943–955.
81 C. Cunha, S. Panseri and F. Gelain, *Methods Mol. Biol.*, 2013, **1058**, 171–182.
82 T. Y. Cheng, M. H. Chen, W. H. Chang, M. Y. Huang and T. W. Wang, *Biomaterials*, 2013, **34**, 2005–2016.
83 N. Bhardwaj and S. C. Kundu, *Biotechnol. Adv.*, 2010, **28**, 325–347.
84 G. T. Christopherson, H. Song and H. Q. Mao, *Biomaterials*, 2009, **30**, 556–564.
85 E. Kang, G. S. Jeong, Y. Y. Choi, K. H. Lee, A. Khademhosseini and S. H. Lee, *Nat. Mater.*, 2011, **10**, 877–883.
86 J. De Waele, K. Reekmans, J. Daans, H. Goossens, Z. Berneman and P. Ponsaerts, *Biomaterials*, 2015, **41**, 122–131.
87 M. K. Horne, D. R. Nisbet, J. S. Forsythe and C. L. Parish, *Stem Cells Dev.*, 2010, **19**, 843–852.
88 K. Zhou, G. Thouas, C. Bernard and J. S. Forsythe, *Nanomedicine*, 2014, **9**, 1239–1251.
89 N. D. Leipzig, R. G. Wylie, H. Kim and M. S. Shoichet, *Biomaterials*, 2011, **32**, 57–64.
90 H. Li, A. M. Koenig, P. Sloan and N. D. Leipzig, *Biomaterials*, 2014, **35**, 9049–9057.
91 R. G. Wylie and M. S. Shoichet, *Biomacromolecules*, 2011, **12**, 3789–3796.
92 W. Lee, J. Pinckney, V. Lee, J. H. Lee, K. Fischer, S. Polio, J. K. Park and S. S. Yoo, *Neuroreport*, 2009, **20**, 798–803.
93 Y. B. Lee, S. Polio, W. Lee, G. Dai, L. Menon, R. S. Carroll and S. S. Yoo, in Exp. Neurol., 2009 Elsevier Inc., USA, 2010, vol. **223**, pp. 645–652.
94 G. R. Souza, J. R. Molina, R. M. Raphael, M. G. Ozawa, D. J. Stark, C. S. Levin, L. F. Bronk, J. S. Ananta, J. Mandelin, M.-M. Georgescu, J. A. Bankson, J. G. Gelovani, T. C. Killian, W. Arap and R. Pasqualini, *Nat. Nanotechnol.*, 2010, **5**, 291–296.
95 M. C. Hu and N. D. Rosenblum, *Pediatr. Res.*, 2003, **54**, 433–438.
96 J. W. Park, H. J. Kim, M. W. Kang and N. L. Jeon, *Lab Chip*, 2013, **13**, 509–521.
97 J. M. Jang, S. H. Tran, S. C. Na and N. L. Jeon, *ACS Appl. Mater. Interfaces*, 2015, **7**, 2183–2188.
98 H. J. Z. R. Costaa, R. F. Bentoa, R. Salomonea, D. Azzi-Nogueirab, D. B. Zanattac, M. P. Costad, C. F. d. Silvae, B. E. Straussc and L. A. Haddadb, *Brain Res.*, 2013, **1510**, 10–21.
99 L. Zheng and H. F. Cui, *J. Mater. Sci. Mater. Med.*, 2010, **21**, 1713–1720.
100 L. Zheng and H. F. Cui, *J. Mater. Sci. Mater. Med.*, 2012, **23**, 2291–2302.
101 S. H. Hsu, W. C. Kuo, Y. T. Chen, C. T. Yen, Y. F. Chen, K. S. Chen, W. C. Huang and H. Cheng, *Acta Biomater.*, 2013, **9**, 6606–6615.
102 J. T. Oliveira, F. M. Almeida, A. Biancalana, A. F. Baptista, M. A. Tomaz, P. A. Melo and A. M. B. Martinez, *Neuroscience*, 2015, **170**, 1295–1303.
103 F. Frattini, F. R. Lopes, F. M. Almeida, R. F. Rodrigues, L. C. Boldrini, M. A. Tomaz, A. F. Baptista, P. A. Melo and A. M. Martinez, *Tissue Eng., Part A*, 2012, **18**, 2030–2039.
104 F. R. Pereira Lopes, L. Camargo de Moura Campos, J. Dias Correa Jr., A. Balduino, S. Lora, F. Langone, R. Borojevic and A. M. Blanco Martinez, *Exp. Neurol.*, 2006, **198**, 457–468.
105 F. R. Pereira Lopes, F. Frattini, S. A. Marques, F. M. Almeida, L. C. de Moura Campos, F. Langone, S. Lora, R. Borojevic and A. M. Martinez, *Micron*, 2010, **41**, 783–790.
106 F. Sun, K. Zhou, W. J. Mi and J. H. Qiu, *Neurosci. Lett.*, 2011, **499**, 104–108.
107 L. G. Dai, G. S. Huang and S. H. Hsu, *Cell Transplant*, 2013, **22**, 2029–2039.
108 N. H. Hassan, A. F. Sulong, M. H. Ng, O. Htwe, R. B. Idrus, S. Roohi, A. S. Naicker and S. Abdullah, *J. Orthop. Res.*, 2012, **30**, 1674–1681.
109 A. F. Sulong, N. H. Hassan, N. M. Hwei, Y. Lokanathan, A. S. Naicker, S. Abdullah, M. R. Yusof, O. Htwe, R. B. Idrus and N. H. Haflah, *Adv. Clin. Exp. Med.*, 2014, **23**, 353–362.
110 F. Sun, K. Zhou, W. J. Mi and J. H. Qiu, *Biomaterials*, 2011, **32**, 8118–8128.
111 Y. Y. Hsueh, Y. J. Chang, T. C. Huang, S. C. Fan, D. H. Wang, J. J. Chen, C. C. Wu and S. C. Lin, *Biomaterials*, 2014, **35**, 2234–2244.
112 K. Y. Fu, L. G. Dai, I. M. Chiu, J. R. Chen and S. H. Hsu, *Artif. Organs*, 2011, **35**, 363–372.
113 S. H. Hsu, C. H. Su and I. M. Chiu, *Artif. Organs*, 2009, **33**, 26–35.
114 D. Yucel, G. T. Kose and V. Hasirci, *Biomacromolecules*, 2010, **11**, 3584–3591.
115 H. C. Ni, T. C. Tseng, J. R. Chen, S. H. Hsu and I. M. Chiu, *Biofabrication*, 2013, **5**, 035010.
116 E. East, J. P. Golding and J. B. Phillips, *J. Tissue Eng. Regener. Med.*, 2009, **3**, 634–646.
117 E. East, J. P. Golding and J. B. Phillips, *Tissue Eng., Part C*, 2012, **18**, 526–536.
118 E. East, N. Johns, M. Georgiou, J. P. Golding, A. J. Loughlin, P. J. Kingham and J. B. Phillips, *Regen. Med.*, 2013, **8**, 739–746.
119 S. Shipp, *Curr. Biol.*, 2007, **17**, R443–R449.
120 Mayo Clinic Staff, Microcephaly, http://www.mayoclinic.org/diseases-conditions/microcephaly/basics/definition/con-20034823, (accessed 9 April, 2015).
121 Alzheimer's Association, Alzheimer's Disease & Dementia, http://www.alz.org/alzheimers_disease_what_is_alzheimers.asp, (accessed 1 June, 2015).

122 S. H. Choi, Y. H. Kim, M. Hebisch, C. Sliwinski, S. Lee, C. D'Avanzo, H. Chen, B. Hooli, C. Asselin, J. Muffat, J. B. Klee, C. Zhang, B. J. Wainger, M. Peitz, D. M. Kovacs, C. J. Woolf, S. L. Wagner, R. E. Tanzi and D. Y. Kim, *Nature*, 2014, **515**, 274–278.
123 J. K. Willmann, N. V. Bruggen, L. M. Dinkelborg and S. S. Gambhir, *Nat. Rev. Drug Discovery*, 2008, **7**, 591–607.
124 R. Finkel, The Drug Development, Testing & Approval Process - Drugsdb.com, http://www.drugsdb.com/blog/drug-development-testing-approval-process.html, (accessed 20 March, 2015).
125 L. Meli, H. S. Barbosa, A. M. Hickey, L. Gasimli, G. Nierode, M. M. Diogo, R. J. Linhardt, J. M. Cabral and J. S. Dordick, *Stem Cell Res.*, 2014, **13**, 36–47.
126 K. J. Lampe, A. L. Antaris and S. C. Heilshorn, *Acta Biomater.*, 2013, **9**, 5590–5599.
127 V. Chiono and C. Tonda-Turo, *Prog. Neurobiol.*, 2015, **131**, 87–104.