Advances in Experimental Medicine and Biology 1212
Cell Biology and Translational Medicine

Kursad Turksen *Editor*

Cell Biology and Translational Medicine, Volume 6

Stem Cells: Their Heterogeneity, Niche and Regenerative Potential

Springer

Advances in Experimental Medicine and Biology

Cell Biology and Translational Medicine

Volume 1212

Subseries Editor
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Stem Cells: Their Heterogeneity, Niche and Regenerative Potential

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISSN 2522-090X ISSN 2522-0918 (electronic)
Cell Biology and Translational Medicine
ISBN 978-3-030-32822-1 ISBN 978-3-030-32823-8 (eBook)
https://doi.org/10.1007/978-3-030-32823-8

Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in regenerative medicine. Chapters in this volume cover a range of topics in the area, including developmental aspects and role of the stem cell niche, function of decellularized scaffolds, and the potential of iPS cells in tissue and organ regeneration. Collectively, the chapters continue to cover crucial aspects in translational studies of tissue and organ regeneration and restoration of function in clinical settings.

I remain very grateful to Peter Butler, Editorial Director, and Meran Lloyd-Owen, Senior Editor, for their support of this series from its inception and for helping to foster its success until now. I would like, in addition, to take the opportunity to welcome Gonzalo Cordova as the Associate Editor of the series and acknowledge his support.

I would also like to acknowledge and thank Sara Germans-Huisman, Assistant Editor, for her outstanding efforts in helping to get this volume to the production stages.

A special thank you also goes to Rathika Ramkumar and Abinay Subramaniam for their outstanding efforts in the production of this volume.

Finally, sincere thanks are due to the contributors not only for their support of the series but also for their insight and effort to capture both the advances and remaining obstacles in their areas of research. I trust readers will find their contributions as interesting and helpful as I have.

Ottawa, ON, Canada — Kursad Turksen

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 1–29
https://doi.org/10.1007/5584_2019_350

Published online: 10 March 2019

Addressing Variability and Heterogeneity of Induced Pluripotent Stem Cell-Derived Cardiomyocytes

Sherri M. Biendarra-Tiegs, Frank J. Secreto, and Timothy J. Nelson

Abstract

Induced pluripotent stem cells (iPSCs) offer great promise in the areas of disease modeling, basic research, drug development, and regenerative medicine. Much of their value comes from the fact that they can be used to create otherwise inaccessible cell types, such as cardiomyocytes, which are genetically matched to a patient or any other individual of interest. A consistent issue plaguing the iPSC platform, however, involves excessive variability exhibited in the differentiated products. This includes discrepancies in genetic, epigenetic, and transcriptional features, cell signalling, the cell types produced from cardiac differentiation, and cardiomyocyte functionality. These properties can result from both the somatic source cells and environmental conditions related to the derivation and handling of these cells. Understanding the potential sources of variability, along with determining which factors are most relevant to a given application, are essential in advancing iPSC-based technologies.

Keywords

Cardiomyocytes · Cellular microenvironment · Differentiation · Induced pluripotent stem cells · Phenotypic variability · Stem cell heterogeneity

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Abbreviations

AP	action potential
APD	action potential duration
cGMP	current Good Manufacturing Practice
CNV	copy number variation
DEG	differentially expressed gene
EB	embryoid body
ECM	extracellular matrix
ESA	etoposide sensitivity assay
ESC	embryonic stem cell
ESC-CM	embryonic stem cell-derived cardiomyocyte
FACS	fluorescence-activated cell sorting
iPSC	induced pluripotent stem cell
iPSC-CM	induced pluripotent stem cell-derived cardiomyocyte
mtDNA	mitochondrial DNA
SNV	single nucleotide variation
XCI	X-chromosome inactivation

1 Introduction

Since the original discovery of human induced pluripotent stem cells (iPSCs) in 2007 (Takahashi et al. 2007; Yu et al. 2007), this revolutionary technology has been adopted in a wide variety of settings including disease modeling, drug discovery and toxicity testing, and cell-based therapies (Musunuru et al. 2018; Yoshida and Yamanaka 2017). Much of the power of this platform lies in the ability to produce iPSCs from individuals with unique genetic backgrounds and subsequently differentiate them into a myriad of cell types, including those difficult to obtain by other means, such as cardiomyocytes. This is highly relevant to the pursuit of individualized medicine, including autologous cell-based therapies. Furthermore, the ability to capture variation in the human population and study genotype-phenotype relationships has led to the use of iPSCs in modeling a wide variety of diseases. For example, iPSC-derived cardiomyocytes (iPSC-CMs) have been applied to model diseases including long QT syndrome (Moretti et al. 2010), other channelopathies such as Timothy syndrome (Yazawa et al. 2011), cardiomyopathies (Wyles et al. 2016), ventricular tachycardia (Jung et al. 2012), mitochondrial diseases including Barth syndrome (Wang et al. 2014), and even structural heart disease (Hrstka et al. 2017).

However, while the natural variation captured by iPSCs and their differentiated cardiomyocyte progeny is of great value, heterogeneity and variability of these cellular populations can also be problematic. Ideally, it would be advantageous to produce iPSC-CMs in a reliable and consistent manner. In terms of disease modeling, it is important to be able to understand the relationship between genotype and phenotype without confounding variables distorting the results. Furthermore, for disease modeling it is often desirable to have cells that closely resemble a particular cardiomyocyte subtype, including cells resembling those found in the ventricular or atrial chambers of the heart (Marczenke et al. 2017). This is also vital for reproducibility of the results, an ongoing concern in scientific research (Osterloh and Mulelane 2018). In terms of drug development applications, it is important to be working with iPSC-CMs of a sufficient developmental state to behave in a manner that is predictive of human heart tissue. Cardiomyocyte subtype is a concern in this setting, as well (Denning et al. 2016). In terms of cell-based therapies, it is critical to minimize any safety concerns such as genetically abnormal cells (Merkle et al. 2017). There has also been concern that mixed subtypes of cardiomyocytes could lead to arrhythmias (Liu et al. 2018).

In this review, we discuss sources and types of variability and heterogeneity in pluripotent stem cells and differentiated cardiomyocytes, as well as approaches which have been proposed to aid in retaining pertinent genetic variability while maximizing consistency. While the focus will be on human iPSC-CMs, much of what has been learned from embryonic stem cells (ESCs) or even murine pluripotent stem cells can be applied to the iPSC-CM platform. Ultimately, the goal will be to determine what facets of heterogeneity or variability are or are not permissible to a particular application, and tailor cell production or the study design accordingly.

2 Intrinsic and Acquired Variation in Pluripotent Stem Cell Lines

2.1 Genetic and Epigenetic Abnormalities

One potentially troubling source of variation in iPSC lines is abnormalities at the genetic or epigenetic level. Such variations have been discovered between different iPSC lines, different passages of the same iPSC line, or even different subpopulations within an iPSC culture. These include aneuploidy, chromosomal rearrangement, sub-chromosomal copy number variation (CNV), single nucleotide variation (SNV), variable X-chromosome inactivation, and aberrant DNA methylation. Any of these could potentially result in unexpected cellular properties such as acquisition or disappearance of disease-related phenotypes (Liang and Zhang 2013; Nguyen et al. 2013).

Large-scale studies have provided much insight into the frequency and specific natures of these abnormalities. For example, karyotypic analysis of over 1,700 iPSC and ESC cultures from 97 investigators in 29 labs revealed that for both cell types, approximately 12% of cultures were abnormal. In terms of the types of abnormalities, there were both similarities and differences between iPSCs and ESCs. Trisomy 12 was predominant for both, partial gain of chromosome 12 and trisomy 20q were also seen in both, trisomy 8 was more common for iPSCs than for ESCs, an additional chromosome X was more common in female ESCs, and trisomy 17 was only seen in ESCs (Taapken et al. 2011). In another study, 12 of 38 (~32%) of ESC lines and 13 of 66 (~20%) of iPSC lines had chromosomal aberrations, with 6 iPSC lines having a full trisomy of chromosome 1, 3, 9, or 12 (Mayshar et al. 2010). Rate of aneuploidy has also been shown to increase with higher passage (Mayshar et al. 2010). It has been suggested that karyotypic abnormalities could be derived from culture adaptation, were present in the parental cells, or arose from selective pressure during the reprogramming process (Mayshar et al. 2010). Studies have indeed demonstrated that chromosomal abnormalities are in some cases present in the original somatic cells (Vitale et al. 2012). Reprogramming method and culturing substrate were not found to have a notable role in some studies (Mayshar et al. 2010; Taapken et al. 2011), while some other studies have found that certain passaging methodologies can lead to chromosomal abnormalities (Mitalipova et al. 2005). Karyotypic abnormalities can have functional consequences, as well. For example, spontaneously differentiated normal and abnormal ESC lines demonstrated differences in expression of differentiation-related genes and different propensities for particular lineages (Fazeli et al. 2011).

CNVs have been observed in pluripotent stem cells as well. For example, one study applied single-cell array-based comparative genomic hybridization to reveal notable fractions of both somatic cells and ESCs with diverse megabase-scale chromosomal abnormalities. The authors identified replication break fork collapse and breakage-induced replication as a potential cause, possibly a result of sub-optimal culture conditions (Jacobs et al. 2014). Genomic analysis of 58 iPSC lines from 10 laboratories revealed CNVs that were donor-specific and others that varied between lines from the same donor. There were some genomic loci that were frequently affected, suggesting a basis in the reprogramming process. In some cases, the deletion of tumor suppressors or duplication of cell growth-related genes suggested a survival or proliferative advantage (Salomonis et al. 2016). Another study similarly concluded that CNVs were produced in the reprogramming process and provided a selective advantage, but also found more CNVs in early-passage iPSCs (Hussein et al. 2011). An evaluation of 711 cell lines from 301 healthy individuals reported lower levels of genetic aberrations than had been detected in some previous studies, likely because the authors also had access to donor-matched reference samples and were thus able to identify germline copy number variations. Most of the aberrations they did find were unique to individual iPSC lines, but some alterations were found in

multiple cell lines from the same donor. The number of these alterations was not associated with passage number, donor age, gender, or the results of the quality control assay PluriTest (Kilpinen et al. 2017). The prevalence of SNVs has also been investigated, and one study identified between 1058 and 1808 heterozygous SNVs in each iPSC line examined, with 50% of these being synonymous changes. Since the SNVs were not shared between iPSC lines from the same donor, the abnormalities were deemed likely to have resulted from the reprogramming process (Cheng et al. 2012).

Epigenetic differences between iPSC lines, including variable levels of aberrant DNA methylation, have also been described. For example, a genomic analysis of 58 iPSC lines from 10 laboratories showed that while ESCs and iPSCs were generally indistinguishable at the level of global gene expression, there were notable differences in methylation profiles (Salomonis et al. 2016). Differences in DNA methylation have also been found for iPSCs derived from distinct source cell types (neonatal dermal fibroblasts, adult dermal fibroblasts, and $CD34^+$ cells from peripheral blood mononuclear cells) via different reprogramming technologies, with this heterogeneity reduced after prolonged culture to a more ESC-like DNA methylation state (Tesarova et al. 2016). Conversely, another group found that epigenetic patterns of different iPSC lines were similar to each other and to ESCs, regardless of source cell, although in that case the same reprogramming approach was used for all the iPSC lines. There was some random aberrant hypermethylation observed at early passages, but this was decreased with additional passaging (Nishino and Umezawa 2016). Transcriptional profiling of 317 human iPSC lines from 101 individuals revealed transcriptional variability in Polycomb repressive complex 2 (PRC2) and H3K27me3 targets, which appeared to be independent of genetic background, suggesting that the reprogramming process could be the source. In this same study, some genes showed allelic imbalance while others demonstrated biallelic expression. These patterns were in some cases consistent within individuals, but different across individuals (Carcamo-Orive et al. 2017). This mixture of genes with monoallelic or biallelic expression had previously been seen for both iPSCs (Pick et al. 2009) and ESCs (Kim et al. 2007). One study reported low frequency loss of imprinting in some iPSC lines, which was stable in culture (Hiura et al. 2013). These epigenetic differences can have functional relevance, since it has been shown that epigenetic features can be used to identify iPSC lines with particular differentiation capacities and perhaps even maturation capacity (Nishizawa et al. 2016). For example, histone modifications H3K27me3 and H3K4me3 at lineage-associated and pluripotency genes in ESCs influence developmental potential towards particular lineages (Hong et al. 2011).

The X-chromosome status of female iPSCs and ESCs has also been an area of extensive characterization (Wutz 2012). This was originally described by Silva et al., who showed that ESCs tend to lose XIST RNA expression during culture, leading to three different classes of cells. Class I is pre X-chromosome inactivation (XCI) with a capacity to recapitulate XCI upon differentiation. Class II cells show elevated XIST-positive cells and XCI status. Class III cells have lost XIST expression but still have an inactivated X-chromosome which is not reactivated upon differentiation. Some of these class III lines demonstrate poor spontaneous differentiation in embryoid bodies (EBs) (Silva et al. 2008). Analysis of dozens of iPSC lines has shown notable variation in XIST expression, H3K27me3, and XCI status (Geens et al. 2016; Mayshar et al. 2010; Salomonis et al. 2016). Single cell-derived iPSC clones from the same donor show various states of XCI right after clonal isolation, with both pre- and post-XCI cells within individual colonies (Andoh-Noda et al. 2017). Other studies have similarly noted a mixture of cells with different XCI status in the same passage or even the same colony (Geens et al. 2016; Tanasijevic et al. 2009). In some cases XCI is acquired over time, with no reactivation with repeated passaging (Andoh-Noda et al. 2017). In other cases erosion of XCI in culture has been reported, with this being a stable

condition that cannot be restored by differentiation or reprogramming (Geens et al. 2016; Mekhoubad et al. 2012). One of these studies which reported some reactivation of X-chromosomes additionally noted that clusters of genes in certain chromosomal areas were being reactivated sooner than those in others (DeBoever et al. 2017). Another study, while reporting activation of X-chromosomes in some iPSCs upon reprogramming, did not note any correlation of XCI status to passage number, culture, conditions, or reprogramming method (Bruck and Benvenisty 2011). X-chromosome status can have functional implications for these iPSCs. Loss of XIST in female iPSCs is correlated with upregulation of X-linked oncogenes, downregulated tumor suppressors, accelerated growth rate in vitro, and poorer differentiation in teratomas (Anguera et al. 2012). Developmental genes are also differentially methylated in female iPSC lines with different XIST expression and XCI status (Salomonis et al. 2016).

2.2 Contribution of Source Cells to iPSC Properties

The main sources of genomic, epigenetic, and transcriptional variation between different iPSC lines remains a major question within the field, although a number of studies have helped to provide insight. Transcriptional profiling of 317 human iPSC lines from 101 individuals revealed that ~50% of genome-wide expression variability could be explained by the variation across individuals (Carcamo-Orive et al. 2017). It was even possible to identify expression quantitative trait loci contributing to this variation, which could be conducive to studying variants identified in genome-wide association studies (Carcamo-Orive et al. 2017; DeBoever et al. 2017). Other variables such as donor age, body mass index, sex, ancestry, reprogramming batch and technician, RNA preparation technician, Sendai virus lot, and reprogramming cell source influenced expression variation for only a small number of genes. There were, however, differences in the degree of similarity between iPSC lines derived from the same individual, with Polycomb targets playing a major role in non-genetic variability both within and between individuals (Carcamo-Orive et al. 2017). When genomic analysis was performed on 58 cell lines from 10 laboratories, donor, sex, reprogramming technology, and originating laboratory, but not passage number, were major driving covariates in mRNA, miRNA, and methylation profiling. In regards to methylation profiling, cell of origin played a contributing role but there was no clear connection to differences in somatic methylation profiles (Salomonis et al. 2016). According to an analysis of 711 lines from 301 healthy individuals, between 5 and 46% of variation in iPSC phenotypes including genome-wide assays, protein immunostaining, differentiation capacity, and cellular morphology was due to differences between individuals, and this donor variance was primarily due to genetic differences (Kilpinen et al. 2017).

Other studies have likewise found that genetic differences between individuals are a major contributing factor to variation between cell lines, such as in mRNA levels, splicing, and imprinting (Rouhani et al. 2014). For example, three iPSC clones from the same individual could not be distinguished by transcriptional profiling and functional pathway analysis, and were distinct from ESCs and iPSCs from different donors. These differences between unique donors were retained after differentiation to all three germ layers in embryoid bodies (Schuster et al. 2015). Generated isogenic ESC and iPSC lines have been shown not to have significantly different gene expression in either an undifferentiation or differentiated state, and have little difference in methylations profiles while undifferentiated (Mallon et al. 2014).

On the other hand, the role of epigenetic memory derived from the somatic cell of origin in the variability between cell lines has been more controversial. It is known that different somatic cells have distinct epigenetic profiles, even for the same cell type from different locations. For example, genome-wide DNA methylation and transcriptome data on matched pairs of dural and

scalp fibroblasts showed strong epigenetic memory based on sampling location. More epigenetic variability was observed with age, especially for the scalp-derived cells (Ivanov et al. 2016). However, it has also been found that epigenetic memory is not necessarily a major contributor to transcriptional variation (DeBoever et al. 2017; Rouhani et al. 2014). One study examined matched iPSCs from fibroblasts and blood from multiple donors and observed that lines from the same donor were highly transcriptionally and epigenetically similar, but that different donors had specific transcriptome and methylation patterns that contribute to distinct differentiation capacities (Kyttala et al. 2016). Similarly, in another study very few differences in DNA methylation states or gene expression patterns were detected between iPSCs derived from lymphoblastoid cell lines and from fibroblasts. Again, genetic variation was found to be the largest contributor to differences between different cell lines (Burrows et al. 2016). In fact, if variation between individuals is not corrected for appropriately, transcriptional differences between iPSCs and ESCs and between iPSCs from different somatic tissues of origin seem much larger than in actuality (Rouhani et al. 2014). When considering genetically matched ESC and iPSC lines, genetic background had a larger impact on transcriptional variation than either somatic origin or Sendai virus reprogramming method (Choi et al. 2015). Putting genetic contributions aside, however, iPSCs derived from murine ventricular cardiomyocytes demonstrate a higher propensity to spontaneously differentiate into ventricular-like cardiomyocytes than genetically matched ESCs or iPSCs from tail-tip fibroblasts. This was thought to potentially be due to distinct transcriptomes and DNA methylation, including at promoters of cardiac genes (Xu et al. 2012). Cardiac differentiation efficiency has also been shown to be higher for cardiac progenitor cell-derived iPSCs than for fibroblast-derived iPSCs, possibly due to differential methylation at the NKX2-5 promoter. However, these epigenetic differences decreased with passaging and there were no significant differences in morphology, calcium handling, or electrophysiology of the resulting cardiomyocytes. Moreover, these cells had a similar therapeutic effect in a murine myocardial infarction model (Sanchez-Freire et al. 2014). In order to address this source of variability, our laboratory devised an approach to negate the influence of somatic origin on methylation and transcriptional profiles of the resultant iPSCs, via comparison of murine iPSC clones against a standardized gene expression profile. Expression levels of two pluripotency genes, Oct4 and Zfp42, were identified to indicate increased cardiogenicity regardless of cell source or reprogramming strategy, thus allowing a way to address clonal variability (Hartjes et al. 2014).

The role of somatic cell source in cellular aberrations is another potential concern. Blood-derived iPSCs have been found to be less likely to acquire aberrant DNA methylations than iPSCs from other somatic sources (Nishizawa et al. 2016). In terms of genomic aberrations, it has been determined that an average iPSC line has two CNVs that are not apparent in the originating fibroblasts, although by using more sensitive techniques it can be seen that at least 50% of those CNVs are actually low frequency somatic genomic variants in the parental fibroblasts which are revealed due to the clonal origin of iPSCs. It has been estimated that about 30% of fibroblasts have somatic CNVs (Abyzov et al. 2012). Examinations of SNVs in murine iPSCs have also suggested that most mutations occur prior to reprogramming (albeit at very low allele frequency) and are captured by the clonal nature of iPSCs, although some mutations can occur later on (Li et al. 2015; Young et al. 2012). One study identified 4 somatic mutation classes: clonal, subclonal (which would have arisen during reprogramming or culturing), UV-damage mutations, and copy number alterations. Most point mutations were found to be in areas of repressed chromatin and thus not influence gene expression in iPSCs, although subclonal mutations were associated with altered gene expression to a greater degree. Furthermore, over a third of the genes overlapped by copy number alterations had altered expression. Still, mutations that did not influence gene expression in iPSCs could still potentially have effects in

differentiated tissues, so they should not necessarily be discounted. As for the UV-damage mutations, these were found in ~50% of iPSCs from skin fibroblast. However, the number of mutations in cancer genes was not significantly different than what would be expected by random chance (D'Antonio et al. 2018).

It has become apparent in the past few years that mutations in mitochondrial DNA (mtDNA) can also vary across iPSC lines. Studies have shown that that individual fibroblasts can carry unique mutations, and that mutations in iPSCs can be found in very low levels in parental fibroblasts (and thus may not even be detectable when analyzing whole tissue). These mutations may even be homoplasmic or present in high heteroplasmy. iPSCs from older adults have been reported to exhibit more mtDNA mutations than those derived from younger individuals, and even blood-derived iPSC lines may harbor mitochondrial mutations. These mutations can subsequently lead to defects in metabolic function and respiration (Kang et al. 2016). It was previously shown that while somatic murine cells with high mtDNA mutation load can be reprogrammed to iPSCs, the resultant cells have slower proliferation and differentiation defects (Wahlestedt et al. 2014). Our laboratory reported that low levels of mtDNA mutations in fibroblasts, even from healthy individuals, are detectable following reprogramming into iPSCs. While cardiac differentiation potential was not impacted by mtDNA mutations, this could lead to impaired mitochondrial respiration in iPSC-CMs. Additionally, we observed that a subset of iPSC clones derived from patients diagnosed with mitochondrial disease exhibit low levels of mtDNA heteroplasmy, and thus do provide a representative model system (Perales-Clemente et al. 2016).

3 The Dynamic Transcriptional State of Pluripotent Stem Cells

Of course, the ultimate uses of iPSCs typically involve the differentiation of these cells into a somatic cell type such as cardiomyocytes. The iPSCs must thus be receptive to developmental cues at the time of initiation of differentiation, and progress fully down a desired trajectory. Therefore, in addition to genetic and epigenetic properties, transcriptional heterogeneity of iPSCs at the time of initiation can have a notable impact upon how the cells respond to those cues (Fig. 1).

Recently, it was discovered that iPSC cultures contain two subtypes of cells which differ in morphology, cell-matrix and cell-cell adhesion, pluripotency, and gene expression. Both of these can differentiate into all 3 germ layers, but have different propensities towards these different germ layers when undergoing spontaneous differentiation (Yu et al. 2018a). Along these lines, it had previously been shown that murine ESCs can be described by one of two transcriptional states, and that DNA methylation plays an important role in maintaining these states (Singer et al. 2014). The Nucleosome Remodeling and Deacetylation (NuRD) complex was also found to modulate transcriptional heterogeneity and the expression of pluripotency of genes in murine ESCs, thus controlling response to differentiation signals (Reynolds et al. 2012). These findings suggest that even within a single culture, different cells can have distinct responses to the same differentiation cues. Moreover, they represent a sampling of a much broader body of work describing heterogeneity in pluripotent and development factors, as well as signalling molecules (Singh 2015).

In particular, studies have focused on the concept of heterogenous pluripotency factor gene expression. For example, it has been reported that some ESCs exhibit high Nanog expression levels, while others display levels considerably lower than expected, with the latter group being particularly prone to undergo spontaneous differentiation. Transitions from the high to the low state were modeled to be rare and stochastic, while transitions in the opposite direction were predicted to be frequent (Kalmar et al. 2009). Model simulations have further shown that low-Nanog cells act as an intermediate state to reduce the barrier of transition in the differentiation process (Yu et al. 2018b). However, it has also been suggested that Nanog heterogeneity

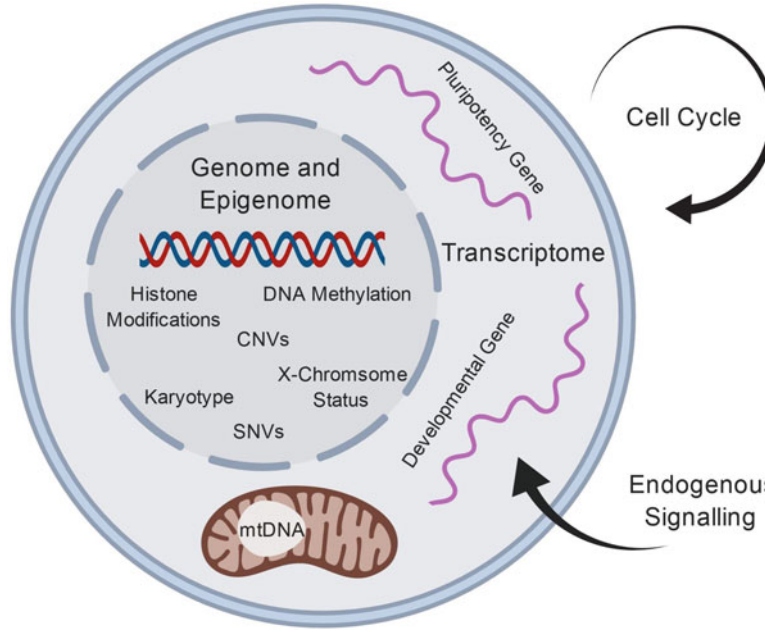

Fig. 1 Sources of variation in iPSCs at the time of initiation of differentiation. Differences in genomes, epigenomes, and transcriptomes between iPSC lines, or even different cells within the same culture, can influence response to differentiation cues. Endogenous signalling and cell cycle position have also been found to exert a noticeable effect upon differentiation trajectories

could be due to specific *in vitro* culture conditions and may not be functionally significant (Smith 2013). The reporters used in such studies can also disturb the cell states they are intended to model. For example, genetic reporters for Nanog can influence behavior of pluripotency-related positive feedback loops and lead to a bifurcation that results in heterogeneous Nanog expression (Smith et al. 2017). Still, some researchers continue to assert that these Nanog fluctuations are real and functionally relevant, based on the fact that similar fluctuations are not seen for some other pluripotency genes, mathematical models have supported bimodal distribution of Nanog, and single-cell RNA seq in murine ESCs suggests bimodal expression (Yu et al. 2018b).

Developmental genes have also been reported to exhibit heterogeneous expression in pluripotent stem cells. For example, Hes1 is a developmental factor that regulates cell proliferation and differentiation in embryogenesis, and along with its downstream gene targets, demonstrates an oscillating expression pattern in murine ESCs. High levels promote mesodermal differentiation whereas low levels promote neural differentiation through modulation of Notch signalling and the cell cycle (Kobayashi and Kageyama 2010; Kobayashi et al. 2009). Modeling approaches have also shown that intrinsic noise in the Hes1 gene regulatory network could explain heterogeneity in murine ESC differentiation (Sturrock et al. 2013). In human ESCs, a Wnt reporter has been used as a read-out of heterogeneity in endogenous Wnt signalling activity, even in cells with similar expression of pluripotency markers. Moreover, the level of Wnt signalling activity in pluripotent stem cells correlates with lineage propensity in differentiation, with high Wnt expression promoting endoderm and cardiac differentiation, and low Wnt enhancing neuroectodermal differentiation (Blauwkamp et al. 2012; Paige et al. 2010).

Interestingly, studies have found that when pluripotent stem cells are exposed to signals which induce differentiation, they activate developmental pathways in an asynchronous manner. Recent evidence has suggested that this is linked to the cell cycle (Dalton 2015). It has been observed that G1 cells are more responsive to differentiation cues, which could help explain heterogeneity in expression of developmental factors. It is possible that developmental genes

are transcriptionally primed in G1 and/or that some pluripotency markers could be diminished in G1, and that a favorable epigenetic and nuclear architecture state in G1 promotes activation of developmental programs (Dalton 2015; Singh et al. 2014). One group used the FUCCI reporter system to show that differentiation capacity of ESCs and iPSCs varies throughout the cell cycle, with early G1 cells having a propensity towards endoderm/mesoderm and late G1 cells tending to differentiate towards neuroectoderm. They found that cells in G2/S/M, on the other hand, responded poorly to differentiation signals. The authors further focused on the differences between early and late G1, and ultimately found that in early G1, level of cyclin D is low, so Smad2/3 can bind and activate endoderm genes, whereas in late G1 cyclin D is high and CDK4/6 is activated and phosphorylates Smad2/3, thus preventing nuclear entry (Pauklin and Vallier 2014). Using the FUCCI system combined with fluorescence-activated cell sorting (FACS) and RNA-seq, other researchers found that heterogeneous expression of developmental regulators in cells which also express pluripotency genes is linked to cell cycle position, with increased expression of these regulators in G1. Major changes in global 5-hydroxymethylcytosine, namely upregulation of 5-hydroxymethylation in G1, were linked to both cell-cycle progression and expression of developmental factors. G1 was seen to be a window of time when the cells could respond to external differentiation signals via gene activation, possibly due to chromatin being in a more permissive state (Singh et al. 2014). Interestingly, it has been reported that when ESCs and iPSCs are cultured with DMSO, this activates the retinoblastoma (Rb) protein, increases proportion of cells in early G1 phase, and improves differentiation efficiency across all germ layers. Such culture manipulation has been used to differentiate cardiomyocytes from an ESC line predicted to be impaired in mesodermal differentiation ability (Chetty et al. 2013). A recent study describing a comparison of various cell cycle inhibitors in human pluripotent stems ultimately identified nocodazole as an efficient and non-toxic means to synchronize these cells in the G2/M phase. This may provide a valuable framework for further investigation into the relationship between cell cycle and differentiation (Yiangou et al. 2019).

Over the past several years, single-cell transcriptional profiling has provided additional insight into heterogeneity of pluripotent stem cells. One such study reported that heterogeneity in expression levels for a number of pluripotency genes was greater in iPSCs than ESCs, and that significant cell-to-cell variability exists even in cells positive for Tra-1-60 and SSEA-4 (Narsinh et al. 2011). From single cell RNA-seq analysis, it has been observed that genes with a higher coefficient of variation in human and murine ESCs form co-expression clusters and partly explain bivalency of gene expression. This data aligns with the idea that pluripotent stem cells alternate between different transient and reversible cell states, although this does not appear to involve lineage priming since genes with a high coefficient of variation were not shown to be enriched for any particular biological process (Mantsoki et al. 2016). Moving forward, it has been suggested that integrative network models—namely gene network models involving epigenetic, transcriptional, and signaling information—from single cell data will be very important for better understanding how self-renewal and differentiation are regulated (Espinosa Angarica and Del Sol 2016).

4 Extrinsic Influences on Pluripotency and Differentiation

Given the extent of reported heterogeneity between pluripotent stem cells, numerous studies have scrutinized the role of extrinsic factors on their properties. One study examining gene expression profiles of 66 iPSC lines found that the lines clustered together according to laboratory and study of origin (Mayshar et al. 2010). Likewise, a reanalysis of microarray gene expression data from seven labs showed strong correlation between gene expression signatures and lab of origin for both ESCs and iPSCs (Newman and

Cooper 2010). Part of this could be due to the culture conditions used. For example, prior to the availability of commercially produced media designed specifically for maintaining pluripotent stem cells, labs employed a variety of in-house developed cell culture media types. Notably, though, a comparison involving eight reported in-house culture methods and two widely available commercial medias (mTeSR1 and STEMPRO) demonstrated that the commercial medias were superior in supporting the maintenance of pluripotent stem cells (International Stem Cell Initiative C 2010). More recently, single-cell RNA-seq of murine ESCs showed enhanced heterogeneity of pluripotency and differentiation marker gene expression for cells cultured in serum as compared to serum free conditions. The most variable of these genes had distinct chromatin state signatures (Guo et al. 2016). A comparison of defined media and media with serum, enzymatic and mechanical passaging, and feeder-free and mouse embryonic fibroblast (MEF) substrates for iPSC and ESC culture demonstrated differences in genomic stability for these different conditions, with more genetic instability in particular for cells subjected to single-cell enzymatic passaging with Accutase (Garitaonandia et al. 2015). Oxygen levels have also been shown to influence properties of pluripotent stem cells, with hypoxic conditions resulting in increased expression of pluripotency markers, reduced chromosomal abnormalities, and reduced transcriptional heterogeneity for ESCs (Forsyth et al. 2008; Lim et al. 2011).

Furthermore, both high and low pH can influence pluripotency of murine and human ESCs, although reports regarding the effects of lactate levels have been more conflicting (Chen et al. 2010; Gupta et al. 2017). One of these reports showed that media acidification due to accumulation of lactic acid from high culture density leads to DNA damage and genomic alterations in ESCs grown on feeders, even over the course of a single passage. This was not seen for a feeder-free system, however (Jacobs et al. 2016). The presence of other metabolites in media can also influence pluripotency. For example, secreted factors from cell culture of ESCs lead to decreased pluripotency marker expression in a system of multiplexed culture chambers (Titmarsh et al. 2013). Build-up of metabolites in media can also influence pluripotency in murine ESCs by priming them for differentiation (Yeo et al. 2013). Some of these effects can be addressed via perfusion culture (Gupta et al. 2017; Yeo et al. 2013). In addition to increasing metabolite levels in media, high density culture results in a higher proportion of cells in G1 (Jacobs et al. 2016; Laco et al. 2018; Wu et al. 2015) and thus could potentially influence differentiation capacity.

Notably, one group found that levels of Wnt fluctuate according to the cell cycle and that higher-density pluripotent stem cells exhibited more cell death and required lower doses of the GSK3β inhibitor CHIR99021 to induce cardiac differentiation. Conversely, cultures consisting of a greater percentage of cells in S/G2/M, along with exhibiting high expression of NANOG and OCT4a, demonstrated an increased propensity for undergoing cardiac differentiation. The authors were therefore able to increase efficiency of more confluent cultures by decreasing concentration of CHIR99021. Ultimately, they discovered that CHIR99021 treatment increased expression of Cyclin D1, promoted cell-cycle progression, and increased genetic instability from acidified media in high-density culture, ultimately leading to cell death. Lower confluence along with increased S/G2/M phase enhanced expression of Wnt inhibitors TCF7L1/2, so those less dense cultures required more CHIR in order to induce higher β-catenin levels via GSK3β inhibition, and ultimately achieve suppression of TCF7L1/2. This then allowed sufficient activation of Wnt target gene expression. Variations in TCF7L1/2 levels and/or cell cycle could thus lead to different differentiation results for the same CHIR concentration. Interestingly, mesoderm development was not found to be as affected by confluency and cell cycle as was full progression to cardiac differentiation (Laco et al. 2018).

In terms of embryoid body (EB) differentiations, outputs can also be influenced by culture conditions, namely colony and EB sizes. Gata6 and Pax6 expression are both impacted by colony size, with

higher input Gata6/Pax6 being connected to more endoderm gene expression. Conversely, there is enhanced mesoderm and cardiac induction at larger EB sizes (Bauwens et al. 2008). Interestingly, the same group later found that efficient cardiac differentiation in EBs is promoted by endogenous extra-embryonic endoderm-like cells which are influenced by aggregate size (Bauwens et al. 2011). A comparison of EB and monolayer cardiac differentiation demonstrated more efficient cardiac differentiation and maturation, as well as homogeneity in cell structure, for the monolayer differentiations (Jeziorowska et al. 2017). Extracellular matrix (ECM) can also potentially influence differentiation ability. For murine ESCs, collagen I and III were individually correlated with decreased cardiac differentiation efficiency, but increased differentiation efficiency when combined. Similar findings were found for the combination of high fibronectin, Wnt2a, and Activin A, suggesting that interactions between growth factors and ECM signalling pathways could modulate stem cell fate (Flaim et al. 2008).

5 The Diverse Nature of Cardiac Differentiations from Pluripotent Stem Cells

5.1 Heterogeneous Cell Populations Resulting from Cardiac Differentiation

Even once iPSCs are successfully differentiated to a cardiac fate, there is still a wide range of variability and heterogeneity in the resultant cell populations. One aspect of this is that cardiac differentiations typically produce a combination of cardiomyocytes and non-cardiomyocytes at varied proportions. There is evidence that these non-cardiomyocytes can impact properties of the cardiomyocytes themselves. For instance, one study reported that when non-cardiomyocytes were removed from a EB-based differentiation of ESCs, development/maturation of electrophysiology and calcium handling were stunted, but these phenotypes were rescued when non-cardiomyocytes were added back (Kim et al. 2010). A second group likewise found that non-cardiomyocytes had an effect upon iPSC-CM electrophysiology and contractility, although they observed optimal properties in several parameters around ~70% cardiomyocytes (Iseoka et al. 2018). However, it is possible that these effects could be cell line-dependent. A study with murine embryonic stem cell-derived cardiomyocytes (ESC-CMs) showed that one line had shortened action potential duration (APD) associated with purification of cardiomyocytes (αMHC+ cells), but another line had a slightly prolonged APD and increased action potential (AP) maximum upstroke velocity when cultured using the same conditions (Hannes et al. 2015). Beyond functional properties, one of these studies also reported an increased proportion of cardiomyocytes expressing ventricular versus atrial myosin light chain for co-cultures with higher cardiomyocyte purity (Iseoka et al. 2018). In an earlier report, it was also found that contaminating non-cardiomyocytes release NRG-1β, which can promote development of working-type (ventricular and atrial) cardiomyocytes (Zhu et al. 2010). Interestingly, one study discovered that BRAF-mutant fibroblast-like cells from cardiac-directed iPSC differentiation promote cardiomyocyte hypertrophy phenotypes via TGFβ paracrine signaling, and that examining purified cardiomyocytes could mask the contributions of non-cardiomyocytes to cardiomyocyte disease processes (Josowitz et al. 2016). This suggests that non-cardiomyocytes may be particularly relevant to some disease modeling applications. There is still much to be learned regarding the interactions between cardiomyocytes and non-cardiomyocytes derived from iPSCs, and this is likely to be an ongoing focus of investigation.

Regarding the cardiomyocytes themselves, there can also be heterogeneity between cultures and within the same culture in when it comes to of properties typically associated with atrial, ventricular, or nodal/pacemaker cardiomyocyte subtypes. Traditionally, it has been asserted that cardiac differentiations produce a heterogeneous population of these subtypes with distinct functional and molecular properties. In order to facilitate

phenotyping of hiPSC-CMs, Kane, et al. have recently proposed a semi-quantitative system for wholistically classifying cardiomyocytes into specific subtypes based on a variety of parameters including AP morphology, gene/protein marker expression, cell morphology, calcium transients, and conduction (Kane and Terracciano 2017).

In terms of electrophysiology, there can be significant variability in APs both between distinct clusters of ESC-CMs and even within the same cluster, with individual clusters frequently having multiple types of APs (Vestergaard et al. 2017; Zhu et al. 2016). Some groups have used signal processing and machine learning to develop platforms to evaluate and classify the electrophysiology of ESC-CMs, and subsequently demonstrated that most cultures exhibit multiple AP phenotypes and even display a continuum of properties between different AP morphologies (Gorospe et al. 2014). However, various studies use different parameters to categorize AP profiles as atrial-like, ventricular-like, or nodal-like, with some researchers questioning whether chamber specificity can be determined via AP morphologies alone (Du et al. 2015; Kane et al. 2016).

One study examined the concordance between electrophysiology and expression of the proposed pacemaker markers HCN4 and Isl1 at Days 40 and 60 of differentiation by acquiring APs of single cells optically, then assessing protein expression via immunofluorescence in the same cell. The researchers saw that HCN4 expression was higher in the cells with pacemaker-like APs initially but that differences decreased with downregulation of HCN4 over time. Conversely, Isl1 expression was initially not different for cells with different AP profiles, but became statistically higher in electrophysiologically pacemaker-like versus ventricular-like cells over time. Therefore, they deemed that neither protein marker was sufficient to identify pacemaker-like cells. Interestingly, they saw that differences in AP properties of the collective groups between Day 40 and 60 seemingly reflected an increase in ventricular- and atrial-like cardiomyocytes, suggesting that subtype may not be determined by Day 40 (Yechikov et al. 2016). Other studies have also found that subtype classification by AP morphology is influenced by time in culture. In one case it was reported that time in culture lead to a transition from nodal-like to ventricular-like APs, with a transient atrial-like phenotype appearing between Days 57–70. That group also performed flow cytometry analysis of cTnT (cardiomyocyte marker), HCN3 (nodal marker), MYL2 (ventricular marker), and MYL7 (atrial marker), which further supported a transition from nodal to atrial/ventricular-like phenotypes from Day 30 to Day 60. Both approaches also revealed some cells with intermediate phenotypes, and ultimately led to the conclusion that AP profiles could not be categorized into three distinct groups (Ben-Ari et al. 2016).

It is possible that culture conditions or microenvironment could further have an impact upon AP properties. For example, AP morphologies of iPSC-CMs seeded at different densities demonstrate distinct distributions, with these differences seemingly not due to gap junction conductance (Du et al. 2015). It has also been observed that similar APs can be found in local regions within clusters of ESC-CMs, with a continuous gradient of AP shapes between regions with distinct AP profiles (Zhu et al. 2016).

5.2 The Quest for Pure Cardiomyocyte Populations

This heterogeneity has led to the development of a variety of approaches to purify cardiomyocytes from the cardiac differentiation process and to enrich for or specifically differentiate cardiomyocytes with the properties associated with a particular cardiomyocyte subtype. These efforts would also aid in addressing variability in cellular distributions between independent differentiations. An early approach to enrich for cardiomyocytes was to use a Percoll density gradient with centrifugation, but this could only enrich to 40–70%(Ban et al. 2017). Mitochondrial staining via the TMRM dye was also proposed fairly early on, but later studies showed that this approach could not robustly discriminate cardiomyocytes early in differentiation from

non-cardiomyocytes and undifferentiated ESCs (Elliott et al. 2011). Other proposed solutions have included expression of a drug resistance gene or fluorescent reporter gene driven by a cardiomyocyte reporter (followed by drug treatment or FACS), but these have the caveat of needing to genetically modify the cells (Ban et al. 2017).

One of the more common, non-invasive approaches is antibody-based enrichment via fluorescent activated cell sorting (FACS) or magnetic-activated cell sorting. Multiple papers from 2011 reported the identification of SIRPA (CD172a) and VCAM1 (CD106) as iPSC and ESC-derived cardiomyocyte cell-surface markers, respectively (Dubois et al. 2011; Elliott et al. 2011; Uosaki et al. 2011). While these markers can be useful, it should be kept in mind that they are not completely specific or selective. One of these papers reported that ~71% of NKX2-5 eGFP^{+} ESC-CMs express VCAM1 and ~85% express SIRPA at Day 14 of differentiation, with only ~37% being dual-positive. Furthermore, only ~67% of VCAM+SIRPA+ cells were also eGFP+, and eGFP+SIRPA+ cells had higher expression of endothelial and smooth muscle markers (Elliott et al. 2011). Later reports have shown that VCAM1 is more highly expressed at earlier stages of differentiation (before Day 25) and that SIRPA expression exists as a continuum, which makes gating based on that alone to be difficult (Veevers et al. 2018).

Another promising non-invasive approach is to take advantage of metabolic differences between cardiomyocytes and non-cardiomyocytes. Differences in glucose and lactate metabolism between non-cardiomyocytes and cardiomyocytes from murine and human pluripotent stem cells allow for cardiomyocyte enrichment in glucose-depleted media with supplementation of lactate (Tohyama et al. 2013). A subsequently-developed protocol involving glucose- and glutamine-depleted media plus lactose was shown to also kill pluripotent stem cells remaining after differentiation (Tohyama et al. 2016). Other methods such as molecular beacons to label cardiomyocyte-specific mRNAs, miRNA-based enrichment, and microfluidic systems are still in relatively early stages of development, but may prove to be useful in the future (Ban et al. 2017).

Likewise, numerous different approaches have been pursued in order to isolate cardiomyocytes with properties of a specific cardiomyocyte subtype. These have included an SLN reporter for atrial-like cardiomyocytes, a cGATA6 reporter for nodal-like cardiomyocytes, and an MLC-2v reporter for ventricular-like cardiomyocytes (Bizy et al. 2013; Josowitz et al. 2014; Zhu et al. 2010). A molecular beacon approach has been investigated in this context as well, namely the use of molecular beacons targeting *Irx4* mRNA in murine ESCs to select for ventricular-like cardiomyocytes. However, a high load of molecular beacons per cell were needed in order to achieve significant signal (Ban et al. 2015). Another group recently used an ESC line for which GFP expression was driven by the MYL2 promoter in order to screen for cell-surface markers of ventricular cardiomyocytes. They found that a CD77+/CD200- population was >97+ cTNI+ with 65% expression MYL2-GFP, allowing for selection of a nearly pure cardiomyocyte population which was enriched for ventricular-like cells. While this approach worked well for the ESC lines they tested, the two iPSC lines they attempted to use interestingly had little-to-no CD77 expression. This enrichment approach was amenable to both EB and monolayer-based differentiations, but with somewhat less efficiency in the monolayer differentiation (Veevers et al. 2018). Other researchers took a unique approach where instead of trying to sort out specific subpopulations, they aimed to identify them *in situ*. To that end, they used subtype-specific promoters (MLC-2v, SLN, and SHOX2) to express a voltage-sensitive fluorescent protein in iPSC-CMs for subtype-specific optical AP recordings (Chen et al. 2017).

Other groups have taken a more developmental biology-informed approach and thereby developed differentiation protocols tailored to the production of particular cardiomyocyte subtypes. Initial work with neonatal rat ventricular myocytes and murine ESCs showed that overexpression of *Tbx18* or *Isl1* transcription

factors was associated with development of the nodal subtype (Dorn et al. 2015; Kapoor et al. 2013). Inhibition of NRG-1β/ErbB signalling can also enhance the proportion of nodal-like cells, as can co-modulation of BMP, RA, and FGF signalling pathways (Protze et al. 2017; Zhu et al. 2010). Interestingly, iPSCs co-cultured with the visceral endoderm-like cell line END-2 produced primarily nodal-like cells, as well (Schweizer et al. 2017).

Protocols have also been proposed for the targeted production of working-type cardiomyocytes. There have been a couple protocols that involved modulation of canonical Wnt signalling by the small molecule IWR-1 in order to produce ventricular-like cardiomyocytes from ESCs and iPSCs (Karakikes et al. 2014; Weng et al. 2014). Gremlin 2 has been reported to upregulate pro-atrial transcription factors and downregulate atrial fate-repressive transcription factors during the differentiation of murine ESCs via stimulation of JNK signaling (Tanwar et al. 2014). More studies, though, have focused on the role of retinoid signaling in atrial verus ventricular development from pluripotent stem cells. Protocols involving retinoic acid treatment can promote atrial-like phenotypes, whereas protocols which include treatment with a retinoic acid receptor antagonist can promote ventricular-like development (Devalla et al. 2015; Lemme et al. 2018; Zhang et al. 2011). A subsequent study showed that atrial and ventricular-like cardiomyocytes develop optimally from specific mesoderm populations (CD235a+/CYP26a1+ for ventricular-like and RALDH2+ for atrial-like), and that these different mesoderms can be specified with different concentrations of BMP4 and Activin A. The RALDH2+ mesoderm responds to retinol to thus make atrial-like cardiomyocytes, since only cells with ALDH expression can synthesize retinoic acid from retinol. Retinoic acid can specify both mesoderms to an atrial fate, but the RALDH2+ mesoderm is more efficient for the production of atrial-like cells. Conversely, without retinoid signalling the RALDH2+ mesoderm can produce ventricular-like cardiomyocytes, but at low efficiency. Importantly, this study also showed that differential cell lines may have variable expression of endogenous Nodal/Activin A and that different cytokine lots can have different activity, and thus optimization of differentiation reagents is necessary (Lee et al. 2017). One group has even used a reporter for the atrial transcription factor COUP-TFII to further enrich atrial-like hESC-CMs from a retinoic acid-directed cardiac differentiation. Interestingly, though, they also found that COUP-TFII was not required for atrial specification of the hESCs, highlighting that the processes associated with development of different cardiomyocyte subtypes have not yet been fully elucidated (Schwach et al. 2017).

5.3 Phenotypic Variability of Cardiomyocytes

Beyond the consideration of different cardiomyocyte subtype-like populations arising from cardiac differentiation, there is also quite a bit of variability and heterogeneity in other aspects of pluripotent stem cell-derived cardiomyocyte properties. A number of studies have focused on evaluating electrophysiological properties in particular, which have been demonstrated to differ between differentiations (with different cell lines or differentiation protocols) for both mouse and human (Hannes et al. 2015; Pekkanen-Mattila et al. 2010). For example, one study reported that there was heterogeneity in electrophysiological phenotypes of ESC-CMs differentiated with two different methods, with approximately one third of cells demonstrating fairly mature electrophysiological properties (maximum diastolic potential < -70 mV and upstroke velocity >140 V/S) but others appearing more embryonic-like (Pekkanen-Mattila et al. 2010). Even cell lines with similar gene expression profiles at the pluripotent cell state can have distinct electrophysiological properties, which was the case for an ESC line and an iPSC line differentiated to cardiomyocytes in one particular study. This comparison revealed great differences in APs and sodium currents at Day 60 of differentiation, with higher sodium currents in the iPSC-CMs and

differential responsiveness to lidocaine and tetrodotoxin. There was also variation in AP frequency and APD, as well as differences in subtype classification between the lines and as a function of time (Sheng et al. 2012). AP profiles can change in numerous ways as the cardiomyocytes undergo maturation with time in culture, due to ongoing development of multiple electrophysiological currents in terms of current density and properties (Sartiani et al. 2007). Such variability can have implications for the application of these cells, where it is often important to elucidate which differences are biologically meaningful. For example, there can be variability in APD and drug responses for iPSC-CMs from LQT3 patients. In response to this, one research group created an *in silico* model to identify plausible mechanisms, and henceforth identified currents with possible differences at baseline or in response to drug treatment (Paci et al. 2017). Culture conditions can have a profound effect upon electrophysiological properties of hiPSC-CMs, which can be a particularly important consideration for drug-screening applications. In one study, it was discovered that more drugs prolonged field potential duration of iPSC-CMs in serum-containing media than in serum-free media, with some drugs also inducing arrhythmias at lower concentrations in the serum-containing media. This was a result of the media formulation impacting both compound availability (dissolved drug concentrations were surprisingly lower in the serum-free media) and baseline electrophysiology (the cells in serum-containing media had longer field potential durations) (Schocken et al. 2018).

Much of the heterogeneity in pluripotent-stem cell-derived cardiomyocytes can be attributed to the maturation status of these cells. With changes in maturation come changes in numerous cardiomyocyte properties including cell morphology (size, shape, nucleation), gene expression, contractility (sarcomere organization, myosin light chain isoforms, troponin T isoforms), electrophysiology (ion channels, APs, cell-cell coupling, conduction velocity), calcium handling, metabolism (including mitochondrial maturity), and proliferation. Numerous different approaches have been taken to modulate and enhance the maturity of these cells and there have been several informative reviews on this topic, including recent reviews by Scuderi et al. and Tu et al. (Scuderi and Butcher 2017; Tu et al. 2018).

One simple approach is to culture the cells for extended periods of time, even months. This can lead to changes in morphology, contractile properties, calcium handling, electrophysiology, and gene expression (Lundy et al. 2013). Culture substrate can also have a notable impact upon cardiomyocyte development, since the use of substrates with physiological stiffness, micro- or nano-patterned surfaces, incorporation of native cardiac extracellular matrix components, and culture in 3D scaffolds can be used to promote advanced maturation (Carson et al. 2016; Fong et al. 2016; Nunes et al. 2013; Ribeiro et al. 2015; Ruan et al. 2015; Tiburcy et al. 2017; Zhang et al. 2013). In an effort to even further mimic physiology, both electrical and mechanical stimulation have been used to promote cardiomyocyte maturation (Mihic et al. 2014; Nunes et al. 2013; Ruan et al. 2015, 2016; Shen et al. 2017). Even increasing the conductivity of the culture system can enhance maturation, for example through the incorporation of trace amounts of electrically conductive silicon nanowires into scaffold-free cardiac spheroids (Tan et al. 2015).

In addition to physical influences upon the cardiomyocytes and their development, chemical influences designed to mimic *in vivo* maturation factors can also be quite impactful. For example, both tri-iodo-L-thyronine and dexamethasone (thyroid and glucocorticoid hormones, respectively) can enhance multiple measures of cardiomyocyte maturation. The combination of the two with a Matrigel mattress protocol is able to promote development of a T-tubule network (Parikh et al. 2017), which has historically been a bottleneck in the maturation of these cells (Scuderi and Butcher 2017). Some miRNAs are also able to impact cardiomyocyte maturation, as was found to be the case for overexpression of Let-7 miRNA family members (Kuppusamy et al. 2015).

A recent examination of cardiac differentiation from human pluripotent stem cells via single-cell RNA-seq was able to provide great insight into

the transcriptional heterogeneity of the cells arising from this differentiation process, in particular revealing the role of HOPX in late stages of cardiac maturation (Friedman et al. 2018). A second recent study applied both single-cell RNA-seq and bulk RNA-seq over the course of a cardiac differentiation of iPSCs, and thereby identified distinct subpopulations of cardiomyocytes which were enriched for specific cardiac transcription factors and represented distinct maturation states. Through a variety of follow-up experiments, the authors furthermore found evidence that two of these transcription factors, *NR2F2* and *HEY2*, can promote atrial and ventricular transcriptional and electrophysiological phenotypes, respectively (Churko et al. 2018). Both of these studies provide a wealth of new information, and the continued use of single-cell RNA-seq will likely provide additional insight into the heterogeneity of iPSC-CMs and allow for generation of new hypotheses regarding how to better control the output of the differentiation process.

6 Looking Ahead: Approaches to Improve Consistency and Reproducibility

6.1 Improving and Validating the Starting Material

Overall, the creation of iPSCs from somatic cells and subsequently differentiation of these cells into cardiomyocytes involves taking mosaic cells from genetically diverse individuals and subjecting them to a wide variety of procedures and environmental conditions over the course of several months. Furthermore, there are no universally defined standards for these processes and culture conditions, which can differ markedly between groups or even individuals (Fig. 2). It should therefore not be surprising that different batches of iPSCs and iPSC-CMs demonstrate considerable variability, and that heterogeneity can even be present within a single population.

Moving forward, there will continue to be a need to reduce undesired variability within the iPSC-derived cardiomyocyte platform, in order to highlight true biological differences that are relevant for the given application. A component of this will be ensuring that the starting iPSCs are of high quality and meet a certain set of desired standards such as pluripotency and differentiation capacity. It has been suggested that one approach to this would be to choose cellular starting material that is less likely to have accumulated mutations or abnormalities (such as multipotent stem cells) (Silva et al. 2015). Quality control assays are also very useful in this regard. The teratoma assay is an established gold-standard for the capacity of pluripotent stem cells to differentiate into all three germ layers. However, it has been shown that it is not necessarily sufficient as a stand-alone means of evaluating pluripotent stem cell quality. For example, one study found that 45/46 evaluated cell lines could form teratomas with all three germ layers, yet 23 of those cell lines had contamination, karyotypic abnormalities, or features suggestive of spontaneous differentiation in culture (Salomonis et al. 2016). It has also been reported that murine iPSCs can demonstrate differences in cardiogenic potential despite a lack of variability in teratoma formation (Hartjes et al. 2014). In response to this, one group created a quantitative scorecard (TeratoScore) based on gene expression data from *in vivo* cell types in order to differentiate pluripotent stem cell-derived teratomas from malignant tumors. This approach could even differentiate between normal and abnormal karyotype (Avior et al. 2015).

A variety of other types of assays are now available to provide additional quality information on pluripotent stem cells. For instance, one group established an unbiased approach to evaluate colony morphology of human pluripotent stem cells using automated live-cell, label-free imaging and analysis algorithms (Kato et al. 2016). The PluriTest was created in order to evaluate pluripotency based on gene expression profiles, using both a "pluripotency score" and a "novelty score", which quantifies how different the gene expression profile of the sample is from the historic data used by the algorithm (Muller et al. 2011). The ScoreCard assay also uses gene

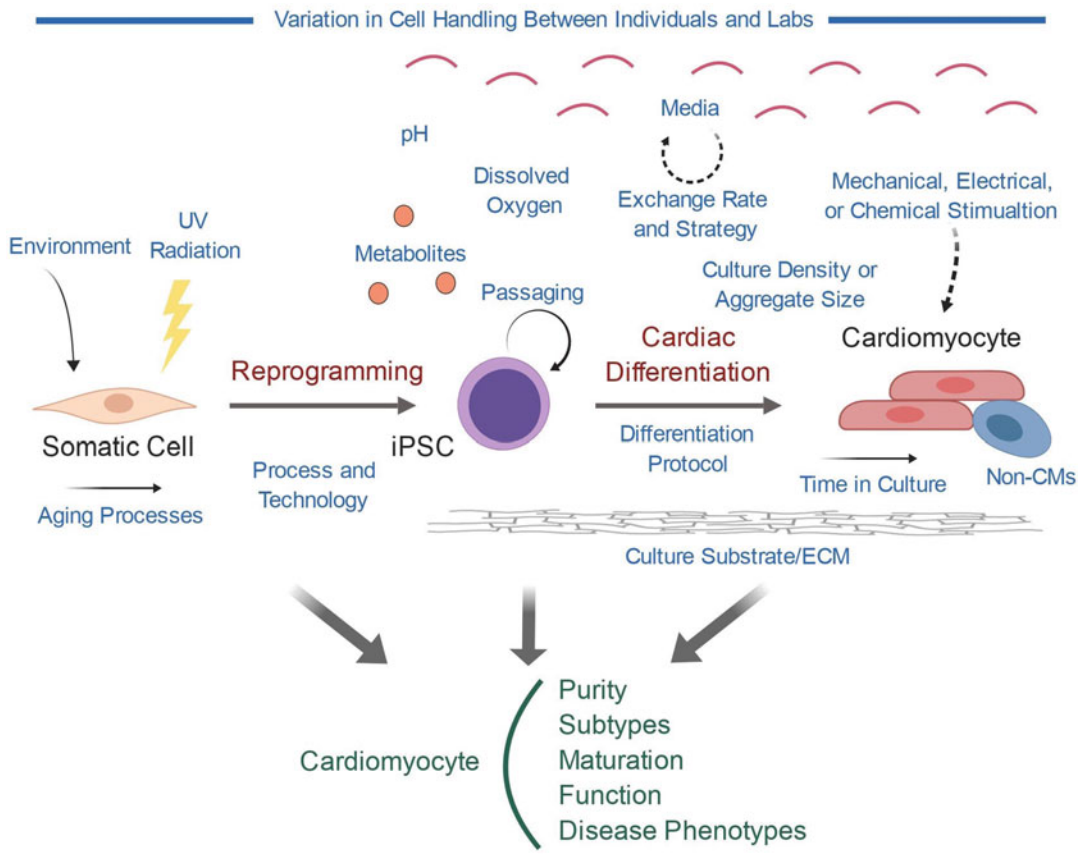

Fig. 2 Role of extrinsic factors in cellular properties. Numerous environmental and technical factors have been shown to influence the molecular and functional properties of somatic cells, iPSCs, and iPSC-derived cardiomyocytes. Altogether, these can ultimately modulate the final cardiomyocyte product and impact its utility for the desired applications

expression signatures, but to determine differentiation capacity (Tsankov et al. 2015). The Etoposide Sensitivity Assay (ESA) developed by our group takes advantage of the fact that pluripotent stem cells are hypersensitive to the topoisomerase inhibitor etoposide, and thus can be used to distinguish good quality iPSC clones from malignant teratocarcinoma clones. This is in contrast to PluriTest, which was shown to be unable to distinguish pluripotent teratocarcinoma cell lines or those with a considerable amount of spontaneous differentiation (Secreto et al. 2017).

Another study, though, highlighted the fact that some of these assays may not be sufficient in isolation. The authors profiled 18 cell lines which had variation in endogenous pluripotency gene expression and other properties, including those which were only partially reprogrammed and had low SSEA4 expression. However, this variability did not have any bearing on other criteria for evaluating pluripotency such as teratoma formation and the PluriTest assay. However, for the lines which did fulfill the most stringent pluripotency criteria, there was low interclonal and inter-individual variability. The authors concluded that thorough analyses of pluripotency are necessary and that proper characterization is vital to be able to distinguish differences between individuals from disease-associated differences (Vitale et al. 2012).

Recently, the International Stem Cell Initiative performed a detailed comparison of several

different quality control assays via blinded analyses by independent experts in iPSCs and ESCs in four laboratories. Of the four methods the evaluated (PluriTest, 'Spin EB' system plus adapted lineage ScoreCard method, histological assessment of teratomas, and TeratoScore assessment of teratomas), all could be used to show pluripotency and each provided some information about differentiation potential. The authors suggested that the particular approach should be chosen based on the final application of the cells. For example, they asserted that a teratoma assay would be vital for cells that are intended for clinical purposes, since only that approach could evaluate both pluripotency and malignant potential (International Stem Cell I 2018).

There has been much focus on figuring out how to maintain pluripotent stem cell quality and consistency through the culture system used, for example by developing fully defined and integration-free conditions for iPSC reprogramming, and implementing pluripotent stem cell culture systems using chemically-defined media, attachment surfaces, and splitting reagents. These approaches have the potential to minimize the batch-to-batch variation that can be seen in culture systems which use serum-containing and Matrigel for cell attachment (Chen et al. 2011). One study reported the creation of current Good Manufacturing Practice (cGMP)-compliant iPSC lines for clinical purposes and tested differentiation capacity into cell types from all 3 germ layers. The authors proposed that the creation of repositories of well-characterized iPSC lines that could be expected to respond predictably to standard differentiation protocols. However, this must be taken with the caveat that they based their assertion that cGMP iPSC lines behave in a predictable manner on only two iPSC lines (Rao et al. 2018). Several chemically-defined differentiation conditions for pluripotent stem cells involving chemically defined medium and small molecules have also been described and subsequently shown to produce reproducible differentiation efficiency across 10+ iPSC lines differentiated repeatedly at multiple passages (Burridge et al. 2014; Lian et al. 2015, 2017). Some researchers even used an albumin-free and chemically-defined medium for ventricular- and atrial-directed differentiations (using either retinoic acid or a retinoic acid inhibitor). They were able to achieve higher efficiency, higher cardiomyocyte yield, and lower inter-experimental variation as compared to differentiations performed using a B27-supplemented medium (Pei et al. 2017).

Significant effort has been devoted in general to the creation of new and improved cardiac differentiation protocols, with the aim of increasing efficiency, yield, and reproducibility. For instance, one early study involved the optimization of >45 different variables for cardiac differentiation of iPSCs and ESCs (Burridge et al. 2011). However, variability between cell lines can hinder the pursuit of universal differentiation protocols, and in some cases modified protocols have been created to enhance cardiac differentiation of specific cell lines which respond poorly to standard protocols (Hrstka et al. 2017; Yassa et al. 2018). Some suggestions have been made as to how to address this challenge in a more systematic way. One group applied a cytokine screening strategy to optimize cardiac output for murine ESC lines with differences in endogenous signaling of Activin/Nodal and BMP (Kattman et al. 2011).

Another group created a high-throughput platform to screen pluripotent stem cells in different microenvironments in order to optimize colony size, cell density, media composition, and substrate, and ultimately quantify endogenous signaling pathways and differentiation bias. They found that endogenous signaling is a major source of variability in how cells respond to exogenous induction conditions, and could therefore use their system to improve differentiation of difficult cell lines, including along a cardiac lineage (Nazareth et al. 2013). A different group focused on addressing the challenges associated with cardiac differentiation which can be posed by high density monolayer culture. They found that using rapamycin (mTOR inhibition) and CHIR99021 together improved efficiency and yield by reducing p53- and DNA damage-depended apoptosis in high density culture through reduction of p53 accumulation and mitochondrial ROS production. A similar effect could potentially be

achieved by hypoxia and control over the nutrients present, instead of rapamycin treatment (Qiu et al. 2017).

6.2 Prioritizing Robust Study Designs and Cellular Manufacturing

Although the variable features of pluripotent stem cell-derived cardiomyocytes can pose a challenge, it should also be kept in mind that these may reflect true biological differences between individuals, an advantage of the iPSC system. For example, not only is beat rate variation present in iPSC-CMs, but heart rate variation can be observed *in vivo* (Binah et al. 2013). Additionally, an examination of ECGs from over 12,000 subjects undergoing routine medical exams for occupation purposes furthermore revealed natural variation in QT interval, down to 335 ms (Gallagher et al. 2006). In the case of diseased populations, electrophysiological recordings from sinus rhythm and chronic atrial fibrillation patients have been shown to exhibit inter-subject variability in AP morphology. Mathematical modeling has been used to determine possible causes of this, and revealing that variability in several different ion currents could modulate variability in APD and triangulation (Sanchez et al. 2014).

Ideally, studies involving iPSC-CMs should be carefully designed in order to highlight relevant differences between healthy and disease states, without being overshadowed by other inter-individual differences. It has been posited that variability among small cohorts of iPSCs could lead to inaccurate conclusions due to inherent differences arising from genetic variability (Kyttala et al. 2016). A number of studies have suggested that it is preferable to utilize cell lines from more individuals, rather than generating and studying multiple iPSC lines from the same individual, in order to differentiate disease mechanisms from the effect of genetic background in disease modeling (Burrows et al. 2016; Rouhani et al. 2014; Schuster et al. 2015).

A recent study concluded that use of more than one clone per individual can actually negatively impact the robustness of findings for transcriptionally-focused studies. Since differences between individuals play large roles in transcriptional variance, comparison of unrelated individuals, as generally done in disease modeling studies, will result in some differentially expressed genes (DEGs) that are not relevant to the disease of interest. They found that using more than one clone per individual actually increased spurious DEGs. While the use of multiple clones per individual can increase sensitivity (although not more than using more individuals), there is a larger loss in specificity. When multiple clones must be used per individual, the authors suggested using analysis methods that take into account the interdependence of the samples, such as an R package that they developed. The choice of controls was found to be another significant issue. An analysis of 77 studies published in 2016 showed that 79% of them used only unrelated controls. This is notable since very few spurious DEGs were found for the comparison of isogenic clones as opposed to a comparison between unrelated individuals. The authors suggested using two clones per individual with a mixed-models approach in order to obtain similar results to the use of isogenic controls, with at least 3 individuals per group in order to reduce false positives. When single clones from unrelated individuals are used, they suggested having at least 4 individuals per group, although having more than 6–7 per group did not improve performance (Germain and Testa 2017).

Other studies have likewise found the choice (or lack-thereof) of controls in iPSC disease modeling studies to be an issue. An analysis of 117 studies revealed that the median and average number of controls in such studies were only 1 and 1.6, respectively, and did not generally account for age, gender, or ethnicity. These authors suggested use of at least 3 controls from 3 separate subjects which are matched for such demographic factors. They proposed that these should be from unaffected family members whenever possible, and when not possible, as many as 12 or more individual donor lines should be used

for controls since it has previously been reported that differences between iPSC and ESCs are negligible when that many lines are evaluated. Furthermore, for differentiated cells it may be essential to compare cells which have been in culture for similar amount of time, in order to reduce effect of maturation-related differences (Johnson et al. 2017).

Some researchers have suggested that when participants in iPSC studies are selected based on the presence or absence of polygenic disease the patients may be genetically heterogeneous and phenotypically variable, thus decreasing statistical power to detect the differences between cases and controls. Instead, they suggested the selection of patients with a known genetic variant with high penetrance and large effect size, or patients with high polygenic risk based on common genetic variants. They proposed an ideal study design with 4 different groups: patients with and without the disease penetrant variant or high polygenic risk, and controls with and without the same. The use of family members as controls could also help to control for genetic heterogeneity (Hoekstra et al. 2017). Finally, regardless of the number of clones per individual used in a study, it may be necessary to produce and screen multiple iPSC lines for chromosomal, nuclear gene, and mtDNA defects, any of which could potentially lead to misleading phenotypes (Kang et al. 2016).

While choice of the number and identity of samples and controls is highly relevant for disease modeling studies involving iPSCs, different considerations exist for the production of iPSCs and iPSC-CMs for therapeutic uses, high-throughput drug screening purposes, or other such applications. In these cases, improving consistency and quality in the cell manufacturing processes is of particular concern. To that end, various approaches to automating aspects of pluripotent stem cell culture have been investigated, including automated approaches to iPSC cell reprogramming, cell seeding, medium changes, passaging, differentiation, imaging, and harvesting (Konagaya et al. 2015; Kowalski et al. 2012; Paull et al. 2015; Serra et al. 2010). Some of these efforts have indeed been shown to reduce well-to-well, plate-to-plate, and line-to-line variability (Crombie et al. 2017; Kowalski et al. 2012; Paull et al. 2015). Automated versus manual cell handling approaches have even been shown to differentially influence expression of pluripotency and differentiation marker expression in iPSCs (Archibald et al. 2016). In addition to adaptation of existing methods to automated approaches, ongoing improvements are being made to the methods themselves. For example, there was a recent report demonstrating that using dextran sulfate during cell seeding was able to control aggregate size and reduce heterogeneity and variability in suspension cultures of pluripotent stem cells. This is due to the fact that greater homogeneity in aggregates allows for more control over nutrient gradients and the prevention of large aggregate formation, in which the cells tend to lose pluripotency and undergo increased apoptosis (Lipsitz et al. 2018). Suspension culture methods will become increasingly valuable as a means to more efficiently produce large batches of pluripotent stem cell-derived cardiomyocytes, and thus improved methods in this area will also be of notable impact.

From a broader standpoint, generating cellular products requires overcoming variability in starting materials, reagents, microenvironment, and stochastic variability. Silva et al. have made some suggestions to help overcome these challenges, such as developing approaches for standardized comparative evaluation of cell product quality during the production process, and the need for robust and scalable standardized platforms for selection, purification, and validation of iPSCs (Silva et al. 2015). French et al. have provided an overview of the types of physical standards (reference materials) which will aid in improving reproducibility and consistency in the creation of differentiated cells from pluripotent stem cells. “Product” reference materials are representative of the product and can aid in evaluating its identity or potency. For example, these could be samples of specific batches, pooled populations from multiple batches, or other cell populations that are biologically equivalent in relevant properties. On the other hand, “method” reference materials, such as fixed cells or RNA

samples, can be used to validate and define criteria for particular assays and perform necessary calibrations (French et al. 2015).

Building upon these ideas, Lipsitz et al. have been proponents of using quality-by-design principles to design cell manufacturing processes, an approach already commonly used by small-molecule pharmaceutical manufacturers. Quality-by-design integrates both scientific knowledge and risk analysis and involves product and process description, characterization, design, and monitoring. It also highlights the need to understand desired characteristics of the end product, attributes that influence safety and efficacy, and what parameters influence those attributes. In the case of pluripotent stem cell-derived cardiomyocytes, potency is now often evaluated via electrophysiological read-outs, but force-of-contraction assays may be of value for applications where they cells are intended to be used as a therapy and ultimately act as new heart tissue. One of the major issues for the use of pluripotent stem cell-derived cardiomyocytes in clinical applications is purity, since nodal cells and non-cardiomyocytes could potentially promote arrhythmias, and undifferentiated pluripotent stem cells can lead to teratomas. Ultimately, these authors highlighted a need to understand the influence of various factors such as dissolved oxygen, pH, metabolic by-products, and media exchange rate and strategy, and then monitor and control them if necessary, with the ability to reduce or at least understand the effect of variability in reagents being of equal importance (Lipsitz et al. 2018).

7 Final Remarks: Strategically Matching Approach to Application

Despite the challenges associated with the derivation and use of iPSC-derived cardiomyocytes, they have proven to be an extremely powerful platform in basic and translational science. Not only have these cells been used to model a wide variety of cardiac diseases, but they show great promise for drug safety testing and have demonstrated efficacy in large animal pre-clinical models (Gao et al. 2018; Ishida et al. 2018; Liu et al. 2018; Musunuru et al. 2018; Yoshida and Yamanaka 2017). Moreover, while there is still much to learn, there has been a progressively detailed understanding of how variability and heterogeneity in the iPSC-CM platform arises, and a number of proposed approaches to further enhance desired characteristics in the final cellular products. However, it should be recognized that it may not be feasible to validate and optimize all possible parameters and cellular properties for every single cell line. Therefore, it will become increasingly necessary to define which criteria are most important for a given study or application, and produce iPSC-CMs with those considerations in mind. This approach of developing purpose-built iPSC-CM products will require identifying which cellular features or functionalities are needed to achieve the ultimate purpose, and choosing the materials, protocols, and quality control measures based on those desired end properties. For instance, disease modeling relies heavily on careful selection of both the patient and control cell lines in order to uncover disease phenotypes and differentiate those from other aspects of inter-individual or inter-clonal variability. Conversely, for drug screening applications, the particular cell lines used may not matter as much as being able to achieve high batch-to-batch consistency.

There must also be an element of being able to balance risk versus benefit, which becomes particularly relevant for more translational or clinical applications. As one reflection of this, it may be appropriate to pursue the first clinical trials of iPSC-CMs in patient populations with severe disease and limited alternative treatment options, since those patients have the potential for achieving the greatest potential benefit despite the risks of a novel therapeutic modality. Then as quality control criteria for achieving maximal safety and efficacy are established, the number of suitable disease indications may grow.

Currently, the systems are in place to successfully create purpose-built iPSC-CMs for many applications. In fact, some of the challenges

associated with these cells can actually be considered assets in expanding their versatility. For example, non-cardiomyocytes can be leveraged to modulate cardiomyocyte properties or reveal disease phenotypes, genetic variability can be used to recapitulate diverse populations *in vitro*, and the ability of these iPSC-CMs to display properties of varied cardiac subtypes and maturation states means that the cells can act as models of distinct regions of the heart across developmental stages. The future will only aid in refining these cells and expanding their utility. Therefore, as the field continues to discover what factors impact cardiomyocyte differentiation, purity, and ultimate phenotypes, as well as develop additional means by which to evaluate and control these factors, the potential of this platform will only grow.

Acknowledgements This work was supported by the Todd and Karen Wanek Family Program for Hypoplastic Left Heart Syndrome at Mayo Clinic. Figures were created with BioRender.

Conflict of interest T.J.N and Mayo Clinic have licensed reprogramming technology to ReGen Theranostics, Inc. in Rochester, MN.

Ethical approval The authors declare that this article does not contain any studies with human participants or animals.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 31–48
https://doi.org/10.1007/5584_2019_374

Published online: 16 April 2019

Direct Lineage Reprogramming in the CNS

Justine Bajohr and Maryam Faiz

Abstract

Direct lineage reprogramming is the conversion of one specialized cell type to another without the need for a pluripotent intermediate. To date, a wide variety of cell types have been successfully generated using direct reprogramming, both *in vitro* and *in vivo*. These newly converted cells have the potential to replace cells that are lost to disease and/or injury. In this chapter, we will focus on direct reprogramming in the central nervous system. We will review current progress in the field with regards to all the major neural cell types and explore how cellular heterogeneity, both in the starter cell and target cell population, may have implications for direct reprogramming. Finally, we will discuss new technologies that will improve our understanding of the reprogramming process and aid the development of more specific and efficient future CNS-based reprogramming strategies.

Keywords

Cellular reprogramming · Direct lineage conversion · Cellular heterogeneity · Neurological disease/Injury · Central nervous system

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Abbreviations

6-OHDA	6-hydroxydopamine
Ascl1	achaete-scute family bHLH transcription factor 1
BAM factors	combination of the transcription factors *Ascl1, Brn2 and Mytl1*
Brn2	POU Class 3 Homeobox 2
CHAT	Choline O-Acetyltransferase
c-Myc	cellular Myc
CNP	2′,3’-Cyclic Nucleotide 3’ Phosphodiesterase
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
DAT	Dopamine transporter
DDC	DOPA Decarboxylase
Dlx2	Distal-Less Homeobox 2
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
E47	transcription factor 3
Ezh2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
Fezf2	FEZ Family Zinc Finger 2
Foxa2	Forkhead Box A2
FoxG1	forkhead box G1
GABA	Gamma-amino butyric acid
GLUT1	glucose transporter protein type 1
GRN	gene regulatory network
Hb9	Motor Neuron And Pancreas Homeobox 1
iPSC	induced pluripotent stem cell

Isl1	Insulin gene enhancer protein ISL-1
ITPR2	Inositol 1,4,5-Trisphosphate Receptor Type 2
Klf4	Kruppel Like Factor 4
Lhx3	LIM Homeobox 3
Lmx1a	LIM Homeobox Transcription Factor 1 Alpha
MBP	myelin basic protein
Mecom	MDS1 And EVI1 Complex Locus
miRNA	microRNA
MOL6	mature oligodendrocytes expressing Grm3 (Glutamate Metabotropic Receptor 3) and Jph4 (Junctophilin 4)
MS	multiple sclerosis
MyoD	myogenic differentiation 1
Myt1l	myelin transcription factor 1 like protein
NANOG	Nanog Homeobox
NeuroD1	Neurogenic Differentiation Factor 1
NFIA	Nuclear Factor I A
NFIB	Nuclear Factor I B
NG2 glia	Neural/glial antigen 2 expressing glial cells
Ngn2	Neurogenin 2
Nkx6.2	NK6 Homeobox 2
NSC	neural stem cell
NSPC	neural stem and progenitor cells
Nurr1	Nuclear receptor related 1 protein
OCT4	octamer-binding transcription factor 4
Olig1	Oligodendrocyte Transcription Factor 1
Olig2	Oligodendrocyte Transcription Factor 2
OPC	oligodendrocyte progenitor cell
Pax6	Paired Box 6
ROS	reactive oxygen species
S1 cortex	primary somatosensory cortex
sc RNA-seq	single cell RNA sequencing
Sox10	SRY-Box 10
Sox2	SRY-Box 2
Sox9	SRY-box 9
VMAT2	Vesicular monoamine transporter 2
VPA	valproic acid
Zfp536	Zinc Finger Protein 536

1 Introduction

Historically, it was believed that cell fate was fixed after the completion of development (Heins et al. 2002; Barker et al. 2018; Faiz and Nagy 2013; Vierbuchen and Wernig 2011). However, discoveries including cell fusion, somatic nuclear transfer, and most recently reprogramming to pluripotency (or the generation of induced pluripotent stem cells, iPSCs) have shown that cell fate is flexible (Faiz and Nagy 2013; Vierbuchen and Wernig 2011; Gurdon 1962; Chen et al. 2015; Graf and Enver 2009; Blau et al. 1983, 1985; Xie et al. 2004; Takahashi and Yamanaka 2006). In this review, we will focus on direct lineage reprogramming, which is the conversion of one specialized cell type to another (Graf and Enver 2009; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016) (Fig. 1). This was first demonstrated by Davis and colleagues, who showed that overexpression of *MyoD* resulted in the conversion of fibroblasts to myoblasts (Davis et al. 1987). More recently, a number of studies have demonstrated successful conversion of various other cell types, both *in vitro* and *in vivo* (for review, see (Barker et al. 2018; Chen et al. 2015; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016)). This ground-breaking technology has had a significant impact on the field of regenerative medicine, as directly reprogrammed cells could be used to replace those lost or damaged to disease or injury (Barker et al. 2018; Faiz and Nagy 2013; Chen et al. 2015; Graf and Enver 2009; Takahashi and Yamanaka 2006; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016).

Direct lineage conversion uses the delivery of specific factors to induce the conversion of cells without the need for a pluripotent intermediate (Graf and Enver 2009; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016). Typically, transcription factors have been used, but the feasibility of using small molecules (Hu et al. 2015; Li et al. 2015),

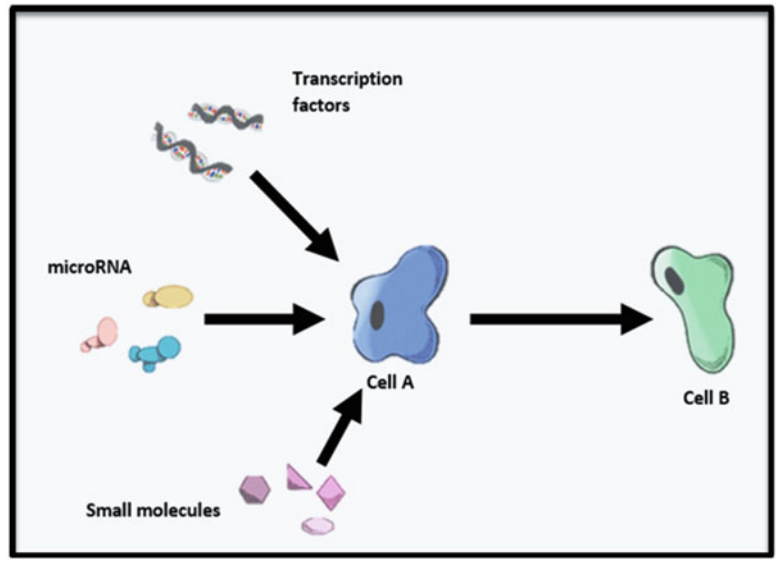

Fig. 1 Direct lineage reprogramming. Direct lineage reprogramming is the conversion of one specialized cell type (Cell A) to another (Cell B) without the need for a pluripotent intermediate. It can be initiated by a variety of methods (small molecules, microRNAs), but is typically achieved by the overexpression of transcription factors. Illustrated by Kayla Hoffman-Rogers

microRNAs (Yoo et al. 2011; Victor et al. 2014), and CRISPRa (Chakraborty et al. 2014; Black et al. 2016) (Fig. 1) has also been demonstrated. To date, most studies have identified reprogramming factors based on their role in specifying a target cell fate during development, and/or uniquely high gene expression in a target cell. For example, Najm and colleagues used microarray data from different central nervous system (CNS) cells to identify a pool of genes that were exclusively upregulated in oligodendrocytes (Najm et al. 2013). These genes were then tested for their ability to convert fibroblasts to oligodendrocytes (Najm et al. 2013).

Many studies have focused on identifying "core" factors that are needed for cellular conversion using a reductionist-additive approach (Ninkovic and Götz 2018). In this paradigm, one factor is removed at a time until the "necessary" factor(s) are found (Ninkovic and Götz 2018). Additional factors are then added back until a desired phenotype or efficiency is achieved (Ninkovic and Götz 2018). For example, following confirmation that a cocktail of eleven reprogramming factors was able to reprogram fibroblasts to motor neurons, Son and colleagues removed one transcription factor at a time and analyzed its effect on the conversion (Son et al. 2011). This allowed them to determine that *Ascl1* or *Lhx3* were crucial for fibroblast to neuron conversion (Son et al. 2011). Then, to determine the optimal combination of transcription factors for a motor neuron phenotype, they added back single transcription factors and identified a "core set" of seven (*Ascl1/Brn2/Myt1l/Lhx3/Hb9/Isl1/Ngn2)* (*Son* et al. 2011). This approach suggests that an end goal is to achieve reprogramming with the smallest number of factors. Indeed, the seminal study by Davis and colleagues used only *MyoD* – highlighting the feasibility of a single factor for direct lineage reprogramming (Davis et al. 1987). One transcription factor for reprogramming may be favorable for future clinical applications, both in terms of feasibility of delivery and patient safety and tolerability. While it has been argued that single-factor reprogramming results in immature cell phenotypes (Morris 2016), neuronal reprogramming strategies using only one factor have resulted in the generation of mature and functional neurons, albeit at times with a slower maturation rate (Chanda et al. 2014; Zhu et al. 2018; Heinrich et al. 2010; Guo et al. 2014).

Interestingly, single-factor lineage reprogramming highlights the ability of certain reprogramming factors to behave as "pioneers"

(Ninkovic and Götz 2018). Pioneer factors can bind to closed areas of chromatin and recruit supporting transcription factors that may be needed to initiate the reprogramming process (Ninkovic and Götz 2018). Further, it has been suggested that the feasibility and efficiency of conversion using single factors may be due to their pioneer activity (Ninkovic and Götz 2018). For example, the pioneer factor *Ascl1*, may endogenously recruit other factors beneficial for fibroblast to neuron conversion, such as *Brn2* and *Myt1l* (Ninkovic and Götz 2018). This ability to bind to closed areas of chromatin demonstrates one way in which a starting cell state can be overridden; as in development, the genes regulating alternate cell fates are epigenetically repressed via chromatin modifications (Ninkovic and Götz 2018). Although a valuable insight into how reprogramming is initiated, many of the mechanisms that drive direct reprogramming have yet to be fully elucidated. It has been suggested that this is a complex process, dependent on many variables, including chromatin remodeling (Ninkovic and Götz 2018; Wapinski et al. 2017) and metabolic changes (Gascón et al. 2016, 2017b), amongst others (see (Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a, b; Masserdotti et al. 2015, 2016; Gascón et al. 2016) for a comprehensive review).

While the mechanisms of reprogramming remain unclear, the applicability of direct reprogramming technology is unmistakable. Direct lineage conversion has been used in many tissue systems and provides a novel therapeutic option for drug-resistant diseases or diseases with no current treatment options (Xu et al. 2015; Berninger 2010). In this review we will use the neural lineage as a model system to explore direct lineage reprogramming. Most studies have focused on direct reprogramming to neurons (reviewed in (Chen et al. 2015; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016)), because of the significant loss or injury to these cells in most neurological conditions. However, other neural lineage cells, for example, oligodendrocytes, may also be of interest. We will discuss the progress and current state of the field of direct lineage reprogramming with regards to all the major CNS cell types. We will explore how cellular heterogeneity, both in the starter cell population and the target cell type, may have implications for direct reprogramming. Finally, we will discuss new technologies that will improve our understanding of direct reprogramming and development of future conversion strategies.

2 Direct Reprogramming to a Neural Cell Fate

2.1 Overview

The first report of direct reprogramming to cells of the neural lineage used the transcription factor *Pax6* to convert astrocytes to neurons *in vitro* (Heins et al. 2002). Subsequent studies showed that the delivery of other transcription factors, such as *Ascl1* (Chanda et al. 2014), *Brn2* (Zhu et al. 2018) and *Ngn2* (Heinrich et al. 2010), could also convert astrocytes to neurons *in vitro*. Direct conversion has also been used to generate other neural cells such as oligodendrocytes (Najm et al. 2013; Yang et al. 2013; Mokhtarzadeh Khanghahi et al. 2018) and astrocytes (Caiazzo et al. 2015; Tian et al. 2016). It has also been shown that a wide variety of cell types, including those of a non-neural lineage, can be converted to the neural lineage. Fibroblasts and hepatocytes, two examples of non-neural cells, were successfully reprogrammed to neurons using a combination of *Brn2*/*Mytl1*/*Ascl1* (Vierbuchen et al. 2010; Marro et al. 2011). There are both advantages and disadvantages in using cells that belong to non-neural lineages as a source population for reprogramming. Veritably, it broadens the potential scope of direct reprogramming, as it does not limit choice of a starting cell type. Conversely, neural lineage cells, such as astrocytes, may already have relevant epigenetic marks and active transcription factors, that may result in easier reprogramming (Faiz and Nagy 2013; Ninkovic and Götz 2018). Thus, future studies must include a functional comparison of cells that are generated from neural versus non-neural starter populations.

Following initial *in vitro* studies, a number of reports demonstrated *in vivo* reprogramming in the brain and spinal cord. This is of particular interest for brain repair, as it enables the targeted generation of new cells at the site of injury and circumvents the need for transplantation of exogenous cells and the associated risks, namely immune-rejection and the potential for cell mutagenesis from long-term cell culture (Faiz and Nagy 2013; Xu et al. 2015; Gascón et al. 2017a). It also provides an alternative to strategies using endogenous neural stem cells that reside within the brain and spinal cord. Attempts to generate neurons from these neural stem cells have resulted in low differentiation into the proper mature neuronal phenotypes, and poor long-term survival (Barker et al. 2018; Gascón et al. 2017a; Arvidsson et al. 2002; Thored et al. 2007).

In 2005, Buffo and colleagues demonstrated for the first time in the CNS that the manipulation of transcription factors could alter cell fate *in vivo* (Buffo et al. 2005). They converted NG2 glia into cells of a neuronal phenotype by inhibiting the expression of *Olig2* (Buffo et al. 2005). This inhibition was achieved through the specific delivery of the dominant negative form of *Olig2* to NG2 glia (Buffo et al. 2005). *In vivo* direct conversion has now been shown in the healthy brains of both young and old mice (Niu et al. 2013; Rouaux and Arlotta 2013). Of clinical relevance, the success of *in vivo* direct reprogramming has also been demonstrated in a number of models of CNS injury and disease, including stroke (Faiz et al. 2015), stab wound injury (Chen et al. 2015; Guo et al. 2014; Heinrich et al. 2014), spinal cord injury (Su et al. 2014), Alzheimer's disease (Chen et al. 2015; Guo et al. 2014) and Parkinson's disease (Rivetti di Val Cervo et al. 2017). Interestingly, it has been suggested that aspects of the injured/diseased environment, such as the increase of beneficial growth factors, increased plasticity of glial cells and increased glycolysis may actually enhance the reprogramming process (Gascón et al. 2017a; Guo et al. 2014; Grande et al. 2013). These disease-induced changes could explain why some reprogramming paradigms have encountered success in an injury context, but no conversion (or a significantly reduced conversion) was observed when the same transcription factor(s) were delivered to the uninjured brain (Heinrich et al. 2014; Grande et al. 2013). Conversely, it has also been noted that an increased production of reactive oxygen species (ROS) during injury could be deleterious to newly generated cells and explain the discrepancy in conversion success between *in vitro* and *in vivo* studies (Gascón et al. 2017a). A better understanding of the mechanisms that underlie each particular injury or disease model will allow for reprogramming strategies that are tailored and optimized for different applications.

2.2 Target Cell Type

Many neurological disorders or conditions have at their core, a significant loss or injury to the cells of the CNS. However, not all disorders implicate the same cells and as such, it is important to generate specific cell types that are needed for a particular disorder. The versatility of direct lineage reprogramming technology is clear – studies have shown the generation of all the main cell types of the CNS, including certain subtypes and progenitors.

2.2.1 Neurons

Generating Neurons In Vitro

Neurons are affected in a wide variety of neurological conditions, and thus direct lineage reprogramming strategies have mainly been focused on regenerating these cells. Since their seminal *Pax6* study, work from Magdalena Götz's lab has also demonstrated that a combination of *Ascl1/Dlx2* or *Ngn2* results in the conversion of astrocytes to GABAergic and glutamatergic neurons, respectively (Vierbuchen and Wernig 2011; Xu et al. 2015; Heinrich et al. 2010). Simultaneously, work done by Vierbuchen and colleagues established the ability of the combination of *Ascl1/Brn2/Mytl1* (referred to as BAM factors) to induce glutamatergic neurons from fibroblasts (Vierbuchen et al. 2010). The

conversion of glial cells (both astrocytes and NG2 glia) to neurons using *NeuroD1* by Gong Chen's lab further demonstrated that transcription factors involved in later stages of neuronal development could also be used to regenerate neurons (Guo et al. 2014).

A number of other starter cell types have also been successfully converted to neurons. Non-neural cell types, such as pericytes, have been reprogrammed to glutamatergic and GABAergic cells (Karow et al. 2018) and the BAM factors have been used to reprogram hepatocytes to glutamatergic-like neuronal cells (Marro et al. 2011). Additionally, it has been shown that microglia can be converted to functional neurons with the delivery of *NeuroD1* alone (Matsuda et al. 2019)**.**

Importantly, the type of neuron lost or affected in a particular disease is often of a specific subtype (i.e.: dopaminergic neurons in Parkinson's disease and motor neurons in Amyotrophic Lateral Sclerosis), and differs across various neurological conditions (Faiz and Nagy 2013; Chen et al. 2015; Xu et al. 2015; Wang and Zhang 2018; Masserdotti et al. 2016). As such, the generation of a random assortment of neuronal subtypes, or the ability to generate only one specific subtype would likely be of minimal therapeutic benefit. For example, generating cholinergic neurons in Alzheimer's disease is likely to confer more benefit than in Parkinson's disease, where dopaminergic neurons are needed. Direct reprogramming must therefore reliably generate subtype specific cell types appropriate for the neurological deficit in question (Faiz and Nagy 2013; Chen et al. 2015; Xu et al. 2015; Wang and Zhang 2018; Masserdotti et al. 2016). Accordingly, *in vitro* studies have shown the generation of dopaminergic (Rivetti di Val Cervo et al. 2017; Kim et al. 2011; Caiazzo et al. 2011; Sheng et al. 2012), motor (Son et al. 2011), serotonergic (Vadodaria et al. 2016), and cholinergic (Liang et al. 2018; Liu et al. 2013) neurons, amongst others (see (Masserdotti et al. 2016) for in depth review) using specific combinations of transcription factors.

In summary, direct lineage reprogramming *in vitro* is clearly feasible, customizable and reliable in generating new neurons. However, *in vitro* lineage conversion still requires transplantation into the brain.

Generating Neurons In Vivo

One of the most exciting features of direct reprogramming is the ability to target endogenous cells at their source. Thus, *in vivo* studies generating novel populations of neurons are of particular interest to the field. Work performed by a number of groups has shown the reliable generation of new neurons *in vivo* using direct reprogramming in healthy and injured environments, and has been extensively reviewed elsewhere.(Chen et al. 2015; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016) What is lacking and of significant interest however, is a systematic comparison of different transcription factors and delivery strategies in various models of disease and injury (Gascón et al. 2017a). Although the transcription factors used in these studies (*Sox2* (Niu et al. 2013*;* Heinrich et al. 2014), BAM factors (Torper et al. 2013), *NeuroD1* (Guo et al. 2014) and *Ascl1/Lmx1a/Nurr1* (Torper et al. 2015)) correspond to *in vitro* studies, there is variation with regards to the delivery system used. It has been proposed that the choice of delivery system may affect the reprogramming paradigm, as there is variance in their temporal kinetics (Gascón et al. 2017a). As such, clear conclusions on the "best" direct reprogramming paradigm for a particular starting cell type, target cell type or disease state cannot yet be made with certainty. Nonetheless, these newly generated neurons are capable of surviving, maturing and integrating into the pre-existing neural circuitry, as shown by electrophysiological and functional assays (Guo et al. 2014; Niu et al. 2013; Heinrich et al. 2014; Torper et al. 2013, 2015).

One hurdle that remains with regards to *in vivo* neuronal reprogramming is subtype specific neuronal regeneration. Success seen in *in vitro* studies of neuronal subtype generation has not been replicated to the same extent *in vivo,* even with

the use of the same transcription factors (Chen et al. 2015; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Masserdotti et al. 2016). The reasons for this are unclear, but as discussed above, could be attributed to the deleterious environment that results from injury (Gascón et al. 2017a). A more complex *in vivo* environment may require multiple transcription factors and/or a combination of both transcription factors and small molecules or microRNA to generate specific neuronal sub-types. In fact, Rivetti di Val Cervo and colleagues successfully obtained a novel population of dopaminergic neurons from astrocytes *in vivo* when they used a combination of both *NeuroD1/Ascl1/Lmx1a* and the microRNA miR218 (Rivetti di Val Cervo et al. 2017).

Neuron to Neuron Reprogramming

While most studies have focused on converting non-neuronal cells to neurons, reports of neuron to neuron reprogramming show that there is cell fate flexibility even within this population. In the cortex, Rouaux and Arlotta were able to successfully convert layer 2/3 callosal projection neurons into layer 5/6 corticofugal projections using only the transcription factor *Fezf2* (Rouaux and Arlotta 2013). More recently, Niu and colleagues used a combination of *Sox2/Nurr1/Foxa2/Lmx1a* paired with valproic acid (VPA) to reprogram striatal neurons to dopaminergic neurons (Niu et al. 2018). Interestingly, they showed that these newly induced dopaminergic cells arise directly from the striatal neurons, without passing through a progenitor stage (Niu et al. 2018). These studies beg the question of whether a shared identity (i.e. neuron) between the starting and target cell is an important consideration for easily generating specific neuronal subtypes *in vivo*.

2.2.2 Oligodendrocytes

Oligodendrocytes play crucial roles in maintaining proper cell signaling in the CNS and many diseases result from their widespread loss or malfunction. Oligodendrocyte death and subsequent de-myelination is characteristic to the pathology of multiple sclerosis (Lassmann et al. 2012; Reich et al. 2018; Sawcer et al. 2014), and a reduction in myelin is seen in multi-system atrophy (Burn and Jaros 2001). Oligodendrocytes have also been implicated in Alzheimer's disease. Although traditionally thought of as a grey matter disease, Alzheimer's disease presents with white matter disruption, impaired myelination patterns and decreased oligodendrocyte and oligodendrocyte progenitor gene expression (Desai et al. 2009, 2010; Roth et al. 2005). Interestingly, a mouse model of Alzheimer's disease showed that impaired myelination and decreased CNPase and MBP expression precedes the onset of tau and amyloid pathology (Desai et al. 2009). Finally, white matter damage and oligodendrocyte dysfunction have been proposed as a risk factor and predictor of stroke (Kuller et al. 2004) and of schizophrenia (Cassoli et al. 2015).

Given the significant implication of oligodendrocytes in disease, strategies to restore or replenish damaged or lost oligodendrocytes are needed. Yet, there is a clear disparity in the number of studies investigating direct reprogramming to neurons versus oligodendrocytes. Only three studies to date have looked at using direct reprogramming to generate new populations of oligodendrocytes and their precursors. Work done by Najm and colleagues, as well as Yang and colleagues produced oligodendrocyte progenitor cells (OPCs) and oligodendrocytes from fibroblasts *in vitro* using combinations of transcription factors involved in OPC development and oligodendrocyte function (*Sox10/Olig2/Nkx6.2* and *Sox10/Olig2/Zfp536*, respectively) (Najm et al. 2013; Yang et al. 2013). The oligodendrocytes generated from both these studies expressed characteristic OPC and oligodendrocyte markers and showed myelination capability in transplantation experiments (Najm et al. 2013; Yang et al. 2013). More recently, Khanghahi and colleagues reported that both *in vitro* and *in vivo* delivery of *Sox10* alone to astrocytes in cuprizone induced de-myelinated mice results in the generation of new oligodendrocyte-like cells (Mokhtarzadeh

Khanghahi et al. 2018). Cells transduced *in vitro* expressed markers of OPC and oligodendrocyte lineage and were transplanted into the corpus callosum of the cuprizone mice (Mokhtarzadeh Khanghahi et al. 2018).

Although promising, there are significantly fewer studies of oligodendrocyte reprogramming in comparison with neuronal reprogramming. Future work examining oligodendrocyte generation and the optimal factors involved are warranted.

2.2.3 Astrocytes

Most reprogramming studies have focused on astrocytes as the starter cell type, rather than the target cell type. Nonetheless, a few reports have shown the feasibility of generating astrocytes. From a pool of 14 genes involved in determining astrocyte fate, Caiazzo and colleagues found that the combination of *NFIA/NFIB/Sox9* could successfully reprogram fibroblasts to astrocytes (Caiazzo et al. 2015). Similarly, work by Tian and colleagues demonstrated that a cocktail of 6 small molecules generated functional astrocytes from fibroblasts (Tian et al. 2016). Given the recent knowledge that a subset of astrocytes, A2 cells, are neuroprotective and conducive to recovery following injury, future studies examining conversion of a starter cell to a beneficial astrocyte subtype, such as the A2 phenotype, may be of interest (Liddelow and Barres 2017; Liddelow et al. 2017; Zamanian et al. 2012; Toft-Hansen et al. 2011).

2.2.4 Stem/Progenitor Cells

To date, studies have generated both neural stem cells and glial progenitors that can give rise to mature neurons and glia. Researchers in the Wernig lab demonstrated that the use of two transcription factors, *FoxG1* and *Brn2,* that are important for neural stem cell (NSC) fate, could convert fibroblasts into tripotent NSCs (Lujan et al. 2012). Not only were these NSCs capable of further differentiation into functional neurons, astrocytes and oligodendrocytes, but they also demonstrated clear proliferative capacity, capable of being passaged up to 17 times, without loss of function (Lujan et al. 2012). Additionally, other studies by Han and colleagues, as well as Ring and colleagues have shown conversion of fibroblasts to NSCs using a combination of *Brn2/Sox2/Klf4/c-Myc/E47* and *Sox2* alone, respectively (Xu et al. 2015; Han et al. 2012; Ring et al. 2012). Similarly, astrocytes have also been successfully converted to NSPCs through delivery of both single factors (*OCT4*, *SOX2* or *NANOG*) (Corti et al. 2012) and combination of factors (*Foxg1/Sox2/Brn2*) (Ma et al. 2018). Finally, OPCs have also been generated in the Tesar lab using a combination of the transcription factors *Sox10/Olig2/Nkx6.2* (Najm et al. 2013). The pathophysiology of a particular disease may determine whether it is advantageous to induce progenitor populations or mature cell types. Given this, future studies comparing the functional outcomes of direct reprogramming to progenitors versus mature cells will be of interest.

2.2.5 Cell Heterogeneity

It is now clear that neurons are not the only cell type in the CNS with delineated subtypes comprising different roles, with recent work demonstrating intra-cellular differences in oligodendrocyte populations. Using single-cell RNA sequencing (sc RNA-seq), Marques and colleagues found unique transcriptome profiles according to the age and region of oligodendrocytes and progenitors in mice (Marques et al. 2016). In addition, they noted a novel population of cells (ITPR2+) hypothesized to be involved in periods of rapid myelination (Marques et al. 2016). Furthermore, they noted that varying regions of the CNS were associated with differing forms of mature oligodendrocytes, such as MOL6 oligodendrocytes specific to the S1 cortex and corpus callosum (Marques et al. 2016). Differences in mature oligodendrocytes in the CNS are also present in the context of disease. Work by Jäkel and colleagues showed that not only is there a similar heterogeneity of oligodendrocytes in humans, but that MS patients had a unique loss of certain mature oligodendrocyte populations (OLIG1+) when compared to controls (Jäkel et al. 2019). These findings will be of particular importance when considering transcription factor cocktails used to create

functional, myelinating oligodendrocytes and how the transcription factor combinations may vary based on disease need.

2.3 Starting Cell Type

When performing direct lineage reprogramming, genetic systems with cell specific promoters can allow for targeting of a precise cellular population (Wang and Zhang 2018). Traditionally, starter cell populations have been chosen based on their developmental closeness to the target cell type (Gascón et al. 2017a; Masserdotti et al. 2016; Waddington 1957). However, developmental closeness may not be the only, or even "best" reason for choosing a particular starting cell type. In the context of injury or disease, it may be more relevant to choose a starting cell based on the role of that cell at the time of reprogramming. For example, cells that contribute to ongoing neuronal death and therefore disease pathology may be the most clinically relevant choice for reprogramming.

2.3.1 Developmental Closeness

The Waddington model, used to explain normal cell fate determination, denotes a linear differentiation and restriction pattern of cell type during development (Waddington 1957). It was hypothesized that more closely related starter and target cells would be easier to convert and reprogram (Gascón et al. 2017a; Masserdotti et al. 2016). Initial studies using starting cell populations that belonged to a non-neuronal lineage, such as fibroblasts, required a combination of transcription factors (Son et al. 2011; Vierbuchen et al. 2010; Kim et al. 2011; Caiazzo et al. 2011; Sheng et al. 2012) for conversion to neurons. In contrast, starting cell populations within the neural lineage, such as astrocytes, could be successfully converted with just one transcription factor (Gascón et al. 2017a; Heinrich et al. 2010; Guo et al. 2014; Niu et al. 2013). However, more recent work has demonstrated the use of single factors to convert non-neural cells neurons. Chanda and colleagues demonstrated the generation of neurons from fibroblasts using only *Ascl1* (Chanda et al. 2014) and *MYT1L* alone has been shown to reprogram pericytes into mature cholinergic neurons (Liang et al. 2018). Given these findings, a new model of reprogramming has been proposed – the Cook's Island model (Masserdotti et al. 2016; Sieweke 2015). In this analogy, the starting cell is a boat and target cell types are the various islands to which the boat can travel (Masserdotti et al. 2016; Sieweke 2015). The boat may face various challenges or hurdles depending on the proximity of the island, but all are potentially accessible (Masserdotti et al. 2016; Sieweke 2015).

2.3.2 Cellular Heterogeneity

Cell heterogeneity within the starter cell population is of particular interest to direct lineage reprogramming. One specific subtype of astrocyte, for instance, may be especially conducive to generating a particular subtype of neuron (Fig. 2). Conversely, as we broaden our scope of potential cells that can be generated by reprogramming, it will be important that those generated are of the correct subtype for the particular disease or injury at hand.

Astrocytes

Recent work by Liddelow and colleagues has shown that astrocytes have at least two defined functional states in the context of disease/injury, termed A1 and A2 (Liddelow and Barres 2017; Liddelow et al. 2017). A1 cells are present in many disease states, including Alzheimer's Disease, Huntington's Disease, Parkinson's Disease and Multiple Sclerosis (Liddelow and Barres 2017; Liddelow et al. 2017). Furthermore, A1 astrocytes lose many normal astrocytic functions, such as phagocytic capacity and the promotion of synaptic formation and become toxic, killing neurons and oligodendrocytes, and impairing oligodendrocyte progenitor cell (OPC) differentiation (Liddelow and Barres 2017; Liddelow et al. 2017). Conversely, A2 cells are thought to be neuroprotective (Liddelow and Barres 2017). They upregulate a number of neurotrophic factors, cytokines and thrombospondins that may help repair and rebuild lost synapses (Liddelow and Barres 2017; Zamanian et al. 2012). In addition, it has also

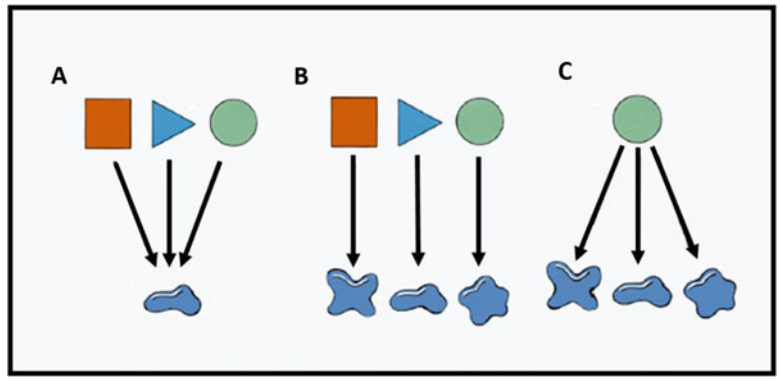

Fig. 2 Cellular heterogeneity. Heterogeneity of both the starting and target cell populations is an important consideration for direct lineage reprogramming. Diversity of the starting cell population may determine what types of target cell types are generated. All subsets of starting cells may give rise to one only type of target cell (**a**). Alternately, specific subtypes of starting cells may only give rise to specific subtypes of target cells (**b**). Or, only one type of starting cell may generate all target cell subtypes (**c**). Illustrated by Kayla Hoffman-Rogers

been postulated that there are many more astrocyte subtypes that have yet to be characterized (Liddelow and Barres 2017).

Given the heterogeneity of astrocytes, it may prove advantageous to reprogram astrocyte subtypes that are detrimental to disease outcome or progression (such as A1 cells), over reprogramming protective subtypes (such as A2 cells) that could lead to worse disease outcomes (Liddelow and Barres 2017; Liddelow et al. 2017; Zamanian et al. 2012; Toft-Hansen et al. 2011). Furthermore, it would be worthwhile investigating whether A1 neurotoxic astrocytes could be reprogrammed to their more beneficial A2 counterparts. This has been suggested in work done by Gong Chen's lab, which noted that astrocytes transduced in their *NeuroD1* mediated astrocyte to neuron paradigm showed a reduction in A1 gene expression prior to their conversion to neurons (Zhang et al. 2018).

Microglia

Microglia have also been shown to have at least two distinct subtypes, termed M1 and M2, with more recent work suggesting that many sub-classes, or even a continuum of microglial states may exist (Liddelow and Barres 2017; Boche et al. 2013; Tang and Le 2016). These subtypes pertain to activation states that correspond with particular functions: M1 microglia are pro-inflammatory and potentially damaging to neighboring cells, whereas M2 microglia are involved in tissue repair and are immunosuppressive (Liddelow and Barres 2017; Boche et al. 2013). Interestingly, this activation pattern is thought to be dependent on the particular injury or disease state (Boche et al. 2013). In fact, work by Tang and Le have shown that changes in M1 and M2 microglia phenotype correspond to different stages of disease (Tang and Le 2016). This knowledge may be of particular relevance in future clinical applications of direct reprogramming, allowing for tailored paradigms based off disease progression.

2.4 Direct Reprogramming: Readouts

In order to ensure the clinical relevance and feasibility of direct reprogramming, there is a need to generate mature cells that can integrate into existing host circuitry, have long-term survival and perform proper cell functions (Barker et al.

2018; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Berninger 2010; Yang et al. 2011). If the cells generated fail to meet these conditions, it is unlikely that they could be utilized as a novel therapy for neurological diseases.

2.4.1 Characterization of Target Cells

To characterize newly reprogrammed cells, many studies have examined the expression of cell type specific proteins and patterns of global gene expression that correspond to native cells (Barker et al. 2018; Faiz and Nagy 2013; Xu et al. 2015; Gascón et al. 2017a; Masserdotti et al. 2016; Yang et al. 2011). Some studies have also used a lack of gene/protein expression, of cells of unwanted lineages or of cells of the starting population to be indicative of proper cell conversion. For example, Niu and colleagues demonstrated that reprogrammed dopaminergic neurons expressed cell-type specific markers [DDC (DOPA Decarboxylase), VMAT2 (Vesicular monoamine transporter 2) and DAT (Dopamine transporter)], but also confirmed that the reprogrammed cells did not express markers associated with other neuronal subtypes (cholinergic or glutamatergic, using CHAT and GLUT1, respectively) (Niu et al. 2018).

Functional assays specific to the desired cell type are also important (Barker et al. 2018; Xu et al. 2015; Wang and Zhang 2018; Gascón et al. 2017a; Berninger 2010; Yang et al. 2011) (Fig. 3). For neurons, there are both general and subtype specific means of assessing neuronal function and integration (Yang et al. 2011). Patch-clamp recording can demonstrate whether reprogrammed neurons exhibit electrophysiological characteristics of neurons – their ability to fire action potentials and their synaptic patterns (Chen et al. 2015; Wang and Zhang 2018; Yang et al. 2011). Most studies to date have demonstrated mature, electrically active neurons, both *in vivo* and *in vitro*. The firing patterns of reprogrammed cells can also be compared to the expected firing patterns of native cells to assess similarity in function, as was done by Niu and colleagues (Niu et al. 2018). Furthermore, fluorescent reporters can be used to trace reprogrammed cells and assess the extent of their integration into host circuitry (Chen et al. 2015; Torper et al. 2015). For example, Torper and colleagues noted that in a mouse model of Parkinson's disease, newly generated neurons did not migrate to alternate regions of the CNS, but rather integrated locally (Torper et al. 2015).

For oligodendrocytes, the ability to myelinate is key. To characterize the reprogrammed OPCs or oligodendrocytes, studies have used mouse models of demyelination or impaired myelination (Najm et al. 2013; Yang et al. 2013; Mokhtarzadeh Khanghahi et al. 2018; Chernoff 1981; Blakemore 1972; Matsushima and Morell 2001). The *Shiverer* mouse, for example, lacks myelin basic protein (MBP) and consequently, the ability to form compact myelin (Chernoff 1981). It has been used in studies such as those done by Najm and colleagues to demonstrate the generation of MBP+ myelin following transplantation of OPCs that were directly reprogrammed from fibroblasts (Najm et al. 2013).

2.4.2 Functional Outcomes

Ultimately, the goal of direct reprogramming is to be a clinical treatment option for neurological diseases. Therefore, it is crucial to employ animal models of disease to determine whether reprogramming can induce functional recovery, slow disease progression or reverse disease progression/impairments all together (Chen et al. 2015; Xu et al. 2015). To date, there has been limited study of the outcomes of reprogramming with regards to disease progression or prevention in disease models. The first report of functional recovery was by Rivetti di Val Cervo and colleagues in a unilateral 6-hydroxydopamine (6-OHDA) mouse model of Parkinson's disease (Rivetti di Val Cervo et al. 2017). Following astrocyte reprogramming to dopaminergic neurons, newly generated neurons were capable of rescuing gait deficits and dopamine-deficient circling behaviors (Rivetti di Val Cervo et al. 2017). A second report by Chen and colleagues, showed improvement in motor and fear memory deficits following ischemia (Chen et al. 2018).

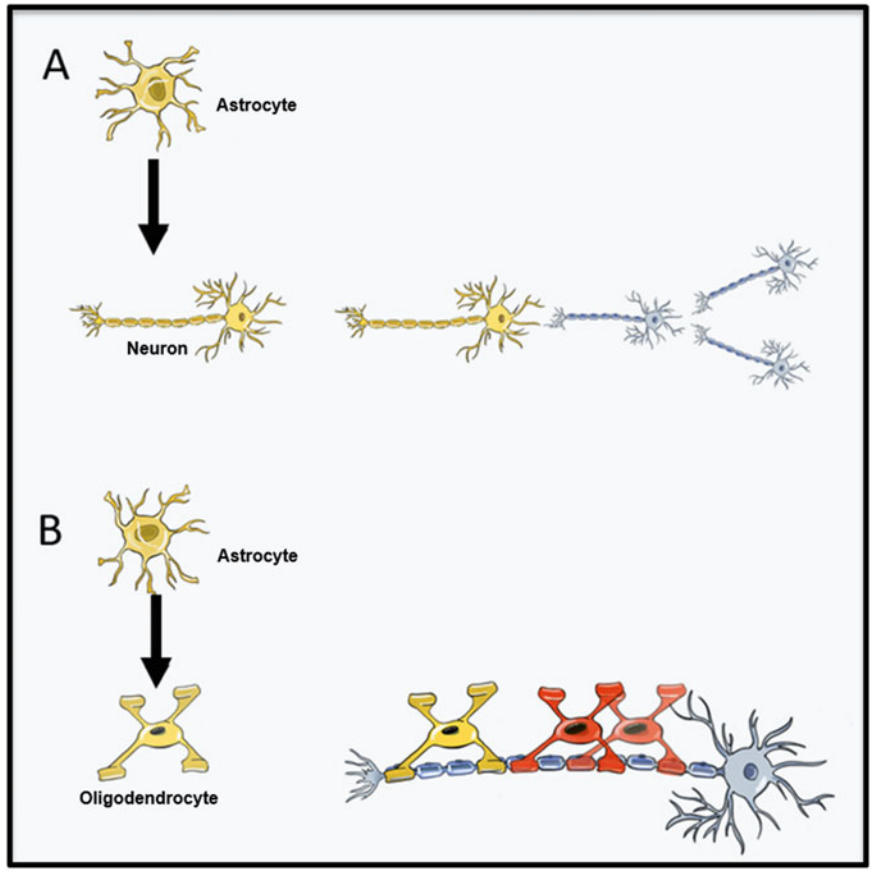

Fig. 3 Functional target cells. The goal of direct lineage reprogramming is to generate functional cells for repair or regeneration. Newly generated cells must recapitulate the function of their endogenous counterparts (blue neurons, red oligodendrocytes). After astrocyte to neuron conversion, new neurons (yellow) must fire action potentials, form synapses and integrate into the exiting host circuitry (blue neurons) (**a**). Similarly, new oligodendrocytes (yellow) must generate myelin and ensheath existing neurons like native oligodendrocytes (red) (**b**). Illustrated by Kayla Hoffman-Rogers

2.4.3 Application of New Technologies

Many exciting and novel technologies have recently emerged that will benefit our understanding of the reprogramming process and cellular outcomes. A new tool for analyzing reprogrammed cell identity is the CellNet database (Xu et al. 2015). It identifies gene regulatory networks (GRNs) in reprogrammed cells, and therefore enables confirmation of reprogramming factor expression in target cells and the comparison of gene expression profiles of experimental and naive cells (Xu et al. 2015; Cahan et al. 2014; Morris et al. 2014). Most striking, however, is the utility of CellNet in predicting how reprogramming paradigms could be improved, which is based on incorrect expression of GRNs (Xu et al. 2015; Morris et al. 2014).

In a pioneering study by Cadwell and colleagues, electrophysiological and single cell RNA-seq readouts were combined to create Patch-seq technology (Cadwell et al. 2016). This technique results in the simultaneous acquisition of cell transcriptomes (sc RNA-seq) and electrophysiological information (Patch-clamp readings), thereby correlating changes in cell function and the transcriptome within a single cell (Cadwell et al. 2016). This is of particular interest, as changes in cell function can be

predicted based on particular transcriptome patterns or modifications (Cadwell et al. 2016). This technology has already been used to identify and predict populations of highly functional reprogrammed neurons generated from iPSCs (Bardy et al. 2016). The application of Patch-seq in direct reprogramming studies is thus greatly warranted, as it would enable a more tailored approach for the creation of specific cell types by identifying reprogramming factors that produce bonafide reprogrammed cells.

CRISPR is another new technology that can be used to determine genes that are involved in cell fate changes and therefore, elucidate optimal transcription factor combinations for direct reprogramming paradigms (Liu et al. 2018). This strategy has unveiled novel factor(s) that result in the conversion of fibroblasts to neurons, such as Ezh2/Mecom, which were not traditionally thought to be key proneural genes, (Liu et al. 2018). As the field strives for subtype specific cell generation, as well as tailorable and translatable therapies, utilizing the power of CRISPR may be of great interest.

Finally, optogenetics and pharmacogenetics provide novel means by which to target populations of cells and manipulate their activity (Deisseroth 2011; Amamoto and Arlotta 2014; Steinbeck and Studer 2015). This technology can be used to specifically analyze whether newly generated cells directly contribute to functional recovery (Amamoto and Arlotta 2014; Steinbeck and Studer 2015). It can also be employed to potentiate the activity of reprogrammed cells (Amamoto and Arlotta 2014; Steinbeck and Studer 2015). Indeed, in a study done by Dell'Anno and colleagues, a DREADD pharmacogenic system was used to selectively activate reprogrammed dopaminergic neurons to enhance their activity (Dell'Anno et al. 2014). Researchers noted that upon activation of these cells, dopamine levels were increased, even up to 5 weeks following reprogramming, supporting the use of chemogenetics as an adjunct strategy to direct reprogramming (Dell'Anno et al. 2014).

3 Conclusions and Future Directions

The field of reprogramming is still in its infancy. Although significant progress has been made in our understanding of direct reprogramming in the CNS since the pioneering work done by Heins and colleagues (2002), new research into the mechanisms underlying direct reprogramming will allow us to tailor better strategies for brain repair. Studies that will determine the optimal starting cell types that are needed for the generation of functional target cells, as well as experiments that systematically compare the efficacy of different reprogramming paradigms are needed. Moreover, research into the impact of cellular heterogeneity in reprogramming will result in better designed reprogramming strategies that cater to a specific disease or injury state. Our progress will only become faster with the implementation of the novel, cutting technologies, such as CellNet and Patch-seq. Given the integrative and multi-faceted nature of direct reprogramming, it seems only fitting to employ an interdisciplinary approach as we move forward.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 49–56
https://doi.org/10.1007/5584_2019_394

Published online: 22 June 2019

Induced Pluripotent Stem Cells for Regenerative Medicine: Quality Control Based on Evaluation of Lipid Composition

Yusuke Nakamura and Yasuo Shimizu

Abstract

Clinical application of induced pluripotent stem cells (iPSCs), which can be differentiated into a wide variety of functional cells, is underway and some clinical trials have already been performed or are ongoing. On the other hand, the risk of carcinogenesis is an issue and the mechanism of cellular reprograming remains unknown. When iPSCs and differentiated cells are used for medical applications, quality control is also important. Here we discuss the possibility of performing quality control of iPSCs by evaluation of phospholipids, which are not just structural components of lipid bilayer membranes, but also have multiple physiological functions. Recently, methods for analysis of lipids have become more widely available and easier to perform. This article reviews the role of iPSCs in regenerative medicine and examines the possibility of using phospholipids for quality control of iPSCs and differentiated cells.

Keywords

Induced pluripotent stem cells · Regenerative medicine · Lipids · Quality control · LC-MS · Imaging mass spectrometry

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Abbreviations

ALS	amyotrophic lateral sclerosis
FIH	first-in-human
FOP	Fibrodysplasia ossificans progressive
IMS	imaging mass spectrometry
iPSCs	induced pluripotent stem cells
KLF4	Kruppel like factor 4
LC-MS	liquid chromatography-mass spectrometry
pPE	plasmalogen phosphatidylethanolamine
SM	sphingomyelin
SOX2	SRY-box 2

1 Introduction

1.1 iPSCs and Regenerative Medicine

Human induced pluripotent stem cells (iPSCs) were first established in 2007 by Yamanaka's group (Takahashi et al. 2007). These cells were generated by adding recombinant factors, such as OCT3/4, SRY-box 2 (SOX2), Kruppel like factor 4 (KLF4), or c-Myc, to cultures of somatic cells. iPSCs can be differentiated into various types of cells with potential clinical uses. However, there is a potential risk of carcinogenesis and mechanism of cellular reprograming is still unknown. It

is generally thought that there is a low risk of rejection of iPSCs, but some research has identified rejection of transplanted iPSCs (Zhao et al. 2011). Another group reported the transplantation of undifferentiated iPSCs, but stated that more detailed investigation was needed. There is the possibility of tumorigenic cells emerging from iPSCs or the immune system eliminating them as foreign cells (Okita et al. 2011). Despite these issues, iPSCs have many possible future clinical applications, including drug development and elucidation of the pathophysiology of various diseases.

Cells undergo various changes in the process of differentiation from undifferentiated pluripotent stem cells and also during the development of organs. To maintain the morphology of cells and organs during these processes, a supporting structure is required that preserves the integrity of each cell and/or organ. The cell membrane, which is composed of a lipid bilayer, plays this role and is an essential determinant of the morphology and function of cells and organs. iPSCs show various changes as these cells undergo differentiation depending on the culture conditions. iPSCs are generally cultured with feeder cells. Undifferentiated iPSCs form colonies that can clearly be identified by visual inspection, and the center of each colony shows squamatization after several days. At this time, the iPSC colonies are doughnut-shaped and the border with the surrounding feeder cells gradually becomes unclear. It is considered that these changes of iPSC colony morphology are associated with changes of membrane lipids.

Intracellular vesicular organs, such as the lysosomes and mitochondria, also have membranes composed of lipid bilayers. The lysosomal membrane plays an important role in formation of autophagosomes (which digest unwanted cellular components for recycling), and the role of membrane fusion in the mechanism of autophagy has recently attracted attention.

Thus, lipids are not only important components of cellular architecture, but are also involved in intracellular signaling and metabolism. When iPSCs are used for regenerative medicine, quality control is essential to prevent carcinogenesis and contamination by unwanted cells. Investigation of lipids could be useful for quality control, including maintenance of undifferentiated pluripotent iPSCs and assessment of cells that have been differentiated from iPSCs.

1.2 Clinical Applications

Differentiated iPSC-derived cells have already been employed for several clinical applications. iPSC-derived cells were first applied clinically to the treatment of age-related macular degeneration. In 2014, transplantation of iPSC-derived retinal pigment epithelium was performed, and the safety of this procedure was confirmed as well as improvement of macular degeneration (Mandai et al. 2017). Treatment of Parkinson's disease with iPSC-derived cells has also been studied. In 2017, efficacy of iPSC-derived cells was confirmed in a primate model (Kikuchi et al. 2017). Subsequently, a clinical study was initiated in Japan in August 2018, with transplantation of iPSC-derived dopamine-producing neuronal precursors being performed in November 2018. Furthermore, the efficacy of transplanted iPSC-derived cells for heart failure has been demonstrated in various animal models (Nakane et al. 2017; Ye et al. 2014; Shiba et al. 2016), with the "first-in-human" (FIH) trial commencing in Japan in May 2018. As another potential clinical application, iPSCs have been employed for creation of platelets (Takayama et al. 2010; Ito et al. 2018). In September 2018, the Japanese Ministry of Health, Labour and Welfare approved clinical research on iPSC-derived platelets for patients with aplastic anemia refractory to platelet transfusion, so safety in humans will be evaluated in the future. Moreover, the effect of iPSC-derived neural stem cells/precursor cells on spinal cord injury has been evaluated in an animal model, with good results being obtained (Okubo et al. 2018). In Japan, approval was granted for a clinical investigation of iPSC-derived neural stem cells/precursor cells in patients with spinal cord injury in February 2019, and the results are eagerly anticipated. Finally, studies on the treatment of sickle-cell anemia by genome editing have been conducted and

clinical application is under consideration in the U.S.A. (Hanna et al. 2007; Zou et al. 2011).

1.3 Creation of Artificial Organs

The ultimate goal of regenerative medicine is to create functional artificial organs for patients with various diseases. There has been marked progress in the fashioning of mini-livers by self-organization of cultured cells, raising the possibility of this becoming the first iPSC-derived artificial organ. Prolongation of survival has already been confirmed in animal models of hepatic insufficiency after mini-liver transplantation (Takebe et al. 2013, 2017). Creation of organs by self-organization has also been applied to the intestines and kidneys (Spence et al. 2011; Takasato et al. 2015). Pancreatic islets have been created by the blastocyst complementation method, in which the organ is prepared in a heterologous animal host, and pancreatic endocrine function was improved by these artificial islets in an animal model (Yamaguchi et al. 2017). Although the creation of artificial lungs is still at an earlier phase of research, efficient differentiation of type 2 alveolar cells has been achieved and application of these cells is anticipated (Yamamoto et al. 2017). In addition to the self-organization and blastocyst complementation methods, organs can be created by decellularization. In this method, pluripotent stem cell-derived cells are seeded into organs or tissues after decellularization has been performed, leaving the extracellular matrix as a biological scaffold. This procedure has already been investigated for the lungs using iPSC-derived cells (Ren et al. 2015).

1.4 Application of Regenerative Medicine Techniques

Application of iPSC-related techniques has already achieved suggestive results in the treatment of several diseases, as well as in drug development and elucidation of disease pathology. Fibrodysplasia ossificans progressiva (FOP) is a disease in which motor dysfunction occurs due to heterotopic ossification. The import role of mTOR signaling in this disease was confirmed by investigation of the underlying molecular pathology using iPSCs derived from FOP patients, suggesting that an mTOR inhibitor could be employed for treatment (Hino et al. 2017). Based on this finding, a physician-initiated clinical study of the mTOR inhibitor rapamycin for FOP has been underway in Japan since September 2017. With regard to Alzheimer's disease, iPSCs derived from patients have been differentiated into cerebral cortical neurons to investigate treatments that decrease accumulation of amyloid-β, which is considered to be central to the pathogenesis of this disease (Kondo et al. 2017). In the case of amyotrophic lateral sclerosis (ALS), iPSCs derived from patients with SOD1 gene abnormalities were differentiated into motor neurons for comparison with genome-edited control cells to identify molecules suppressing cell death, revealing that phosphorylation of Src/c-Ab1 is involved in motor neuron death (Egawa et al. 2012). Bosutinib (a treatment for chronic myelocytic leukemia) was found to suppress Src/c-Ab1 phosphorylation and a Phase I study assessing its effectiveness for ALS was commenced in March 2019. Moreover, disease-specific iPSCs were created and differentiated into chondrocytes to investigate the pathology of achondroplasia, leading to the possibility of statin treatment being used to improve cartilage formation in this condition (Yamashita et al. 2014). Clinical application of regenerative medicine techniques is also being employed in the treatment of hypertonic cardiomyopathy (Tanaka et al. 2014) and myotonic dystrophy (Ueki et al. 2017). As outlined above, iPSCs are not only useful for direct regenerative medicine applications such as cell transplantation and organ creation, but also for identification of candidate drugs and investigation of disease pathology.

2 Quality Control

It is important to evaluate the quality of cells and tissues used in basic research on iPSCs or clinical applications. When differentiated cells are

employed clinically, their properties are evaluated by immunostaining of surface markers and assessment of function, e.g., contractility of the myocardium or secretion of target hormones by endocrine organs. However, quality control has rarely been performed by assessing cellular lipids and the importance of lipids has generally been overlooked. Because lipids have essential embryological and physiological roles, we will next discuss evaluation of lipids for quality control of iPSCs.

3 iPSCs and Lipids

Lipids have been reported to have an important role in differentiation of pluripotent stem cells, normal development, and various diseases, suggesting that evaluation of lipids should be taken into consideration when clinical application of iPSCs is performed. We found that expression of plasmalogen increased during the process of iPSC differentiation into vascular endothelial cells (Nakamura et al. 2017) (Fig. 1). Plasmalogens are glycerophospholipids with a vinyl-ether linkage to a fatty acid at the sn-1 position and an ester linkage at the sn-2 position that are reported to have an antioxidant effect (Braverman and Moser 2012; Zoeller et al. 1988, 2002). Because plasmalogen synthetase deficiency has been reported to cause optic nerve hypoplasia and male sterility in animals, it has been suggested that plasmalogens are involved in the differentiation and maturation of cells (Rodemer et al. 2003). A decrease of plasmalogen has been reported in Alzheimer's disease (Han et al. 2001) and aging (Maeba et al. 2007), as well as in cardiorespiratory diseases such as myocardial infarction (Park et al. 2015) and chronic obstructive pulmonary disease (Wang-Sattler et al. 2008).

Expression of sphingomyelin also changes during the differentiation process. We found a transient decrease of sphingomyelin during the process of iPSC differentiation into vascular endothelial cells and subsequent maturation (Nakamura et al. 2017) (Fig. 1). Sphingosine 1-phosphate is a metabolite of sphingomyelin that is essential for vessel formation in the fetus and its deficiency is thought to be lethal (Liu et al. 2000). Sphingosine 1-phosphate has a role in regulating the permeability of vascular endothelial cells, and is also related to acute respiratory distress syndrome (Sun et al. 2013) and anaphylaxis (Camerer et al. 2009).

As indicated above, lipids have various roles in the differentiation, maturation, and aging of cells and lipid abnormalities are related to various

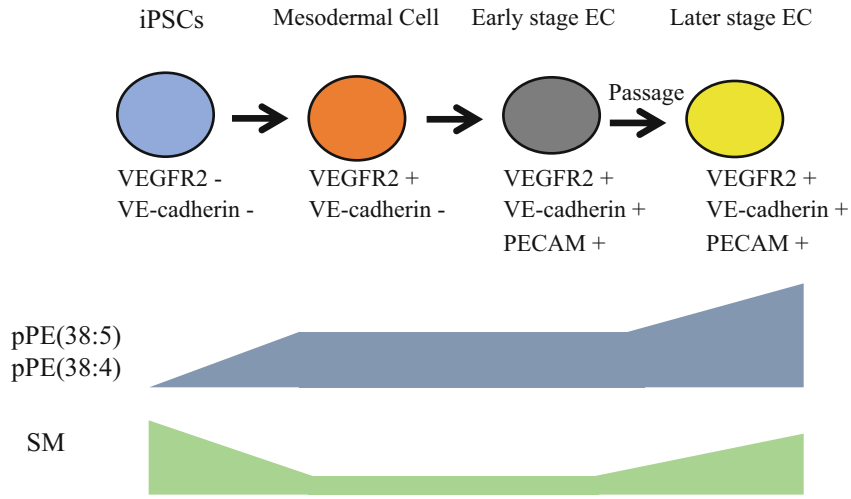

Fig. 1 Changes of lipid components during differentiation of iPSCs into vascular endothelial cells. *pPE* Plasmalogen phosphatidylethanolamine, *SM* Sphingomyelin

diseases. Accordingly, lipid components may determine the physiological characteristics of differentiated cells, suggesting that further investigation of lipids is warranted in relation to regenerative medicine using iPSCs.

4 Evaluation of Lipids

Some lipids can be evaluated indirectly by labeling, such as detection of phosphatidylserine to identify cells undergoing apoptosis. However, lipids cannot be easily labeled with antibodies, unlike proteins and nucleic acids. Recently developed analytical methods have made it possible to analyze lipids in more detail. Lipid components are generally extracted from cells or tissues for evaluation by chromatography-mass spectrometry or imaging mass spectrometry (IMS), which can directly assess two-dimensional structures.

Liquid chromatography-mass spectrometry (LC-MS) is a common method for analysis of lipids, which requires extraction of the target lipids before measurement. Among various lipid extraction methods, the Bligh-Dyer method employing methanol and chloroform can be used to easily extract phospholipids for analysis (Bligh and Dyer 1959). When LC-MS is performed, the mass-to-charge ratio (m/z) of an ionized substance is specified using a mass detector, after which the ionized substance is fragmented with argon gas and identified by analysis of the fragment ions. According to reports concerning the methods of ionization and chromatography, identification of a target substance is simple if m/z is specified with reference to previous data (Shui et al. 2011). We have used LC-MS to analyze the changes of lipids during the process of iPSC differentiation into vascular endothelial cells (Fig. 1).

IMS can be used to evaluate the secondary structure of lipids in tissues or cultured cells. After a tissue or cell specimen is placed on an electrically conductive slide, lipid analysis is performed by mass spectrometry (Fig. 2), with matrix-assisted laser desorption/ionization

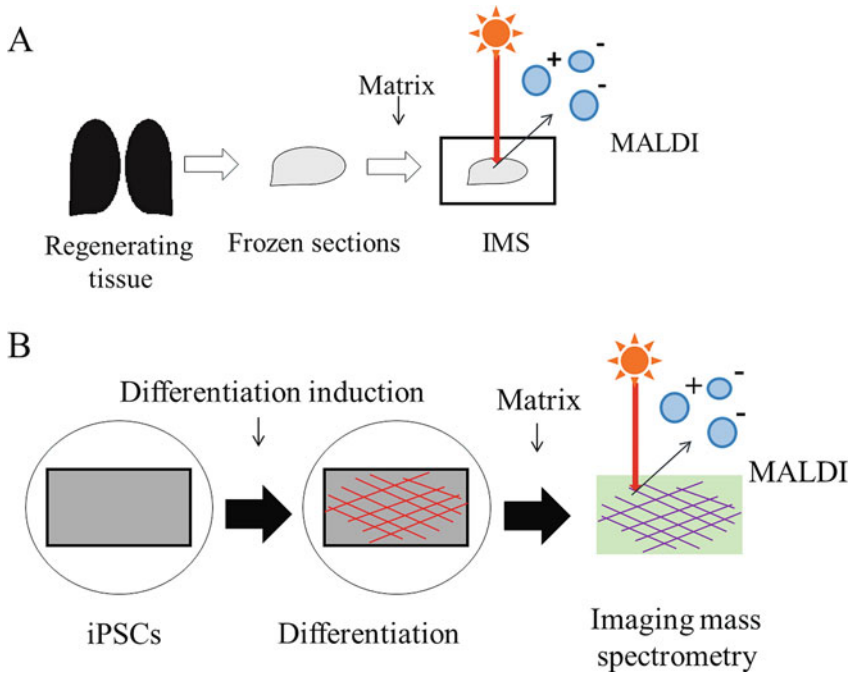

Fig. 2 Application of imaging mass spectrometry (IMS) for evaluation of iPSCs. (**a**) Evaluation of tissue created from differentiated cells. (**b**) Inducing the differentiation of iPSCs on an electrically conductive slide

generally being employed for molecular ionization (Tanaka et al. 1988). We have confirmed that IMS is applicable to cultured iPSCs and have used this method to show that the distribution of phosphatidylcholine differs between the differentiated and undifferentiated parts of iPSC colonies (Shimizu et al. 2017).

5 Conclusions

iPSCs are associated with a low risk of rejection and are currently being used clinically for various regenerative medicine applications. Although some problems still remain to be solved, such as the potential risk of cancer and the mechanisms for initialization of differentiation, iPSCs seem to be a promising tool that will be increasingly used for regenerative medicine. If iPSC-derived cells are widely employed for clinical applications in the future, quality control will become more important. iPSC-derived cells have previously been evaluated on the basis of function and surface marker expression, with little attention being paid to assessment of cellular lipids. However, lipids are not only important structural components of membranes (lipid bilayer), but are also required for differentiation and development as well as physiological cellular functions. In this review, we outlined the clinical applications of iPSCs and suggested that evaluation of lipids could be employed for quality control. It has recently become possible to perform detailed analysis of lipids by methods such as LC-MS and IMS. These methods may become useful tools for quality control based on evaluation of lipids when iPSC-derived cells and tissues are used for clinical applications in the future.

Acknowledgements This work was supported by a Japan Society for the Promotion of Science (JSPS) KAKENHI Grant, Japan to Y.S (16 K09553).

Author Contributions Y.N. wrote the manuscript. Y.S. designed and wrote the manuscript. Both authors reviewed the manuscript.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 57–70
https://doi.org/10.1007/5584_2019_371

Published online: 16 April 2019

Decellularized Adipose Tissue: Biochemical Composition, *in vivo* Analysis and Potential Clinical Applications

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Abstract

Decellularized tissues are gaining popularity as scaffolds for tissue engineering; they allow cell attachment, proliferation, differentiation, and are non-immunogenic. Adipose tissue is an abundant resource that can be decellularized and converted in to a bio-scaffold. Several methods have been developed for adipose tissue decellularization, typically starting with freeze thaw cycles, followed by washes with hypotonic/hypertonic sodium chloride solution, isopropanol, detergent (SDS, SDC and Triton X-100) and trypsin digestion. After decellularization, decellularized adipose tissue (DAT) can be converted into a powder, solution, foam, or sheet to allow for convenient subcutaneous implantation or to repair external injuries. Additionally, DAT bio-ink can be used to 3D print structures that closely resemble physiological tissues and organs. Proteomic analysis of DAT reveals that it is composed of collagens (I, III, IV, VI and VII), glycosaminoglycans, laminin, elastin, and fibronectin. It has also been found to retain growth factors like VEGF and bFGF after decellularization. DAT inherently promotes adipogenesis when seeded with adipose stem cells *in vitro*, and when DAT is implanted subcutaneously it is capable of recruiting host stem cells and forming adipose tissue in rodents. Furthermore, DAT has promoted healing in rat models of full-thickness skin wounds and peripheral nerve injury. These findings suggest that DAT is a promising candidate for repair of

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soft tissue defects, and is suitable for breast reconstruction post-mastectomy, wound healing, and adipose tissue regeneration. Moreover, since DAT's form and stiffness can be altered by physicochemical manipulation, it may prove suitable for engineering of additional soft and hard tissues.

Keywords
Biochemical composition · Biological scaffold · Clinical applications · Decellularized adipose tissue · Tissue engineering

Abbreviations

ASC	adipose stem cell
bFGF	basic fibroblast growth factor
DAT	decellularized adipose tissue
ECM	extra cellular matrix
EDC	1-Ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
GAG	glycosaminoglycan
GPDH	glycerol-3-pohosphate dehydrogenase
hDAT	human decellularized adipose tissue
IHC	immuno-histochemistry
MCS	methacrylated chondroitin sulfate
MGC	methacrylated glycol chitosan
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NHS	*N*-hydroxysuccinimide
OEhMSC	Osteogenically enhanced human mesenchymal stem cell
PEG	Polyethylene glycol
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
VEGF	vascular endothelial growth factor

1 Introduction

The persistent increase in demand for donor tissues for transplants over the last few decades has stimulated the development of *in vitro* tissue engineering and regenerative strategies (Parmaksiz et al. 2016). Tissue engineering requires a minimum of two primary components; viable cells and a minimally immunogenic scaffold that can support cell proliferation and local delivery of growth factors and cytokines. The scaffolds established thus far are categorized as synthetic scaffolds, extracellular matrix (ECM) protein (e.g. collagen, laminin etc.) derived scaffolds, decellularized tissue-based scaffolds or a combination thereof (Costa et al. 2017). Decellularized tissue-based scaffolds are useful tools for tissue engineering, primarily because they can mimic the native tissue microenvironment better than alternative synthetic scaffolds.

While autologous, allogeneic, or xenogeneic grafts have been used previously as transplants for heart valves, skin and coronary arteries, the presence of foreign antigens in these grafts often has caused adverse immune reactions (Costa et al. 2017). Decellularized tissue is processed to remove immunogenic epitopes including cell surface antigens, cytoplasmic components and nucleic acids (Crapo et al. 2011; Gilbert et al. 2006). The retained ECM, is generally conserved between species and does not elicit an immune response (Gilbert et al. 2006). ECM consists of collagens, glycosaminoglycans (GAGs), laminins, growth factors and other biochemical components (Gilpin and Yang 2017). These components serve as important survival, proliferation and migration cues for stem cells and provide signaling that leads to differentiation along specific lineages (Agmon and Christman 2016; Gilbert et al. 2006). Decellularized tissue-based scaffolds actively communicate with cells seeded on them, as evidenced by the scaffolds ability to influence cell differentiation and tissue remodeling, features not typically associated with synthetic scaffolds (Costa et al. 2017). These characteristics make decellularized tissue-based scaffolds superior biomaterials for tissue engineering.

Decellularized tissue-based scaffolds have been generated from dermis, intestinal mucosa, urinary bladder, pericardium, and adipose, among other tissues (Costa et al. 2017; Parmaksiz et al. 2016). The potential clinical uses of these

scaffolds are under investigation for a wide range of applications. Various 'off-the-shelf' tissue-engineered skin substitutes for dermal and epidermal regeneration have become available (Costa et al. 2017; Parmaksiz et al. 2016); however, there remains a void in composites for subdermal adipose tissue-engineering. Although skin grafts and tissue-engineered epidermal substitutes are useful for regenerating the superficial tissue they do not adequately restore the subdermal adipose tissue (Chung et al. 2017). Thus, decellularized adipose tissue (DAT) a promising scaffold for plastic surgery applications, it can serve as a non-immunogenic substitute to fat tissue grafting for augmentation or reconstruction of soft tissues (Han et al. 2015).

Human adipose tissue obtained after lipectomy, abdominoplasty or breast reduction is discarded as medical waste (Schneider et al. 2017), therefore unlike other human tissues, it is an abundant resource for bio-scaffolds (Flynn 2010; Song et al. 2018). Consequently, recent research has focused on preparing DAT that can be utilized for tissue engineering. Several studies have shown that DAT can be used for *in vitro* adipose tissue engineering and *in vivo* adipose tissue regeneration (Han et al. 2015; Wang et al. 2013). The role of DAT is not limited to adipose tissue engineering since it has potential application to heal wounds, regenerate cartilage, support breast tissue reconstruction and promote nerve repair (Choi et al. 2012; Haddad et al. 2016; Lin et al. 2011; Woo et al. 2015).

Decellularization of adipose tissue was first described in 2010 (Flynn 2010). Subsequently, several published articles have reported alternative methods for efficiently decellularizing adipose tissue (Brown et al. 2011; Choi et al. 2011; Song et al. 2018). The majority of publications have focused on converting DAT into diverse physicochemical forms. These modifications are aimed at making a scaffold that allows for easier encapsulation of cells as well as better cell proliferation and differentiation. Attempts have also been made to transform DAT into an injectable scaffold which can be implanted in a non-invasive manner (Young et al. 2011).

This review will summarize the methods utilized for adipose tissue decellularization and the biochemical composition of DAT. Furthermore, physical forms of DAT developed thus far will be discussed, with emphasis on pre-clinical studies and potential clinical applications.

2 Adipose Tissue Decellularization and Physicochemical Manipulation

The ECM of the human body is a gel-like, fibrous network that provides mechanical support and biochemical signals to cells that make up the body's tissues. ECM includes structural proteins, growth factors, proteoglycans, GAGs, and proteolytic enzymes (Costa et al. 2017). It is currently being harvested, processed, and utilized in a clinical capacity for a variety of tissue regeneration applications (Flowers et al. 2017). Removal of parenchymal and stromal cells from the whole tissue by various decellularization techniques minimizes the potential for *in vivo* rejection of the resultant ECM (Crapo et al. 2011). Adipose tissue can be harvested from both xenogeneic and allogeneic sources. Porcine DAT is being explored as a xenograft, while DAT from allogeneic human donors is routinely harvested via abdominoplasty, liposuction, or breast reduction procedures (Flynn 2010).

Once acquired, adipose tissue is physically, chemically and/or enzymatically processed to produce DAT (Fig. 1). Methods for tissue decellularization differ based on the intended application of the DAT. The initial stages for adipose tissue decellularization often involve methods to lyse cells within the tissue, including repeated freeze-thaw cycles or multiple washes in hypotonic or hypertonic solutions (Flynn 2010; Morissette Martin et al. 2018; Roehm et al. 2016). Adipose tissue can be minced by mechanical means or frozen and ground into a powder in order to increase its surface area for further disruption (Choi et al. 2012; Pati et al. 2015). To remove lipids from the proteinaceous ECM, polar extraction with isopropanol is frequently

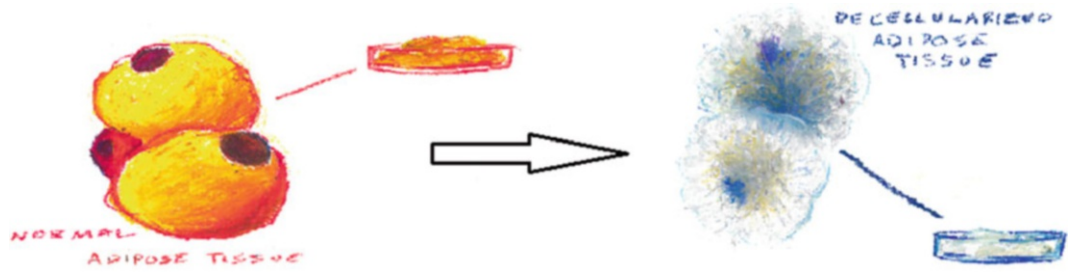

Fig. 1 Native adipose tissue vs decellularized adipose tissue

employed (Flynn 2010; Zhang et al. 2016a). Centrifugation or enzymatic digestion using lipase are also employed to accomplish the same goal (Brown et al. 2011; Choi et al. 2011; Thomas-Porch et al. 2018). Ionic detergents, such as sodium dodecyl sulfate (SDS) or sodium deoxycholate (SDC), can be utilized to disrupt cellular membrane components, while non-ionic detergents, such as Triton X-100, act to remove residual lipids by disturbing lipid-lipid and lipid-protein interactions (Costa et al. 2017; Giatsidis et al. 2018; Tan et al. 2017; Young et al. 2011; Yu et al. 2013). The highly specific proteolytic enzyme, trypsin, is commonly used in conjunction with the chelating agent, EDTA, for further breakdown of cell membrane proteins (Dunne et al. 2014; Wang et al. 2013); however, overexposure to trypsin can disrupt the ECM (Costa et al. 2017). Since the resultant DAT is intended for use for *in vivo* grafting, it is routinely treated with nucleases, such as RNAse and DNAse, to limit the scope of potential immunogenic responses (Lin et al. 2011; Yu et al. 2017). The culmination of a successful decellularization procedure yields a stable, biologically active polymer with little immunogenicity.

While some studies have characterized DAT without further physicochemical manipulation, several groups have improved its mechanical property's tunability by conversion to a hydrogel (Young et al. 2011). After digestion in a protease solution, such as pepsin or alpha-amylase (dissolved in acetic or hydrochloric acid), solubilized DAT can self-polymerize under physiological pH and temperature (Tan et al. 2017; Young et al. 2011; Yu et al. 2013). Furthermore, the DAT hydrogel displays thermosensitive gelation properties, remaining in a liquid state at cooler temperatures. It can, therefore, be used in applications that require biomaterial polymerization *in vivo*. A DAT-hydrogel can also be used as bio-ink to print 3D constructs for *in vitro* or *in vivo* tissue engineering. While the mechanisms involved in the polymerization of collagens, the principle components of DAT-hydrogels, are poorly understood, it is believed that hydrogen bonding, Vander Waals' forces, and ionic interactions are primarily responsible (Tan et al. 2017). The mechanical properties of a hydrogel, such as stiffness, porosity, storage modulus, and gelation time, can be altered based on ECM concentration (Ghuman et al. 2016).

Further improvement in mechanical tunability of DAT-derived hydrogels can be achieved by functionalization of the DAT components prior to polymerization through a variety of chemical reactions. Combination of DAT with biologic or synthetic polymers can yield greater mechanical strength (Cheung et al. 2014; Dong et al. 2012). For example, DAT can be cross-linked using 1-Ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), resulting in increased resistance to enzymatic degradation *in vivo* while maintaining cytocompatibility (Wu et al. 2012). A composite hydrogel made from DAT and polyethylene glycol (PEG) displayed tunable mechanical and degradation properties based on varying DAT concentrations (Li et al. 2018). Another method of synthesizing cross-linked hydrogels includes the combination of DAT with either methacrylated glycol chitosan (MGC) or methacrylated chondroitin sulfate (MCS) which polymerizes through a photo-

catalyzed reaction induced by low-intensity UV light (Cheung et al. 2014). Increasing concentrations of the methacrylated polymers can lead to greater hydrogel stiffness (Cheung et al. 2014). Hypothetically, incorporation of these cross-linking strategies, copolymers, and polymerization methods DAT-hydrogel fabrication will increase the final product's applicability to an expanded range of tissue engineering challenges.

3 Biochemical Composition of DAT

4 Mechanical Properties of DAT

Decellularized tissue needs to possess a particular mechanical strength to retain biological activity. There is evidence to suggest that tissue stiffness is one of the factors that drives the differentiation of stem cells to specific cell lineages (Breuls et al. 2008; Gilpin and Yang 2017; Levy-Mishali et al. 2009). Therefore, creation of a tunable scaffold that can be tailored in terms of stiffness to support the formation of different tissue types is likely to prove pivotal.

Chemical component	Tissue source and form	Assay method	Result	References
Total collagen	C57BL/6 mouse adipose tissue	Sircol collagen assay kit Biocolor	1211.1 ± 223.7 μg/100 mg hydrated DAT	Lu et al. (2014)
	Porcine adipose tissue		332.9 ± 12.1 μg/mg ECM dry weight	Choi et al. (2012)
GAGs	C57BL/6 mouse adipose tissue	Blyscan GAG assay kit	15.6 ± 1.4 μg/100 mg hydrated DAT	Lu et al. (2014)
	Porcine adipose tissue		85 ± 0.7 μg/mg ECM dry weight	Choi et al. (2012)
			768.3 ± 52.2–11.09 ± 43.1 μg/g ECM dry weight	Brown et al. (2011)
	Human adipose tissue		39.67 ± 2.31 μg/g ECM dry weight	Song et al. (2018)
	Human adipose tissue derived hydrogel		2.18 ± 0.32 μg/mg ECM dry weight	Young et al. (2011)
	Human adipose tissue microparticles	Alcian blue colorimetric assay kit	1.72 ± 0.64 μg/mg ECM dry weight	Wang et al. (2013)
	Fischer 344 rat adipose tissue		0.54 ± 0.58 μg/mg ECM dry weight	Zhang et al. (2016a)
Collagen I	Human adipose tissue	IHC	Present	He et al. (2018)
	Porcine adipose tissue		Present	Brown et al. (2011 and Zhao et al. (2018)
	Human adipose tissue derived hydrogel		Present	Young et al. (2011)
Collagen III	Human adipose tissue derived hydrogel	IHC	Present	Young et al. (2011)
	Porcine adipose tissue		Present	Brown et al. (2011)

(continued)

Chemical component	Tissue source and form	Assay method	Result	References
Collagen IV	C57BL/6 mouse adipose tissue	IHC	Present	Lu et al. (2014)
	Human adipose tissue		Present	Flynn (2010), Giatsidis et al. (2018), He et al. (2018), Song et al. (2018) and Zhao et al. (2018)
	Porcine adipose tissue		Present	Brown et al. (2011)
	Human adipose tissue derived hydrogel		Present	Young et al. (2011)
Collagen VI	Human adipose tissue	IHC	Present	Thomas-Porch et al. (2018)
Collagen VII	Porcine adipose tissue	IHC	Present	Brown et al. (2011)
Laminin	C57BL/6 mouse adipose tissue	IHC	Present	Lu et al. (2014)
	Human adipose tissue		Present	Flynn (2010), Giatsidis et al. (2018), He et al. (2018), Song et al. (2018) and Zhao et al. (2018)
	Porcine adipose tissue		Present	Brown et al. (2011)
	Fischer 344 rat adipose tissue		Present	Zhang et al. (2016a)
	Human adipose tissue derived hydrogel		Present	Young et al. (2011)
Elastin	Porcine adipose tissue	Fastin elastin assay kit	152.6 ± 4.5 μg/mg ECM dry weight	Choi et al. (2012)
Fibronectin	Human adipose tissue	IHC	Present	Giatsidis et al. (2018 and Zhao et al. (2018)
Vascular endothelial growth factor (VEGF)	C57BL/6 mouse adipose tissue	ELISA	42.9 ± 25.2 pg/100 mg hydrated DAT	Lu et al. (2014)
	Porcine adipose tissue		15.2 ± 130–27.6 ± 1.2 pg/g ECM dry weight	Brown et al. (2011)
	Human adipose tissue microparticles	IHC	Present	Wang et al. (2013)
	Fischer 344 rat adipose tissue		Present	Zhang et al. (2016a)
Basic fibroblast growth factor (bFGF)	C57BL/6 mouse adipose tissue	ELISA	360.7 ± 120.5 pg/100 mg hydrated DATs.	Lu et al. (2014)
	Porcine adipose tissue		1840.5 ± 92.3–2551.8 ± 148.1 pg/g ECM dry weight	Brown et al. (2011)
	Fischer 344 rat adipose tissue	IHC	Present	Zhang et al. (2016a)

Common tests conducted to determine DAT mechanical strength measure Young's modulus, elastic modulus, and tensile strength. Independent studies have reported Young's modulus of human DAT (hDAT) as 8.312 MPa (Choi et al. 2011) and 65.7 ± 5.97 MPa (Song et al. 2018), whereas DAT microcarriers were found to have Young's modulus of 0.73 ± 0.23 KPa (Yu et al. 2017). Human DAT-MGC and DAT-MCS composite hydrogels exhibited Young's modulus of 30.1 ± 4.0 KPa and 37.1 ± 5.0 KPa respectively (Cheung et al. 2014). Young's modulus of hDAT derived porous foams formulated with different concentrations of DAT was found to range from 2.42 ± 0.65 to 4.01 ± 0.46 KPa (Yu et al. 2013). Tensile strength was determined to be 87.4 ± 23.1 KPa (Roehm et al. 2016) and 128.57 ± 13.15 KPa (Choi et al. 2012) for porcine DAT and 220 ± 50 KPa for hDAT (Song et al. 2018).

Omidi et al. compared the mechanical properties of DAT sourced from different fat depots. Young's modulus calculated for several different source tissues including breast, abdomen, pericardium, omentum and thymic remnant, was found to range from 2.109 ± 0.685 to 3.46 ± 1.21 KPa (Omidi et al. 2014).

5 Applications of DAT

5.1 Modified DAT Forms for *in vivo* Applications

5.1.1 Powder

A powdered form of DAT has been developed for subcutaneous injection. DAT was freeze-dried, and manually milled into a powder and suspended in cell culture medium. The suspension permitted cell invasion, proliferation, and survival *in vitro* (Choi et al. 2009). Suspensions with and without adipose stem cells (ASC) were injected subcutaneously into nude mice. Histology and RT-qPCR for adipogenic genes showed both experimental groups induced adipogenesis with organized intracellular lipid droplets and angiogenesis; however, the changes were more prominent in the suspensions seeded with ASC (Choi et al. 2009).

5.1.2 Injectable Liquid

Likewise, an injectable form of DAT has been established and tested for soft tissue defect applications. After being lyophilized, DAT can be digested with pepsin to form a viscous

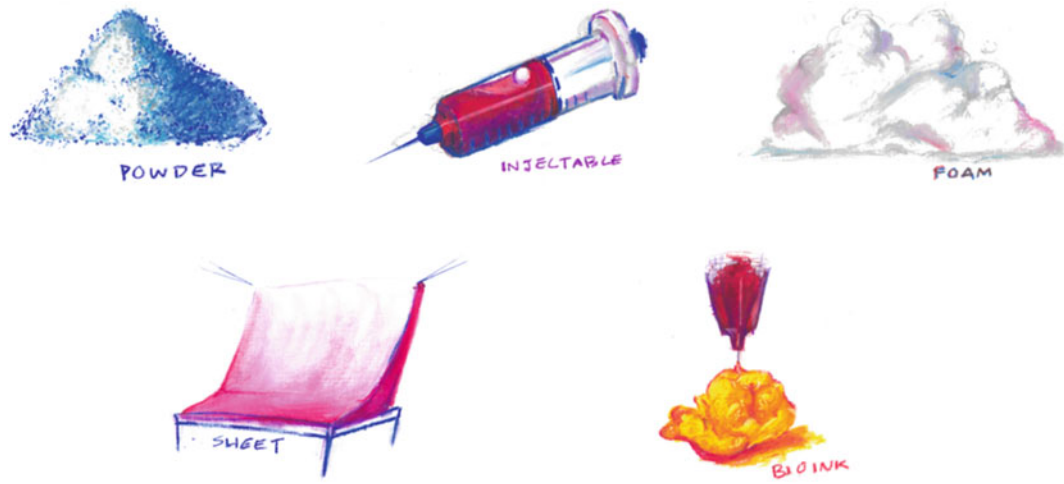

Fig. 2 Modified forms of DAT for *in vivo* applications; powder, injectable liquid, foam, scaffold sheet, bio-ink

suspension. When injected into rats or mice, the scaffold has displayed the potential to promote angiogenesis (Giatsidis et al. 2019) and adipogenesis (Zhao et al. 2018). It has also been shown that injectable DAT alone can instruct host cells to remodel the tissue and thus, the scaffold has the potential for stimulating adipose tissue regeneration (Choi et al. 2012; Tan et al. 2017; Wu et al. 2012; Young et al. 2011; Zhang et al. 2016b; Zhao et al. 2018). Injectable DAT undergoes rapid volume loss after *in vivo* implantation, whereas crosslinking liquid DAT with synthetic polymers reduces degradation of the scaffold while retaining its capability to allow cell attachment and adipo-induction (Cheung et al. 2014; Li et al. 2018; Wu et al. 2012). However, cross-linking introduces a trade-off between biocompatibility and volume persistence.

5.1.3 Foam

DAT was converted in to a porous foam by solubilization using α-amylase followed by freeze-drying. The α-amylase treatment is milder than the pepsin treatment, thus retaining the structural integrity of collagens, and removing the need for a chemical crosslinker (Yu et al. 2013). The porous property of the foam improves cellular infiltration, viability, and intracellular signaling. Another procedure was established to construct "bead foams" which are fused interconnected networks of porous DAT beads. This was accomplished by electrospraying DAT solution followed by freezing and lyophilization. Glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity, RT-qPCR analysis of adipogenic gene expression, and intracellular lipid accumulation studies served as strong evidence that the porous foam and bead foams supported adipogenesis (Yu et al. 2013). Subcutaneous implants in a rat model further demonstrated that the porous foam and bead foams were inherently adipogenic and biocompatible (Yu et al. 2013), suggesting that the composites are a viable soft defect filler.

5.1.4 Scaffold Sheet

While injections and foams have several potential applications for subcutaneous tissue regeneration, a bandage-like sheet could be useful for the treatment of topical injuries. A thin scaffold sheet was fabricated from DAT by casting the acellular tissue in a shallow mold and lyophilizing it. Based on mechanical tests the sheets were determined to have sufficient mechanical integrity for easy handling (Kim et al. 2012). The scaffold sheets were seeded with different cell types to establish their potential to support cell adhesion and proliferation. Live/dead assays confirmed that several different human cells types successfully integrated into the sheet. (Kim et al. 2012). The potential applications of this sheet are considerable due to its ability to support a wide variety of cell types. Furthermore, a DAT sheet could be applied non-invasively and topically (Fig. 2).

5.1.5 Bio-ink

Apart from the biochemical composition of the scaffold and the inherent presence of growth factors, another key regulator of cell differentiation and tissue remodeling is its three-dimensional architecture. The advent of 3-D printing has allowed custom designed fabrication of scaffolds in specific shapes and structures to promote the formation of specific tissue types (Pati et al. 2014). DAT can be enzymatically digested and converted into liquid bio-ink for 3D printing. Pati et al. developed dome-shaped tissue constructs using DAT bio-ink and ASC. *In vitro* experiments showed that the cells remained highly viable in the constructs and displayed adipogenic differentiation. The tissue constructs also exhibited remodeling and adipose tissue formation when implanted subcutaneously in mice (Pati et al. 2014, 2015).

5.2 *In vivo* Applications

5.2.1 Adipose Tissue Engineering

DAT's most obvious clinical use is as a filler or implant in situations where soft tissue damage has occurred due to traumatic injury or tumor resection. By providing important microenvironmental signals that promote adipogenesis and angiogenesis, DAT can resolve soft tissue defects.

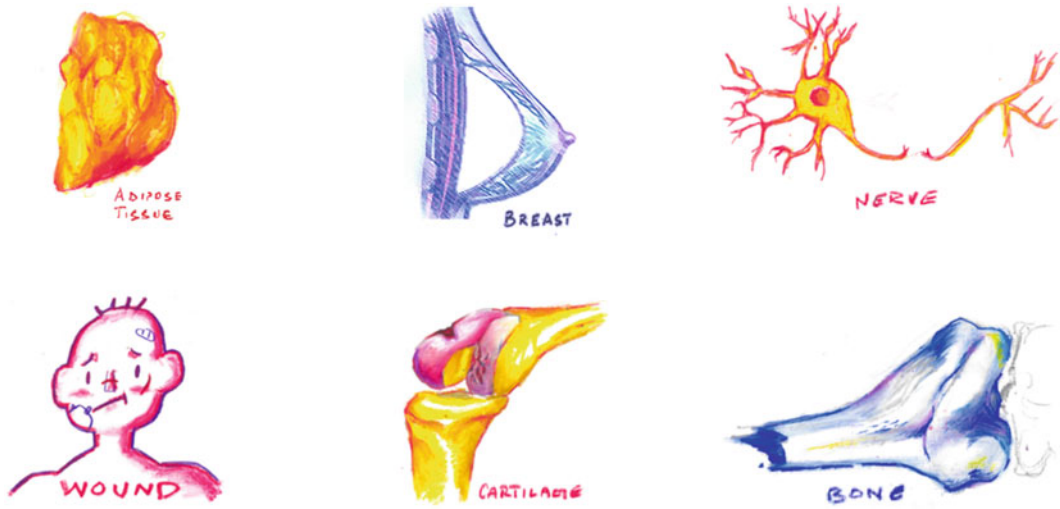

Fig. 3 *In vivo* applications of DAT; adipose tissue engineering, wound healing, breast reconstruction, cartilage engineering, nerve repair, bone regeneration

The capability of DAT to form adipose tissue after subcutaneous implantation has been demonstrated in different animal models. Additionally, DAT in its native form or any of the modified forms (discussed in Sect. 5.1) has been found to be adipo-inductive *in vivo* and recruits host stem cells (Han et al. 2015). DAT microparticles seeded with ASC formed adipose tissue within 30 days of implantation in Fischer rats (Wang et al. 2013). DAT-MCS composite hydrogels and DAT foam, seeded with ASC, displayed significant adipogenesis in Wistar rats after 12 weeks (Cheung et al. 2014; Han et al. 2015; Yu et al. 2013). ASC seeded on 3D printed polycaprolactone-DAT composite scaffolds underwent adipogenic differentiation in nude mice (Pati et al. 2015). Viscous DAT suspension with ASC caused adipose tissue formation 4–12 weeks after implantation in mice (Choi et al. 2012; Young et al. 2011; Zhang et al. 2016b). There is also evidence that DAT alone in the absence of cells possesses the potential to promote adipogenesis through the recruitment of host stromal/stem cells (Tan et al. 2017).

5.2.2 Wound Healing

Lacerations that penetrate to the depth of the subdermal tissue and full-thickness third-degree burns may require surgical intervention and skin grafting. Without an intervention to repair the defect, the patient is at risk of infection, unable to regulate body temperature, and likely to develop excess fluid loss and electrolyte imbalance. DAT fabrications have the potential to limit these complications as well as eliminate the disadvantages of conventional treatment methods.

To address this issue, a bilayer sheet was developed for wound dressing. The top layer was composed of a chitosan membrane integrated with titanium dioxide prepared by electrospinning chitosan solution with titanium dioxide solution. This top sheet provided an external bacterial barrier while a DAT bottom layer served to promote subdermal tissue regeneration (Woo et al. 2015). *In vivo* studies using a full-thickness wound in rats showed that the bilayer sheets induced faster regeneration of granulation tissue and reduced epidermal scar formation compared to controls (Woo et al. 2015). This bilayer sheet, as well as the bandage-like sheet (Kim et al. 2012), are promising wound dressings, especially in clinical settings with damaged subdermal tissue and increased risk of bacterial penetration.

5.2.3 Breast Reconstruction

With 1 in 8 women in the United States facing a lifetime risk of breast cancer development

(Schneider et al. 2014) and an increasing annual number of mastectomies being performed, there is a clear need for advances in breast reconstruction biomaterials. Haddad et al. evaluated the biomechanical efficacy of DAT as a reconstructive material from breast defects using a computational approach. The study used numerical models to compare deformation that a normal breast endures due to gravitational loading conditions versus deformation in DAT under the same conditions. These numerical models were developed using magnetic resonance imaging (MRI) data of breasts from female volunteers. DAT from several different physiological depots were analyzed and the results demonstrated that breasts reconstructed with these DAT materials did not show contour defects. Furthermore, the breasts reconstructed with DAT sourced from breast and abdominal sites demonstrated more similar deformation compared to native breast tissue than those breasts reconstructed with DAT sourced from other depots. (Haddad et al. 2016). These results are encouraging for expanded use of DAT in plastic surgery applications. DAT may prove to be a practical biomaterial for mammoplasty in which the goal is to reshape or modify the appearance of the breast. Theoretically, a DAT scaffold will prove superior to a synthetic implant by allowing the breast to retain its natural texture.

5.2.4 Cartilage Tissue Engineering

Cartilage acts as a cushion to protect the bones from impact and allows joints to easily glide and bend without pain. Damage to cartilage can occur from trauma such as a meniscus tear in the knee or from degenerative changes due to osteoarthritis (Beaufils et al. 2017). Joint surface defects are ubiquitous and difficult to renew, especially full-thickness defects. These injuries have limited capability of healing due to the avascular nature of cartilage and declining chondrocytes function with age and injury (Sophia Fox et al. 2009). Current treatment strategies such as microfracture lead to the replacement of the original hyaline cartilage with a less durable, less resilient, and sub-optimal fibrocartilage (Goldberg et al. 2017). Autologous chondrocyte implantation is an alternative therapy involving harvesting, culturing, and re-implanting a patient's cells into the defect (Goldberg et al. 2017). The use of cartilage-derived ECM for cartilage regeneration does not have promising clinical application because of limited cartilage sources. While investigators have conjectured that a wider application of DAT includes cartilage regeneration, research currently remains limited to *in vitro* studies. Choi et al. investigated whether adipose stem cells seeded on DAT could be induced to undergo chondrogenic differentiation. ASC and DAT scaffolds cultured in chondrogenic medium with 10 ng/mL TGF-β1 for 45 days successfully formed cartilaginous tissue with cartilage-specific collagens II and XI, proteoglycans, and GAGs (Choi et al. 2012). These experiments indicate that a DAT can support chondrogenic differentiation of human ASC and has potential utility in synthesizing a cartilage-like tissue *in vitro*. Further studies will be necessary to determine whether a DAT scaffold with chondrogenic-induced ASC can generate a cartilage-like tissue *in vivo* with volume persistence or can promote healing in a cartilage defect model.

5.2.5 Nerve Repair

The utility of DAT for nerve repair was examined in a rat model of peripheral nerve injury. A 5 mm long nerve section was resected from the cavernous nerves, which mediate erectile function. DAT seeded with ASC was grafted into the site of nerve injury and erectile function was assessed 3 months later. DAT treated rats displayed better erectile function than controls, the results were not statistically significant (Lin et al. 2011).

5.2.6 Bone Regeneration

Although bone has a remarkable capacity for regeneration, critical-size fractures are unable to heal (Clough et al. 2015a). These defects generally require an invasive surgical procedure or autologous bone grafting. Bone grafting involves the harvesting of bone, which often leads to prolonged donor site pain, hospitalization, and risk of infection (Clough et al. 2015a; Strong et al. 2016). Scaffolds that have successfully promoted cell adhesion, proliferation, and osteogenic

differentiation of ASC include natural polymers such as chitosan/gelatin, decellularized bovine tendon and decellularized bovine trabecular bone (Clough et al. 2015b; Elgali et al. 2017). Often, synthetic bone scaffolds are not biologically compatible and do not effectively support angiogenesis or proliferation (Amini et al. 2012). Additionally, the source of tendon and bone-derived scaffolds is not abundant enough for wide orthopedic use. Consequently, there remains an orthopedic demand for a biocompatible composite with translatable clinical relevancy.

Mesenchymal stem cells (MSC) derived ECM scaffolds have displayed improved bone healing in mouse model of calvarial defect (Zeitouni et al. 2012). Additionally, osteogenically enhanced MSC (OEhMSC) seeded on gelatin foam promoted bone regeneration in mouse model of femoral defect (Clough et al. 2015a). While there are presently no *in vivo* studies that have analyzed the use of DAT for bone regeneration, ASC seeded on DAT have displayed osteogenic differentiation when cultured in osteogenic media (Guneta et al. 2017). Future research needs to be performed to demonstrate osteogenically enhanced ASC adhere and proliferate in DAT. Furthermore, experiments validating DATs efficacy as a promoter of bone growth in a bone defect model would be essential (Fig. 3).

5.3 3-Dimensional Cell Culture

In addition to clinical *in vivo* applications, DAT has potential use for *in vitro* research. DAT's biomimetic properties make it an ideal 3D cell culturing system because of its ability to very closely simulate the *in vivo* microenvironment.

It has been shown by several studies that ASC can maintain high viability and proliferation when seeded on native DAT (Choi et al. 2012; Fan et al. 2014; Flynn 2010; Kim et al. 2012; Song et al. 2018; Young et al. 2011; Yu et al. 2017; Zhang et al. 2016a) or chemically modified DAT (Brown et al. 2015; Cheung et al. 2014; Li et al. 2018; Pati et al. 2015; Turner et al. 2012; Wu et al. 2012). DAT powder displayed lower attachment of ASC in comparison to 2D culture plate, but exhibited increased proliferation rates (Choi et al. 2009). Apart from ASC, DAT also allows attachment and proliferation of human aortic smooth muscle cells, human chondrocytes, human umbilical vein endothelial cells (Kim et al. 2012; Zhang et al. 2016a), human dermal fibroblasts (Choi et al. 2012) and neuroblasts (Roehm et al. 2016). DAT has been found to be adipo-inductive *in vitro* and ASC seeded on DAT are able to differentiate in to adipocytes without adipogenic induction (Brown et al. 2015; Cheung et al. 2014; Flynn 2010; Lin et al. 2016; Pati et al. 2015; Tan et al. 2017; Turner et al. 2012; Yu et al. 2013).

5.3.1 In Vitro Cancer Research

DAT has been used as a 3D cell culturing system to study breast cancer growth and drug response (Dunne et al. 2014). DAT scaffold was compared to standard 2D cell culture and 3D Matrigel™ scaffolds. The proliferation profile of the *in vivo* xenografts was found to be similar to that of the cells in DAT scaffolds *in vitro*, with a long lag phase and a significantly slower proliferation compared to that of the 2D and Matrigel™ culture proliferation profiles (Dunne et al. 2014). These results demonstrated the superiority of DAT as a cell culturing system to mimic *in vivo* conditions.

6 Conclusions

The easy accessibility of source tissue makes DAT an attractive candidate as a regenerative medical bio-scaffold and *in vitro* research tool. Decellularization of adipose tissue has been optimized through repeated studies and it has been shown that DAT retains most of its structural proteins as well as functional molecules such as VEGF and bFGF. These biochemical components make DAT a bioactive system that is capable of recruiting and housing cells, providing them with adipogenic signals and promoting neovascularization. DAT is an adaptable scaffold, as it can be modified in to an array of different chemical compositions and physical forms. This theoretically means that a wide variety of tissue types could be engineered in DAT.

The obvious clinical application of DAT rests on its homologous use for regeneration of soft tissue defects. However, based on pre-clinical animal studies and *in vitro* work, DAT has the potential for wider ranging applications including peripheral nerve, cartilage, and bone regeneration.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 71–85
https://doi.org/10.1007/5584_2019_338

Published online: 2 February 2019

Decellularization Concept in Regenerative Medicine

Özge Sezin Somuncu

Abstract

Decellularized organs and tissues are effectively utilized in a diversity of regenerative medicine purposes, and the decellularization approaches employed differ as broadly as the tissues/organs of concern. Biological scaffold substances formed by extracellular matrix (ECM) are mostly produced with methods that include decellularization of tissues. Conservation of the multifaceted arrangement and three-dimensional (3D) construction of the ECM is very wanted but it is documented that almost every approach of decellularization cause disturbance of the organization and possible forfeiture of surface organization and conformation. The competence of cell elimination from a tissue is reliant on the basis of the tissue and the precise physical, chemical, and enzymatic approaches that are utilized. Here, the most frequently applied and newly developed decellularization techniques are designated, organ engineering with decellularized scaffolds for different organs, recent knowledge in the field are explained.

Keywords

Decellularization · Regenerative medicine · Recellularization · Tissue engineering

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Abbreviation

3D	Three dimensional
ADSCs	Adipose-derived stem cells
CC10	Secretoglobin Family 1A
CCSP	Clara cell secretory protein
CD 31	Cluster of differentiation 31
CK18	Keratin 18
ECM	Extracellular matrix
ESCs	Embryonic stem cells
FOXJ1	Forkhead box protein J1
GAG	Glycosaminoglycans
Mg	Miligram
MIN-6	Mouse insulinoma 6
Ng	Nano gram
Nkx2.1	NK2 Homeobox 1
PBS	Phosphate buffered saline
PDGFR	Platelet derived growth factor receptor
ProSPC	Alveolar type 2 cell marker
SDS	Sodium dodecyl sulfate
SPC	Pulmonary-associated surfactant protein C
TNF	Tumor necrosis factor
TTF-1	Transcription termination factor 1

1 Introduction

The antiquity of medical expedient manufacturing is oversupplied with power-driven materials designed for organ utility replacement;

nevertheless, these non-biological tools have been mainly ineffective, since they were proposed to imitate one or more occupations of a specific organ, yet not the organ in its wholeness. Beings, dissimilar to medicinal tools, are not produced from metal and nylon. Medicinal tools have aimed the management of an illness, whereas regenerative medicine has the prospective to compromise a treatment. If a person really desires to substitute the occupation of a human organ, why not begin with a completely practical, vigorous organ afterwards (Seetapun and Ross 2017)?

Regenerative medicine embraces the potential to substitute or restore human cells, tissue or organs so as to reestablish the healthy function lost because of disease and injury. Through the arrangement of innovative biomaterials with cells, one of the purposes of regenerative medicine is to generate autologous tissue grafts for future replacement therapies (Garreta et al. 2017). Innately reproduced constituents have demonstrated to be greater to artificial polymers as matrix scaffolds, which may preserve the hierarchical complication of natural tissues. Indeed, these constituents can be utilized to form environments of enlarged complexity: from micro-tissues to organ scaffolds shaped through decellularization of complete tissues (Taylor et al. 2018). Studies show the existence of significant bioactive constituents covering collagen types, basic fibroblastic growth factor, transforming growth factor-β, vascular endothelial growth factor upon decellularization of dissimilar tissues. Preserving greater quantities of active molecules upsurges the chance of the medical usage of that decellularized tissue (Parmaksiz et al. 2016). In this present work, we assess the newest progressions in the practice of decellularization and recellularization technologies for the production of autologous tissue grafts and organ replacement therapies.

2 Concept of Decellularization

Decellularization is a technique which comprises the elimination of cellular apparatuses from the tissue to obtain extracellular matrix (ECM) patterns with a multifaceted combination of physical and practical proteins for maintaining architecturally systematized entities to work as bio-derivative scaffold (Rana et al. 2017). Decellularization may be custom-made with the specific properties of an idyllic scaffold fulfilling bio-comformity, non-immunogenicity and the capacity to deliver physical, mechanical, chemical and biotic signals for cell connection, propagation, relocation, differentiation and sustained utility (Rana et al. 2017).

It has been stated that decellularized scaffolds display their organ-precise attitude pertaining to the foundation of the original tissue ECM for organ renovation. Decellularized scaffolds have been presented to be convenient applicants for consignment undertaking practicalities through procuring the mechanical force of collagen filaments, the springiness of elastin backbones and the hydration-attachment occupations of proteoglycans from their non-natural ECM matrices (Rana et al. 2017). The usage of innate vasculature and fast partition of debris and excess liquid from tissue meaningfully expands the superiority of scaffold for reconstruction of novel organ/tissue (Khan et al. 2014).

By way of expending biological matrices which recapitulate innate tissue to variable levels, it is likely to boost the chemical, mechanical and vascular environment of injured tissues possibly both to enhance cell preservation or endurance and to review natural signals for cell conductivity, improving the efficiency of cell-level overhaul (Taylor et al. 2018). Decellularized ECM has been effectively utilized to restructure numerous varieties of tissues and organs covering lung, heart valve, blood vessel, esophagus, urinary bladder, kidney, cornea, trachea (Gilpin and Yang 2017). Concept of decellularization is stated in Fig. 1.

3 Decelllularization Methods

The method of decellularization necessitates the separation of the ECM from any particular tissue with negligible forfeiture, injury or disturbance, whereas exploiting the elimination of cellular substance. This can be accomplished through

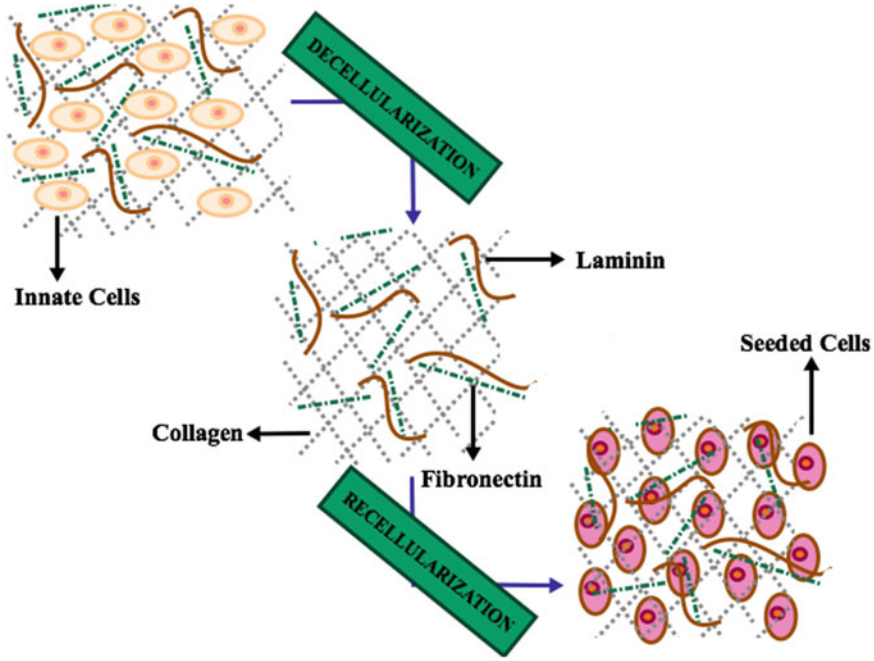

Fig. 1 Model of tissue decellularization. Artificial tissues may be produced by decellularization of native tissue. ECM then may be reseeded with cells that "maturate" the substance (e.g., to improve its renovation or vascularization) or "get matured" in the direction of a precise utility (e.g., to multiply or differentiate)

the joint implementation of physical, chemical, enzymatic and apoptosis-inducing approaches (Tapias and Ott 2014). Cell and genetic constituent exclusion is serious in avoiding immune rejection of the structure to seeded cells. The standards for evaluating the efficiency of elimination of these constituents is proposed like this: the decellularized ECM must carry fewer than 50 ng double stranded DNA per mg ECM dry heaviness, a smaller amount of 200 base pair DNA fragment size, no detectable nuclear material (Gilpin and Yang 2017).

While effective decellularization was attained for different organs, still much energy should be focused on the description of normalized decellularization procedures alongside the ultimate aim to develop well matched and tailored organ scaffolds for medical purposes. In order to achieve that, subjects covering degradation, bioconformity, pathogenicity and immunogenic responses should be further deliberated (Garreta et al. 2017).

3.1 Chemical and Enzymatic Methods

Different kinds of chemicals have been utilized in decellularization, containing surfactants, acids and bases. Surfactants, the most usual decellularizing materials, characteristically employ via lysing cells over disturbing the phospholipid cell membrane. These agents are categorized attribute to their charge, as they are ionic, nonionic, or zwitterionic. Acids such as peracetic acid and bases covering sodium hydroxide degrade the cell membrane and nuclear material by using their intrinsically charged features (Gilpin and Yang 2017).

Sodium dodecyl sulfate (SDS) has been effective in a quantity of submissions in decellularization according to its capability to proficiently eliminate cells and genetic content. SDS therapies have encountered the optimal necessities of whole cell elimination and removal of 90% of host DNA in numerous categories of tissues and organs, comprising rat arm, porcine

cornea, procine myocardium, porcine heart valve, porcine small intestine, porcine kidney, human vein, rat-porcine-human lungs and human heart (Gilpin and Yang 2017).

While SDS can effectively eradicate unsolicited innate components of the tissue, it may be harmful to the organizational and signaling proteins. Destructive structural proteins and factors not only may avert the cells from occupying the tissue as earlier but similarly stops the full maintenance of its mechanical features. This consequence is mainly established in tinnier tissues and cell sheets. Although most surfactant-treated tissues must classically be rinsed with solutions like phosphate buffered saline (PBS), SDS is more challenging to be detached because of its ionic nature. Triton-X is a non-ionic less harsh and less damaging surfactant. It is oftentimes utilized to remove the remnant SDS. Not only is Triton-X advantageous in rinsing, but also it is regularly employed as a decellularizing agent unaccompanied (Gilpin and Yang 2017).

Enzymes can be employed to take aim at the leftover nucleic acids upon cell fraction or the peptide bonds that bind the cells to the ECM. They incline to endure in noteworthy numbers in the tissue and may inflame a further immune retort (Bourgine et al. 2013). Trypsin is an enzyme frequently utilized with EDTA that functions by pleating the cell-matrix linkages. Utilized in the treatment of porcine pulmonary valves, whole cell and genetic material elimination was detected after 24 h (Gilpin and Yang 2017).

Acids and bases counter with and denature proteins, solubilize cell constituents and change nucleic acids, therefore rupture the cells. They are not choosy and so modify also ECM apparatuses, particularly collagens, glycosaminoglycans (GAG) and growth factors (Bourgine et al. 2013).

3.2 Mechanical Methods

Approaches that can physically/mechanically decellularize the ECMs contain temperature and compression actions that operate to remove cells over mishmash of lysing the cells and abolishing cell-matrix adhesive proteins. Especially, physical therapies comprise the utilization of freeze-thaw, high hydrostatic pressure, or supercritical carbon dioxide to entirely eradicate constituent cells and genetic materials (Gilpin and Yang 2017).

Freeze-thaw operation effectually lyses cells inside tissues and organs. However, the consequential membranous and intracellular substances endure if not detached via consequent dispensation. A solitary freeze-thaw cycle may decrease opposing immune reactions covering leukocyte permeation in vascular ECM scaffolds. Repetitive freeze-thaw cycles can be utilized throughout decellularization and do not suggestively upsurge the forfeiture of ECM proteins from tissue (Crapo et al. 2011).

Hydrostatic force necessitates moderately slight time period and may be more efficient than detergents or enzymes for eradicating cells from vasculature and corneal tissues, while the baric construction of ice crystals can disturb ECM structure. Augmented temperature through pressure decellularization averts ice crystal construction but then again disturb ECM regarding to the related upsurge in entropy (Crapo et al. 2011).

3.3 Decellularization by Programmed Cell Death

Current decellularization approaches necessitate a compromise amongst effective cellular elimination and ECM conservation, as enhancing the action harshness for a more comprehensive decellularization seriously fallouts in a heightened ECM disturbance. The apoptotic paths may be intentionally stimulated over a clean and coordinated procedure via the conveyance of suitable signals. Throughout the entire apoptotic progression, the cellular substance is retained precisely inside the plasma membrane and the apoptotic bodies. The immunogenic cellular components do not outflow into the nearby environment, therefore averting a redundant inflammatory response. This is contrary to present decellularization methods, encouraging necrosis, cell exploding and the discharge of immunogenic material in the adjacent surroundings (Bourgine et al. 2013).

Kiss-of-death approach completely depends on the extrinsic pathway initiation by the distribution of precise ligands that attach their analogous death receptors of the TNF (Tumor necrosis factor) superfamily. Lethal-environmental-conditioning method is persuaded by moderating environmental influences such as temperature, pH in addition to carbon dioxide/oxygen, nitric oxide and hydrogen peroxide content. Contrasting to the physical freeze&thaw method triggering necrosis, induction of apoptosis through temperature variations necessitates low differences, in either hyperthermic or hypothermic ranges. Death-engineering approach depends on the instigation of any of the two apoptotic pathways by the routine of a genetic approach. Apoptosis stimulation could theoretically be attained by controlling the expression level of crucial genes elaborated in the pathway (Bourgine et al. 2013).

4 Pros and Cons of Decellularization

Decellularization may harm the ECM and membrane material of the organ. Most effective technique for the exact tissue form ought to be assessed for overpowering the difficulty. One other key problem with decellularized scaffold is to cultivate the practical anticipated cell types inside this and further to transplant *in vivo* to acquire purposeful organs. Therefore, there is a requirement for cells suitable to be transplanted, apposite medium and skill (Khan et al. 2014). The effects of each and individual decellularization method and their operation of actions are shown in Fig. 2.

Utilizing xenogenic scaffolds have a misgiving about antigen that can trigger the refusal. Although the decellularization is completed

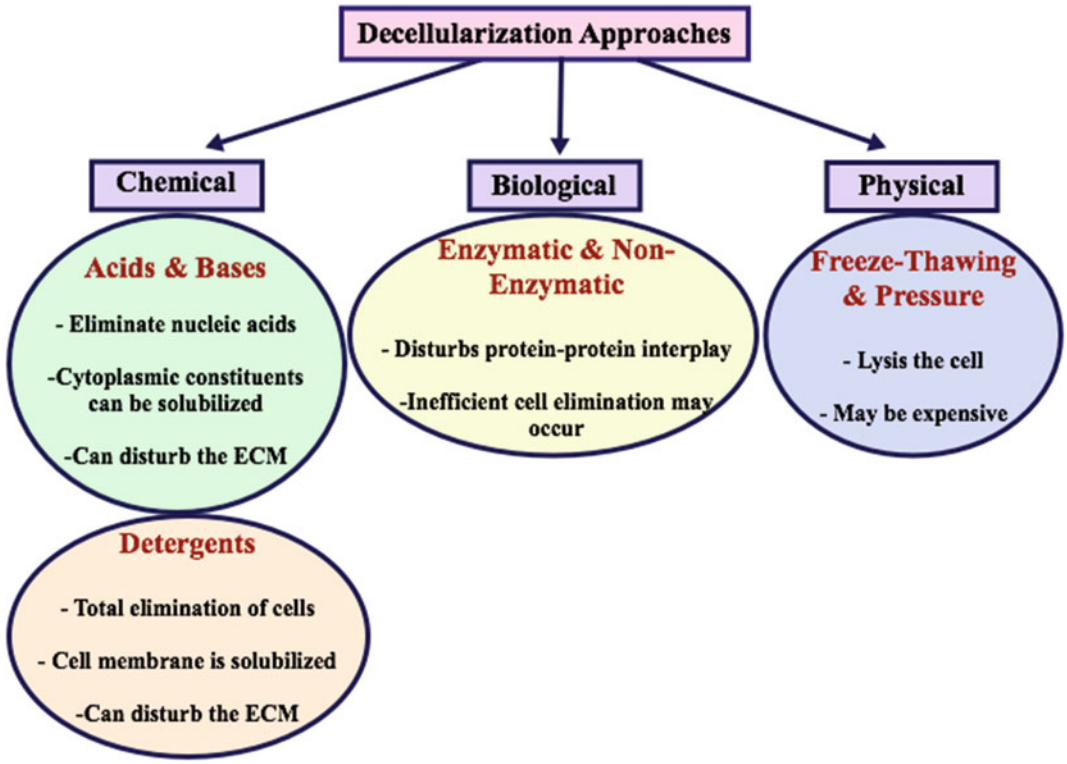

Fig. 2 Methods of decellularization. Their operation of actions, along with the advantages and disadvantages of each method

faultlessly, a specific surface antigen stays at nominal level called galactosyl α (1,3) galactose in xenogenic tissues and is inattentive in human that may activate severe refusal (Khan et al. 2014). Decellularized scaffold maintain the vasculature and reinstating blood stream precisely is less puzzling, nonetheless the problem stays to be replied of how long it can be hold. Since any partly re-endothelialized vasculature carries danger of thrombosis prominent to confined organ failure (Khan et al. 2014).

Even though the decellularized tissue and organ-derived matrices are like the in vivo ECM, they cannot imitate the expansion progression of tissue or body. In order to simulate the modelling of the ECM through expansion, the utilization of stem cells which are regulated at diverse periods of growth is essential. Hypothetically, the provision of ECM imitating the entire growth is conceivable if embryonic stem cells or induced pluripotent cells are expended (Hoshiba et al. 2010). The probable outcomes of decellularization is specified in Fig. 3, respectively.

The main pro of decellularized ECM is maintenance of the organizational entirety at the macro and micro measure, such that the subsequent scaffolds carry parallel tensile forte to innate tissues and preserve maximum of the connatural natural vasculature (Taylor et al. 2018). Decellularized ECM may moderate cell attitude: adherence, relocation and differentiation. Conservation of ultrastructure and conformation persuade advantageous tissue organization and remodeling (Garreta et al. 2017).

5 Recellularization

Recellularization is a progression of producing practical cells inside the decellularized organ scaffold to generate entirely operative simulated organ. Seeding of precise sort of cells with high propagation and regulated differentiation capacity is essential to recellularize the decellularized organ scaffold. Every organ has dissimilar compositions with divergent utilities. Thus,

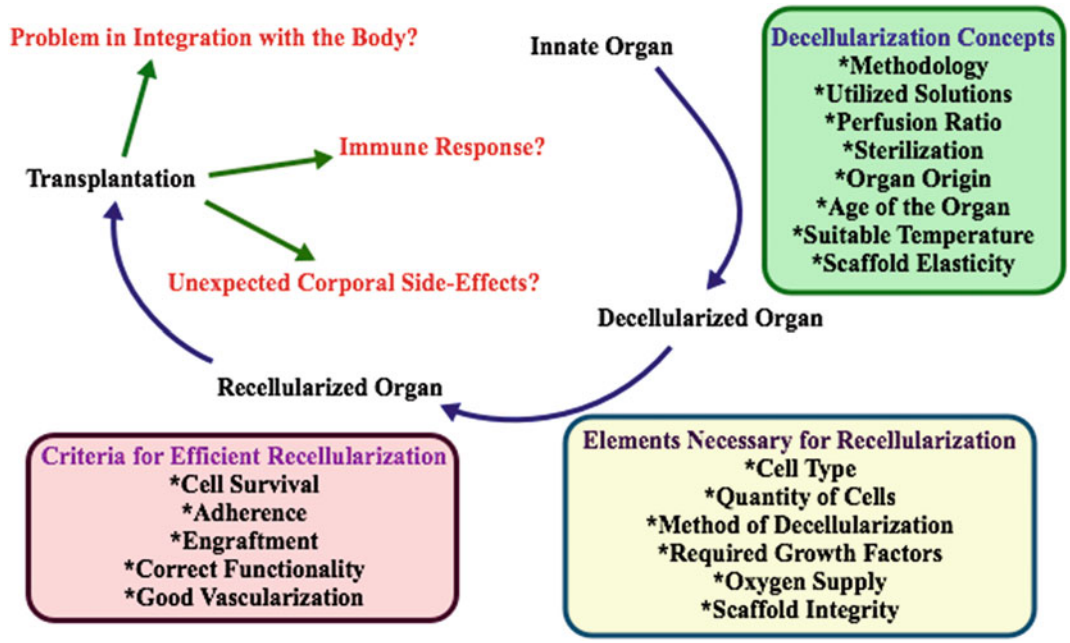

Fig. 3 Schematic demonstration indicating bioartificial organ expansion utilizing decellularization and recellularization methods and their probable application

utilization of more suitable cells stays as an obstacle to restore a whole organ (Khan et al. 2014).

To accurately form an organ or tissue necessitates producing the particular assemblies of that tissue together with any vascular or ductal constituents and secondary assemblies supplied with cells, counting inhabitants of occupant stem or progenitor cells for constant organ conservation (Fig. 4). Rejuvenation of an organ needs both parenchymal and non-parenchymal cell foundations. Preferably both would be originating from non-immunogenic bases and would bring matured functioning tissue. Therefore, a naive foundation of cells to reconstruct any particular organ would appear to be that real organ. Conversely, most adult organs either do not cover cells which are adequately proliferative to produce the quantities of cells required for reconstructing the entire structure or do not comprise cells that may be simply collected. For each organ type dissimilar approaches and practical features are required to consider before emerging a healthier solution for the existing difficulties (Khan et al. 2014; Badylak et al. 2011). The factors that should be estimated during recellularization is indicated in Fig. 5.

6 Stem Cells Deliver an Innovative Aspect to Decellularized Scaffold-Related Tissue Engineering

Stem cells have turned into an essential segment of tissue engineering and deliver a novel aspect to decellularized scaffold-related tissue engineering especially, either as a cell foundation for recellularization or as a basis for producing ECM matrix (Badylak et al. 2011). Stem cells are stated as undifferentiated cells which can turn into precise lineages (potency) and can split by themselves to generate more stem cells (self-regeneration) (Clevers 2015). Fortified with their innate features, decellularized scaffolds coated with stem cells are inviting more courtesy in essential research and medical studies and are now being operated for interpreting practical organs. Nevertheless, the appliances underlying the interface of stem cells with decellularized scaffolds have still not been fully understood (Rana et al. 2017; Agmon and Christman 2016). The demonstration of stem cell seeding on decellularized scaffolds is presented in Fig. 6.

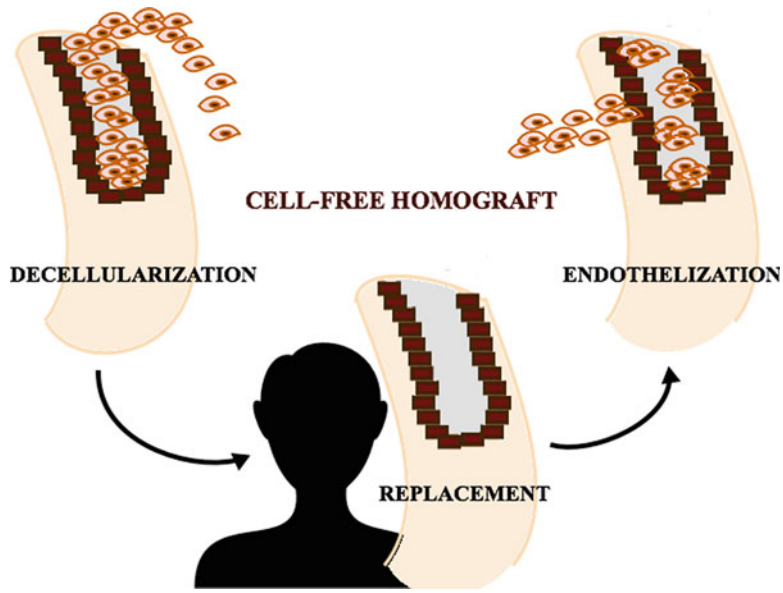

Fig. 4 Concepts of endothelialized biomaterials. Decellularized scaffolds may be implanted to generate an elementary internal vasculature. Upon implantation, the embedded endothelial cells are expected to connect with the host tissue to generate a perusable construct

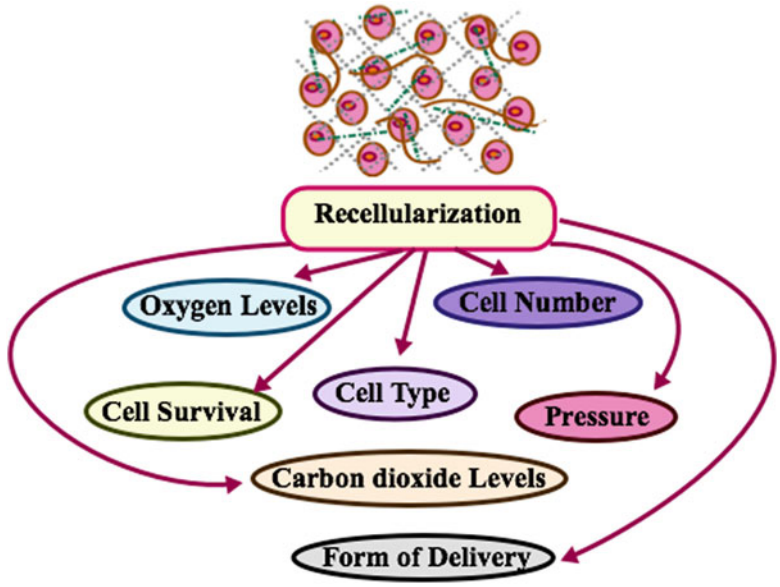

Fig. 5 Recellularization factors that are important for suitable integration with the decellularized scaffold

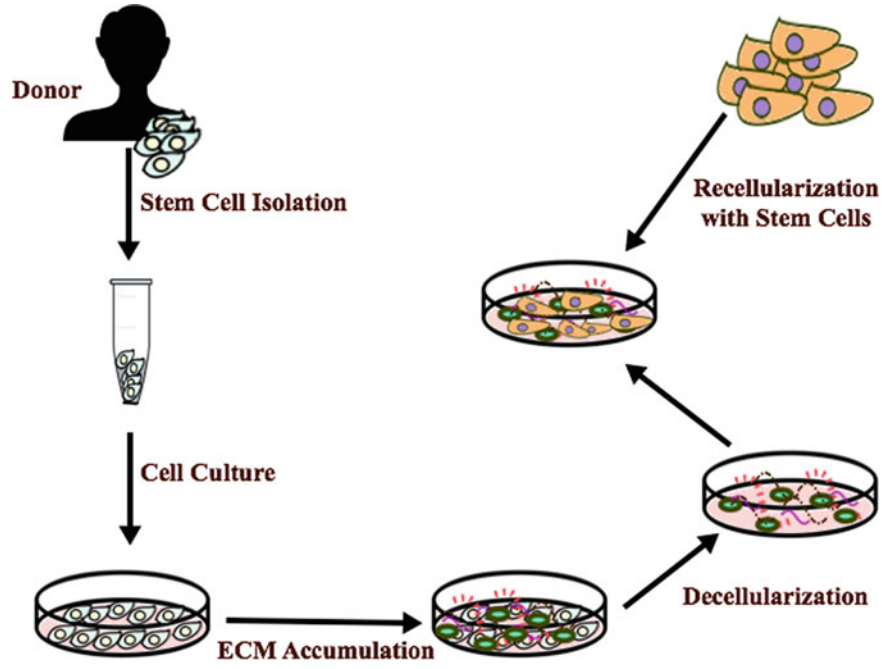

Fig. 6 Diagram demonstration for the expansion of practical tissues by decellularization and recellularization through stem cell seeding

Murine embryonic stem cells have been persuaded to positively expressing lung epithelial phenotype comprising alveolar (TTF-1 and SPC) and airway (CCSP) epithelium later to differentiation of definitive endoderm utilizing Activin A to simulate the Nodal signaling

followed through adherent cell culture in small airways growth medium or use of more discerning differentiation medium. Cellularizing decellularized rat scaffolds with mouse ESCs led to superior survival compared to cells seeded onto non-lung matrices and also deceptive differentiation in the direction of various lineages that showed zone-precise distribution covering club cells (CC10+), Alveolar epithelial type II (AT II) cells (CK18+, proSPC+), endothelial cells (CD31+) and mesenchymal cells (PDGFRa +). Therefore, it is hypothetically conceivable to detect differentiation of the most nascent of stem cells along the lines of growth only by seeding them on acellular scaffolds. Correspondingly, mouse ESCs differentiated to Nkx2.1+, proSPC + ATII-like cells seeded onto mouse decellularized scaffolds that were injected subcutaneously distributed to airways ($FoxJ1^{pos}$) and alveolar regions (proSPC+ or PDGFRa+ cells) and conserved phenotype expression for 14 days. Separately, host-derived endothelial cells infiltrated the scaffolds signifying that practical vascularization could happen (Wagner et al. 2013).

Studies established the viability of decellularized scaffolds seeded with autologous adipose-derived stem cells (ADSCs) for cartilage fault overhaul in rabbits. The results indicated that the ADSC-seeded decellularized scaffolds prompted cartilage tissue restoration similar to natural cartilage in point of physical features and chemical constituents. Hence, the expansion of decellularized scaffolds, together with stem cells, is an enthusing field of research that unlocks a novel area in stem cell-related tissue engineering (Kang et al. 2014).

Several cell types including bone marrow stem cells, umbilical stem cells, embryonic stem cells, fetal cardiomyocytes, smooth muscle cells, and dermal fibroblasts, were examined as cell foundations for spot recellularization with the purpose of fabrication of porcine cardiac patches. Between them, bone marrow stem cells were discovered to have a capability to renew myocardium, persuade angiogenesis, and be permitted from ethical issues (Wang et al. 2010).

7 Regenerative Medicine

Encouraging preclinical and experimental data thus far upkeep the opportunity for handling both chronic acute illnesses, and for regenerative medicine to abet diseases happening through an extensive group of organ systems and contexts covering skin wounds, cardiovascular diseases and traumas, managements for specific forms of cancer, etc. The existing treatment of transplantation of intact organs and tissues to cure organ and tissue insufficiency and forfeiture agonizes from restricted donor quantity and frequently serious immune difficulties, but these problems may possibly be avoided by the utilization of regenerative medicine approaches (Mao and Mooney 2015). Over the last 20 years, regenerative medicine has developed with a goal of directly restoring/renewing damaged or unhealthy tissue instead of treating disease indications (Taylor et al. 2018; Rijal 2017). Decellularized ECM coming from tissues or complete organs has significant regenerative benefits. Regardless of numerous stated compensations and the accomplishment in premature clinical studies, this know-how has not entirely been interpreted into standard medical practice so far (Parmaksiz et al. 2016).

8 Recent Research on Decellularized Tissue in Regenerative Medicine

8.1 Heart

Heart failure is one of the foremost reasons of hospitalization and mortality, effecting almost 15 million people in Europe (Heidenreich et al. 2011). A progressive phase of heart failure, also recognized as end-stage heart failure, is rising and this propensity grants itself as a difficulty to both medical doctors and healthcare organizations. Heart failure is described as having cardiac output that is incompetent for living, and diagnosis is predominantly poor, with around 25–50% of patients dying in 5 years after diagnosis (Zia et al. 2016).

Ott et al. have indicated an original technique of perfusion decellularization that can produce complete organ scaffolds. The extension of a cannula through the ascending aorta permitted backward coronary perfusion by detergents. This type of process attained the elimination of the cellular compartment of an entire heart. That was then recellularized with newborn rat cardiomyocytes. The construct was distributed inside of the heart scaffold over transmural injection, whereas endothelial cells were inserted through the aorta. The final structure was capable of contract up to 2% of the usual contractile function (Ott et al. 2008).

Ng et al. stated a parallel method where they seeded the decellularized heart with embryonic stem cells that botched to produce practical heart regarding to poor cardiac myocyte differentiation competence (Ng et al. 2011). Then, Lu et al. established the manufacturing efficient human heart tissues via recellularization of decellularized mouse hearts by human induced pluripotent stem cell-derived cardiac myocyte progenitors. Cells were transported over coronary vessels of the heart which also showed cardiomyocyte propagation, differentiation, myofilament development, impulsive contraction, production of mechanical strength and response to medications (Lu et al. 2013).

8.2 Liver

Liver is the biggest interior organ carrying vital physiological utilities, comprising vitamin storing, protein synthesis and detoxification. On behalf of people with end-stage liver failures, that are frequently linked with hepatitis, bile duct illnesses or liver cancers, liver transplantation has turned into first-rate management. It delivers improved excellence of life and affordability. The 5 year survival ratio after liver replacement is over 70%. Nonetheless, the number of patients who require transplantation surpasses suitable donors. In only United States of America, it was stated that over 16,000 patients demand transplantation every year but just 6000 liver replacements are accomplished because of lack of apposite donors. The lack of suitable donors consequences in eighteen deceases daily. There is a necessity for inventive methods to develop practical liver substitutes for end-stage liver failure patients (Meng et al. 2017).

Uygun et al. have indicated an exhaustive perspective of decellularizing rat livers and seeding them with rat primary hepatocytes, presenting encouraging hepatic utility and the capacity of heterotopically transplant these engineered livers into animals for almost 8 h (Uygun et al. 2010).

Hassanein et al. have showed recellularization by the bile duct supported practical allogenic and xenogenic cell proliferation on a decellularized rat liver scaffold. Replacement of the recellularized paradigm has resulted in fast implant thrombosis, likely secondary to exposed collagen in the deendothelialized vasculature. Development in the field is restricted by the incapability to produce a completely endothelialized structure covering different cell lines similar to an innate organ (Hassanein et al. 2017).

8.3 Pancreas

More than 1500 islet replacement practices have been implemented globally since 2000. However only about 7.5% of the transferred patients persist insulin independent 5 years after procedure. Existing transplantation methods cover the infusion of islets into the liver portal vein. The main difficulties of intraportal islet infusion are hemorrhage and thrombosis whereas other factors like anoikis, hypoxia, and inflammation related immune response also consequence in damage of islet utility and graft loss (Salvatori et al. 2014).

Claudius et al. have presented the perfusion decellularization of cadaveric rat and human pancreases for production of ECM scaffolds aiming to maintain the innate islet cell niche to enhance islet endurance and utility after replacement. Recellularization with human islets created practical endocrine tissue *in vitro* and retreated the diabetic phase after transplantation in rat models (Conrad et al. 2010).

Wu et al. have tried to create a microenvironment mimicking the innate pancreas that is suitable for not only cell development but also

cellular utility exertion. They utilized a decellularized mouse pancreas as 3D scaffold in the experimentation. MIN-6 (Mouse Insulinoma 6) cells were seeded on the bioscaffold. Moreover, *in vivo* plantation of the recellularized bioscaffold presented its capacity of regulating blood glucose. Nevertheless, they detected the progressively swelling blood glucose, which suggested the seeded cells might perish because of the absence of incessant source of nourishment (Wu et al. 2015).

8.4 Trachea

Idyllic approaches for renovating the tracheal structure and reestablishing tracheal utility upon tracheal injury or subtraction have not been settled. Artificial constituents have been utilized as unconventional alternates. Studies stated good outcomes for different tracheal engineering techniques with countless compatible materials. Conversely, the restorations have motivated on preserving the unity of the tubular organization, and difficulties covering body reactions and infections have not been completely addressed, with the long-term healing effect of this approach lasting unclear (Hung et al. 2016).

Gray et al. composed a tissue-engineered scaffold from xenologous decellularized leporine tracheal segment seeded with autologous amniotic mesenchymal stem cells and equated it with decellularized scaffolds. The consequences of the animal study established supreme endurance with full epithelization for the engineered scaffolds with amplified elastin plane later to transplantation (Gray et al. 2012).

Zang et al. have collected from Brown Norway rats (donor) and Lewis rats (receiver) were decellularized with frequent detergent/enzymatic methods. Decellularized Brown Norway tracheal matrix scaffolds were recellularized with Lewis rat stem cell–derived chondrocytes from outside and tracheal epithelial cells from inside to produce a layered tracheal structure. Decellularized tracheal matrix scaffold did not provoke noteworthy allograft refusal or remote body answer *in vivo*. While the structure sustained reepithelialization, stem cell–induced chondrocytes unsuccessful for engrafting in the heterotopic background (Zang et al. 2013).

8.5 Lung

In only United Stated, more than 24 million patients have indication of compromised lung utility, triggering extensive physical and financial weight. Presently, lung transplantation is the solitary absolute cure for people with end-stage lung disease. Nevertheless, vigorous organ unavailability averts transplantation from being a useful answer for the mainstream of these patients. The present donor lung unavailability is also worsened by the delicateness of lung itself. Lung tissues are simply injured and frequently conceded throughout the progression of transplantation. Along with these restrictions, patients are obligatory to be on permanent immunosuppressive drugs after lung transplantation, causing intense lessening in standard of life and an improved susceptibility to pulmonary contaminations. The formation of a disinfected, autologously cell sourced artificial lung would reduce the disease allied with immunosuppression and the lack of donor organ, and it would also deliver tailor lung substitutes for patients with an extensive variety of functional requirements (Balestrini et al. 2015).

Pulmonary tissue engineering has concentrated on renewal endorsed by decellularized scaffold *in vivo* and *in vitro*. Throughout decellularization, the organizational proteins and pertinent cytokines of extracellular matrix were preserved, while cellular constituents were detached. Epithelial and endothelial cells were seeded onto trachea and vessels, two groups discovered that efficient gas conversation may be produced 6 h later in rats with recellularized lungs (Yu et al. 2016).

8.6 Bone

Treatment options to serious bone deficiencies because of trauma, corruptions, tumors or

hereditary disorders are based on autologous or allogeneic bone grafts. Conversely, severe donor-site indisposition, elevated risk of contagions and location inadequacies hamper them as maintainable alternatives. Unconventional tissue engineering methods, in spite of the important developments of the field, are not yet capable of delivering reliably effective clarifications for repetitive therapeutic use. Evidently, there is the necessity to establish original or superior approaches able to encounter patients' requests (Papadimitropoulos et al. 2015).

Fröhlich et al. established an *in vitro* 0.5 cm sized bone structure utilizing human adipose derived stem cells, decellularized bone scaffolds and perfusion bioreactors. Later to 5 weeks of refinement, introduction of osteogenic supplements to the culture medium meaningfully improved the construct cellularity and the aggregates of bone matrix constituents covering collagen, bone sialoprotein and bone osteopontin (Frohlich et al. 2010).

8.7 Kidney

Regarding the universal upsurge of patients with renal failure, the expansion of practical renal replacement treatments have gained substantial attention and new tools are promptly advancing. Currently expended renal replacement therapies inefficiently eliminate accumulating waste products, resulting in the uremic syndrome. A more desired management choice is kidney trans plantation, but the lack of donor organs and the rising amount of patients remaining for a transplant permit the expansion of original technologies (Jansen et al. 2014).

Porcine kidneys were effectively decellularized, suggesting the probability of utilizing these transplantable scaffolds to build engineered kidney clinically pertinent. Entire porcine kidneys were decellularized and orthotopically *in vivo* relocated, then prophylaxis was controlled with anticoagulant therapy. Immune cells in the pericapsular area and thrombosis happened owing to the absence of endothelial cells (Yu et al. 2016).

9 Workbench to Bedside Interpretation of Repopulated Decellularized Scaffolds

Conveying scaffolds to medical excellence and measure is only one of numerous phases headed for the renewal of feasible and operative organs. At the modern level of expertise, an effort to review the whole progression of embryogenesis from a single cell to organogenesis in a laboratory looks barely convincing. This kind of an accomplishment would necessitate broad culture intervals to produce tissues of quantifiable dimension and a diversity of tissues to allow interplay, and would cause obvious ethical problems (Song and Ott 2011).

There have been couple of attempts for the production of innate ECM with practical retrieval. Ott et al. and Petersen et al. established transplant endurance for limited hours of recellularized lung scaffold into rats with passable oxygen and carbon dioxide interchange with suitable pressure/volume interactions. The rat passed away owing to pulmonary edema subsequent to respiratory failure (Khan et al. 2014).

Song et al. produced an engineered rat kidney by decellularization and recellularization method by human umbilical cord blood-derived endothelial cells and presented urine making accompanied by minor macromolecular sieving and reabsorption capacity (Khan et al. 2014). Laronda et al. instigated sexual maturity in mice following decellularized ovary transplant. They established that ovarian transplant seeded with primary ovarian cells on a decellularized matrix may deliver a niche for steroid and peptide hormone assembly that can start adolescence in ovariectomized mice (Laronda et al. 2015).

Hung et al. performed segmental organ decellularization on trachea and employed autotransplantation on rabbit models. Even though the respirational epithelium renewal on the internal surface seemed to be pleasing, the tubular assemblies were not able to be preserved later to replacement that eventually led to the decease of the animals (Hung et al. 2016).

Hopkins, et al. evaluated in a baboon model the hemodynamics and human leukocyte antigen immunogenicity of recurrently inserted engineered human and baboon heart valve scaffolds. Valve impairment interrelated with markers for more powerful inflammatory incitement. The verified bioengineering techniques condensed antigenicity of both human and baboon valves. Replacement valves from both species were found hemodynamically equal to innate valves (Hopkins et al. 2013).

Duisit et al. formerly stated the use of the perfusion-decellularization method in porcine ear and human face models, with effective implant decellularization, conservation and convenience of the vascular system, appropriate for seeding with fresh cells and conformity. They then theorized that this knowledge may be pertained to human ear grafts. They found that vascularized and multifaceted auricular scaffolds may be acquired from human basis to deliver a platform for additional useful auricular tissue engineered creates, thus offering a perfect path to the vascularized complex tissue engineering method (Duisit et al. 2018).

10 Conclusion

Regenerative medicine embraces the potential of renewing tissues and organs by either encouraging formerly irremediable tissues to overhaul themselves or engineering them ex vivo. Decellularized constituents have the capacity to grasp the exclusive principles presented by autografts that elucidates the many dissimilar areas of utilization for these knowledges. According to the tissue form, allogeneic or xenogeneic tissues maintain their tissue precise features and bioactive composites following decellularization. Consequently, decellularized ECM materials have the capability to enable reformative renovation of the injured tissue. Regulation of construction, organization of donor tissues and organs, efficacy and welfare assessment, superiority control, application proprieties and strategies, calibration and consciences are serious subjects for the expansion and commercialization of decellularized ECM scaffolds. The suitable approach for producing a 3D structure is displayed in Fig. 7.

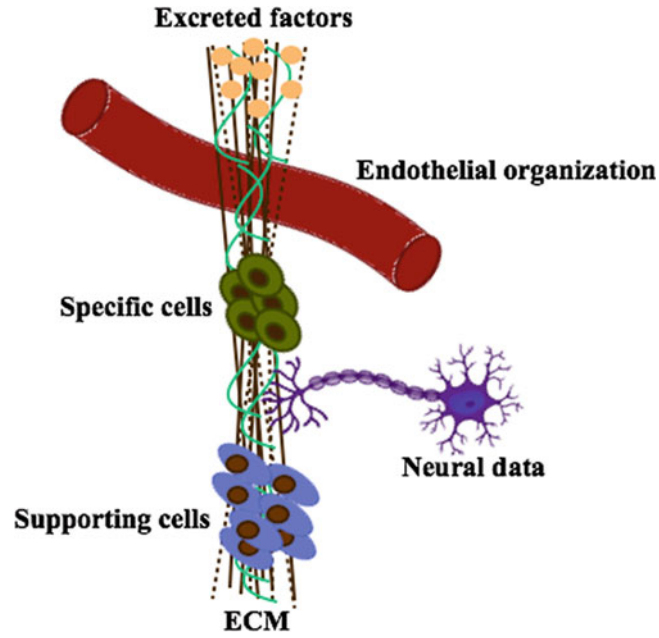

Fig. 7 Common approach for creating a suitable 3D structure. The precise range of ECM features, emblematic for designated normal tissue can be established. A decellularized scaffold with toning factors and appropriate cells can be identified and united in vitro for growth of specific, custom-made 3D system

Many of the existing efforts target emerging approaches for growth and stemness might convey innovative solutions relevant to organ engineering in the anticipatable future. Solid organ redevelopment founded on perfusion-decellularized innate ECM scaffolds carries excessive aptitude for patients distressing end-organ failure, nonetheless evidently remains an aspiring aim. Effort in the direction of that target will include numerous fields and construct intermediate remedial products and highpoints that expand our knowledge on stem and progenitor cell fate in organ expansion and disease.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 87–106
https://doi.org/10.1007/5584_2019_381

Published online: 9 May 2019

Synovium-Derived Mesenchymal Stem/Stromal Cells and their Promise for Cartilage Regeneration

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Abstract

Adult tissues are reservoirs of rare populations of cells known as mesenchymal stem/stromal cells (MSCs) that have tissue-regenerating features retained from embryonic development. As well as building up the musculoskeletal system in early life, MSCs also replenish and repair tissues in adult life, such as bone, cartilage, muscle, and adipose tissue. Cells that show regenerative features at least *in vitro* have been identified from several connective tissues. Bone marrow and adipose tissue are the most well recognized sources of MSCs that are already used widely in clinical practice. Regenerative medicine aims to exploit MSCs and their tissue regeneration even though the underlying mechanisms for their beneficial effects are largely unknown. Despite many studies that have used various tissue-derived MSCs, the most effective tissue source for orthopedic procedures still remains to be identified. Another question that needs to be addressed is how to evaluate autologous MSCs (i.e., patient derived). Previous studies have suggested the features of bone-marrow-derived MSCs can differ widely between individuals, and can be changed in particular in patients suffering from some forms of degenerative disorder, such as osteoarthritis. The synovium is a thin membrane that protects the synovial joints, and it is a rich source of MSCs that show great potential for regenerative medicine. Here, we review synovium-derived MSCs from reports on basic and clinical studies. We discuss their potential to treat cartilage defects caused by either degeneration or trauma, and what needs to be done in further research toward their better exploitation for joint regeneration.

Keywords

Animal studies · Clinical studies · *In-vitro* studies · Mesenchymal stem/stromal cells · Synovium

1 Introduction

Mesenchymal stem/stromal cells (MSCs) are heterogeneous populations of stem cells (Sacchetti et al. 2016) that reside in adult tissues that have the unique ability for multilineage differentiation into bone, cartilage, muscle, and adipose tissues (Fellows et al. 2016). MSCs were first described for bone marrow by Friedenstein and colleagues (Friedenstein et al. 1970), and have since been found in many other adult tissues, such as bone, muscle, adipose tissue, synovium, skin, and other

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connective tissues. The scientific pursuit of these cells has been long hampered by their rare frequency in adult tissues and their lack of specific markers *in vivo*. Their propensity for plastic adherence allowed a series of *in-vitro* studies to evaluate cultures of MSCs and their potency for cartilage, bone and adipose tissue differentiation. It is likely that *in-vitro* expanded MSCs represent a population comprised of multiple types of distinct stem cells, as well as mature cells (Chan et al. 2018).

Transgenic animal models that can trace MSCs from early development through adult life and through health and disease (Fuchs and Horsley 2011) have led the *in-vivo* scientific research of MSCs. Use of transgenic animal models in combination with tissue injury models has provided priceless information about the identities and regenerative abilities of MSCs. Using these approaches, specific MSC populations have been identified in bone marrow (Zhou et al. 2014; Worthley et al. 2015; Chan et al. 2018) and synovium (Roelofs et al. 2017). It has also been clearly shown that these MSCs replenish bone and cartilage in adult life, and can contribute to tissue repair following injury; some can even form rudimentary joints *de novo* (Roelofs et al. 2017).

As MSCs are endogenously present in several tissues and contribute to tissue repair in adult life, they show great promise for regenerative medicine in degenerative joint disorders. Osteoarthritis, in particular, is the most common joint disorder, and it results from a combination of the breakdown of a joint and the attempt by the body to repair the damage. In humans, endogenous cartilage repair is ineffective, and the poor healing capacity of cartilage after injury can lead to osteoarthritis. The risk of developing osteoarthritis also increases with age (Arthritis Research UK 2018). Stem-cell exhaustion and their decreased regeneration potential have been proposed as hallmarks of aging in humans (Partridge et al. 2018). It has been shown that bone-marrow-derived MSCs in patients with hip osteoarthritis have low proliferative potential, and they are less active in chondrogenic and adipogenic differentiation (Murphy et al. 2002). Moreover, another study reported that MSCs in subchondral bone from patients with late-stage hip osteoarthritis increase in number in areas of damage, but show perturbations that can lead to damage escalation (Campbell et al. 2016).

Controversy remains over whether articular cartilage itself has MSCs and which tissue-derived MSCs, if any, contribute to cartilage repair in humans. Recently, increasing evidence has indicated that chondrogenic abilities and cartilage repair features can be attributed to synovium-derived MSCs (Mak et al. 2016; Roelofs et al. 2017; Yao et al. 2018; De Bari and Roelofs 2018; Zayed et al. 2018; Enomoto et al. 2018; Shimomura et al. 2018; Murata et al. 2018; Jia et al. 2018b). Synovium is the soft tissue that lines the spaces of diarthrodial joints, tendon sheaths, and bursae. It includes the continuous surface layer of cells (i.e., the intima) and the underlying tissue (i.e., the subintima). The intima consists of macrophages and fibroblasts, while the subintima includes blood and lymphatic vessels, where both resident fibroblasts and infiltrating cells are found in a collagenous extracellular matrix. Between the intimal surfaces there is a small amount of fluid, which is usually rich in hyaluronan (Smith 2011).

Synovial hyperplasia is a very common phenomenon following joint injury. However, it is not only inflammation in the joint that sustains synovial hyperplasia. It has been shown that hyperplasia is also underpinned by proliferation of MSCs that respond upon joint injury (Kurth et al. 2011). Recently, a subpopulation of synovial MSCs (defined as a Gdf5-lineage derived from the interzone of the early embryonic joint structure) was shown to contribute to cartilage repair postnatally (Roelofs et al. 2017).

There is gathering evidence that this tiny membrane wrapped around the synovial joints is a rich source of MSCs, which has been little recognized in comparison to bone marrow and adipose tissue. Here, we provide an overview of MSCs derived from synovium. We start with basic and clinical studies, with a focus on their potential for regeneration of cartilage defects caused by either degeneration or trauma. We also discuss the best

lines for further research toward better exploitation of synovium MSCs in regenerative medicine.

2 Basic Studies

Here we provide an overview of the basic studies that have investigated synovium-derived MSCs. *In vitro*, human and animal synovium tissues have been used to characterize MSCs using laboratory methods. *In vivo* in animal studies, synovium-derived MSCs have been implanted in different animal models of osteoarthritis, to define their regenerative features.

2.1 In-Vitro Studies

The *in-vitro* studies to date are summarized in Table 1. Minimal criteria to define cells cultured *in vitro* as MSCs were suggested by the International Society for Cell Therapy (ISCT) in 2006 (Dominici et al. 2006). These criteria included plastic adherence, more than 95% positivity for CD73, CD90, and CD105, and less than 2% positivity for CD45, CD34, CD11b/14, CD79α/19, and human leukocyte antigen (HLA)-DR, combined with trilineage differentiation potential; i.e., chondrogenic, adipogenic, and osteogenic. In investigations into these three criteria, the majority of *in-vitro* studies into synovium and synovial-fluid-derived MSCs have been performed in humans. Despite the minimal criteria set by the ISCT, the cell isolation and cultivation methods used still vary widely, with a general lack of standard identification (Dominici et al. 2006; Lv et al. 2014). Therefore, it is difficult to compare the data across these studies.

Most of these human studies have investigated synovium from the knee joint, with synovium from the hip joint less well recognized (Hermida-Gómez et al. 2011; Murata et al. 2018). The sites of synovium in the hip joint that have been shown to be sources of MSCs are given in Fig. 1.

De Bari et al. (2001) were the first to demonstrate that MSC-like cells can be isolated from synovium of knee joints. Sakaguchi et al. (2005) showed this synovium to be a superior reservoir of MSCs in comparison with bone marrow, adipose tissue, periosteum, and muscle in patients with anterior cruciate ligament injury. The advantage of their study was that five different tissue sources of MSCs were compared within the same patient (Sakaguchi et al. 2005). Using this approach, the influence of concomitant conditions and other variables such as age, sex, and body mass index, among others, can be eliminated. Similar findings were reported in mice, where infrapatellar fat-pad-derived MSCs were shown to have higher proliferative potential and similar or higher multilineage potential in comparison with bone-marrow and muscle-derived MSCs. A recent study compared two different synovium sources of MSCs in patients with femoracetabular impingement syndrome (Murata et al. 2018). They observed that MSCs from the cotyloid fossa synovium have higher proliferation and differentiation potential than those from the paralabral synovium. Hence, they suggested the use of cotyloid-fossa-synovium-derived MSCs for stem-cell therapy.

Efforts have also been made to identify the optimal tissue source of MSCs in particular joint disorders. Kohno et al. (2017) compared synovium-derived MSCs in patients with osteoarthritis and rheumatoid arthritis. They showed that cell yields, surface markers, and the chondrogenic potentials of synovial MSCs from both of these patient groups were comparable, and so they indicated that synovium derived from patients with rheumatoid arthritis represents a promising source of MSCs for cartilage and meniscus regeneration. As mentioned above, in such comparisons of two groups of patients, it is imperative to exclude other sources of donor-to-donor variations that might influence the features of the MSCs, such as age, sex, concomitant conditions, and disorders.

Some studies have compared synovium-derived MSCs from patients with osteoarthritis to those from healthy controls. The aim was to determine whether osteoarthritis affects MSCs in synovium in the same way as it affects, for instance, MSCs in bone-marrow (Murphy et al. 2002; Campbell et al. 2016). Generally, the

Table 1 Summary of *in-vitro* studies using synovium-derived MSCs

Species	MSC source	Methods used	Outcomes	Reference
Human	Paralabral and cotyloid fossa synovium from 18 patients with femoroacetabular impingement syndrome during hip arthroscopy	CFU-F, trilineage differentiation	Higher CFU-F, adipogenesis, osteogenesis and chondrogenesis in cotyloid fossa compared to paralabral synovium	Murata et al. (2018)
Rabbit	Knee synovium and bone marrow from tibia	Macroscopic analysis, histology	More MSCs observed at site of defective ligament in the knee	Morito et al. (2008)
Human	Synovial fluid from 22 patients with meniscus injury and eight controls with no history of knee injury	*In-vitro* expansion, CFU-F, trilineage differentiation, surface epitopes	Higher CFU-F of MSCs from patients. CFU-F correlated with post-injury period. No difference in trilineage potential and surface epitopes	Matsukura et al. (2014)
Human	Fibrous synovium from 28 patients and fibrous and adipose synovium from six patients during total-knee arthroplasty	Suspended synovium culture model: CFU-F, trilineage differentiation, surface epitopes	MSCs from suspended synovium culture model formed multipotent colonies and expressed CD73, CD90, CD105, CD44. Higher numbers of MSCs from fibrous synovium in comparison with adipose synovium	Katagiri et al. (2017)
Human	Synovial fluid from 17 patients and synovium from eight patients with temporomandibular joint osteoarthritis, during surgical debridement or joint disk perforation	*In-vitro* expansion, CFU-F, trilineage differentiation, surface epitopes	Similar proliferative and differentiation potentials between synovial fluid and synovium-derived MSCs. Higher expression of CD73, CD90, CD105, CD44 in synovial fluid MSCs	Yao et al. (2018)
Human	Surface, stromal and perivascular regions of synovium from 10 patients during total knee arthroplasty	Immunostaining for 19 markers, cell sorting, proliferation, surface epitopes, trilineage differentiation	Surface MSCs, CD55+ CD271−; stromal MSCs, CD55− CD271−; perivascular MSCs, CD55− CD271+. Highest proliferation, chondrogenic, osteogenic potential of perivascular MSCs	Mizuno et al. (2018)
Human	Synovium collected by direct biopsy and arthroscopic trocar shaver-blade filtrate, and synovial fluid MSCs from 19 patients with traumatic injuries, traumatic inflammation, osteoarthritis	Isolation efficacy, growth kinetics, surface epitopes, culture expansion under hypoxic conditions, trilineage differentiation	Isolation efficacy >75% for all three groups. No difference in surface markers CD73, CD90, CD105. No difference in trilineage potential. Higher growth kinetics of arthroscopic shaver-blade-derived MSCs, also under hypoxic conditions	Ferro et al. (2019)

(continued)

Table 1 (continued)

Species	MSC source	Methods used	Outcomes	Reference
Mouse	Synovium from the infrapatellar fat pad, bone marrow flushed from femur and tibia, and muscle from quadriceps	*In-vitro* expansion, CFU-F, trilineage differentiation, surface epitopes	Higher proliferative potential, growth kinetics, CFU-F, PDGFRα expression of synovium MSCs compared to muscle and bone-marrow MSCs. Similar or higher osteogenic, adipogenic, and chondrogenic potential of synovium MSCs compared to muscle and bone marrow MSCs	Futami et al. (2012)
Human	Synovium from 20 osteoarthritis patients during hip arthroplasty and six healthy donors during organ donation	Immunostaining of tissue sections, trilineage differentiation, surface epitopes	Higher expression of CD44, CD90, CD105 in intimal lining in healthy donors, and diffused expression in osteoarthritic patients. Higher expression of CD44, CD90, CD105 in MSCs from patients with osteoarthritis. No comparison of trilineage potential	Hermida-Gómez et al. (2011)
Human	Synovium from eught patients with rheumatoid arthritis and eight patients with osteoarthritis, during total knee arthroplasty	Cell yields, surface markers, trilineage differentiation	No difference in cell yields, surface markers CD73, CD90, CD105, CD44 expression, and trilineage potential	Kohno et al. (2017)
Human	Synovium, bone marrow, adipose tissue, periosteum and muscle from eight donors during anterior cruciate ligament reconstruction surgery for ligament injury	Cell yields, culture expansion, trilineage differentiation, surface epitopes	Highest colony numbers and cell numbers per colony in bone marrow compared to other MSCs. MSCs from all tissues retained proliferation ability even at passage 10. Higher chondrogenic potential in synovium MSCs. No difference in surface epitopes	Sakaguchi et al. (2005)
Human	Synovium from six donors post mortem and from patients during knee arthroplasty	*In-vitro* expansion, CFU-F, senescence staining, telomerase activity, trilineage differentiation	Synovium MSCs expanded intensively in monolayers, with limited senescence, showed trilineage differentiation even at single clone level. Donor age, cell passaging, and cryopreservation did not affect multilineage potential of MSCs	De Bari et al. (2001)
Human	Infrapatellar fat pad and synovial fluid from six patients undergoing total knee replacement or anterior cruciate ligament surgery	Tissue histology, growth kinetics, trilineage differentiation, surface epitopes, stimulation with interferon-γ	MSCs from both tissues positive for CD73, CD90, CD105, and showed multipotency. Synovium-derived MSCs had significantly faster proliferation rates. MSCs from both tissues increased production of human leukocyte antigen-DR (HLA-DR) following interferon-γ stimulation	Garcia et al. (2016)

(continued)

Table 1 (continued)

Species	MSC source	Methods used	Outcomes	Reference
Human	Synovium from patients undergoing knee replacement for degenerative arthritis	CFU-F, surface epitopes, chondrogenesis	CD105, CD166, CD10, CD13, CD44, CD49a, CD73 positive and chondrogenic	Jo et al. (2007)
Human	Synovial MSCs collected during knee arthroscopy as irrigation fluid at inception, after initial inspection of the joint, after agitation of the synovium (mobilized MSCs)	CFU-F, surface epitopes, trilineage differentiation, adherence to blood clots and fibrin scaffolds	Mobilized synovial MSCs showed the highest CFU-F trilineage differentiation, standard MSC phenotype, and adhered to various fibrin scaffolds	Baboolal et al. (2018)
Rabbit	Synovial fluid from articular cavity of knee	*In-vitro* expansion, CD90 sorting, trilineage differentiation	CD90 sorted *in vitro* expanded MSCs showed expression of CD44, CD90, and trilineage potential	Jia et al. (2018a)
Human	Synovium and bone marrow from same patients undergoing total knee arthroplasty	Low-affinity nerve growth factor receptor and THY-1 sorting, CFU-F, trilineage differentation	Low-affinity nerve growth factor receptor and THY-1 sorted synovial MSCs showed higher CFU-F and enhanced adipogenic and chondrogenic differentiation in comparison with bone-marrow-derived MSCs	Ogata et al. (2015)

CFU-F, colony forming unit in fibroblast assay; PDGFRα, platelet-derived growth factor receptor α.

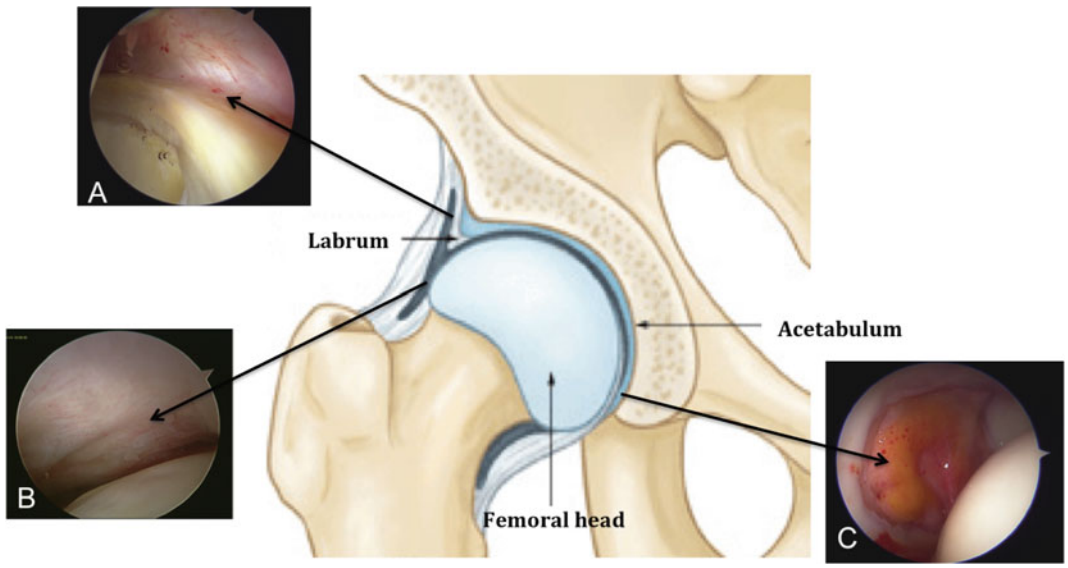

Fig. 1 Sites of synovium in the hip joint shown to be reservoirs of MSCs, accessible by arthroscopy
A – periacetabular sulcus
B – inner surface of the joint capsule
C – cotyloid fossa

healthy controls were post-mortem donors with no evidence of joint disorders (De Bari et al. 2001; Hermida-Gómez et al. 2011). Interestingly, Hermida-Gómez et al. (2011) showed that synovium in patients with osteoarthritis who were undergoing total hip replacement contained more cells that expressed MSC markers than synovium from joints with no cartilage damage. These latter cells were obtained from healthy donors who were undergoing organ donation (Hermida-Gómez et al. 2011). No differences in multilineage potential was seen between these groups, which would suggest that MSCs from patients with osteoarthritis have functional deficiencies. Ferro et al. (2019) investigated whether different methods of synovium biopsy affect MSCs, here as direct biopsy *versus* arthroscopic trocar shaver-blade filtrate. They compared MSCs derived from synovium using these two methods of synovium collection with MSCs isolated from synovial fluid. All of their samples showed MSC-like characteristics, while arthroscopic shaver-blade-derived MSCs also showed higher proliferation (Ferro et al. 2019). Apart from synovial tissue, MSCs can also be isolated from synovial fluid from the knee (Morito et al. 2008; Matsukura et al. 2014; Garcia et al. 2016) and temoporomandibular joint (Yao et al. 2018). Similar to synovium, synovial fluid in patients with osteoarthritis has also been shown to contain greater numbers of MSCs than for healthy volunteers, although with no difference in their multipotency (Morito et al. 2008; Matsukura et al. 2014).

In addition to the MSC markers CD73, CD90, and CD105 that were suggested by the ISCT (Dominici et al. 2006), CD44 (i.e., the hyaluronan receptor) is frequently analyzed in *in-vitro* studies. Hyaluronan and hyaluronic acid bind to CD44, which appears to be the main factor responsible for the constant volume of the synovial fluid that serves as a cushion for synovial tissue and as a reservoir of lubricant for cartilage (Smith 2011). Synovial MSCs express high levels of CD44 (Hermida-Gómez et al. 2011; Kohno et al. 2017; Katagiri et al. 2017; Yao et al. 2018). CD44 has an important role during synovial joint development, prior to cavitation. It is expressed in the interzone and the articular surfaces. After cavitation, hyaluronic acid binds to CD44 on synovium and articular surfaces. This facilitates tissue separation and helps to create a functional joint cavity (de Sousa et al. 2014). On this basis, CD44 might be a true marker of synovial MSCs, which are retained from early embryonic development through adult life. However, as with other MSC markers, CD44 is not exclusive to synovial MSCs.

Mizuno et al. (2018) compared MSCs derived from different regions of synovium; i.e., from the surface, stromal, and perivascular regions. They reported that combination of CD55 and CD271 as markers can differentiate between synovial MSCs from these three regions. Moreover, they also showed that MSCs from the perivascular region that were CD55 negative and CD271 positive had the highest proliferative and chondrogenic potentials. Garcia et al. (2016) also challenged MSCs from infrapatellar fad pads and synovial fluid with interferon γ in an *in-vitro* setting. Both MSCs responded with increased levels of HLA-DR, thus showing an immunomodulatory capacity that confers further therapeutic value to these cells with regard to treatment of the inflammatory aspect of osteoarthritis.

Synovium-derived MSCs have also been isolated from knees of rabbits (Jia et al. 2018a) and mice (Futami et al. 2012). The most challenging step here was the dissection of synovium from the knees of these small animal species. Indeed, larger animals are more commonly used for *in-vivo* studies of synovium-derived MSCs.

In summary, most of the *in-vitro* studies to demonstrate that MSC-like cells can be isolated from adult synovium have investigated the proliferation and trilineage potential, and the limited set of standard markers of CD73, CD90, and CD105, plus the additional marker CD44, of synovium or synovial fluid-derived MSCs in human knee joints. There is a lack of studies that have compared synovium of patients with osteoarthritis and healthy donors to identify changes in these MSCs that would provide better understanding of the mechanisms underlying joint degeneration in osteoarthritis. Future research on synovial MSCs requires verification of the MSCs markers

identified in animal studies (Zhou et al. 2014; Worthley et al. 2015; Roelofs et al. 2017) and in tissue sources other than synovium (Chan et al. 2018). Immunomodulation of synovial MSCs, and in particular their immunosuppression, needs to be evaluated using appropriate *in-vitro* tests. It might also be of interest to investigate the potential of synovial MSCs for tendinocyte differentiation, due to the increasing prevalence of tendon and ligament degeneration, and for myocyte differentiation, due to their potential for muscle repair.

2.2 In-Vivo Animal Studies

The *in-vivo* animal studies carried out to date using synovium-derived MSCs are summarized in Table 2. Synovium-derived cell therapies have most commonly been tested in mouse, rat, rabbit, pig, and equine models of osteoarthritis. To define the regenerative features of MSCs *in vivo*, joint defects must first be created in these animals. Osteoarthritis in animal models is simulated by creating full-thickness cartilage defects or ligament injuries (Kuyinu et al. 2016). Due to the ease of access for surgical induction of osteoarthritis and implantation of MSCs, the knee is the preferred joint in all of these animal species.

In addition to determination of the *in-vivo* effects of implanted MSCs on joint regeneration, the major advantage of these studies is that they also provide some *in-vitro* assessment of the implanted cells, to demonstrate that they were indeed MSCs. This is feasible because the majority of the *in-vivo* studies with animals have used *in-vitro* expanded cells. Due to the experimental nature of these studies, no strict standards are required, such as good manufacturing practice, unlike for clinical studies using *in-vitro* expanded MSCs. The majority of these expanded MSCs are used for implantation, while the standard MSC tests are performed on a small aliquot (i.e., for surface MSC markers, trilineage differentiation, chondrogenesis).

In most of these studies, pure preparations of *in-vitro* expanded MSCs have been implanted, while studies that have embedded MSCs in some kind of a scaffold or have preconditioning them in chondrogenic media have been infrequent. Jia et al. (2018a, b) showed that prechondrogenesis of synovial-fluid-derived MSCs did not improve cartilage repair in a rabbit chondral-defect model. This observation might be explained by the loss of paracrine function of the MSCs by pre-differentiation.

There is some evidence that rather than contributing to tissue repair themselves, MSCs have an immunomodulatory action and stimulate the endogenous cells of the recipients toward tissue repair (for review, see Čamernik et al. 2018). However, apart from rare *in-vitro* studies (e.g., Garcia et al. 2016), little is known about the immunomodulation of synovial MSCs and their potential to supress inflammation.

To evaluate the *in-vivo* effects of implanted MSCs on cartilage regeneration following joint injury, the preferable methods of assessment are arthroscopy, histology, and magnetic resonance imaging. Most studies have investigated these effects at 4–12 weeks post injury or post MSC implantation. Koga et al. (2008) compared the *in-vivo* cartilage regeneration of MSCs isolated from bone marrow, synovium, adipose tissue, and muscle of adult rabbits. They embedded these MSCs in collagen gel and transplanted them into full-thickness cartilage defects of rabbit knees, where synovium-derived and bone marrow-derived MSCs formed a more abundant cartilage matrix than adipose-derived and muscle-derived MSCs. When implanting synovium-derived MSCs into osteochondral defects of pigs, Nakamura et al. (2012) reported that leaving a suspension of synovial MSCs on the cartilage defect for 10 min before closure of the wound allowed the cells to adhere within the defect first, which promoted improved cartilage repair.

In the light of the dilemma of whether to use autologous or allogeneic MSCs for tissue regeneration, there have also been studies where xenogenic cell therapies were used (Ozeki et al. 2016; Zayed et al. 2018; Neybecker et al. 2018). None of these studies observed adverse events associated with xenogenic cell therapies. Ozeki et al. (2016) and Zayed et al. (2018) also observed some degree of chondroprotection in their animal

Table 2 Summary of the *in-vivo* animal studies

Species	Number (n)	Model of osteoarthritis	Cell therapy used	Methods used	Duration	Outcomes	Reference
Rabbit	NA	Full thickness osteochondral defects in trochlear groove	Bone marrow, synovium, adipose tissue and muscle-derived MSCs embedded in collagen gel	*In vitro*: Proliferation and chondrogenesis	4, 12 weeks	*In vitro*: Higher proliferation of synovium and muscle-derived MSCs, more cartilage formed by bone marrow and synovium-derived MSCs	Koga et al. (2008)
				In vivo: Histology of the cartilage matrix		*In vivo*: More cartilage produced by synovium and bone marrow-derived MSCs	
Pig	16	Full thickness osteochondral defects in weight-bearing area of medial femoral condyles	Bone marrow, periosteum, muscle, adipose tissue, suprapatellar pouch synovium-derived MSCs	*In vitro*: Chondrogenesis	4, 12 weeks	*In vitro*: Higher chondrogenic potential of synovial MSCs	Nakamura et al. (2012)
				In vivo: Arthroscopy, histology, MRI		*In vivo*: Oswestry arthroscopy, ICRS and modified Wakitani score higher with synovial MSCs. Placing suspension of synovial MSCs on cartilage defect 10 min before wound closure promoted cartilage repair	
Rabbit	NA	Patellar groove cartilage defects	Autologous synovial fluid MSCs cultured for 3 weeks *in vitro* in either chondrogenic or normal medium, intra-articular injection once a week for 4 weeks	*In vitro*: Surface epitopes, trilineage differentiation	4 weeks	*In vitro*: Expression of CD73, CD90, CD44, and trilineage differentiation	Jia et al. (2018b)
				In vivo: Arthroscopy, histology, MRI		*In vivo*: Hyaline-like cartilage was observed in defects treated with synovium-fluid-derived MSCs in comparison with fibrocartilage formed in the defects treated with MSCs in chondrogenic medium	

(continued)

Table 2 (continued)

Species	Number (n)	Model of osteoarthritis	Cell therapy used	Methods used	Duration	Outcomes	Reference
Mouse	NA	Joint surface knee injury (medial parapatellar arthrotomy)	C57BL/6 mice received two different nucleosides in drinking water to label MSCs in synovium *in vivo*	Histology and immunohistochemistry	4, 8, 12 days post-injury	First evidence of resident MSCs in knee joint synovium that undergo proliferation and chondrogenic differentiation following injury *in vivo*	Kurth et al. (2011)
Equine, rat	NA	Full thickness articular cartilage defects in trochlear grooves of distal femur in rats	Xenogenic implantation of equine-derived bone marrow and synovial fluid MSCs encapsulated in neutral agarose scaffold	*In vitro*: Proliferation, viability, chondrogenesis	1, 12 weeks	*In vitro*: Higher chondrogenic potential of synovium fluid MSCs. MSCs seeded on agarose construct were metabolically active and viable	Zayed et al. (2018)
				In vivo: Macroscopic and histology		*In vivo:* Better macroscopic and histological result of articular cartilage in knee treated with MSCs than in control	
Rats	40	Partial thickness cartilage defect on the medial femoral condyle	Intra-articular injection of allogeneic synovium-derived *in-vitro* expanded MSCs at three different times (injury, 1, 2 weeks post injury)	*In vitro*: Surface markers, trilineage differentiation	6 weeks post injury	*In vitro*: MSCs expressed CD90 and demonstrated trilineage differentiation potential	Enomoto et al. (2018)
				In vivo: Histology		*In vivo*: Significantly higher histological score in group with MSCs at time of injury. MSCs distributed in synovium, not in cartilage surrounding defective area	
Mouse	52	Focal, full-thickness knee cartilage defect	Sca-1+ synovial MSCs from MRL/MpJ "super-healer" and C57BL6 mice	*In vitro*: Trilineage differentiation	At injury, 2, 4 weeks post injury	*In vitro*: Similar trilineage potential of synovial MSCs from MRL and C57BL6 mice	Mak et al. (2016)
				In vivo: Histology, MRI		*In vivo*: Increased cartilage repair 4 weeks post injury with MSCs from both mice. C57BL6 mice injected with MRL-derived MSCs showed greatest change in MRI signal intensity in defect site, in comparison to imaging directly after injury	

Rat	76	Anterior cruciate ligament transection in Lewis rats	Xenogenic implantation of human synovial MSCs (10^6); periodic *versus* single injection	*In vivo*: Histology and flow cytometry	12 weeks	Periodic injections maintained MSCs in knees and showed higher chondroprotective effects in comparison with single MSCs migrated mainly into synovium and retained undifferentiated	Ozeki et al. (2016)
Mouse	12	Joint surface knee injury (medial parapatellar arthrotomy)	Gdf5-lineage of synovial MSCs (endogenous and after allogeneic transplantation)	*In vitro*: Trilineage differentiation, synoviogenesis	4, 8 weeks	Gdf5-lineage cells contribute to cartilage repair via yes-associated protein (yap)	Roelofs et al. (2017)
				In vivo: Histology			
Rat	16	Anterior cruciate ligament transection	Xenogenic injections of human synovial fluid-derived MSCs obtained from donors with advanced knee osteoarthritis in rat knee at day 7 and 14 following anterior cruciate ligament transection	*In vitro*: Immunophenotype, trilineage differentiation, chondrogenic induction in collagen sponges	4, 8 weeks post anterior cruciate ligament transection	*In vitro*: CD73+, CD90+, CD105+, CD34−, CD45− immunophenotype and multilineage. Potency, chondrogenic induction (TGF-ß1 ± BMP-2) in collagen sponges induced expression of chondrogenic and extracellular matrix genes	Neybecker et al. (2018)
				In vivo: Macroscopic, histology		*In vivo*: No chondroprotection nor inflammation in rat knees injected with MSCs	

NA, not assigned; ICRS, International Cartilage Regeneration & Joint Preservation Society; MRI, magnetic resonance imaging; TGF-ß1, transforming growth factor ß1; BMP-2, bone morphogenetic factor 2.

models of osteoarthritis, while in contrast to chondroprotection, Neybecker et al. (2018) observed lack of inflammation, which suggested immunosuppressive effects of this cell therapy. Mak et al. (2016) investigated Sca-1 positive, chondrogenesis-capable mouse synovial MSCs that were derived from an MRL/MpJ 'super-healer' mouse strain, to determine whether these might regenerate cartilage injury better than those derived from 'nonhealer' C57BL6 mice. However, as no differences were seen, they suggested that regardless of strain background, synovial MSCs have beneficial effects when injected into an injured joint.

Enomoto et al. (2018) evaluated the effects of timing of intra-articular injection of MSCs on healing of a partial-thickness cartilage defect in rat. Synovium of infrapatella fat-pad-derived MSCs were implanted at three different times: time of injury, and 1 week and 2 weeks post injury. In their hands, the early intra-articular injection of MSCs enhanced cartilage healing. They also traced fluorescently labeled MSCs 1 day after implantation, and interestingly, they found them distributed in synovium, not in the cartilage surrounding the defect. Kurth et al. (2011) were the first to evaluate endogenous MSCs, and they showed that adult synovium is a site of functional MSCs that contributes to cartilage repair following joint surface injury (Kurth et al. 2011).

It has also been shown that the subpopulation of synovial MSCs with a Gdf5 lineage underpins the synovial hyperplasia and contributes to cartilage repair in a mouse model of join surface injury (Roelofs et al. 2017). The transcriptional co-factor known as Yes associated protein (Yap) was up-regulated after injury, and its conditional ablation in the Gdf5-lineage cells prevented synovial lining hyperplasia and decreased the contribution of the Gdf5-lineage cells to cartilage repair.

Together, these studies indicated the pivotal role of the resident MSCs in the joint, and in particular in synovium, which become activated upon a stimulus such as articular cartilage injury. These then respond by either contributing to cartilage repair themselves or through their paracrine functions, such as immunosuppression, to promote joint regeneration. Immunomodulatory potential of human synovium-derived MSCs has been shown in collagen-induced arthritis in mice (Yan et al. 2017). Human synovium-derived MSCs injected into inflamed joints of mice suppressed immune responses via immunoregulatory cell expansion. Further animal studies are awaited to demonstrate the same for osteoarthritis.

Another study similar to that of Enomoto et al. (2018) investigated the effects of single and repetitive intra-articular injections of MSCs in a rat osteoarthritis model (Ozeki et al. 2016). Histological analysis of the femoral and tibial cartilage showed that a single injection of the MSCs was ineffective, while weekly injections for 12 weeks had significant chondroprotective effects.

In summary, most of the *in-vivo* studies have investigated the effects of *in-vitro* expanded synovial MSCs on regeneration of cartilage injuries in models from small animals, such as mice and rats. Due to the experimental nature of these studies, these can provide the evidence that the implanted cells are indeed MSC-like cells. Moreover, they allow detailed analysis of the regenerated tissue, such as the presence of collagen type II. They can also provide the proof of concept that xenogeneic cell implantation also works for cartilage regeneration. However, most of the implanted MSCs were collected from 'healthy' animal donors. It would be particularly interesting to determine whether synovial MSCs derived from mouse models of osteoarthritis or from 'aged' mice can still regenerate cartilage. Further animal studies that investigate the cartilage regeneration capabilities of recently identified specific MSC subpopulations (Chan et al. 2018) are also awaited.

Finally, what needs to be learnt from the preclinical studies is not only the optimal tissue source(s) of MSCs, but also the timing and frequency of the implanted cell therapies. High quality experimental studies and efforts for effective translation from preclinical studies to clinical trials are still required (Xing et al. 2018).

3 Clinical Studies

Several clinical studies have shown the promising potential of MSC-based therapies in the treatment of osteoarthritis and traumatic cartilage lesions, with minimal adverse effects seen (Harrell et al. 2019; Ha et al. 2019; Reissis et al. 2016). Most of these studies have been conducted on knees using MSCs derived from bone marrow or adipose tissue. Unfortunately, the present critical systematic reviews do not provided sufficient level of evidence that intra-articular–derived MSCs are indeed effective for hyaline cartilage regeneration or repair, for symptoms attenuation, and for functional restoration (Ha et al. 2019).

It has been estimated that the great majority of the available clinical studies have some risk of reporting bias. Further, the optimum source and concentration of MSCs to fulfill clinical expectations remain to be identified. Also, subpopulations of MSCs differ in their chondrogenic differentiation potential and immunomodulatory capability (Im et al. 2005; Waldner et al. 2018).

These clinical studies are also difficult to compare due to the inconsistency in their delivery methods into the affected joints (Ha et al. 2019). MSCs applied directly to the site of a lesion using three-dimensional (3D) scaffolds are believed to have better healing potential, compared to MSCs injected percutaneously (Coelho et al. 2012). Furthermore, percutaneous injections of MSCs might represent a risk for undesired dissemination of the cells into noncartilage tissue, although this remains to be shown (Roffi et al. 2018).

Some clinical studies include adjuvant procedures that can have significant influence on the outcome of the treatment, and might also increase the risk of bias; e.g., concomitant injections of platelet-rich plasma or hyaluronic acid, microfracture of the subchondral bone, or corrective osteotomies (Ha et al. 2019). Similarly, to evaluate the outcomes of treatments, inconsistent, and on several occasions irregular, tools have been used (Ha et al. 2019). Some studies have provided information about the postoperative conditions of the cartilage using MRI or even histological evaluation of biopsies obtained during second-look arthroscopy, although the long-term fate of the treated cartilage remains unclear.

Autologous MSCs have also been used in elderly patients, which again results in outcome inconsistencies between trials due to the age-related decrease in MSC proliferation. Unlike pharmaceutical drugs with defined chemical structures and functions, identification and functional characterisation of MSCs have not yet been standardized, thus making it difficult to produce MSCs with consistent biological activities on a large scale for clinical trials (Lee and Wang 2017).

Clinical research on synovium-derived MSCs is still in its infancy. Review of the currently available literature revealed only three reports of their clinical use in the knee (Table 3). Only two of these reports are clinical studies on case series, while the other is a case report.

Sekiya et al. (2015) reported on the injection of synovium-derived MSCs that had been expanded in 10% autologous human serum. This study included 10 adult patients with International Cartilage Repair Society grade 3 or 4 focal lesions of the femoral condyle, but no control group (Sekiya et al. 2015). Suspensions of synovium-derived MSCs were applied arthroscopically directly into the lesion, which was positioned facing upward to allow 10 min of static exposure. At the final follow-up (median, 48 months), all 10 of these patients reported significant symptomatic improvements. Their MRI scores also significantly increased after the treatments. Biopsy specimens of cartilage were obtained arthroscopically in only four of these patients, and histological evaluation revealed hyaline cartilage in three of them. In five patients, concomitant anterior cruciate ligament reconstruction was carried out, and in two, meniscal repair, and these might have had a significant influence on the final outcome, thus presenting high risk of bias. Here, for implantation, the authors prepared passage 0 MSCs that were expanded with autologous human serum over 14 days. In agreement with their previous study, these synovium MSCs showed better potential for expansion, compared to bone-derived MSCs (Nimura et al. 2008).

Table 3 Summary of clinical studies using synovium-derived MSCs

Study design	Diagnosis	Number of patients (n)	Age (years)	Cell therapy	Route of transplantation	Follow-up	Treatment outcome	Reference
Clinical study	Trauma-induced femoral condyle defects	10	20–43	Synovial MSCs expanded with autologous serum	Arthroscopic implantation of cell suspension for 10 min using syringe	37–80 months	MRI score: Significantly increased after cell therapy. Arthroscopy: Improved quality of cartilage defect. Histology: Hyaline cartilage in three and fibrous in one patient. Lysholm score: Significantly increased. Tegner activity level scale: Did not decrease	Sekiya et al. (2015)
Case series (level of evidence, 4)	Isolated full-thickness cartilage defects of knee (<5cm^2; ICRS grade III, IV)	5	28–46	Autologous synovial membrane-derived MSCs, cultured, scaffold free	Implantation without use of sutures or fixation glue	24 months	Safety: No adverse events recorded. Self-assessed clinical scores: Significantly improved. Arthroscopy and MOCART: Secure defect filling confirmed. Tissue biopsy: Repair tissue with composition and structure of hyaline cartilage formed	Shimomura et al. (2018)
Case report, controlled laboratory study	Isolated full-thickness cartilage defects of knee (<5cm^2, ICRS grade III or IV)	1	34	Autologous synovial membrane-derived MSCs, cultured, scaffold free, and high dose steroid therapy	Implantation without sutures or fixation glue	3, 7 weeks	MSCs at 3 weeks post high-dose steroid therapy failed to generate functional construct *in vitro*, and recovered 7 weeks after therapy	Yasui et al. (2018)

ICRS, International Cartilage Regeneration & Joint Preservation Society; MOCART, magnetic resonance observation of cartilage repair tissue scoring.

Passage 0 cells are also safer than cells passaged several times, in terms of potential development of chromosome abnormalities (Ermis et al. 1995). The possibility to use allogeneic MSCs might offer huge advantages in terms of the concentration of potent cells in suspension, although further studies are needed to demonstrate the safety of this alternative to autologous products (Vangsness Jr et al. 2014).

More recently, Shimomura et al. (2018) reported on a pilot study of implantation of a scaffold-free tissue-engineered construct that was generated from autologous synovium-derived MSCs, for the repair of focal chondral lesions in the knee (Shimomura et al. 2018). Although this study only included five patients, their 2-year follow-up revealed evidence of secure defect filling. The histology of the biopsy specimens obtained during second-look arthroscopy indicated repair of the lesion by hyaline-like constructs.

Additionally, a case report was published by Yasui et al. (2018). They showed that high-dose steroid therapies can compromise synovial MSCs. Hence, they suggested that the drug-use profiles of MSC donors and recipients must be carefully monitored to optimize the opportunities for successful repair of damaged tissues.

Due to the scarce clinical experience and poor quality of available reports, at this stage it is not possible to form any conclusions on the clinical potential of synovium-derived MSCs in regeneration or repair of chondral lesions in human joints. However, these early clinical experiences have yielded positive data with minimal adverse effects.

Just recently, an innovative approach for cartilage regeneration was proposed based on articular injection of the bioactive cell-free formulation BIOF2, which can promote expansion and chondrogenic differentiation of endogenous synovial MSCs. However, the clinical relevance of this new concept remains to be determined (Delgado-Enciso et al. 2018).

Randomised controlled trials are required to objectively compare clinical efficacy and long-term safety of various treatment protocols. With clinical research continuing to evolve and address these challenges, it is likely that MSCs will become integrated into routine clinical practice in the near future (Kon et al. 2015). Randomised control trials are required to objectively compare clinical efficacy and long-term safety of various treatment protocols. As clinical research continues to evolve and address these challenges, it is likely that MSCs will become integrated into routine clinical practice in the near future (Kon et al. 2015).

4 Synovium-Derived MSCs – What Lies Ahead?

Based on the current evidence from basic and clinical studies, it is reasonable to have expectations that MSCs from synovium and from other tissue sources will be (part of) the future therapy for degenerative joint disorders. Their advantages provide solid grounds for further clinical trials, which include ease of access, isolation, cultivation, and expansion, along with their regenerative, anti-inflammatory, and immunomodulatory properties. However, before reaching this stage, several problems need to be addressed.

First, we need to translate the knowledge from animal studies to humans. There is evidence from animal studies that specific populations of MSCs exist in adult synovium that can repair cartilage, and can even form new joints. In humans, these populations are largely undefined, and further studies to identify human markers of these populations are awaited.

Secondly, future studies will need to focus on the development of new techniques for the minimal invasive harvesting of these cells, and for their transplantation to damaged joints. The majority of studies to date require cell harvesting and transplantation that are associated with high costs, even if culture expansion and good manufacturing practice can be avoided. Currently, intra-operative cell therapies represent the easiest route of cell implantation, although more remains to be done for better selection of the MSC-like cells for these therapies (Coelho et al. 2012). Alternatively, less invasive and more cost

effective ways need to be found to stimulate the endogenous MSCs toward tissue regeneration. Biophysical stimulation such as extracorporeal shock-wave treatments and pulsed electromagnetic fields can be used to enhance endogenous MSCs to maintain healthy joints and prevent osteoarthritis (Viganò et al. 2016). Another possibility is to stimulate the differentiation potential of the endogenous MSCs with pharmacological approaches, as has been shown for bone-marrow MSCs (Johnson et al. 2012; Heck et al. 2017). The prerequisite to elicit repair through activation of endogenous MSCs is, of course, to understand them fully first, both in health and disease.

Thirdly, MSC identification needs to be standardized, particularly for clinical use. Currently, hundreds of clinics and clinical trials are using the term 'human MSCs' with very few, if any, that have focused on the *in-vitro* multipotent capacities of these cells. Hence the term 'stem cells' is easily and largely misused for the direct-to-consumer marketing of unapproved stem-cell treatments for numerous medical conditions (Sipp et al. 2018). The term 'stem cells' is indeed misleading, as it implies that the patients will receive direct medical benefits because these cells will differentiate into regenerating tissue-producing cells. This has been acknowledged recently by the very father of the name MSCs, Arnold Caplan (Caplan 2017). Based on his suggestions, here the term MSCs should be replaced by 'medicinal signaling cells', to more accurately reflect that these cells home in on sites of injury and disease and secrete bioactive factors that are immunomodulatory and trophic (i.e., regenerative). This thus means that these cells release therapeutic drugs *in situ* that are medicinal. It is, indeed, the patient's own site-specific and tissue-specific resident stem cells that construct the new tissue following stimulation by the bioactive factors secreted by the exogenously supplied MSCs (Caplan 2017).

Fourthly, and certainly not the last of the issues associated with MSCs, the procedures and methods of their *in-vitro* expansion, storage, and transport need to be further optimized (and standardized) to develop safe and effective cell therapies.

To summarize, MSCs are truly the 'newcomers to the Club' (Bianco et al. 2013). More time and serious efforts are needed to produce evidence-based regenerative medicine for their use in degenerative joint disorders. Furthermore, synovium-derived MSCs represent the most recent newcomers to the 'MSC Club', and more basic and clinical studies are awaited before we can anticipate the exploitation of their full potential as a treatment for regeneration of cartilage and other joint structures.

Acknowledgments Janja Zupan was funded by UK Arthritis Research (2016–2018) and is currently part of the P3-0298 Research Programme 'Genes, hormones and personality changes in metabolic disorders', funded by the Slovenian Research Agency, and ARTE Project EU INTERREG Italia-Slovenija 2014–2020. The authors would like to thank Chris Berrie for scientific English editing of the manuscript.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 107–126
https://doi.org/10.1007/5584_2019_380

Published online: 8 May 2019

Skin Stem Cells, Their Niche and Tissue Engineering Approach for Skin Regeneration

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Abstract

Skin is the main organ that covers the human body and acts as a protective barrier between the human body and the environment. Skin tissue as a stem cell source can be used for transplantation in therapeutic application in terms of its properties such as abundant, easy to access, high plasticity and high ability to regenerate. The immunological profile of these cells makes it a suitable resource for autologous and allogeneic applications. The lack of major histo-compatibility complex 1 is also advantageous in its use. Epidermal stem cells are the main stem cells in the skin and are suitable cells in tissue engineering studies for their important role in wound repair. In the last 30 years, many studies have been conducted to develop substitutions that mimic human skin. Stem cell-based skin substitutions have been developed to be used in clinical applications, to support the healing of acute and chronic wounds and as test systems for dermatological and pharmacological applications. In this chapter, tissue specific properties of epidermal stem cells, composition of their niche, regenerative approaches and repair of tissue degeneration have been examined.

Keywords

Epidermal stem cells · Niche · Skin · Stem cells · Tissue engineering

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Abbreviations

3D	Three dimensional
ATRA	All-Trans Retinoic Acid
BM	Basement Membrane
BM MSC	Bone Marrow Mesenchymal Stem Cell
BMP	Bone Morphogenic Protein
CD	Cluster of Differentiation
DNA	Deoxyribonucleic acid
DP	Dermal Papilla
ECM	Extracellular matrix
EB	Epidermolysis Bullosa
EGF	Epidermal Growth Factor
EPU	Epidermal Proliferative Unit
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
hASCs	Human Adipose Tissue Derived Stem/Stromal Cells
HF	Hair Follicle Bulge
IFE	Interfollicular Epidermis
IRS	Inner Root Sheath
Krt15+	Keratin15

MRNA	Messenger RNA
miRNAs	MicroRNAs
MMP	Matrix Metalloproteinase
Muse	Multilineage Differentiating Stress Enduring
ORS	Outer Root Sheath
Ptch	Patch
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic acid
Shh	Sonic Hedgehog
SSEA	Stage-Spesific Embryonic Antigen
TGF-β	Transforming Growth Factor-beta
UCPC	Umbilical cord pericyte cell

1 Introduction

Human skin is composed of two layers; epidermis and dermis and dermo-epidermal junction which is usually known as subcutaneous tissues or hypodermis (Proksch et al. 2008). The outher part of the skin, epidermis, is an important part of the skin that prevents water and body fluid loss, protects the body from bacteria, viruses and parasitic infections and shows resistance to mechanical and chemical injuries. The major cell group inside of the epidermis is keratinocytes. They form a sequence of layers during movement from the basal layer to the skin surface. These layers are arranged from top to bottom; stratum corneum, stratum granulosum, stratum spinosum and basal layer. Other prominent cells in the epidermis layer are melanocytes, merkel cells, and immune cells such as langerhans cells. The basal epidermal layer contains undifferentiated proliferative progenitors expressing keratin K14 and K5. In addition to the regeneration of the basal layer, these progenitor cells also form outer layers of terminally differentiated dead stratum corneum cells and non-proliferative, transcriptionally active spinous and granular layers expressing K1, K10 (Figs. 1 and 2). These progenitors also form the dead layer barrier between the granular layer and the terminally differentiated cells. Second part of the skin that is named dermis composed of fibroblasts, which are involved in the construction of collagen, elastin and other structural molecules. The dermis consists of physically and functionally two layers. These are; *stratum papillare* and *stratum reticulare*. Dermis provides architecture and support against mechanical injuries. Instead of providing static support, the dynamic interaction between cells and extracellular matrix (ECM) affects cell behavior and fate.

In the dermis, collagen type I and type III are the common fibrous forming collagens within the ECM. They form a network with other proteins in the ECM such as laminins, nidogens, fibronectin, proteoglycans etc.. Hyaluronan, is one type of proteoglycan proteins found in the basal lamina of the skin, especially in the dermis in large amounts. It is also found more in basal regions containing the proliferating cells, but gradually decreasing in the upper layers of the skin, towards the stratum corneum (Brizzi et al. 2012; Chermnykh et al. 2018). Hypodermis is under the dermis. There is no clear boundary between these two layers, extending to the underlying muscle layer. Basic function of the hypodermis is carrying and connect. It also acts as an energy store and mechanical buffer and protects the body from temperature fluctuations.

2 Embryology of the Skin

After the fertilization, the zygote is divided into successive cleavages, forming blastomeres and blastocyst structure. During the gastrulation event which starts in the third week of early development, epiblast cells invade through the primitive streak and organize the formation of three germ layers, ectoderm, mesoderm, and endoderm. The ectoderm then form structures of nervous system (central nervous system from neural plate and pheripheral nervous system from the neural crest) and the skin epidermis is developed from the surface ectoderm. During the embryogenesis, a multistep process consist of epidermal specification, epidermal commitment, stratification, terminal differentiation and generation of epidermal appendages is required for skin development (Fig. 3) (Hu et al. 2018).

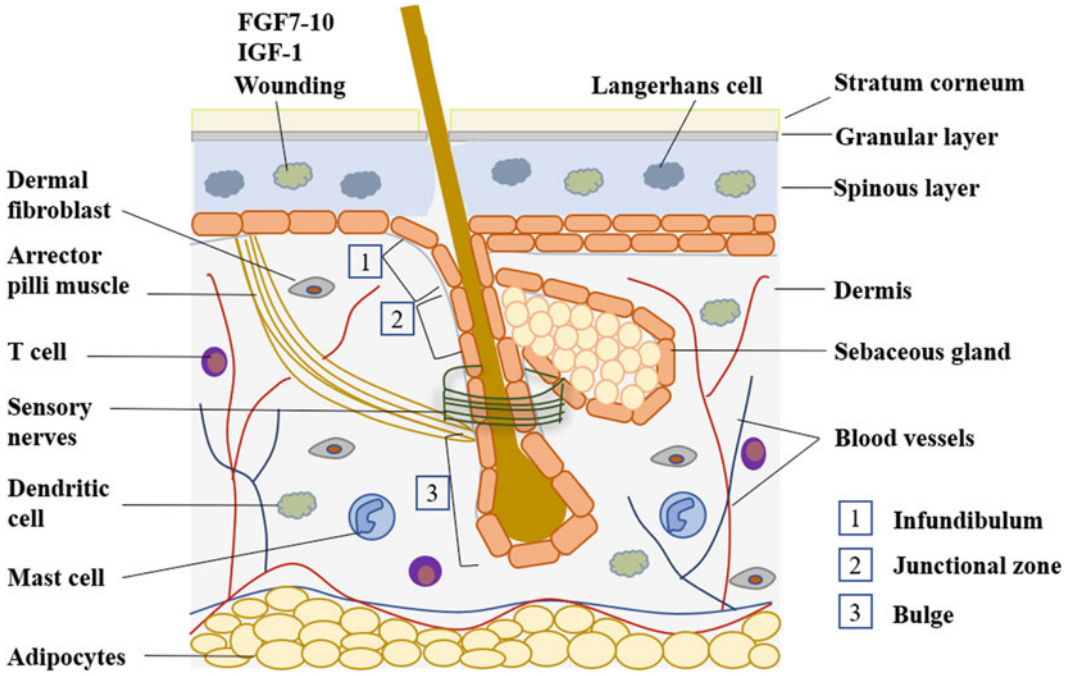

Fig. 1 Composition of the skin. (It is adapted from Hsu et al. (2014))

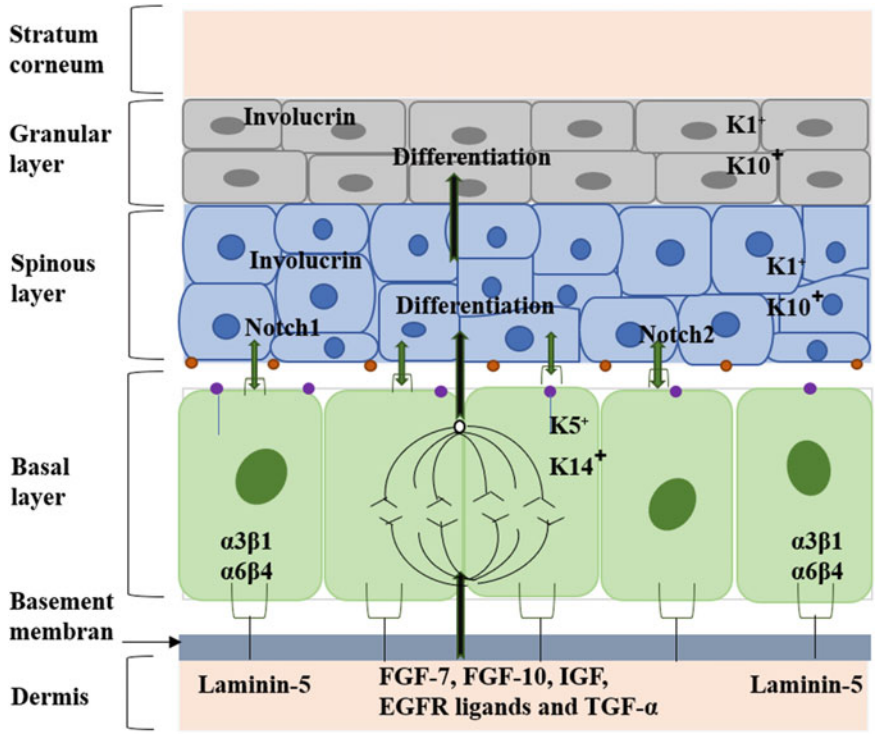

Fig. 2 Interfollicular epidermis: structure, signaling and progenies. (It is adapted from Hsu et al. (2014))

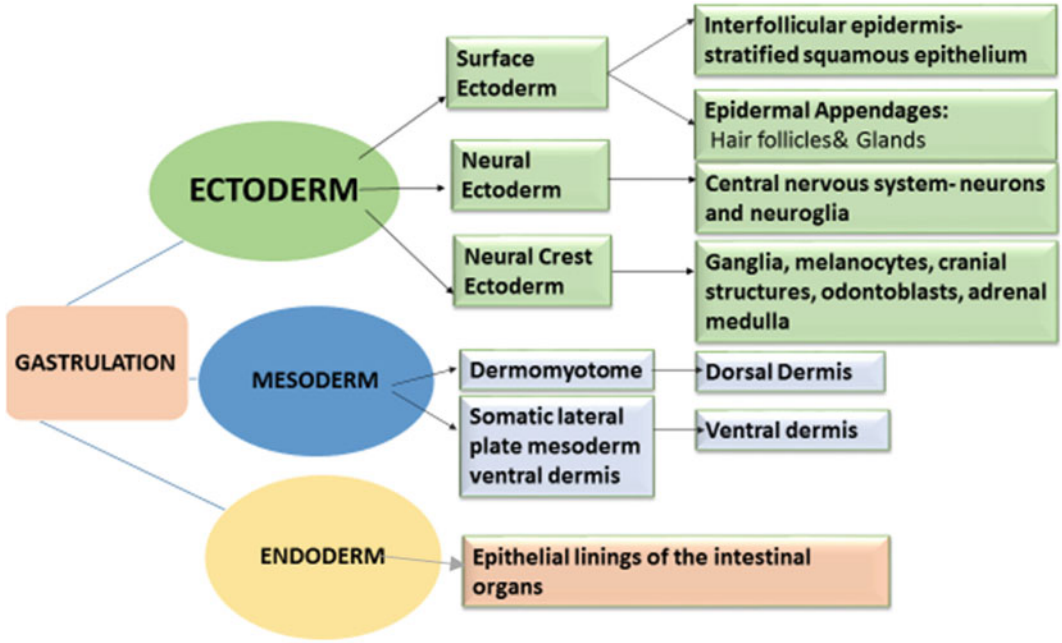

Fig. 3 Embryonic development of ectodermal lineages. After fertilization and the successive cleavages, at the third week of the embryonic development, the epiblast cells of the embryo give rise to three different germ layers (Ectoderm, Mesoderm, Endoderm) by gastrulation. From mesoderm, dorsal and ventral dermis of the embryo is formed. Ectoderm lineage has potential to generate both nervous system and the stratified epithelium of the skin&epidermal appendages, from the surface ectoderm

During the gestation, at the five-eight weeks period, the early skin is observed as mesenchymal dermis. The first trimester is the phase of transition to fibrous dermal connective structure and during second& third trimesters, fetal dermis is specialized. The presence of the certain cell types, the distribution of extracellular matrix, structure and organization of the cellullar and biochemical components, enzymes, and structural proteins, especially collagen types contributes to this process. Dermal mesenchyma contains a high proportion of hyaluronic acid compared to adult skin, and sulfated glycosaminoglycan (GAG) is in scarce (Smith and Holbrook 1986). As the age of the early embryo increases, the amount of collagen accumulated in the extracellular space increases and the cells with the round shape lose their shape. The collagen fibers in the dermal mesenchymal stage are more elastic and resist to the mechanical stress, and are largely made up of collagen types I, III and V (Riddle 1986). There is no presence of a clear distinction of dermis, and along with the basement membrane, there is a high rate of collagen type IV around the small-sized capillary arterioles and venules. At the nineth week of development, the fibrillary composition is increased in the matrix and the dermal-subdermal line becomes evident. At this stage, the collagen fibers increase their diameters and they are in the matrix by forming the bundle organization. The advanced development of papillary and reticular regions in the dermis is seen at the 14th week of the fetal development. As a result of this process, the skin thickens with the organization and contribution of collagen fibers.

Ectoderm has the potential to form both the nervous system and the skin epithelium. There are important signal molecules and pathways that determine cell fate in early embryonic period. During gastrulation, neuroectoderm-derived cells constitute the nervous system with increased Fibroblast Growth Factor (FGF) expression and suppression of Bone Morphogenic Protein (BMP) expression (Gaspard and Vanderhaeghen 2010).

In addition, BMP and Wnt expressions are necessary for the formation of skin epithelium (Wilson and Hemmati-Brivanlou 1995). As a result of this process, multipotent cells are epithelialized as a single layer. At fifth-sixth weeks of embryonic development, the embryonic dermis, called cellular dermis, consists of mesenchymal cells and there is no clear boundary with the dermis. These multicellular cells are characterized by higher nucleus/cytoplasmic volume ratio and have cell-to-cell contacts, tight connections, glycogen stores and a less developed Rough Endoplasmic Reticulum (RER)-golgi complex (Riddle 1986). The keratinocyte cells found in the skin produce keratin, and the type of keratin varies depending on the embryonic period. Initially generated multipotent-single layer epithelial cells express largely K8 and K18, while upon the stratification, the major keratin constituent is replaced with K5 and K14 (Byrne et al. 1994). Epithelial cells originating from the surface ectoderm generates epidermis and with further differentiation, it gives rise to the formation of future epidermal appendages.

The stratification of the hair follicle occurs in three stages, namely, the placode formation, organogenesis of the hair follicle and cytodifferentiation. During the first stage, the keratinocytes show clustering, extend and expand and form hair placodes, the precursor of the hair follicle, at E14.5 (Embryonic day 14.5) (Driskell et al. 2013). After the formation of specialized placode constructs, proliferation is observed as a result of cross-talk signaling with fibroblast cells (Michno et al. 2003). In the second stage, rapidly proliferating cells show downward growth and form the dermal papilla structure. At E15.5, hair germ is formed. Following E16.5–17.6, terminal differantiation of the inner root sheath and formation of hair shaft is observed. Formation of outer root sheath occurs at E18.5 (Muller-Rover et al. 2001). The dermis has multiple embryonic origins and is a result of 3 different anatomical organizations, anterior-posterior, proximal-distal and dermal-nondermal, fibroblasts are directed to different lineages. Each fibroblast type produces, secretes and contributes to the protein composition of the dermis (Driskell et al. 2013).

Sebacous glands are located at the end of the hair follicle and prevent the water loss of the skin with the sebum it produces. Apocrine sweat glands are found near the hair follicles and are only seen in these regions throughout the body. Eccrine sweat glands are another epidermal derivative, spread across various parts of the body and are surruounded by adipose tissue. In human, both sebaceous glands and sweat glands develops at 13 and 14 weeks of gestation, from the ectoderm.

3 Skin Stem Cells and Their Niche

The mammalian skin consists of two structural layers: the outermost structural component is epidermis and the collagen-rich dermis (rich connective tissue) separated from it by a Basement Membrane (BM). Stem cells are in close contact with the BM, which has specialized sheat-like structure plus with ECM for the epidermal cells. As stem cells deteached from the BM and migrate towards the overlying zones, they become progenitor cells and undergo terminal differantiation (*cornification)* (Chermnykh et al. 2018; Tadeu and Horsley 2014). Epidermal cells shed and replaced with new ones thanks to the multipotent and unipotent differentiation potential of skin stem cells (Tadeu and Horsley 2014). The microenvironmental components of the BM is provided by the secretuar activity of basal keratinocytes and dermal fibroblasts within the skin (Blanpain et al. 2004). Beneath the BM, firstly uppermost papillary dermis comprises delicate matrix fibers. Secondly reticular dermis includes large fibers and thirdly dermal adipocytes are found to be within the intradermal adipocytes (Arwert et al. 2012; Zouboulis et al. 2008). Among these layers, except from acellular the matrix fibers, there are additional cell types that support the epidermal stem cells by providing a microenvironment: inflammatory cells, neurons, muscle cells and the blood vessels of the skin (Brizzi et al. 2012; Kretzschmar and Watt 2014). Keratinocyte is the epidermal cell lineage which mainly constitutes the skin. Other cell types such as Merkel cells, melanocytes, Langerhan cells found in

mammalian epidermis (Kretzschmar and Watt 2014). Merkel cells reside in specialized compartments of the IFE, called touch domes, and function in the touch sensation. Langerhans cells are the dendritic cells of the epidermis and contribute to the adaptive immune responses. Melanocyte cells are responsible for the formation of melanin granules, and these granules provide protection to the keratinoctyes against Deoxyribonucleic Acid (DNA) damage from sun light (Sorg et al. 2017). Merkel cells reside in the touch domes in the Interfollicular Epidermis (IFE) and function in the touch sensation of skin. They are in close contact with somatosensory nerve fibers in the specialized region of skin, which is the borderline between dermal-epidermal regions (Brizzi et al. 2012; Arwert et al. 2012). Melanocytes are responsible for the protection of skin against exposue of sun light ultraviolet, radiation by expression of melanin granules/production of melanin pigment which are transported to keratinocytes and function against DNA damage. Langerhans cells, epidermal dendritic cells: part of adaptive immune response (Chermnykh et al. 2018). Arrector pili muscle function as protector against the heat loss and it interconnects Hair Follicle Bulge (HF) and IFE (Sorg et al. 2017; Wong et al. 2012).

There seems to exist three different niche regions defined for epidermal stem cells to date: **interfollicular epidermis**, **hair follicle bulge and the sebaceous gland** (Chermnykh et al. 2018; Levy et al. 2007) (Table 1). In the skin, each niche regions counted previously contain stem cells at the basal layer, in association with the basement membrane (Sadowski et al. 2017). The sebaceous gland and hair follicle forming basal cells within the **"pilosebaceous unit"** is formed during epidermal development (Levy et al. 2007).

3.1 Interfollicular Epidermis (IFE)

The mammalian skin outermost region: IFE is tightly connected to its underlying basement membrane via integrin-receptor and ligand adhesion. Its top layer is called stratum corneum and constantly shed and proliferate. The cells which are anchored and dinamically interacting the BM are basal keratinoctyes. These cells are continually shed and proliferate in order to regenerate the skin. Keratinocytes are formed from the surface ectoderm during embriyonic development and cells further differentiate to form later epidermal constituents of the skin via asymmetric cell divisions. Each asymmetric division gives rise to a progenitor suprabasal and basal daughter cell (Blanpain et al. 2004; Gillespie and Owens 2018). The interfollicular epidermis, which is located between the hair follicles, is important in the replacement of terminally differentiated cells that continuously spilled from the skin surface during adult life and in the regeneration of the hair follicle (Abbas and Mahalingam 2009). The hair region of the hair follicle represents the most well-characterized epidermal stem cell population today (Abbas and Mahalingam 2009). Lineage-tracing experiments have done to clarify the proposal of the existence of Epidermal Proliferative Unit (EPU) in the IFE. EPU suggests that there are central slow cycling cells serve as pre-progenitor cells for later rapidly cycling progenitor cells to differantiate as units. These units are resembled as a single stem cell and its surruounder committed progenitor cells (Transit amplifying cells) (Purba et al. 2014; Terskikh et al. 2012). Thus, there is a heterogeneity and hierarchy in the basal cells. Merkel cells within the IFE has a seperate specialized compartment called "touch domes". Merkel cells function to serve touch sensation and response to mechanical stimuli to an organism. The afferent somatosensory nerve fibers at dermal-epidermal border provide the mechanical stimulus to Merkel cells (Doucet et al. 2013).

3.2 Pilosebaceous Unit

Several lineage tracing experiments illustrates that several type of cells are capable of production of the lineages of hair follicle and sebaceous gland as well as the epidermis after wounding (Levy et al. 2007). Sebaceous gland produces specialized lipid sebum for skin via lysis of

Table 1 Skin stem cells inside of the niche and their markers

Stem Cells	Niche	Markers
Interfollicular epidermal stem cells	Epidermal basal layer	β1high/melanoma chondroitin sulfate
		Proteoglycan+(MCSP+), P63 α6high/CD71dim
Hair follicle stem cells	Bulge region	CD34, Lgr5, Sox9, CD200 Lhx2, NFATC1, Bromodeoxyuridine dye retention
		NFIB, K15, PHLDA1, Lhx2, K19.
	Isthmus	Lrig1, MST24, Lgr6, Gli1
	Hair germ at base of hair follicle	Gli1, Lgr5, K15
Melanocyte stem cells	Hair follicle bulge region, hair germ	Pax3, Dct, Sox
Sebaceous gland stem cells	Sebacesus glands, infundibulum	Blimp1Pax3, Dct, Sox
Neuronal progenitor cells	Bulge region	Nestin

It is adapted from Ojeh et al. (2015)

sebocytes and the lipid produced is excreted to the skin surface via hair canal. Hair follicle generates hair in a three-step cycle firstly begins with hair follicle growth, secondly destruction of its lower portion remaining with bulge, and the last step is the resting phase of hair follicle for later regenerative cycles; anagen, catagen and telogen, respectively (Tadeu and Horsley 2014).

3.3 Hair Follicle

3.3.1 Hair Follicle Embryogenesis and Hair Cycle in Adults

The hair follicle maintains its development with a series of epithelial-mesenchymal interplays. First, the dermis sends a signal to the upper epidermis to form a supplement, and in response to the epidermis, conducting Dermal Papilla (DP) signaling to the underlying dermal cells. The DP contains a densely packed small mesenchymal cell cluster. The first morphological symptom of hair follicule development is the formation of a hair plate where the basal epithelium is prolonged, and the dermal density occurs.

The developing follicle extends downwards to surround the DP and the underlying cells (matrix) begin to multiply. These cells, which proliferate during follicle maturation, begin to differentiate into the Inner Root Sheath (IRS) of the hair tissue to be formed in later stages. The outer cell layer became the Outer Root Sheath (ORS) which is wrapped by the basement membrane, which expresses ORS, K5 and K14. As the follicle expands, a new inner core is formed in the cells and the keratin genes of the hair begin to appear. The IRS, which is degenerated closer the skin surface of the hair root, allows the hair strand to overflow itself. On the 16th day of the cycle, proliferation is terminated in the matrix and a rapid deterioration occurs with apoptosis (catagen stage) in a large region beneath the hair follicle. DP is stimulated with the withdrawal of the epithelial hairs encircled by the basement membrane and enters the resting phase. This resting phase is called telogen. In the first hair cycle, the telogen lasts for one day, but in later cycles, this phase is gradually extended. The molecular mechanisms involved in the pathogenesis of hair follicles are not clearly understood, but genetic studies in mice have shown that Wnt/β-catenin, BMP, FGF, Sonic Hedgehog (Shh), Epidermal Growth Factor (EGF), NFkB and Notch indicates the importance of the signal path. Hair follicle occurs in the process of epithelial-mesenchymal interactions that begin with the formation of hair plaques in dermal mesenchymal cell regions (Hardy 1992).

This hair placode allows hair to grow and differentiate. An adult hair follicle consists of a stable top part and an under portion which is in continuous construction. The bulge region of the hair follicle is currently the best characterized site of epidermal stem cell populations (Abbas and Mahalingam 2009). Laminins, together with

collagen type IV are essential components of the BM and Laminin-511 (laminin-10) and Laminin-332 (laminin 5) are mostly found in the adult skin and are important for the regeneration of hair follicule and its development. Hair follicles are particularly well-defined niche regions with their known molecular and developmental mechanisms (Wong et al. 2012). The regeneration of the hair follicle comprises three phases: (1) growth phase, (2) regression phase (3) rest phase. Each cycle results in the production of the hair shaft that is grown over the skin surface (Rompolas and Greco 2014).

3.3.2 Bulge

Bulge is the region of hair follicle where the stem cells reside. In vivo genetic lineage tracing experiments have clarified that bulge stem cell progeny is Keratin15 (Krt15+). Bulge stem cells are known as their quiescence and long-lived nature compared with the other microenvironments within the skin (Ito et al. 2005). For mouse hair follicle stem cells, bulge markers are Krt19, Lgr5, Cluster of Differentiation (CD) 34 together with the transcription factors Gli1, Hopx, Lhx2, Sox9, Tcf3 and Nfatc1 (Rompolas and Greco 2014). Hair follicule stem cells demonstrate regenerative response after wounding by re-construction of epithelium (temporary function) and capacity of generating both hair follicule and sebaceous gland lineages (primary function). The multipotency of hair follicle bulge is related with its stem cell heterogeneity and it is demonstrated recently via lineage tracing experiments. The resident stem cells have slow-cycling property compared with other epithelial cells of the skin. Bulge stem cells are sorted by alpha 6-integrin and CD34+ identity (Brizzi et al. 2012; Choi et al. 2015).

3.3.3 Isthmus, Infundibulum

The compartment between the hair follicle bulge and the base of the sebaceous gland is called isthmus and cells inthere are Krt15- and CD34- yet they express Gli1, MTS24 and Lgr6 in high amounts. Ishtmus stem cells have capabilty to generate hair follicle lineages in homeostasis and have regenerative power in the case of injury (Gaspard and Vanderhaeghen 2010). Within isthmus, there are Lrig1+ cells which do not contribute to hair follicle formation in normal conditions but promote maintenance of infundibulum. After wounding, cells of the infundibulum (upper follicle region) migrate towards the upper layers for regeneration, and in order to replace them, the stem cells in the bulge migrate to infundibulum (Rompolas and Greco 2014; Ito et al. 2005; Clevers et al. 2014). In contrast with the hair follicle bulge stem cells, hair germ cells express P-cadherin instead of CD34 and Nfatc1 (Kretzschmar and Watt 2014; Nowak et al. 2008).

3.4 Sweat Gland

Sweat glands are another form of the epidermal appandages of the skin which function in thermoregulation (Ji et al. 2017). The secretory structure of the sweat gland consists of outer basal layer of myoepithelial cells expressing K5, K14 and smooth muscle actin. Inner suprabasal layer of luminal cells is positive for K8, K18 and K19 (Gillespie and Owens 2018). Unlike the mammalian gland, sweat gland has a little ability for regeneration (Tadeu and Horsley 2014). In the wounding, the progenitor cells of sweat gland participate in the replenishment of damaged tissue and regeneration of skin epithelium however, sweat gland itself is quiescent during this period (Wong et al. 2012).

3.5 Sebaceous Gland

The underlying mechanism of the maintanance of stem cells in sebaceous gland is an issue to be further investigation.The peripheral unipotent stem cells or the hair follicle bulge stem cells function in the renewal of the gland (Chermnykh et al. 2018). Sebocytes are marked by the presence of B lymphocyte-induced maturation protein (Blimp1) and this protein is expressed by the *Prdm1* gene. It is demonstrated by the lineage tracing experiments that Blimp1+ cells are

generating the sebum-producing sebocyte lineage (Sorg et al. 2017).

The basic stem cells in the skin can be listed as follows; keratinocytes, melanocytes, follicular stem cells, sebaceous stem stem cells, mesenchymal stem cell-like stem cells, nerve progenitor cells, and hematopoietic stem cells. Epidermal stem cells, which are among these different subtypes of skin stem cells, are the most effective cells in tissue repair and skin regeneration. Research shows that the epidermis has a small number of stem cells, is uncommonly divided and short-lived. The majority of epidermal stem cells are found in the basal layer of the epidermis and the rest is located at the base of the hair follicle and sebaceous glands (Watt et al. 2006). Epidermal stem cells circulate between two different cell phases throughout their life cycle. During the slow cell phase, the epidermal stem cells are inactive, but in the transition to the transformed cell phase, these cells begin to split rapidly and increase the density of the skin cells to regenerate the skin tissue. In the final stage, it undergoes many cell divisions before it becomes terminal (Chu et al. 2018). Most of them are found in the basal layer of the epidermis and may differentiate into transient amplifying cells and terminal differentiated epidermal cells. Important cell markers are β1 integrin, α6 integrin, K15, p63 and nestin. For the proliferative basal cells of the skin, high levels of α6-integrin expression, Delta1 expression (Notch ligand), CD200 expression together with the low levels of CD71 expression are indicative. The skin stem cell is linked to these laminin proteins via specific integrin units: mostly via α3β1, α6β1 and α6β4 integrins (Chermnykh et al. 2018). Among the previous receptors, α6β4-integrin is the most common one for the connection between the basal membrane and the basal keratinocytes as a part of hemidesmosomes. Integrins expressed by the basal keratinocytes are categorised differently: firstly depending on their expression levels, secondly depending on whether they are expressed constitively or transiently, and lastly depending on pathological and homeostatic conditions (Sorg et al. 2017). Also, for the quiescent basal keratinocyte cells, non-actively cycling stem cells, Lrig1 expression level is high and it is related with the promotion of epidermal stem cell maintenance. Studies demonstrated the putative contribution of with type IV collagen to basal stem cell proliferation capacity in the skin and promoting the maintanence (Hsu et al. 2014; Chermnykh et al. 2018; Kumar et al. 2017). Follicular stem cells are in the follicular bulge region. The outer root sheath, inner root sheath, and cells in the hair hole can be derived from the hair follicle epithelium. The known surface markers are K15, K19, Sox9, CD34, Lgr5, Lhx2, NFIB NFATC1, CD200, PHLDA1. CD34 expression, a specific marker for hematopoietic progenitor cells, has also been demonstrated in the protrusion region of murine hair follicles. CD34-positive cells are CK15-negative, which may represent transgenic amplifier cells or progeny of the protrusion stem cell (Abbas and Mahalingam 2009). These cells have a high proliferative potential. In the dermis, each region has fibroblast cells which are functionally specialized, especially the ones in the DP. DP cells induce the epidermal growth towards to the formation of hair follicle and once it is formed, DP stays inside the hair follicular structure throughout hair cycle (Chermnykh et al. 2018; Gattazzo et al. 2014). These cells in the dermis can be divided into mesodermal-like cells and some nerve cells and express important cell surface markers such as CD70, CD90 and CD105. The sebaceous glands, which play a role in the formation of differentiated sebocytes as terminal, begin with the formation of progenitor cells towards the end of embryogenesis, and maturation occurs in the postnatal period (Fuchs 2007). Blimp1, located in the follicle protrusion region and hair germ, represents the progenitor cell population found in the sebaceous glands of mice (Abbas and Mahalingam 2009). Melanocyte stem cell markers are Pax3, Dct, Sox and MITF, also known as melanocyte host transcription regulator. It was reported that Pax3 was involved in the initiation of melanogenic cascade and showed undifferentiated stem cell status (Abbas and Mahalingam 2009). In recent years, neural crest-derived stem cell populations have been found in the murine hair follicle bulge. These stem cells

can differentiate in vitro from melanocytes, smooth muscle cells, keratinocytes, glial cells, neurons, and adipocytes. Nestin, which is the surface marker of neural stem cells, is an intermediate filament protein (Abbas and Mahalingam 2009).

4 Regulation of Stem Cell Behavior in the Skin and Signalling Pathways

Since stem cells exhibit their functional properties within the specialized microenvironments, characterizing the components of stem cell niche is important in order to understand the cell fate dynamics. There are several molecular mechanisms which mediate cell fate decisions and play a role in the skin homeostasis and morphogenesis (Tadeu and Horsley 2014; Arwert et al. 2012).

4.1 Shh Signalling

Shh is an important signaling pathway involved in cell fate and proliferation during animal development. In the absence of Shh, a mutation in the activated Patch (Ptch) resulted in basal cell carcinomas, a type of skin cancer that is commonly seen in humans, and these results suggest that Ptch has served as a tumor suppressor gene (Rubin et al. 2005). Derivation of basal cell carcinomas from the hair follicle indicates that the Shh signal is expressed in hair plaques in embryonic skin and is a signal required for follicle formation (St-Jacques et al. 1998; Oro and Higgins 2003). The Shh signal is additionally important for follicle regeneration throughout the adult hair cycle. Shh is expressed in the matrix and developing germ, where the anagen is polarized to one side during its advance (Blanpain and Fuchs 2006). Anti-Shh antibodies to postnatal follicles obstruct anagen progression (Wang et al. 2000) and hair regeneration (Silva-Vargas et al. 2005). In contrast, Shh or small molecule Shh agonists accelerate the make progress from the telogene to the anagen (Paladini et al. 2005).

4.2 Wnt Signalling

The Wnt/β-catenin signaling pathway controls cellular events along embryonic and postnatal developmental processes.Wnt deregulation often give a leads to cancer-induced proliferation and differentiation instability (Reya et al. 2001). Wnt and β-catenin in the skin induce hair follicle morphogenesis, stem cell maintenance or activation, hair shaft differentiation (Alonso and Fuchs 2003). It is thought that the Wnt signalling might be the first dermal stimulus to instruct the epidermal stem cells to produce hair. Wnt signals that temporarily regulate follicle stem cell lines, promote β-catenin stabilization that encourage stem cell activation, proliferation and stimulation of follicle regeneration; supports the morphogenesis of de novo hair follicle; enhances the specification of matrix cells to terminally differentiate across the hair cell line (Blanpain and Fuchs 2006).

4.3 Bone Morphogenetic Protein Signalling

BMP binds to a transmembrane receptor complex via Bmpr1a and Bmpr1b receptors to activate signal transduction. Bmpr1a is expressed in most of the developing skin epithelium but is particularly emphasized in the hair follicle. In adult hair follicules, Bmp's also function in epithelial-mesenchymal interplays (Kratochwil et al. 1996; Lyons et al. 1989, 1990). Bmp signaling begins with neuroepithelia, where the epidermis indicates incomplete ectodermal cells (Nikaido et al. 1999). Bmp signaling was found to play an important role in the differentiation of matrix cells into IRS and hair shaft lines, and inhibition was shown to support stem cell activation (Andl et al. 2004; Kobielak et al. 2003; Ming Kwan et al. 2004). At the end of the usual hair cycle, the growth stops and the hair follicle passes

through the disruptive phase (katagen). However, Bmpr1a-null ORS continues to expand, matrix cells accumulate and cause the creation of follicular tumors (Andl et al. 2004; Ming Kwan et al. 2004).

4.4 Notch Signaling

Notch signal is expressed in embryonic and adult epidermis and is involved in various cell fate processes. At the beginning of epidermal stratification, Notch1 is expressed from the basal and suprabasal cells of the epidermis and sebaceous glands (Okuyama et al. 2004; Rangarajan et al. 2001). In the final phases of epidermal stratification, Notch1 activity is reduced in the basal layer (Okuyama et al. 2004). The loss of the Notch1 function leads to problems in the IFE differentiation phase (Rangarajan et al. 2001). In hair follicule, Notch1–3 is expressed in matrix cells with proliferative capacity and in differentiating HF cells (Kopan and Weintraub 1993). When Notch 1 and Notch2 are conditionally eliminated, hair follicules disappear quantitatively and epidermal cysts emerge that emphasize the role of the Notch signal in follicle maturation and differentiation (Pan et al. 2004). In the lack of both Notch1 and Notch2, the sebaceous glands cannot form (Pan et al. 2004). The loss of Notch1 in the epidermis does not disrupt the early follicle morphogenesis, but decreases the number of hair follicule in time (Vauclair et al. 2005). Understanding the downstream genes regulated by Notch signals in the epidermis and their role in cellular functions needs further investigation.

5 Skin Tissue Regeneration

Injuries that damage the body and the internal epithelium endanger the integrity of the body. Skin wound healing is a process that results in repair of tissue integrity and tasks. A rapid reepalization is achieved by preventing or limiting threats from the environment. Reepithelization of the skin is carried out by the migration of epidermal cells and keratinocytes to the wound site. In order to ensure regeneration of the epidermis, the dermoepidermal composition, which allows the epidermis to differentiate into the dermis and keratinocytes into a protective layer, needs to be reconstructed (Stanley et al. 1981; Rigal et al. 1991; Demarchez et al. 1987; Odland and Ross 1968). In the epidermis, mitotically active keratinocytes are present in the basal layer of the dermis, where they come into direct communication with the basal lamina and proliferate slowly. Keratinocytes in the supra basal layer migrate to the surface and the terminal begins to differentiate (Tomic-Canic et al. 1998; Bartkova et al. 2003). Keratinocytes are an significant part of the wound healing response with autocrine and paracrine communications. Several hours after injury, keratinocytes begin to migrate without proliferation (Bartkova et al. 2003). If the wound is superficial, the epithelial cells will appear as normal skin within 3 days. However, if the wound is damaged deep and dermal extensions, keratinocytes and fibroblasts on the edge of the wound will migrate to the wound. During the migration processes of re-epithelialization, keratinocytes can be grouped in three different groups (1) high proliferative cells with wound edge; (2) cells expressing integrins; (3) cells that are responsible for the regeneration of the basement membrane and epidermis (Saarialho-Kere et al. 1995). In order for these cells to migrate, some chemical signaling molecules, such as nitric oxide, must be formed with the inhibition of cell contact (Lee et al. 2001).

However, fibroblasts migrate from the edges of the damaged tissue to the wound area and begin to produce collagen, thus initiating the proliferative phase. The granulation tissue begins to form with the proliferative phase. Fibronectin and HA, the major components of the granulation tissue, facilitate cell migration by providing a well-hydrated transient matrix for subsequent healing events (Krawczyk 1971). Fibroblasts adhere to fibronectin and migrate through the wound bed. An increase in chondrotin sulphate and reduction in HA production decrease the migration and proliferation of fibroblast cells. The collagen provides a scaffold to promote and

facilitate the proliferation and differentiation of the cells related in inflammation angiogenesis and connective tissue making (Eichler and Carlson 2006). Fibroblasts play significant roles in the production of basal membranes by secreting cytokines such as collagen types IV and VII, laminin 5, nidogen and transforming growth factor (TGF-β).

Any complications during wound healing may cause wounds to become chronic wounds that prolong the healing process. Factors such as venous/arterial insufficiency, diabetes, kidney disease, trauma, advanced age and local pressure effects may cause delay in wound healing. In addition to these factors, systemic factors such as tissue hypoxia, ischemia, infection, dysregulation of the inflammatory process, irregular nutrition or immune status may also prevent healing. It is known that the spread of diabetes, obesity and vascular diseases cause chronic wounds. In major skin injuries that occur as a result of extensive burns, infection or trauma, the skin often cannot repair it itself and requires medical attention. Autologous skin graft is the most appropriate and aesthetically effective technique. However, this approach has several barriers, such as being effective only in a specific part of the skin and causing additionally injuries in the donor. Due to these obstacles, scientists have searched for alternate ways of treating serious skin damage and found that the in vitro colonies of adult epidermal stem cells in the epidermis and hair follicles were capable of forming a functional skin barrier (Green et al. 1979; Rheinwald and Green 1975; Sun et al. 2013).

During the inflammatory phase, the wound is closed by fibrin, which acts as a transient matrix, circulating immune cells occupy the new matrix, cleans the dead tissue and prevents infection. Proliferative fibroblasts come together and support angiogenesis by secreting collagen to form granulation tissue. Myofibroblasts derived from fibroblasts in that region express a-smooth muscle actin and initiate contraction in the wound area. Re-epithelialization begins with local stem cell populations migrating from the edge of the wound. In the last step, fibroblasts and keratinocytes provide fresh ECM components and Matrix Metalloproteinases (MMPs) to reshape the matrix. After 3–4 months of injury, IFE cells migrate to the wound area and provide a healthy skin appearance at a rate of 80% (Ito et al. 2005; Mascre et al. 2012; Page et al. 2013). It was observed that K15-positive hair follicle stem cells temporarily contributed to wound epithelialization immediately after injury but disappeared after a few weeks. In a study by Jimenez et al. (2012), 10 patients showed that chronic leg ulcers reduce the area of ulcerative scarring of autologous scalp follicular grafts transplanted into the wound site. It is predicted that hair follicle grafts may be a hopeful treatment alternative for difficult chronic wounds due to epithelization, neovascularization and increased dermal reorganization in the wound area (Jimenez et al. 2012).

Among the important goals of stem cell applications are collecting stem cells from the patient, replicating by making modifications on it, and treating many genetic and acquired disease by giving the patient again. In short, epidermal stem cells can be used as a potential therapeutic source in regenerative medicine applications (Fig. 4). Epithelial stem cells that have been replaced in vitro to repair genetic disorders can be used as a new tool in gene therapy.

Epidermal stem cells with clonogenicity and long-term lastingness properties, can provide permanent treatment for skin diseases like Epidermolysis Bullosa (EB) (De Rosa et al. 2014) and vitiligo (Falabella et al. 1992). EB is a skin disease that occurs with a genetic mutation in any of the genes involved in the binding of basal epidermal cells and basement membrane, leading to a destructive effect on the skin and often death (Jackson et al. 2017). Clones of primary epidermal stem cells from biopsy taken from a patient with EB caused by a mutation in the Laminin-5 gene were corrected with a retrovirus and applied to the legs of nine patients (Mavilio et al. 2006). One year after the application, it was seen that the levels of laminin-5 synthesis were similar to the normal epidermis and at the end of 6.5 years there was no disease in the transplanted areas and a healthy epidermis was formed. In a study conducted by Amoh et al. in 2005, stem cells in the root sheath of hairs

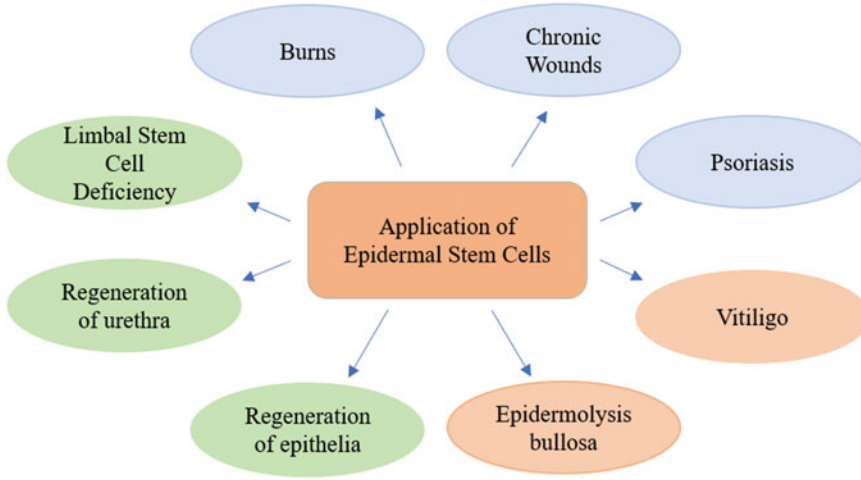

Fig. 4 Application of epidermal stem cells. (It is adapted from Jackson et al. (2017))

during anagen were cultured in the form of ex vivo organotypic culture and obtained a multi-layer epidermal-like structure. As a result of the treatment applied to 23 patients, positive responses were obtained in 18 patients. Total wound closure with a single transplantation was around 33% (4 cases) (Amoh et al. 2005, 2008). A successful in vitro epidermis was obtained by using autologous epidermal stem cells in undamaged skin biopsy to treat large skin burns (Lapouge and Blanpain 2008). The skin biopsy specimen is separated by trypsin and the epidermal stem cells are isolated. Epidermal stem cells are developed on fibroblasts which make the ECM and growth factors suitable for keratinocyte proliferation (Green et al. 1979). Keratinocytes are cultured until they form a layered epithelium covering the wound. On the other hand, this technique has two main disadvantages: (1) the long time required for in vitro expansion of keratinocytes and (2) the high price of the process (Clark et al. 2007).

Micro Ribonucleic Acids (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression by suppressing or reversing Messenger RNA (mRNA) translation (Ambros 2004). One miRNA can target hundreds of genes, and a gene can be rearranged with various miRNAs. The miRNAs, which are the primary regulators of gene expression, play an important role in numerous biological processes such as homeostasis, differentiation and survival. Abnormal expressions of miRNAs can cause diseases. In the epidermis of mice, several miRNAs expressed as distinct from other skin lines have been identified. The miR-200 family such as a, b and c, miR-141, miR-429 and miR-19/miR-20 family (miR-19b, miR-20, miR-17-5p and miR-93) expressed in the epidermal line while the miR-199 family is present only in the hair follicles (Andl et al. 2006; Yi et al. 2006).

Hildebrand et al. (2011) compared expression profiles of calcium-derived keratinocytes with TA keratinocytes and terminally differentiated keratinocytes. At the end of the comparison, 8 up-regulated miRNAs (miR-23b, miR-95, miR-210, miR-224, miR-26a, miR-200a, miR-27b and miR-328) and one down-regulated miRNA (miR-376a) were found to be involved in the epidermal differentiation duration (Koster et al. 2004). The expression of miR-203 during acute wound healing showed that it contributed to the wound epithelization with the upward expression of p63, RAN (member of the G-protein super family), and RAPH1 (lamellipoid) at the anterior edge of the epithelial migrating extension (Viticchie et al. 2012).

miRNAs modulate cellular action of target cells by secretion by various cell types. miRNAs

enter into the circulation through exosomes, where they modulate the cellular movement of the target cells (Lo Cicero et al. 2015; Shabbir et al. 2015; Valadi et al. 2007). Studies have shown that exosomes derived from Bone Marrow Mesenchymal Stem Cells (BM MSCs) show an increase in miRNAs in proliferation and migration of normal and diabetic scar fibroblasts (Shabbir et al. 2015). It has been shown that exosomes secreted by keratinocytes also have an effect on the regulation of melanin synthesis and provide important data for better understanding of the role of miRNAs mediated by exosomes in the protection of epidermal stem cells (Lo Cicero et al. 2015).

In 2010, Kuroda et al. defined Multilineage Differentiating Stress Enduring (Muse) cells (Kuroda et al. 2010) expressing pluripotency markers and being able to differentiate into three germ leaves. Muse cells are sporadically multiplied in the fascia of most organs and are not associated with a structural niche and are also found in the bone marrow cavity (Wakao et al. 2011).Muse cells are the pluripotent subpopulation of mesenchymal stem cells and express the following markers with mesenchymal markers; Stage-Spesific Embryonic Antigen (SSEA-3), CD105, CD29, CD90, Oct 3/4, Nanog, Sox2 and Rex1. When Muse cells identify damage signals and are delivered to the target tissue, they can differentiate into compatible cells and cross the mesodermal boundary between mesodermal and ectodermal cells. Thanks to these critical features, muse cells can be used to repair cells in cells, tissues and organs. Thus, Muse cells can undertake tasks such as repairing cells in various tissues and organs. Muse cells may be preferred over other cells since they are integrated in the damaged tissue or organ and have little risk of developing teratomas. So far, Muse cells have been isolated from human skin fibroblasts, adipose stem cells, and bone marrow stem cells (Yamauchi et al. 2017). In the process of skin renewal, clinicians are looking for new ways for bioengineered skin by using cells that can be obtained more easily. Muse cells are an exciting type of cell because it has the potential to bypass certain limitations of embryonic stem cells and induced pluripotent stem cells for skin regeneration (Yamauchi et al. 2017).

It has been shown that muse cells can differentiate into melanocytes as well as fibroblasts and keratinocytes and produce reconstructed skins derived from muse cells (Yamauchi et al. 2017). Itoh and his team (2011) have managed to produce reconstructed skins containing stimulated pluripotent stem cell-derived keratinocytes and fibroblasts. For keratinocyte differentiation, we have found that BMP4 and All-Trans Retinoic Acid (ATRA) are required molecules for stem cells, including the muse, induced pluripotent stem cells and embryonic stem cells (Itoh et al. 2011). In the study, it has been declared that somatic muse cells may be a promising source for regenerative medicine in the skin. In another study, SSEA-3 + Muse cells were isolated from human Adipose Tissue Derived Stem/Stromal Cells (hASCs) (Kinoshita et al. 2015)., In the study, adipose-derived Muse cells were characterized, and their therapeutic potential was evaluated in the treatment of diabetic skin ulcers. Skin ulcers under ischemic conditions indicate long-term wound healing and use mesenchymal stem cells or endothelial progenitor cells to treat (Kinoshita et al. 2015). To investigate the efficacy of Muse cells in comparison to other cells, two separate cell populations were compared between treatments in wound healing processes in diabetic rats. Muse-derived fat-derived cells supported wound healing in DM-SCID mice, while Muse cells in ASC populations showed a more effective outcome on tissue repair. Histological analyzes showed that Muse-rich ASCs were integrated into the repaired dermis. Although the proliferative capacity of Muse cells is not high, it is thought to be a powerful vehicle for clinical use because they are nontumorogenic (Kuroda et al. 2010; Heneidi et al. 2013; Ogura et al. 2014; Wakao et al. 2014).

5.1 Advantages of Intelligent Matrices

Advances in skin reconstruction, which were developed in tissue engineering applications, were recorded along with the development of reconstructed living equivalents. The provision of tissue homoeostasis in restructured substitutions is very significant for self-renewing tissues such as the skin. Tissue-engineered substitutions are useful vehicles for learning about cell interplays and regulation of tissue homoeostasis. In addition to the use of such substitutions in the treatment of skin defects, it is important to understand the contribution of stromal-epithelial interplays to differentiation (Boa et al. 2013; Carrier et al. 2009).

Dermal matrices provide the appropriate environment and conditions to promote cell proliferation and regeneration. They are available in a variety of forms such as natural, synthetic and hybrid matrices and are arranged by different technics such as (a) electrospinning, (b) phase separation, (c) freeze drying and (d) self-assembly. Dermal matrices as simple equivalents of ECM may be cellular or acellular, biodegradable or non-degradable polymers. In natural matrices, disease transmission and immunogenic risks are higher than synthetic matrices. In addition, synthetic matrices can be produced in larger quantities, standardized and thus have a minimum risk of disease by reducing their variability. Despite all these advantages, these skin scaffolds have limitations such as the inability to perform all the functions of the skin or the inability to produce skin supplements after in vivo application. Moreover, cell-cultivated matrices did not produce the desired result in low cell proliferation, survival rates. Demarchez et al. 1987 indicated that allogenic cells in the Food and Drug Administration (FDA)-approved Apligraf, a therapeutic product for chronic wounds, were not long-lasting in vivo and acted as a temporary biological bandaging that provides growth factors to deep wounds Therefore, studies on cell survival in scaffolds formed by tissue engineering to get better results in wound treatments are ongoing. Electrospinning and three dimensional (3D) bioprinting are new methods for cell survival and scaffold standardization. Studies have reported that electrospun scaffolds support fibroblast viability, enhance cellular organization, and reduce wound shrinkage in a mouse full-thickness wound model compared to freeze-dried scaffolds (Powell et al. 2008). Lee et al. (2014) successfully modeled human skin based on a layer of 3D bioprinting using collagen type 1, fibroblasts and keratinocytes. These cells are at a promising point in scaffold-based therapeutic research ensure new therapeutic approaches.

Commercially available epidermal substitutions are available in various forms, such as combined or pre-fluid, autologous, allogenic keratinocytes or cells used in combination with aerosol spray methods to facilitate access to the wound. EpiDex, which is used in chronic wound treatments, consists of ORS keratinocytes from hair follicles and shows positive results in the treatment of chronic leg ulcers (Ortega-Zilic et al. 2010). Examples of allogenic keratinocyte layers include Cryoskin (Beele et al. 2005). To preserve a proliferative phenotype in vitro, cells were grown up to pre-flow in delivery systems such as Laserskin® and Myskin, which facilitate transplantation (Moustafa et al. 2007; Andreassi et al. 1998). It has also been reported that fibrin glue is used in combination with aerosol method for the transmission of cell suspension to keratinocytes in BioSeed-S and CellSpray wounds. These epidermal substitutes have successful clinical applications in the treatment of diabetic ulcers, venous and burn wounds.

The curative effects of BM MSCs planted on collagen scaffolds applied to various wound styles in patients have been shown to positively affect wound healing (Yoshikawa et al. 2008). Badiava and Falang achieved successful results by applying autologous BM MSCs embedded in collagen matrix to chronic leg ulcers (Badiavas and Falanga 2003). AlloDerm and ASCs embedded in a fibrin-chitosan scaffold have been shown to increase wound healing by releasing angiogenic factors that trigger the development of the

vascular system (Altman et al. 2008). In a study with newborn mice, the epidermal and dermal stem cell suspension was developed at the back of the mice with Integra Artificial Skin before implementation to full thickness injuries in vitro to form a gel-like matrix (Lee et al. 2011). The data obtained from the studies show that the prospective use of stem cells in new wound therapies based on cell/scaffold is promising.

Pericytes have major tasks in vascular development, maturation, stabilization and remodeling processes. Other important features of pericytes are wound healing, physiological repair and renewal of organs etc. (Armulik et al. 2005; Rajkumar et al. 2006; Corselli et al. 2010; Davidoff et al. 2009). The biggest obstruction to in the clinical applications of tissue engineering structures is the formation of necrosis or ischemic conditions due to lack of oxygen and nutrition. This is a very important limitation for major and vital organs such as the kidney, heart and liver. Considering that the diffusion limit of oxygen is 150–200 μm, appropriate vascularity support should be provided for survival in large structures (Avolio et al. 2017). The growth of the vessels in vivo, the colonization of the structure by host cells providing the construction is a very slow process. Thus, the cellular combination of fibroblasts, smooth muscle cells and pericytes in the vascular niche, which physiologically constitute blood vessels, is a preferred solution to support vascularization. Because of the failure to achieve optimal improvement in the treatment of skin diseases, direct injection or scaffold cell based applications are used in the treatment of cutaneous wounds by dermal tissue engineering. The treatment feature of pericyte cells obtained from human umbilical cord (UCPC) in skin wounds is very interesting today (Zebardast et al. 2010). UCPCs showed a higher proliferative rate compared to human BM MSC used in clinical therapies. Placing UCPCs on the wound site as a polymeric fibrin patch promoted the cure of full-thickness mouse skin defects and thus showed that UCPCs could be a potential source of cells for skin tissue engineering and repair (Zebardast et al. 2010).

Wound healing is a dynamic cyclic event including the migration of different cell types such as keratinocytes, macrophages, fibroblasts, extracellular matrix, leukocytes, endothelial cells and pericytes (Bodnar et al. 2016). Coagulation occurs after skin injury to provide homeostasis. The platelets and fibrinogens play a critical role in the development of the inflammatory response. The platelets stimulate the pericyt activity by releasing platelet derived growth factor and Transforming Growth Factor-beta (TGF-β) from the endothelial cells that support the separation of pericytes and migration into the parenchyma (Bodnar et al. 2016). Pericytes provide the secretion of many chemokines, growth factors and matrix proteins involved in the functioning of parenchymal cells in the skin (Dulmovits and Herman 2012). Paquet-Fifield et al. (2009) investigated pericytes associated with the proliferative basal layer of the epidermis and showed that pericytes were mesenchymal stem cells. They found that pericytes form a strong cell population in the skin and are important micro-environment regulators in skin regeneration. In a recent study by the same group, the use of pericytes in murine wounds did not significantly affect the re-epithelization of the dermal wound. The team stated that this may be related to the pro-inflammatory effects of pericytes in paracrine signaling and that the experiments should be performed more repetitive. Pericytes are an important group of cells with properties such as the umbilical cord, adipose tissue and placenta, which can be obtained from many different tissues, improve re-epithelialization and rebuild dermis (Zebardast et al. 2010). In one study, pericytes were applied to full-thickness wounds on a fibrin gel, and full thickness wounds formed a higher tensile strength, denser collagen fibers and a dermis with enhanced angiogenic effect (Zebardast et al. 2010). In another study using an organotypic skin culture, it was observed that pericytes increased epidermal regeneration through the secretion of laminin α5 and thus a potential cell in the therapy of non-healing wounds (Paquet-Fifield et al. 2009).

In a study by Tomoko and his team (Yamazaki et al. 2017), tissue localized myeloid progenitors have been shown to contribute to the development of pericytes in the embryonic skin vessels.

Myeloid cells isolated by flow cytometry and their progenitors in embryonic skin have been shown to induce pericidal differentiation in culture with TGF-β. The role of TGF-β signaling in pericyte development will not be fully known but will provide important information to better understand the mechanisms controlling neovascularization. Evidence has been found in BMP-2 organotypic cultures, a protein secreted by pericytes, that provides cell polarity and planar sections on epidermal cells (Paquet-Fifield et al. 2009). At the end of his study of Zhuang et al. (2018), It was found that the extrinsic dermal signals provided by the pericytes have the ability to regulate the orientation of the keratinocyte layer in the proliferative basal cell section of the epidermis. Asymmetric divisions in the epidermis cause signals to support cell proliferation against differentiation. Therefore, the balance of symmetrical and asymmetric sections in the basal layer is of great importance for the preservation of the homeostatic balance in the interfollicular epidermis (Zhuang et al. 2018).

6 Conclusions and Perspectives

Skin tissue as a stem cell source can be used for transplantation in terms of properties such as abundant, easy to access, high plasticity and high ability to regenerate. The immunological profile of these cells makes it a suitable resource for autologous and allogeneic applications. There is evidence that they generate strong immunosuppressants such as α-melanocyte-stimulating hormone, interleukin 10 and transforming growth factor beta 1. The lack of major histocompatibility complex 1 is also advantageous in its use. Epidermal stem cells are the main stem cells in the skin and are suitable cells in tissue engineering studies for their important role in wound repair. However, epidermal stem cells do not have specific markers, and there is no effective method for its purification. Therefore, its use in tissue engineering studies is limited. Other stem cells can be used as an alternative. Recently, organ on a chip technology has been started to be used both as skin substitute and drug testing. It is estimated that the studies in this area will gain even more success in the future.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 127–153
https://doi.org/10.1007/5584_2019_398

Published online: 25 July 2019

Neurological Regulation of the Bone Marrow Niche

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Abstract

The bone marrow (BM) hematopoietic niche is the microenvironment where in the adult hematopoietic stem and progenitor cells (HSPCs) are maintained and regulated. This regulation is tightly controlled through direct cell-cell interactions with mesenchymal stromal stem (MSCs) and reticular cells, adipocytes, osteoblasts and endothelial cells, through binding to extracellular matrix molecules and through signaling by cytokines and hematopoietic growth factors. These interactions provide a healthy environment and secure the maintenance of the HSPC pool, their proliferation, differentiation and migration. Recent studies have shown that innervation of the BM and interactions with the peripheral sympathetic neural system are important for maintenance of the hematopoietic niche, through direct interactions with HSCPs or via interactions with other cells of the HSPC microenvironment. Signaling through adrenergic receptors (ARs), opioid receptors (ORs), endocannabinoid receptors (CRs) on HSPCs and MSCs has been shown to play an important role in HSPC homeostasis and mobilization. In addition, a wide range of neuropeptides and neurotransmitters, such as Neuropeptide Y (NPY), Substance P (SP) and Tachykinins, as well as neurotrophins and neuropoietic growth factors have been shown to be involved in regulation of the hematopoietic niche. Here, a comprehensive overview is given of their role and interactions with important cells in the hematopoietic niche, including HSPCs and MSCs, and their effect on HSPC maintenance, regulation and mobilization.

Keywords

Bone Marrow Niche · Endocannabinoids · Hematopoiesis · Neuropeptides · Opioids · Tachykinins

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Abbreviations

2-AG	2-ArachidonoylGlycerol
ACh	acetylcholine
AEA	Anandamide
AGM	aorta-gonad-mesonephros
ARs	adrenergic receptors
BDNF	Brain-Derived Growth factor
BM	bone marrow
CBD	Cannabidiol
CD271	Low affinity nerve growth factor receptor
CFU-F	Colony Forming Unit-Fibroblast
CFU-GEMM	Colony Forming Unit-Granulocyte/Erythrocyte/Monocyte/Megakaryocyte
ChAT	choline acetyltransferase
CKs	cytokines
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRs	endocannabinoid receptors
CT-1	cardiotrophin-1
CXCL12/SDF1	Stromal Derived Factor-1
D	Dopamine
DCs	Dendritic cells
DRs	dopamine receptors
E	Epinephrine/Adrenaline
ECB	Endocannabinoid
ECM	extracellular matrix
ECs	endothelial cells
EK	Endokinin
FAAH	fatty acid amide hydrolase
FGF	Fibroblasts growth factor
FTOC	fetal thymus organ cultures
G-CSF	Granulocyte-Colony Stimulating Factor
GDNF	glial cell-line derived neurotrophic factor
GDNF	Glial-derived Neurotrophic Factor
GFLs	GDNF family of ligands
GM-CSF	Granulocyte/Macrophage-Colony Stimulating Factor
GPCRs	G-protein coupled receptors
HGFs	hematopoietic growth factors
HK-1	Hemokinin-1
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem and progenitor cells
IL	Interleukin
LIF	Leukemia inhibiting factor
LSK cells	$Lin^{-}Sca^{+}c\text{-}kit^{+}$ cells
MIP1α	Macrophage Inflammatory Protein-1alpha
MMP	Metalloproteinase
MSCs	mesenchymal stromal/stem cells
NE	Norepinephrine/Noradrenaline
NGF	Nerve growth factor
NK cell	Natural Killer cell
NKA	Neurokinin A
NKB	Neurokinin B
NK-Rs	Neurokinin receptors
NPY	Neuropeptide Y
NTs	Neurotrophins
OBs	osteoblasts
ORs	opioid receptors
OSM	Oncostatin M
PAA	periarterial adventitial cells
PDGF	Platelet-derived growth factor
RET	rearranged during transfection receptor
RTK	receptor tyrosine kinase
SCF	Stem Cell Factor
SNS	sympathetic nervous system
SP	Substance P
TGFβ	Transforming Growth Factor
TH	tyrosine hydrolase
THC	Tetrahydrocannabinol
TK	Tachykinins
TLRs	Toll-like receptors
TNFR	Tumor necrosis factor receptor
TNFα	Tumor Necrosis Factor alpha
TPO	Thrombopoietin
Trk	tropomyosin receptor kinase
UCB	Umbilical Cord Blood
VEGF	Vascular endothelial growth factor
WT	Wild type

1 Introduction

In the adult, Hematopoietic Stem Cells (HSCs) and progenitor cells are located in a specialized microenvironment called the bone marrow (BM) niche. This microenvironment contains all the requirements for the support and maintenance of healthy hematopoiesis. The functions of these HSCs, as well as their fate are determined by tightly regulated interactions with this BM niche, which directs HSC survival, quiescence, self-renewal, proliferation, differentiation and migration. The BM niche contains many different types of supporting cells, such as Nestin+ Mesenchymal Stem Cells (MSCs), osteoblasts (OBs), macrophages, adipocytes, fibroblasts, osteoclasts, CXCL12-abundant reticular (CAR) cells, endothelial cells (ECs) and perivascular cells, but also sympathetic nerves and non-myelinated GFAP+ Schwann cells (Morrison and Scadden 2014). In addition to cell-cell interactions, regulation and maintenance of HSCs is heavily affected by signals transferred from the extracellular matrix (ECM), such as fibronectin, collagen, laminin and proteoglycans; by gradients of hormones, hematopoietic growth factors (HGFs), cytokines (CKs) and chemokines; by pH, O_2 tension and calcium gradients; as well as by interactions with adhesion molecules for anchoring and retention in and release of HSCs from the niche. Thus, the BM niche provides a complex shelter to protect HSCs from harmful conditions and environmental damage.

The sympathetic nervous system (SNS) has also been implicated in the physiological regulation of the BM niche and direction of hematopoietic stress responses. Anatomically, both myelinated and unmyelinated nerve fibers enter the marrow cavity alongside nutrient blood vessels (Kuntz and Richins 1945) and provide an extensive network of innervation of the BM (Artico et al. 2002). The nerve fibers can be visualized using staining for tyrosine hydrolase (TH), which is involved in catecholamine synthesis. TH+ noradrenergic nerve fibers have been found to be closely associated with blood vessels, but can also be found in the periosteum, mineralized bone and BM (Jung et al. 2017). The nerve fibers secrete different classes of neurotransmitters, including the catecholamines Norepinephrine (NE) and Dopamine (D), and a range of neurotransmitters, neuropeptides and neurotrophic factors (Tabarowski et al. 1996). Both afferent and efferent sympathetic nerves can be found adjacent to the vasculature of the BM (Calvo and Forteza-Vila 1969). Furthermore, sympathetic nerves synapse with perivascular cells to regulate BM homeostasis and are associated with HSC quiescence and regulation of self-renewal (Park et al. 2015a; Yamazaki et al. 2011). These perivascular cells include a specialized group of periarterial adventitial (PAA) cells, that are attached to each other through gap-junctions and are found in close proximity of nerve terminals (Yamazaki and Allen 1990). This network was appropriately termed the "neuro-reticular complex". The role of the parasympathetic innervation of the BM is less well established. However, parasympathetic choline acetyltransferase (ChAT) + nerve fibers which synthesize acetylcholine (ACh) have been detected around hematopoietic islets in the BM of rats (Artico et al. 2002). The significance of BM innervation gains even more importance after realizing that neuronal control of hematopoiesis already commences during early embryogenesis, and that only after the beginning of BM innervation the onset of hematopoiesis is initiated (Fitch et al. 2012).

Nerve endings releasing Substance P (SP) and Neurokinin A (NKA) have been shown to control secretion of hematopoietic growth factors and cytokines from stromal and parenchymal cells, BM stromal cells and monocytes (Rameshwar and Gascon 1995, 1996), whereas Granulocyte-Colony Stimulating Factor (G-CSF) and Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) were shown to upregulate neuronal receptor expression on human HSCs, further increasing sensitivity of these cells for neuronal cues (Kalinkovich et al. 2009). BM nerve damage and physical or chemical denervation of the BM was shown to severely impact the survival of HSCs and BM regeneration and treatment with neuroprotective agents, such as Neuropeptide Y(NPY), or neuroregeneration supporting

growth factors, such as Glial-derived Neurotrophic Factor (GDNF) restored BM dysfunction and promoted hematopoietic recovery (Park et al. 2015a; Lucas et al. 2013). Non-myelinating Schwann cells were shown to maintain HSC quiescence through production of TGFβ and induction of Smad signaling. Autonomic denervation reduced the number of these glial cells, resulting in rapid loss of HSCs from the BM (Yamazaki et al. 2011; Bruckner 2011).

The SNS also plays an important role in HSC mobilization (Katayama et al. 2006). Studies using the UDP-galactose ceramide galactosyl transferase-deficient ($Cgt^{-/-}$) mouse model, elegantly showed that defects in nerve conduction and absence of adrenergic neurotransmission are directly responsible for the lack of HSC egress from BM following G-CSF stimulation (Katayama et al. 2006). Furthermore, important evidence that supports the role of the SNS in HSC mobilization came from a study that showed that HSC release from the BM is regulated by circadian oscillations (Mendez-Ferrer et al. 2008). In mice, numbers of circulating HSCs were shown to fluctuate in antiphase with BM Cxcl12 concentrations. These circadian changes in levels of Cxcl12 and circulating HSCs was shown to be synchronized through Norepinephrine (NE) secretion. NE secreted from BM-SNS fibers interacted with β-adrenergic receptors expressed by osteoblasts, MSCs and HSCs, and caused a decrease in the expression of the transcription factor Sp1 and downregulation of Cxcl12 (Mendez-Ferrer et al. 2008; Mendez-Ferrer et al. 2010). G-CSF and GM-CSF treatment has been shown to increase the expression of DR3 and DR5 dopamine receptors and b2-adrenergic receptors on immature CD34+ cells in mice and humans (Spiegel et al. 2007; Kose et al. 2018). Furthermore, treatment with neurotransmitters increased the motility, proliferation and colony formation of human HSCs, as well as expression and activity of the metalloproteinases MT1-MMP and MMP-2, respectively (Spiegel et al. 2007).

Thus, the regulation of HSC homeostasis, self-renewal, differentiation and mobilization appears to be a cooperative interaction between the nervous system and the bone marrow, through direct communication with HSPCs or indirect effects through other cells of the bone marrow niche, as well as the involvement of hormones, cytokines, neurotrophic factors, neurotransmitters, and neuropeptides, which will be discussed in detail below. In line with the above data, Lapidot and colleagues proposed the presence of a blood-brain-blood triad (Lapidot and Kollet 2010).

2 Adrenergic Regulation of the Hematopoietic Bone Marrow Niche

Interactions between neural signals and hematopoiesis occur early during embryogenesis where sympathetic signaling contributes to the emergence of primitive HSCs from the aorta-gonad-mesonephros (AGM) region (Fitch et al. 2012). Microarray analysis showed that expression of the transcription factor *Gata3* was upregulated in the AGM and deletion of *Gata3* caused a significant reduction in the numbers of AGM resident HSPCs at E10.5 and E11.5 days. Interestingly, local *Gata3* expression was found not to co-localize with AGM-HSPCs, but with SNS fibers and *Gata3* was shown to regulate HSC numbers and development via the production of catecholamines. Similarly, deficiency of the sympathoadrenal marker Tyrosine Hydroxylase (Th) which is normally expressed in the SNS and is responsible for the production of catecholamines, was shown to result in a similar phenotype with a dramatic reduction in the numbers of AGM-HSPCs (Fitch et al. 2012).

Dopamine (D) is an important neurotransmitter in the central nervous system, where it mainly regulates movement, cognition, motivation-reward responses. However, it has also been shown to be released from sympathetic nerve endings in the peripheral nervous system. Epinephrine (E, adrenaline) is mainly derived from the adrenal medulla, whereas norepinephrine (NE, noradrenaline) is secreted from sympathetic nerves. All three neurotransmitters are closely related molecules, that are derived from the non-essential amino acid Tyrosine and act through activation of 7-transmembrane G-protein coupled receptors

(GPCRs). Dopaminergic receptors (DR) consist of five different subtypes, i.e. the D1-like (D1 and D5) receptors, which activate adenylate cyclase and the D2-like (D2, D3 and D4) receptors, which inhibit adenylate cyclase (Cosentino et al. 2015). DRs are expressed on most hematopoietic cells, including T and B cells, dendritic cells, macrophages, neutrophils and NK cells (Cosentino et al. 2015) and have been shown to play an important role in the regulation of immune responses. The adrenergic receptors (AR) are classified into alpha-adrenergic receptors (α-AR) and beta-adrenergic receptors (β-AR). The effects of the SNS on HSCs have been suggested to be mediated by signaling via both α-AR and β-AR. Surface expression of α1-AR, α2-AR, β1-AR, β2-AR and β3-AR was detected on human adult CD34+ HSCs; on murine embryonic HSCs β2-AR was found to be predominantly expressed; expression of α1-AR, α2-AR and β2-AR was shown on different subsets of adult murine myeloid progenitors, multipotent progenitors and HSCs, and β3-AR expression was found on BM-MSCs (Kose et al. 2018; Spiegel et al. 2007; Mendez-Ferrer et al. 2008; Fitch et al. 2012; Muthu et al. 2007).

Maestroni and colleagues were among the first to show the involvement of the adrenal system in the modulation of hematopoiesis (Maestroni et al. 1992). They showed that chemical sympathectomy using B-hydroxydopamine (6-OHDA) and the α-adrenergic antagonist prazosin significantly increased the number of PB granulocytes, monocytes and platelet counts after BMT, whereas addition of the β-adrenergic blocker propranolol completely abolished the effects on blood platelets, but not on white blood cell count (Maestroni et al. 1992). In contrast, α-adrenergic agonists seem to exert an inhibitory effect on myelopoiesis (Maestroni and Conti 1994) and it has been suggested that inhibition of the α1-AR might increase interactions of NE with beta-adrenergic receptors. Not only do catecholamines play an important role in the regulation of hematopoiesis through α1-AR, they are also secreted in high levels by BM sympathetic postganglionic fibers and BM resident cells (Marino et al. 1997).

Also (circadian) release of HSCs from the BM has been shown to be regulated through involvement of the adrenergic system (Katayama et al. 2006). Adrenergic signals are directly delivered to the hematopoietic niche through secretion from sympathetic nerve endings and transmitted to β3-AR expressing BM-MSCs. This results in a decrease of the transcription factor Sp1 and rapid downregulation of SDF-1 (Mendez-Ferrer et al. 2008). Furthermore, these circadian oscillations were shown to occur through changes in parasympathetic and sympathetic activity. At night, when parasympathetic cholinergic signals are dominant, the sympathetic noradrenergic tone is suppressed and egress of HSPCs from the BM decreases. In contrast, during daytime, increased sympathetic noradrenergic activity causes HSPC mobilization through activation of β3-AR (Garcia-Garcia et al. 2019). β2-AR, but not β3-AR stimulation was shown to induce sequential gene expression of clock genes *per1*, *bmal1* and *clock,* showing that although both β2-AR and β3-AR are both required for G-CSF induced mobilization of HSPCs, their roles are functionally distinct. In addition, a double deficiency of both β2-AR and β3-AR negatively affects HSPCs mobilization, showing that although β2- and β3-ARs have distinct roles in stromal cells, during HSPC mobilization both are needed (Mendez-Ferrer et al. 2010).

Further proof that the adrenergic system is heavily involved in modulation of HSPCs trafficking was shown using the UDP-galactose ceramide galactosyltransferase-deficient ($Cgty^{-/-}$) mouse model. These mice were shown to exhibit impaired nerve conduction and lack of HSPC mobilization in response to G-CSF stimulation. In addition, the adrenergic tone, OB function, and bone SDF-1 were all shown to be dysfunctional in this mouse model (Katayama et al. 2006). Inhibition of adrenergic signaling showed that NE regulates G-CSF-induced OB suppression, bone SDF-1 downregulation, and HSPC mobilization, and administration of a β2-AR agonist enhanced mobilization in both control and NE-deficient mice (Katayama et al. 2006).

3 Opioids

Opiates were first isolated as natural extracts from the poppy plant (*Papaver Somniverum*) and have been used medically for centuries for mediation of nociception and sedation. The most well-known opiate Morphine binds to Opioid receptors (Opr) in the central and peripheral nervous system. Opioid receptors belong to the superfamily of the GPCRs (Satoh and Minami 1995). In vertebrates, the opioid receptors are encoded by the μ (Oprm1), κ (Oprk1) and δ (Oprd1) genes and are respectively named MOR, KOR and DOR (Waldhoer et al. 2004). Endogenous opioid signaling plays an important role in the regulation of analgesia, euphoria, homeostasis, anorexia/obesity, immune responses and the cardiovascular system. The opioid peptides are further classified in three genetically distinct families, i.e. the endorphins, enkephalins and dynorphins (Kieffer and Evans 2009). These endogenous opioids function as neurotransmitters or neuromodulators. Similar to other small peptide molecules, endogenous opioids are synthesized through proteolytic cleavage of large protein precursors. These precursors include pro-opiomelanocortin (POMC), the precursor of β-endorphin; pre-pro-enkephalin (PENK), the precursor of leucine (Leu)- and methionine (Met)-enkephalins; and pre-pro-dynorphin (PDYN), the precursor of the dynorphins (Benarroch 2012). Each opioid peptide is derived from a pre-pro and a pro-form, which is then further cleaved and processed through post-translational modifications. As a result of these modifications, receptor binding, affinity and potency of the responses towards opioids is context and tissue-dependent. All opioids share a common five amino acid motif at their N-terminus consisting of Tyr-Gly-Gly-Phe-Met/Leu. POMC is first cleaved into adrenocorticotropin-releasing hormone (ACTH) and β-Lipotropin (βLPH). The latter is further cleaved into α-melanocyte-stimulating hormone (αMSH) and β-endorphin. Beta-endorphins have been shown to both serve as locally active neuromodulators and as paracrine hormones after release into the peripheral circulation. Cleavage of pro-enkephalin results in the release of four met-enkephalins, one leu-enkephalin, one octapeptide and one heptapeptide. As a consequence of the rapid metabolism by peptidases enkephalinase-A and enkephalinase-B, the enkephalins are known to have a short half-life. Cleavage of pro-dynorphin results in the formation of dynorphin A, dynorphin B and neoendorphin (Benarroch 2012).

Although opioid peptides such as β-endorphin and the dynorphin have been shown to play a role in the modulation of the immune system and function as cytokines (Bidlack 2000), not much is known about their role in hematopoiesis. Opioid receptor expression has been detected on many hematopoietic cells, including DOR expression by T-cells, B-cells, macrophages and BM dendritic cells (DCs) and BM-MSCs (Benard et al. 2008; Higuchi et al. 2012); KOR expression by lymphocytes, PB-CD34+ cells (Steidl et al. 2004), BM neutrophils (Kulkarni-Narla et al. 2001), BM stromal cells and BM macrophages (Maestroni 1998, 1999); and MOR expression by lymphocyte subsets, monocytes/macrophages, granulocytes (Sharp et al. 1998) and PB-CD34+ (Steidl et al. 2004) and UCB-CD34+ cells (Rozenfeld-Granot et al. 2002). Agonists of in particularly KOR and to a lesser extent MOR and DOR were shown to inhibit chemotaxis of BM neutrophils towards macrophage inflammatory protein-2 (MIP-2) through downregulation of β2-integrin CD11b/CD18 in a dose-dependent, and naloxone-reversible fashion (Kulkarni-Narla et al. 2001). However, enkephalins themselves appear to have chemotactic properties on DOR expressing mature BM-DCs, although the migratory effect on mature DCs decreased when the concentration of other chemokines, such as CCL19 and CCL21 increased (Benard et al. 2008). When the effects of opioid receptor agonists on colony forming capacity of BM were tested, agonists of KOR, such as Dynorphin A, increased numbers of colony forming unit-granulocyte/macrophage (CFU-GM) in synergy with GM-CSF (Maestroni et al. 1999). In contrast, DOR agonists, such as met-enkephalin, decreased numbers of CFU-GM in a circadian pattern when HSPCs were cultured in presence of adherent BM stromal cells, whereas leu-enkephalin predominantly

suppressed lymphoid cell proliferation (Krizanac-Bengez et al. 1996). Mice subjected to stress induced by immobilization, were shown to develop BM hyperplasia with increased production of neutrophils, monocytes and erythrocytes, which could be completely inhibited by injection of the DOR agonists Leu-enkephalin and its synthetic analog Dalargin (Gol'dberg et al. 1987). MOR-deficient mice displayed increased proliferation of granulocyte-macrophage, erythroid, and hematopoietic progenitor cells in both BM and spleen (Tian et al. 1997). The neutral endopeptidase CD10 (NEP, CALLA, enkephalinase) is expressed by immature lymphoid, myeloid and BM stromal cells and through cleavage can activate or inactivate opioid peptides and a range of neuropeptides, such as Substance P and tachykinins (Boranic et al. 1997).

4 Endocannabinoids

The endocannabinoid (ECB) system consists of two G protein-coupled classical Cannabinoid Receptors (CB1 and CB2), the endogenous Cannabis ligands Anandamide (AEA) and 2-ArachidonoylGlycerol (2-AG), who have an unsaturated fatty acid structure, and the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, respectively (Diaz-Laviada and Ruiz-Llorente 2005). ECBs and their receptors are distributed and act on many systems, predominantly, but not exclusively, the central nervous system (CNS) (Diaz-Laviada and Ruiz-Llorente 2005). Members of the ECB system are involved in many physiological processes, such as hunger control, pain perception, motor function, bone metabolism and regulation of the immune response and may be involved in the development of a range of pathological conditions, including certain inflammatory diseases and the development or progression of cancer (Maccarrone et al. 2015). Furthermore, ECBs play important roles in the regulation of proliferation, differentiation, migration and apoptosis of germ cells and many somatic cells.

4.1 Endocannabinoids and Hematopoietic Stem Cells

The expression of ECBs in hematopoietic tissues, including BM, spleen and thymus, has been reported in a limited number of studies. CB receptor expression has been detected in macrophages, erythroid, B and T lymphoid, mast cell lines (Valk et al. 1997), murine BM HSCs and MSCs (Scutt and Williamson 2007). CB receptor mediated cell migration has been demonstrated in rodent myeloid leukemia cells (Jiang et al. 2011b), mouse mononuclear cells (MNCs) (Patinkin et al. 2008) and endothelial cells (Mo et al. 2004). The interactions between the ECB system and BM cells were investigated by assessing the presence of CB receptors on rodent BM cells and hematopoietic growth factor (HGF, IL-3, GM-CSF, G-CSF, EPO) dependent cell lines. Expression of the CB1 receptor was found on a T lymphoid cell line, whereas expression of the CB2 receptor could be detected more broadly on a variety of myeloid, macrophage, erythroid, B-lymphoid, T-lymphoid and mast cell lines (Valk et al. 1997). The stimulatory effect of AEA on proliferation of these cell lines was further enhanced by addition of IL-3, GM-CSF, G-CSF and EPO. Therefore, it was proposed that AEA could be used to synergistically to enhance proliferation of hematopoietic cells in combination with known hematopoietic growth factors and cytokines (Yamaguchi and Levy 2016). Furthermore, stimulation of CB2 overexpressing rodent myeloid leukemia cells was shown to induce migration of the cells in the direction of AEA, 2-AG and other cannabinoids, thus revealing a role for ECBs in migration and chemotaxis of hematopoietic cells (Jiang et al. 2011a). It has also been suggested that the primary function of CB2 receptor expression on B lymphocytes might be regulation of migration of these cells (Jorda et al. 2002). Administration of AEA and 2-AG to mice was shown to increase the numbers of BM CFU-GEMM colonies in culture two-fold. Furthermore, an increase in circulating mouse MNCs was shown within 4 h of administration of 2-AG

and AEA. Studies using CB1 and CB2 receptor antagonists showed that the effect of 2-AG is mainly mediated by interaction with CB1 (Patinkin et al. 2008). The importance of CB2 for hematopoiesis was emphasized in a study where hematopoietic recovery was assessed in CB2 knockout ($Cnr2^{-/-}$) mice and WT mice subjected to sublethal irradiation. In comparison to the WT mice, the $Cnr2^{-/-}$ mice displayed considerably decreased hematopoietic recovery with low colony formation and a prolonged period of low PB counts. When the WT mice were administered CB2 agonists after sublethal irradiation, HSC survival increased and resulted in accelerated BM recovery. Additionally, when WT mice were administered CB2 agonists 12 days prior to sublethal irradiation, HSC apoptosis decreased, c-kit positive and total BM cell numbers increased, resulting in an overall increased survival of the mice (Jiang et al. 2011b). Interestingly, during and after transplantation of HSCs, peripheral blood levels of IL-6 and 2-AG increased. This increase in 2-AG was linked to a general increase in stress and inflammation in the receiver (Knight et al. 2015).

4.2 Endocannabinoids and Mesenchymal Stem Cells

In addition to their effect on HSC, ECBs have also been shown to be involved in the regulation of migration, differentiation, survival and activity of bone cells. In addition, the ECB system has been shown to play a critical role in the regulation of bone mass maintenance (Gowran et al. 2013; Idris et al. 2005; Idris et al. 2008; Idris et al. 2009). *Ex vivo* studies have shown that bone cells express both CB1 and CB2 receptors, as well as the Transient Receptor Potential Vanilloid type 1 (TRPV1) receptors and the G-coupled protein receptor 55 (GPR55), which have been shown to oppositely modulate the effects of ECBs *in vitro* human osteoblast activity (Whyte et al. 2009). In addition, they were also shown to possess the machinery required for synthesis and metabolism of ECBs (Smith et al. 2015). Although CB1 and CB2 receptors could not be detected on the general population of BM stromal cells; both receptors have been shown to be present in a small fraction of the BM stromal cell side population, which is typically enriched for stem cells (Scutt and Williamson 2007; Kose et al. 2018).

In mice, CB1 receptor inactivation was shown to initially result in increased bone mass and protection from ovariectomy-induced bone loss due to increased osteoclast activity (Idris et al. 2005). However, prolonged CB1 receptor deficiency eventually results in osteoporosis due to preferential differentiation of the BM stromal cells towards adipogenic lineage (Idris et al. 2009). CB2 receptor activation plays a role in bone turnover and CB2 agonists were shown to enhance bone mass by increasing osteoblasts numbers and activity, by inhibition of osteoclast proliferation and by increasing CFU-F numbers (Ofek et al. 2006; Scutt and Williamson 2007; Idris et al. 2008). To further assess the effect of ECBs on BM migration and colony forming unit-fibroblast (CFU-F) formation, 2-AG was applied to rat BM cultures. Addition of 2-AG to these cultures resulted in a dose-dependent increase in colony size and colony formation. In addition, it is thought that activation of the CB2 receptor plays an important role in osteogenic differentiation of BM-MSCs (Yamaguchi and Levy 2016). Tetrahydrocannabinol (THC) is the psychoactive component of cannabis and functions as a partial agonist of the CB1 receptor and an antagonist of the CB2 receptor. Addition of THC *in vitro* to cultures of rat BM-MSCs resulted in a negative effect on MSC survival and osteogenic capacity of the cells (Gowran et al. 2013). BM-MSCs have been shown to secrete the CB ligands AEA and 2-AG (Kose et al. 2018). In response to the locally produced AEA and 2-AG, activation of in particular CB2 signaling has been shown to result in an altered cytokine release profile in immune cells and immunosuppressive effects on activated T lymphocytes (Rossi et al. 2013; Xie et al. 2016). The non-psychoactive phytocannabinoid Cannabidiol (CBD) displays a suppressive effect on inflammatory processes through activation of both CR-dependent and independent mechanisms. In an *in vitro* model of inflammation where extended exposure to LPS was used activate

Toll-like receptors (TLRs) on adipose tissue derived MSCs, application of CBD was shown to decrease oxidative stress and restore adipogenic and chondrogenic differentiation potential of these cells (Ruhl et al. 2018). Similarly, the CB2 agonist AM1241 was shown to protect adipose tissue derived MSCs from H_2O_2 induced oxidative stress and promote their regenerative potential when transplanted *in vivo* into the ischemic myocardium of mice (Han et al. 2017).

Using Boyden chamber assays, both CBD and THC were found to increase the migration of adipose tissue-derived MSCs in a time- and concentration-dependent manner. This effect of ECBs on MSC migration was shown to be modulated by activation of the p42/44 MAPK pathway. Furthermore, CBD-induced migration inhibited by the CB2 antagonist AM-630 and GRP55 agonist O-1602 and PD98059, an inhibitor of the p42/44 MAPK pathway (Luder et al. 2017), whereas THC-induced migration was almost completely blocked by addition of CB1 receptor antagonist AM-251 and AM-630 (Schmuhl et al. 2014). These data were further confirmed by other studies that showed that inhibition of FAAH, which catalyzes the degradation of AEA and 2-AG, increased stem cell migration via PPARα (Wollank et al. 2015). Similarly, CB1 receptor agonist ACEA promoted migration of murine BM-MSCs *in vitro* and *in vivo*, whereas CB2 agonist JWH133 had no effect. Accordingly, pharmacological or genetic ablation of CB1 reduced ACEA-induced migration, whereas inhibition of CB2 activation did not noticeably affect migration (Wang et al. 2017).

4.3 The Role of Endocannabinoids in the Brain-Bone-Blood Triad

Recent studies have established that HPSCs/HSCs dynamically change their features and location, shifting from quiescent and stationary cells anchored in the BM to cycling and motile cells entering the circulation. These changes are controlled by brain-bone-blood triad via stress signals controls (Lapidot and Kollet 2010). However, how and why HSCs enter and exit the BM is not fully understood. ECBs may act as mobilizers of HSC from the BM under stress conditions similar to stimulation of beta-adrenergic receptors (β-AR). Our group previously demonstrated that BM-MSCs secrete both AEA and 2-AG, and AEA and 2-AG was detected in both PB and BM plasma samples of healthy donors (Kose et al. 2018). BM mononuclear cells (MNCs) and CD34+ HSCs were both shown to express CB1, CB2 and β-AR subtypes. When compared to BM-MSCs, CD34+ HSCs showed a higher CB1 and CB2 receptor expression. AEA and 2-AG induced HSC migration was blocked by ECB receptor antagonists in *in vitro* migration assays. In addition, components of the ECB system and β-AR subtypes were shown to interact with HSCs and MSCs of G-CSF treated and untreated healthy donors in vitro, revealing that ECBs might be potential candidates to enhance or facilitate G-CSF-mediated HSC migration under stress conditions (Kose et al. 2018). The effect of the ECB system in the migration/mobilization of HSC has been summarized in Fig. 1.

Thus, ECB stimulation increases proliferation, migration and especially osteogenic differentiation of human and rodent MSCs, albeit with different potencies, depending on the agonist used, and promotes their anti-inflammatory properties.

5 Neuropeptides

Neuropeptides have been defined as small peptides produced and secreted by neurons and acting on neural substrates. The neuropeptides consist of a large group of diverse signaling molecules that can act directly as neurotransmitters, as modulators of neurotransmission, as autocrine or paracrine regulators of a close microenvironment or as hormones, affecting other systems at a long distance (Burbach 2011; Burbach 2010).

5.1 Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is a neurotransmitter composed of 36 amino acids that is secreted from the

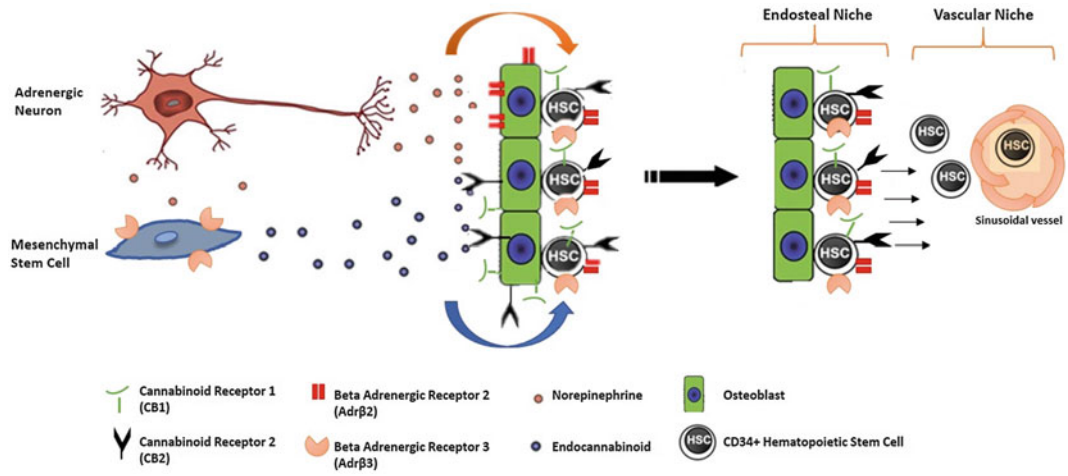

Fig. 1 The effect of the ECB system in the migration/mobilization of HSC. (i) ECBs, AEA and 2-AG are secreted from BM-MSCs; (ii) BM-MNCs and CD34 + HSCs express the cannabinoid receptors CB1, CB2 and beta adrenergic receptors β2-AR, β3-AR; (iii) CD34 + HSCs migrate towards SDF-1, Norepinephrine, AEA and 2-AG is mediated by CB receptors; (iv) CD34 + HSCs migration toward MSCs is inhibited by cannabinoid receptors CB1, CB2 and β-AR antagonists. Courtesy of S. Köse

central nervous system and sympathetic nerve ends (Park et al. 2015b). NPY regulates a range of physiological processes such as appetite, energy storage, emotional regulation and pain through activation of 5 different G-protein coupled receptors (Y1R-Y5R), which are highly expressed in mammals (Brothers and Wahlestedt 2010). While the expression levels of the NPY receptors in the CNS have been extensively studied, their distribution and function in peripheral tissues remains largely unexplored (Table 1). NPY has been shown to be involved in the regulation of immune cell homeostasis (Wheway et al. 2005), bone homeostasis (Sousa et al. 2009; Baldock et al. 2009) and vascular remodeling (Kuo et al. 2007) through NPY receptors expressed in BM cells, osteoblasts, macrophages and endothelial cells (Lee and Herzog 2009; Singh et al. 2017; Park et al. 2016). Furthermore, the fact that NPY deficient mice have decreased numbers of HSCs and a significantly impaired bone marrow function (Lin et al. 2004; Park et al. 2015a), as well as the fact that treatment with NPY through activation of the Y1-receptor results in hematopoietic regeneration, suggest that NPY plays a protective role in the bone marrow microenvironment. Even more, HSC transplantation into NPY deficient mice was shown to result in delayed and impaired engraftment, whereas treatment with NPY restored bone marrow function. However, the differentiation capacity and the maturation of HSCs from NPY deficient mice were not affected (Park et al. 2015a).

5.1.1 NPY and Mesenchymal Stem Cells

NPY was shown to exert multiple regulatory effects on a range of stem cells, as reviewed by Peng et al. (2017). Outside of the CNS, NPY plays an important role on bone homeostasis through direct stimulation of proliferation (via the NPY-Y5 receptor), migration (CXCR4) and differentiation (via the NPY-Y1 and Y2 receptors) of BM-MSCs (Peng et al. 2017).

NPY significantly increased rat BM-MSC proliferation, prevented apoptosis and promoted osteogenic differentiation in a dose-dependent through upregulation of β-catenin, p-GSK-3β and c-myc and activation of canonical Wnt signaling (Liu et al. 2016; Wu et al. 2017). Furthermore, NPY treatment of rat BM-MSCs also caused an upregulation in the expression of VEGF and SDF-1α and genes involved in the regulation of proliferation, including aurora B

Table 1 Tissue expression of NPY receptors and their function

NPY receptor	Alternative name	Chromo-some	Ligand	Tissue expression	Function
Y1		4q32.2	PYY > NPY	**Spleen, placenta, bone marrow, lymphnodes,** adipose tissue, adrenal glands, kidney, brain, heart	Modulation of anxiety, depression and nociception, regulation of the circadian rhythm and cardiovascular sympathetic tone, bone homeostasis, proliferation of neuronal stem cells and smooth muscle cells, angiogenesis, nutrient absorption.
Y2		4q32.1	NPY = PYY	Brain, testis, gastrointestinal system, adipose tissue	Modulation of anxiety, depression, nociception and addiction, regulation of the circadian rhythm and cardio-vascular sympathetic tone, bone homeostasis, proliferation of neuronal stem cells and smooth muscle cells, angiogenesis.
Y3	CXCR4, CD184	2q22.1	CXCL12, NPY > > PYY	**Bone marrow, lymphnodes, spleen, placenta**	**Role of NPY on CXCR4 signaling is currently unknown**
Y4	PYY-R1, PPR-1	10q11.22	PP > PYY > NPY	Gastrointestinal system, skin, prostate, lungs, pancreas	Nutrient absorption, regulation of acid secretion
Y5		4q32.2	NPY > PYY	**Spleen, placenta, lymphnodes,** adipose tissue, testis, kidneys, brain, adrenal glands	Cardiac hypertrophy, regulation of food intake and obesity, modulation of addiction, proliferation of mesenchymal stem cells

kinase, bFGF, cyclin A2 and EIF-4E (Wang et al. 2010; Liu et al. 2016). The proliferative effects of NPY on BM-MSCs were shown to be directly mediated through interactions with the NPY-Y5 receptor: BM-MSCs from old rats showed decreased expression of Y5 and a lower proliferative response to NPY than BM-MSCs from young rats, who had high Y5 expression and an increased proliferative response (Igura et al. 2011).

In addition to proliferation, NPY also plays a role in the regulation of BM-MSC differentiation. For example, NPY-deficient murine BM-MSCs showed an increase in osteogenic differentiation as apparent from prominent alkaline phosphatase staining and increased *bsp* (Bone Sialoprotein) and *ocn* (Osteocalcin) gene expression (Wee et al. 2019). Y2 receptor knockout ($Y2^{-/-}$) mice were shown to control bone volume via modulation of osteoblastic activity (Baldock et al. 2002). *In vivo* $Y2^{-/-}$ mice displayed an increase in the numbers of BM osteoprogenitor cells and *in vitro* these BM-MSCs showed increased mineralization and adipocyte formation (Lundberg et al. 2007). Similarly, antagonists of the NPY Y1 receptor promoted anabolic activity of osteoblasts in mice resulting in increased bone mass (Sousa et al. 2012) and BM-MSCs isolated from Y1 knockout ($Y1^{-/-}$) mice showed increased mRNA levels of the osteogenic transcription factors, *runx*2 (Runx2) and *osx* (Osterix), and adipogenic transcription factor *pparg* (PPAR-γ) (Lee et al. 2010). Thus, Y2 and Y1 receptor signaling appears to have similar effects on the regulation of bone mass and osteoprogenitor cell numbers. Interestingly, Y2 receptor deletion resulted in a simultaneous decrease in the expression of Y1 transcripts,

possibly through a lack of feedback inhibition, thus further increasing bone density and aggravating the skeletal phenotype in these mice (Lundberg et al. 2007). Chronic exposure to NPY resulted in downregulation of both Y2 and Y1 receptor expression (Teixeira et al. 2009).

Furthermore, NPY was shown to promote migration of BM-MSCs through upregulation of CXCR4 expression and induce endothelial differentiation and tube formation of BM-MSCs (Wang et al. 2010).

5.1.2 NPY and Hematopoietic Stem Cells

NPY was shown to play an important role in the regulation and maintenance of HSPCs (Ulum 2019). Using the Y1 knockout ($Y1^{-/-}$) mice Park and colleagues showed that NPY deficiency causes a severe impairment of HSC survival and BM regeneration (Park et al. 2015a). In the absence of NPY/NPY-Y1 signaling, the researchers found increased SNS nerve fiber destruction and a reduction in the numbers of ECs. Treatment with NPY or other Y1 agonists was shown to protect the BM nerves from Cisplatin-induced toxicity. In addition, TGF-β secreted by NPY-mediated Y1 receptor stimulation in macrophages was shown to play a key role in neuroprotection by NPY and HSC survival in the BM (Park et al. 2015b). Further evidence of the importance of NPY for the maintenance of HSPCs came from another study using $NPY^{-/-}$ and $NPY\text{-}Y1^{-/-}$ mouse models. $NPY^{-/-}$ mice did not exhibit HSPC mobilization in response to common mobilizing agents such as AMD3100 or G-CSF (Park et al. 2016). This lack of mobilization was found to be associated with an increased level of SDF-1α in the BM. Since NPY deficient HSCPs were shown to exhibit a normal migratory activity *in vitro* (Park et al. 2015a), the lack of mobilization *in vivo* was thought to be related to the effects of NPY on the BM niche. Indeed, $NPY^{-/-}$ mice were shown to have decreased levels of MMP-9, which is required for the degradation of SDF-1α in the BM by MMP-9 (Park et al. 2016) and NPY treatment of WT mice resulted in increased MMP-9 levels and rapid mobilization of HSPCs. In contrast, HSPCs from WT mice did not display significant migration in response to NPY *in vitro*, thus confirming that NPY has no chemotactic effect itself and requires a functional BM niche to exert its effects on HSPCs. Similar to the $NPY^{-/-}$ mice, WT mice treated with NPY-Y1 receptor antagonists showed inhibition of NPY induced mobilization, whereas treatment with NPY or NPY-Y1 receptor agonist resulted in increased HSPC mobilization. The effects of NPY on HSPC mobilization was shown to be mediated by Y1 expressing OBs, and NPY treatment of WT mice led to an increase in MMP-9 activation by OBs and a subsequent reduction in the levels of SDF-1α followed by rapid HSPC mobilization (Park et al. 2016). Others pathways through which NPY appears to affect mobilization is through regulation of transendothelial migration of HSPCs (Singh et al. 2017). NPY was shown to reduce VE-Cadherin and CD31 expression along EC junctions, thus causing increased vascular permeability and HSPC egress. Regulation of vascular integrity was shown to be mediated by interactions with NPY-Y2 and Y5 receptors, and Y2 and Y5 receptor antagonists restored vascular integrity and prevented HSPC egress from the BM (Singh et al. 2017). The CD26 (DPPIV/dipeptidyl-peptidase IV) antigen plays an important role in mobilization of HSCPs. CD26 is a membrane-bound peptidase that is expressed on the surface of a subpopulation of stem and mononuclear cells and degrades several chemokines, including SDF-1. In response to G-CSF the HSPCs are stimulated to expand and differentiate into neutrophils. The latter produce a variety of enzymes including Neutrophil Elastase, Pepsin G and MMP-9, which are activated inside the BM and cleave the SDF-1 protein, inhibiting its ability to interact with HSPCs. When the interaction between SDF-1 and CXCR4 is broken, the HSPCs are no longer held in place inside the BM and are released into the PB. Recently it was shown that CD26 on ECs increases in response to G-CSF and cleaves the NPY peptide, which through signaling via NPY-Y2 and Y5 receptors increased vascular permeability of the BM niche (Singh et al. 2017). In addition, mice lacking either CD26 or NPY were shown to exhibit impaired HSPC mobilization, that could be restored by treatment with truncated NPY (Singh et al. 2017).

The alternative name for the NPY-Y3 receptor is CXCR4. CXCR4 is a chemokine receptor crucial for the anchoring of HSCs in the BM and necessary for homing after HSPC mobilization and transplantation. The actual ligand of CXCR4 is SDF-1 and there is no homology at the nucleotide and amino acid level between SDF1 and NPY (NCBI BLAST and NCBI pBLAST). Due to SDF-1/CXCR4 interactions, HSCs are retained in the BM niche and HSC mobilization occurs when G-CSF or SDF-1 antagonists, such as AMD3100 are administered. Although NPY increases CXCR4 in BM-MSCs, NPY does not appear to affect CXCR4 expression by HSPCs (Park et al. 2016). The effect of NPY on CXCR4-expressing hematopoietic cells is not yet known, but studies indicate that NPY may not effectively bind to or activate the chemokine receptor (Herzog et al. 1993). However, NPY/NPY-Y3 interactions have been shown to increase vascular permeability in the pulmonary circulation and in rat aorta endothelial cells (Nan et al. 2004; Hirabayashi et al. 1994). It is highly likely that a similar effect of NPY may be found on the BM sinus endothelium. The NPY-Y4 receptor has not been shown to play an important role in the hematopoietic and immune system until now and is expressed mostly in the gastrointestinal tract. NPY-Y5 is expressed in hematopoietic tissues such as spleen, lymph nodes and placenta, and its role in hematopoiesis has not yet been elucidated.

Interestingly, it has been recently shown that all NPY receptors are present in varying degrees on HSPCs, although the highest expression was observed for NPY-Y1 (Ulum 2019). Thus far the effects of NPY on maintenance, self-renewal, proliferation and migration have been attributed to interactions with cells from the hematopoietic niche. However, new data indicate that NPY may directly interact with NPY receptors on HSCs as well and affect their potential for self-renewal through modulation of quiescence (Ulum 2019). Current knowledge of NPY receptor-mediated effects on hematopoiesis and their role in the regulation of the hematopoietic niche are summarized in Fig. 2. The effects of NPY on hematopoiesis under physiological conditions and after G-CSF mobilization are depicted in Fig. 3.

5.2 Tachykinins

Tachykinins belong to a peptide family that is characterized by a common C-terminal sequence, consisting of Phe-X-Gly-Leu-Met-NH_2. This family of peptides is predominantly active in the brain and gut and owns its name to their ability to rapidly induce contraction of gut and bladder smooth muscle. Similar to the opioids, the tachykinin genes encode large precursor proteins called pre-pro-tachykinins, that are further spliced into sets of peptides and precursor proteins and posttranslationally processed by several proteases. The Neurokinin family includes substance P (SP), the two structurally related peptides neurokinin A (NKA) and neurokinin B (NKB), and Hemokinin-1 (HK-1). These peptides are encoded by three different pre-pro-tachykinin genes, i.e. the *TAC1* (PPT-A) gene, which encodes the sequences of SP, NKA, and neuropeptide K; the *TAC3* (PPT-B) gene, which encodes NKB and the *TAC4* (PPT-C) gene, which encodes HK-1, Endokinin A and B (EKA and EKB) and the Tachykinin-related peptides Endokinin C and D (EKC and EKD) (Nowicki et al. 2007; Klassert et al. 2010). The effects of the neurokinins are mediated through the family 1 (rhodopsin-like) GPCR neurokinin receptors NK-1R, NK-2R and NK-3R (Gerard et al. 1993; Klassert et al. 2010). Whereas SP and HK-1 bind preferentially to NK-1R, NK-A binds with highest affinity to NK-2R (Pennefather et al. 2004). NK-Rs are broadly expressed in both neural and non-neural tissues, including hematopoietic cells, immune cells and BM stroma (Beaujouan et al. 2004). However, the distribution of the NK receptors is quite different, with the NK-1R being ubiquitously expressed in vascular endothelium, BM, immune cells, muscle and neurons, NK-2R expression predominantly being found in peripheral tissues, while NK-3R is preferentially expressed in the CNS (Pennefather et al. 2004). Similarly, expression of the neurokinins has also been detected in many non-neural tissues, such as the cardiovascular gastrointestinal and immune system, although neurons are the most prominent source of SP,

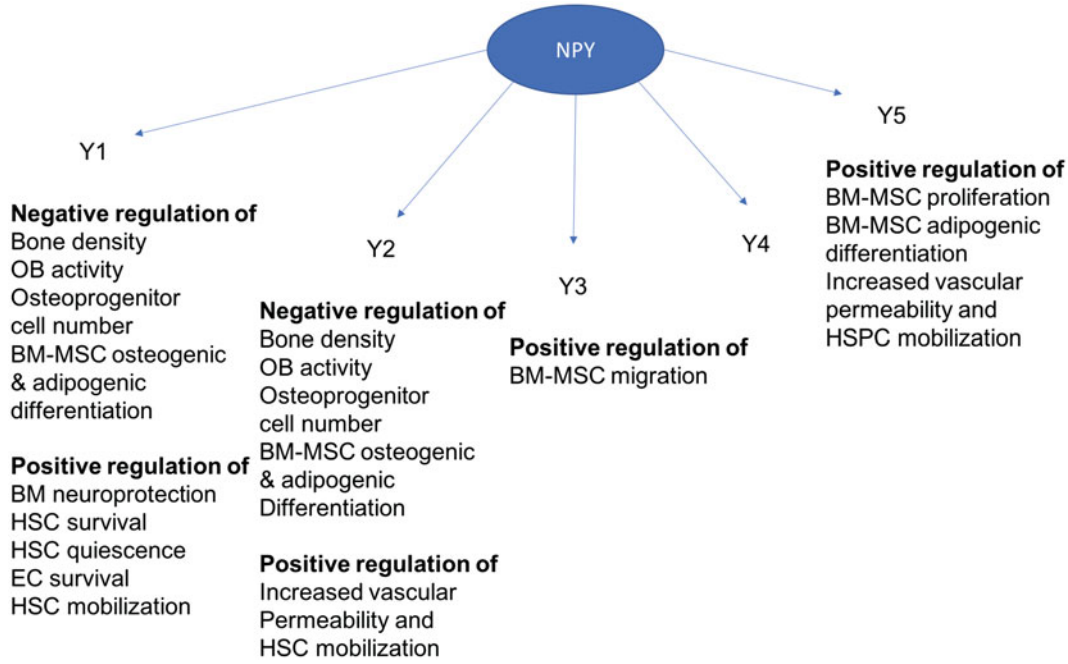

Fig. 2 Role of the NPY receptors in the regulation of the hematopoietic niche. NPY-Y1 and Y2 show overlapping actions and are highly expressed in the BM on MSCs, OBs, ECs and HSCs. NPY-3 is highly expressed on BM-MSCs and HSCs, but appears to be playing a minor role in NPY signaling. The function of NPY-Y4 in the BM has not been assessed and NPY-Y5 appear to play an important role in regulation of HSC egress

NKA and NKB (Nowicki et al. 2007). Whereas NKA, SP and HK-1 are known to be involved in the regulation of hematopoiesis, NKB plays a predominant role in nociception.

5.2.1 Substance P

Substance P is not only widely expressed throughout the central and peripheral nervous system, expression and local production of SP was also be detected in bone marrow innervating fibers, T and B cells, (vascular) endothelial cells, eosinophils, fibroblasts and monocytes/macrophages (Rameshwar et al. 1993a). SP was shown to induce the production of Interleukin-1 (IL-1), IL-6, and Tumor Necrosis Factor-alpha (TNFα) by monocytes and mast cells, IL-3 and GM-CSF production from BM-MNCs, IL-2 expression by activated T-cells, Interferon gamma (IFNγ) by PB-MNCs, and IL-1 and SCF synthesis by BM stromal cells (Nowicki et al. 2007). In addition, BM stromal cells were shown to participate in the local synthesis of IL-1 and SCF (Rameshwar and Gascon 1995).

In vitro, SP alone, in the absence of HGFs, was able to support hematopoiesis and formation of myeloid and erythroid colonies in semi-solid cultures. Using SP antagonists, the effect of SP on hematopoietic cultures was completely abrogated, whereas addition of antibodies against IL-3 and GM-CSF resulted in a reduction of the effect of SP, indicating that the effects of SP on hematopoiesis are mediated by IL-3 and GM-CSF (Rameshwar et al. 1993a). This and other studies show that SP affects both murine and human HSPCs during the gradual stages of differentiation (Liu et al. 2007; Greco et al. 2004). In response to treatment with SP, expression of IL-2 was induced in murine T-cell lines, PB lymphocytes and spleen lymphocytes and purified CD4+ T cells (Rameshwar et al. 1993b). Degradation of SP by neuroendopeptidase (NEP/CD10) expressed by hematopoietic cells into the smaller SP(1–4) has

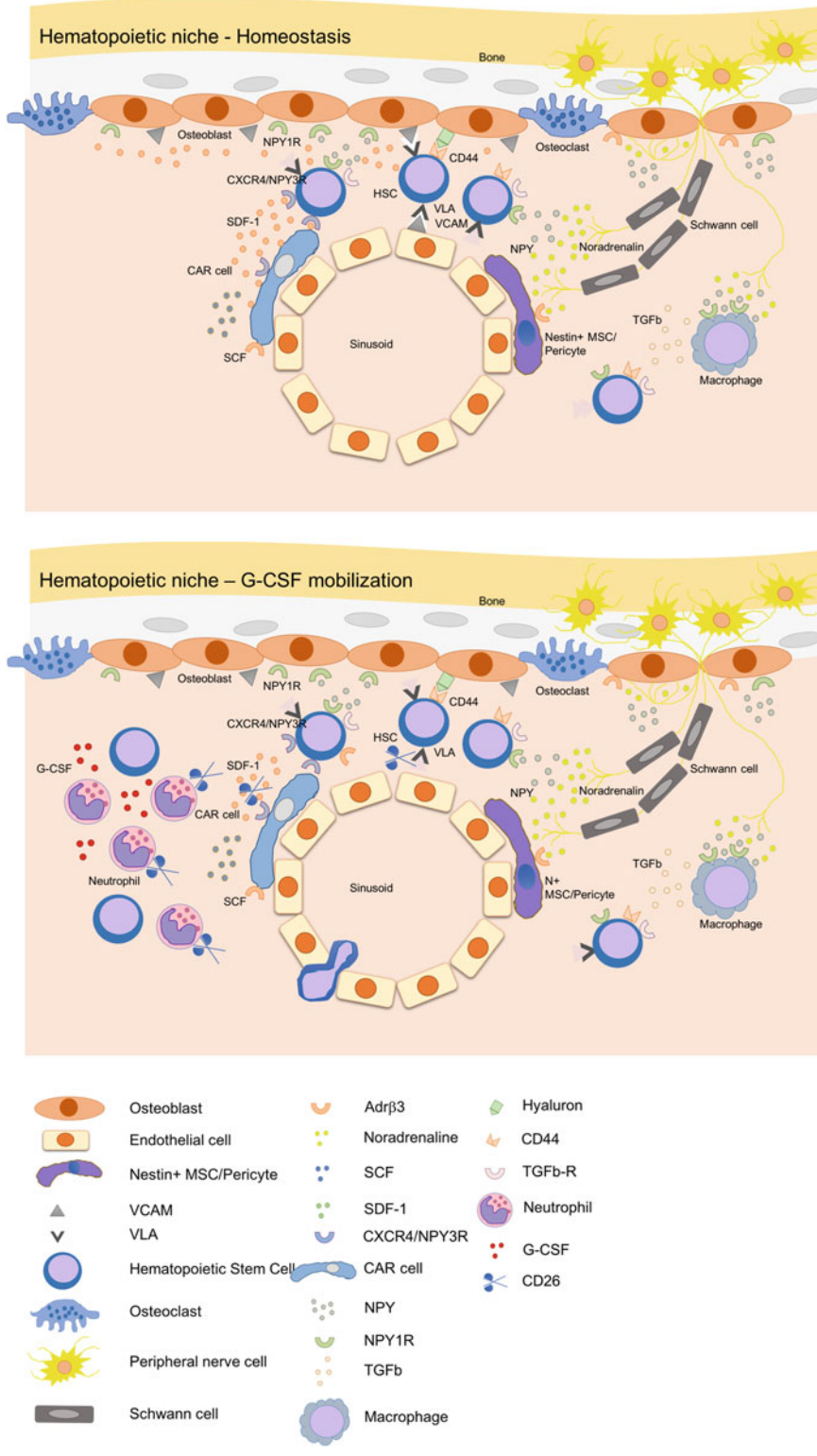

Fig. 3 The role of NPY in the hematopoietic bone marrow niche. Upper panel: NPY protects the hematopoietic environment through interactions with Y1, Y2 receptors on BM-MSCs, OBs, macrophages and ECs. NPY is secreted from sympathetic nerve endings, macrophages, OBs and ECs. Lower panel: HSPCs are retained in the BM by firm interactions between CXCR4 and SDF-1. G-CSF induced mobilization causes upregulation of CD26 and MMP-9, cleavage of SDF-1/ CXCR4 interactions, cleavage and binding of truncated NPY to NPY-Y2 and Y5, increased vascular permeability and mobilization of HSPCs

been shown to modify its function, inhibit proliferation of mature myeloid progenitors and induce the production of Transforming Growth Factor-beta (TGFβ) and TNFα in BM stroma (Joshi et al. 2001). Further stimulation of BM stroma with SCF, increases NEP transcripts and degradation of SP. Since SP and SP(1–4) compete for binding to NK-1R but have opposite functions, SCF stimulation of BM stroma results in a negative feedback on hematopoiesis mediated by SP.

In vivo, SP was shown to be released from the sensory nerve endings of C-type nerve fibers in response to stress factors and tissue injury (Holzer 1988). These peptidergic nerve endings are often found in close proximity to immune cells in mucosal membranes (Stead et al. 1987) and in BM, affecting both hematopoietic and stromal cell populations (Felten et al. 1992). The SP receptor NK-1R was found to be expressed by most BM cells, including hematopoietic cells, stromal cells and T-lymphocytes (Nowicki et al. 2007).

SP plays a fundamental role as a regulator of synaptic transmission in sympathetic peptidergic nerve endings. As discussed above, the sympathetic nervous system has been shown to steer mobilization of HSPCs from their BM niche (Katayama et al. 2006). Thus, it has been suggested that by modulating the sympathetic tone of the BM microenvironment, SP may be involved in the regulation of mobilization efficacy in response to G-CSF treatment and potentially be used to improve clinical protocols of mobilization and stem cell harvests. These intriguing thoughts are further supported by the fact that SDF-1/CXCR4 interactions are essential for HSPC retaining, quiescence and mobilization (Petit et al. 2002) and that SDF-1 may induce expression of the *TAC1* gene and production of SP and NKA *in vitro* (Klassert et al. 2010). As mentioned above, SP was also shown to stimulate production of GM-CSF by BM-MNCs, which also plays a role in mobilization of HSPCs (Rameshwar et al. 1993a). In addition, SP was shown to upregulate gene and cell surface expression of CXCR4 on CD34+ cord blood cells (Shahrokhi et al. 2010).

In addition to its effect on hematopoiesis, SP has also been shown to have important angiogenic properties and stimulate endothelial cell proliferation through interaction with its receptor NK-1R (Pelletier et al. 2002)

5.2.2 Neurokinin A

In contrast to SP, NKA has been shown to inhibit the development of granulocyte/monocyte precursors, but stimulates erythrocyte progenitors (Nowicki et al. 2007; Liu et al. 2007). These opposing effects of NKA are the result of signaling through NK-2R and its stimulation of the production of the hematopoietic suppressors, Macrophage Inflammatory Protein-1a (MIP1α) and TGFβ by BM stromal cells (Rameshwar and Gascon 1996). Under physiologic conditions, SP and NKA, promote the production of distinct cytokines, exerting contrasting effects on the proliferation of hematopoietic progenitors. It has been suggested that the contrasting effects of NKA and SP may serve to protect HSPCs from exhaustive proliferation (Klassert et al. 2010) and that the *TAC1* gene can both stimulate and suppress HSPCs through a negative feedback system (Rameshwar et al. 1997; Rameshwar et al. 1993a). Furthermore, a protective role for NKA has been predicted based on the levels of the predominant types of TAC1 transcripts found in healthy BM and leukemic cells (Nowicki et al. 2003): Whereas healthy BM stromal cells were shown to express transcript b, encoding both SP and NKA, leukemic cells predominantly expressed transcript a, which only produces SP (Nowicki et al. 2007). In leukemic cells, where predominantly SP production takes place, the autoregulatory feedback regulated by NKA is absent, and as a consequence cells may not be able to inhibit their proliferative responses (Nowicki and Miskowiak 2003).

5.2.3 Hemokinin-1

HK-1 is encoded by the *TAC4* (*PPT-C*) gene and its expression was exclusively outside the neural system and predominantly detected in hematopoietic cells, in contrast to PPT-A and B genes (Zhang et al. 2000). HL-1 was shown to have a particularly prominent role in the regulation of lymphopoiesis. Based on the structural similarity of SP and HK-1, it was suggested that HK-1

might exert its hematopoietic effects through binding of the NK-1R. Indeed, it was shown that HK-1 could bind to NK-1R with equal affinity to that of SP (Morteau et al. 2001). Thus, as expected, HK-1 has been shown to display a range of biological activities that closely resemble those of SP. HK-1 was shown to be a critical regulator of B cell development in mice, and induced proliferation of IL-7 expanded B cell precursors, whereas SP was not effective. In addition, HK-1, but not SP, promoted the survival and expansion, and suppressed apoptosis of fresh and cultured B cell precursors (Zhang et al. 2000; Morteau et al. 2001). Similarly, HK-1 was shown to support and regulate T cell development and use of antagonists in fetal thymus organ cultures (FTOC) resulted in an early blockage of differentiation of T cell precursors at the double negative (DN, CD44-,CD25+) or double positive (DP, CD4+/CD8+) stage (Zhang and Paige 2003). Thus, HK-1 may provide a proliferative stimulus for T and B cell precursors, and decreased TAC4 mRNA expression has been suggested to support progression into more mature stages. Expression of HK-1 was observed in murine monocyte and macrophage cells lines and freshly isolated human PB granulocyte, eosinophil, monocyte and lymphocyte subsets. Stimulation of these cells with either proinflammatory cytokines or PHA decreased HK-1 and NK-1 receptor mRNA (Klassert et al. 2008; Berger et al. 2013). Interestingly in the same cell subsets also NKB expression was detected.

5.2.4 Effect of NK Receptor Signaling on Hematopoiesis

Using competitive BM repopulation assays it was shown that BM from NK-1R deficient mice ($Tacr1^{-/-}$) showed decreased lymphoid lineage specific engraftment potential, whereas myeloid and erythroid lineages were not affected. However, BM cells from Tachykinin-knockout mice lacking either SP, HK-1 or both ($Tac1^{-/-}$, $Tac4^{-/-}$ and $Tac1^{-/-}$/$Tac4^{-/-}$ mice) were able to repopulate lethally irradiated recipients with normal efficiency, presumably due to Tachykinin peptide release by the host or competitor cells, indicating that Tachykinin signaling plays an important role in engraftment and normal hematopoiesis and functions through a paracrine or endocrine mode of action (Berger et al. 2013). However, as discussed above, aberrant regulation of TAC1 expression with predominant expression of SP and loss of NKA, may contribute to the phenotype of leukemic transformation (Nowicki et al. 2007).

The effects of NK-1R and NK-2R agonists on hematopoiesis are distinctly different (Klassert et al. 2010; Rameshwar et al. 1997), with stimulation of NK-1R mediating production of hematopoiesis-supporting cytokines, and NK-2R stimulation resulting in the synthesis of inhibitory cytokines (Greco et al. 2004; Rameshwar and Gascon 1996). The balance between the expression and activation of NK-1R and NK-2R in the hematopoietic microenvironment is most likely to direct the effects and outcome for the HSPCs. The opposite nature of the effects of NK-1R and NK-2R stimulation makes it unlikely that both receptors are present on the same cells at the same time. Indeed, co-expression of these receptors could not be detected on BM stromal cells (Klassert et al. 2010). Evenmore, BM stromal cells expressing high levels of NK-1R showed downregulation of NK-2R expression and *vice versa* (Kang et al. 2004; Rameshwar et al. 1997; Bandari et al. 2003). These opposing expressions of the NK-1R and NK-2R are thus in line with the stimulatory effects of NK-1R agonists SP and HK-1 and the suppressive effects of NK-2R ligand NKA (Bandari et al. 2003; Kang et al. 2004).

The role of tachykinins on hematopoiesis during stress or injury (model 1), during SCF treatment (model 2), during mobilization (model 3) and during physiological situations, where the majority of HSCs are quiescent (model 4), are summarized in Fig. 4.

6 Neurotrophins and Neuropoietic Cytokines

The neurotrophins (NTs) are a family of growth and survival factors of which the members are related to Nerve Growth Factor (NGF). NTs were initially thought to mainly regulate the

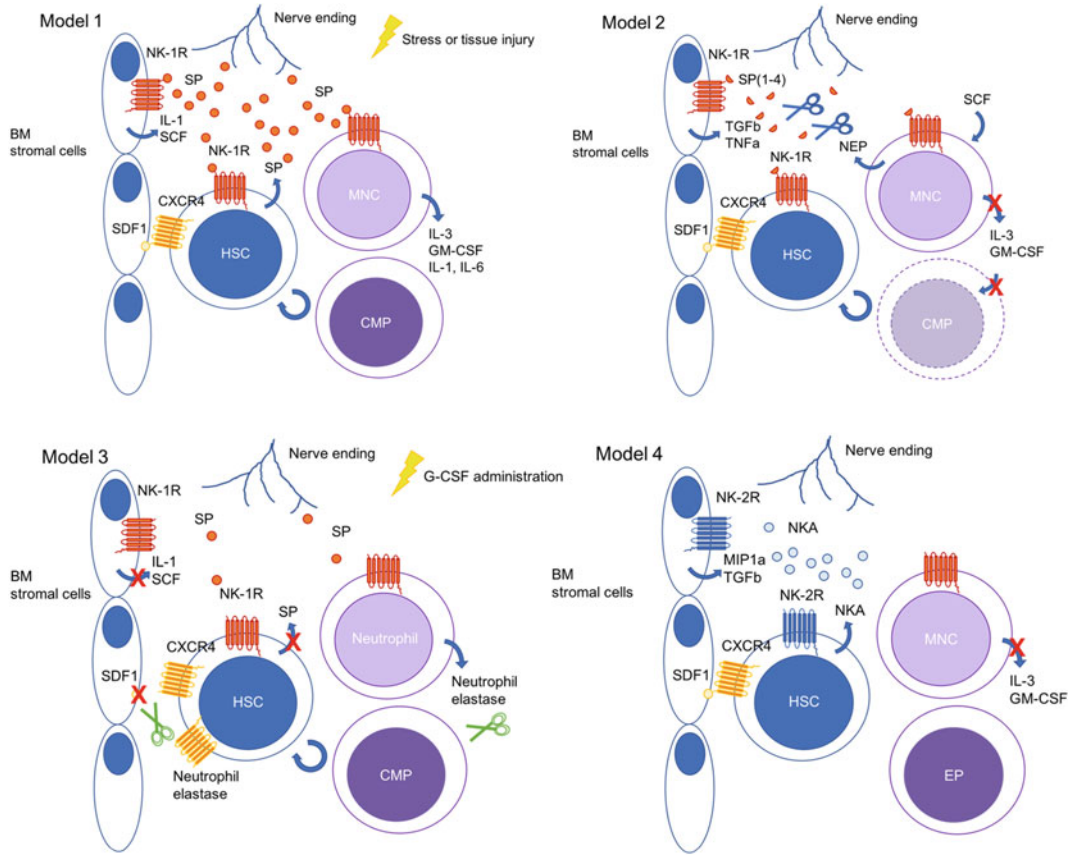

Fig. 4 Effects of Tachykinins on hematopoiesis. *Model 1:* In response to stress or tissue injury, SP is secreted from BM nerve endings. SP stimulates NK-1Rs on HSCs and BM stromal cells, leading in increased self-renewal of HSCs and secretion of IL-1 and SCF by BM stromal cells. Stimulation of NK-1R on BM-MNCs results in local production of IL-1, IL-3, IL-6 and GM-CSF and differentiation of HSCs into common myeloid progenitors (CMP). *Model 2:* SCF production increases expression of neuroendopeptidase (NEP), which cuts SP into the smaller peptide SP(1–4). SP(1–4) induces TGFβ and TNFα production by BM stromal cells. Since SP and SP(1–4) compete for the same receptor, but have opposite actions, differentiation of HSCs into CMPs is inhibited. *Model 3:* SDF-1 binding to CXCR4 retains HSCs in the BM and induces SP production by HSCs. In response to G-CSF, HSCs produce neutrophils. G-CSF also increases CXCR4 expression by HSCs. SDF-1 degradation by neutrophil elastase results in loss of SP production and release of the HSCs from the BM. *Model 4:* NKA binds to NK-2R on BM stromal cells and HSCs. NKA stimulates erythrocyte progenitors at the expense of the granulocyte/monocyte precursors and induces the production of hematopoiesis suppressing cytokines MIP1α and TGFβ by BM stromal cells. HSCs enter quiescence and are protected from exhaustive proliferation. Leukemic cells do not express NKA and lose their ability to suppress their proliferative responses

development and the maintenance of neuronal cells (McAllister 2001; Ip 1998), but some of its members have now been shown to play a significant role in hematopoiesis as well. Similar to opioids and neurokinins, the NTs are generated by enzymatic processing of their proneurotrophin precursors. Their effects are mediated by a subgroup of the receptor tyrosine kinase (RTK) family of receptors, known as the tropomyosin receptor kinases (Trk). The Trk receptors function similarly to other subgroups of the RTK family including PDGF, VEGF and FGF. Members of the NT family include NGF, Brain-Derived Growth factor (BDNF), Neurotrophin 3 (NT3)

and Neurotrophin 4/5 (NT4/5) (Ip 1998). Although the Trk receptors TrkA, TrkB and TrkC display overlapping specificities for these NTs, NGF preferentially binds to TrkA (high affinity NGF receptor), BDNF and NT4/5 to TrkB, and NT3 to TrkC (Bothwell 1995; Ip et al. 1993).

CD271 (p75NTR, low affinity NGF receptor) is a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily of transmembrane proteins (Rogers et al. 2008). Members of the TNFR family consist of a ligand-binding extracellular domain and an intracellular death domain (Lu et al. 2005; Nykjaer et al. 2005). CD271 is an atypical member of the TNFR family because distinct from the other members, 1) CD271 has a different intracellular structure and downstream signaling partners; 2) it binds dimeric, rather than trimeric ligands; and 3) CD271 can bind both pro-neurotrophins and mature NTs (Rogers et al. 2008). CD271 is involved in both the regulation of growth and differentiation, and death of cells in the nervous system and can associate with any of the Trk proteins TrkA, TrkB and TrkC. These opposing effects of CD271 are mediated through interactions with different receptor partners. When CD271 forms a complex with Trk it supports cell survival and growth. However, in the absence of Trk expression and signaling it induces cell death or apoptosis (Lu et al. 2005; Nykjaer et al. 2005; Hempstead et al. 1991). Thus interaction between CD271 and NGF plays an important role in the survival and protection of sympathetic and sensory neurons (Levi-Montalcini and Angeletti 1963).

The CD271 antigen is expressed in both neuronal and non-neuronal tissues. CD271 is expressed in the bone marrow (Cattoretti et al. 1993; Caneva et al. 1995), in BM-MSCs (Kuci et al. 2010; Quirici et al. 2002) and in the trabecular bone cavity (Jones et al. 2010; Cox et al. 2012). CD271 is also expressed by other BM resident cells, including endothelial cells, perivascular fibroblasts and B cells (Chesa et al. 1988), but its exact role in the bone marrow has not yet been revealed. Nevertheless, the role of CD271 must be substantial, given that expression of CD271+ stromal reticular cells already appears in the fetal BM in a perivascular pattern and lining the sinus endothelial cells even before the beginning of hematopoietic activity (Cattoretti et al. 1993). Furthermore, the presence and distribution of CD271+ cells in the BM stroma also supports evidence of a co-stimulatory effect of NGF on early hematopoiesis (Caneva et al. 1995).

Prospective isolation of multipotent stromal BM progenitor cells using anti-CD271 has been shown to be feasible and result in the enrichment of a population of MSCs with a high clonogenic and proliferative potential, and similar if not better immune modulatory properties and hematopoiesis supporting activity (Kuci et al. 2010; Aydin 2018). However, since murine CD271 knockout models ($Ngfr^{tm1.1Vk}$ mice) were not associated with a major deficit of hematopoiesis, the role of NGF signaling in hematopoietic differentiation has been thought to be mediated through activation of the Trk receptors, rather than through CD271 (Bracci-Laudiero et al. 2003). Indeed, although high expression of CD271, TrkA, TrkB and TrkC has been observed on BM stromal cells (Rezaee et al. 2010), CD34+ HSCs do not express the pan-neurotrophin receptor CD271. Even more, human umbilical cord blood HSCs were shown to highly express both the Trk receptor and NGF, and their expression was strongly downregulated during differentiation of cells (Bracci-Laudiero et al. 2003). NGF is also synthesized by neurons, Schwann cells, fibroblasts, smooth muscle cells and B-cells in the bone marrow niche and has been shown to support human hematopoietic colony growth and differentiation *in vitro* (Matsuda et al. 1988) and to have a synergistic effect on the development of basophilic cells when used in combination with GM-CSF (Tsuda et al. 1991). Further evidence of the effect of NGF is provided by a study that showed that both normal primitive human BM $CD34^{+}/CD38^{-}$ HSCs and a HGF-dependent leukemic cell line UT-7 could be maintained in the presence of stem cell factor (SCF) and NGF only, and that NGF promoted survival and

differentiation of $CD34^+/CD38^-$ mature erythroid progenitors (Auffray et al. 1996). Additionally, NGF was also shown to promote human granulopoiesis in synergy with GM-CSF, IL-1, M-CSF, IL-3 and SCF (Simone et al. 1999).

Another neurotrophic factor receptor implicated in hematopoiesis is the RET (rearranged during transfection) receptor (Fonseca-Pereira et al. 2014). The RET receptor is activated after interaction with the glial cell-line derived neurotrophic factor (GDNF) family of ligands (GFLs). Fonseca-Pereira and colleagues showed that RET and its co-receptors Gfra1, Gfra2 and Gfra3 were highly expressed on murine fetal liver $Lin^-Sca^+c\text{-}kit^+$ (LSK) cells, but less on multipotent myeloid progenitor cells. However, whereas RET expression was found to be substantial in fetal liver LSK cells, BM HSCs were shown to express only low levels of RET (Fonseca-Pereira et al. 2014). Interestingly, in both fetal liver and the bone marrow niche, high expression of GFLs was detected, indicating an active interaction between GFL expressing niche cells and RET expressing HSCs. Although $RET^{-/-}$ LSK cells were shown to normally differentiate, they completely lost their potential for long-term engraftment (Fonseca-Pereira et al. 2014).

In addition to the neurotrophins, also the neuropoietic cytokines play a dual role with effects on the neural system and neural development and direct effects on the hematopoietic system and hematopoietic stem cells. The neuropoietic cytokines share their gp130 chain with a number of known hematopoietic cytokines belonging to the class I Hematopoietic growth factor/cytokine receptor family. The gp130 chain is used for transduction of the signals of IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (Taga 1996). The functions of these cytokines are all pleiotropic, displaying overlapping biological activities for hematopoietic and neuronal regulation (Ip 1998). IL-6, CT-1, CNTF and LIF have all been shown to possess the ability to stimulate hematopoiesis and neuropoiesis and their effects are mainly directed by the expression of their ligand-specific chain.

7 Conclusions

The data summarized here are merely a reflection of the wide impact of the neural system on the bone marrow niche and do not give a complete overview of all the factors and players involved. However, it can be easily understood that the involvement of the brain and in particular the sympathetic nervous system in the regulation of hematopoiesis does not involve a single neurotransmitter or neurotrophic factor, but the interaction between the innervation of the bone marrow, a multitude of factors, including catecholamines, opioids, endocannabinoids, neurotransmitters, neuropeptides and neurotrophic factors on one side and the bone marrow niche stromal cells, its vasculature and the hematopoietic stem and progenitor cells on the other side. It also becomes clear that not only maintenance of the stem cell pool through retention and self-renewal of HSPCs, but also their physiological circadian and forced mobilization in response to G-CSF and GM-CSF are affected and regulated by neuronal involvement. Chemical or physical denervation of the bone marrow have shown a direct effect on HSC function, HSPC numbers, hematopoietic regeneration and BM impairment. In conclusion, a better understanding of the innervation of the bone marrow and the role of the nervous system on the hematopoietic niche will pave the way for the development of new procedures for *ex vivo* expansion of HSPCs, *in vivo* mobilization of HSPCs and development of new drugs that can be used in the treatment of BM failure syndromes.

Acknowledgements This manuscript was supported by grants from the Scientific and Technological Research Council of Turkey, project no 318S073 and the Hacettepe University, Scientific Research Project Coordination Unit TYL-2018-17435 and THD-2018-17209.

Conflicts of Interest The authors declare no conflict of interest.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 155–178
https://doi.org/10.1007/5584_2019_349

Published online: 4 April 2019

Homeobox Genes and Homeodomain Proteins: New Insights into Cardiac Development, Degeneration and Regeneration

Rokas Miksiunas, Ali Mobasheri, and Daiva Bironaite

Abstract

Cardiovascular diseases are the most common cause of human death in the developing world. Extensive evidence indicates that various toxic environmental factors and unhealthy lifestyle choices contribute to the risk, incidence and severity of cardiovascular diseases. Alterations in the genetic level of myocardium affects normal heart development and initiates pathological processes leading to various types of cardiac diseases. Homeobox genes are a large and highly specialized family of closely related genes that direct the formation of body structure, including cardiac development. Homeobox genes encode homeodomain proteins that function as transcription factors with characteristic structures that allow them to bind to DNA, regulate gene expression and subsequently control the proper physiological function of cells, tissues and organs. Mutations in homeobox genes are rare and usually lethal with evident alterations in cardiac function at or soon after the birth. Our understanding of homeobox gene family expression and function has expanded significantly during the recent years. However, the involvement of homeobox genes in the development of human and animal cardiac tissue requires further investigation. The phenotype of human congenital heart defects unveils only some aspects of human heart development. Therefore, mouse models are often used to gain a better understanding of human heart function, pathology and regeneration. In this review, we have focused on the role of homeobox genes in the development and pathology of human heart as potential tools for the future development of targeted regenerative strategies for various heart malfunctions.

Keywords

Cardiac development · Cardiac regeneration · Heart disease · Homeobox genes

Abbreviations

AMHC1	atrial myosin heavy chain-1
ANTP	Antennapedia
BMP	bone morphogenetic protein
Cdh2	cadherin 2
CDK	cyclin-dependent kinases
Cited2	Cbp/P300 interacting transactivator with Glu/Asp Rich Carboxy-Terminal Domain 2
CNS	central nerve system

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ESC	embryonic stem cells
FGF	fibroblast growth factor
FHF	first heart field
Flk1	fetal liver kinase 1
GJA5	gap junction protein alpha 5
GSC	goosecoid
H3K27me3	histone H3 methylation on the amino (N) terminal tail
Hcn4	hyperpolarization-activated cyclic nucleotide-gated channel 4 gene
HOXL	homeobox transcription factor Hox-like
Irx	Iroquois family of homeobox genes
ISL1	LIM-homeodomain transcription factor islet 1/insulin gene enhancer protein ISL-1
JMJD3	JmjC domain-containing protein 3
MEF2C	myocyte-specific enhancer factor 2C
MESP1	mesoderm posterior BHLH transcription factor 1
MSCs	mesenchymal stem cells
Myocd	myocardin
NKL	NK-like
Nkx2-5	homeobox protein NK-2 homolog E
Nodal	nodal growth differentiation factor
Nppa	natriuretic peptide A
OFT	outflow tract
PCBP2	poly(rC)-binding protein 2
Pitx2	paired like homeodomain 2
Pitx2c	paired-like homeodomain transcription factor 2
PROS	prospero
RA	retinoic acid
SAN	sinoatrial node
SHF	second heart field
Shox2	short stature homeobox 2
SMAD	main signal transducers for receptors of the transforming growth factor beta (TGF-β) superfamily;
TALE	three-amino-acid loop extension
Tbx5	T-box transcription factor 5
TF	transcription factors
TGF-β	transforming growth factor beta;
VCS	ventricular conduction system
ZEB2	zinc finger E-box binding homeobox 2
ZF	zinc finger
Ziro	zebrafish iroquois homeobox genes
ZO-3	tight junction protein 3

1 Introduction

Homeobox genes are a large family of genes that direct the formation of body structures along the head-tail axis in multicellular animal species (Innis 1997; Shashikant et al. 1991). It is also known that homeobox genes (Hox genes), as an ancient class of transcription factors, are important for the body patterning during embryo development (Innis 1997; Shashikant et al. 1991). Many of the homeobox genes play very important part in the spatiotemporal development of human heart (Lage et al. 2010). Likewise, some of these genes shape the human heart and control its multistep developmental process from simple crescent cells to a fully functional organ. For example, homeobox genes like homeobox protein NK-2 homolog E (Nkx2-5), LIM-homeodomain transcription factor islet 1 (Isl1), paired like homeodomain 2 (Pitx2) are widely known to be important for the proper development of human heart (Akazawa and Komuro 2005; Luo et al. 2014; Franco et al. 2017). However, there are many more homeobox genes that play substantial roles in cardiac function but thus far, there are less known and/or less investigated.

Specific inherited gene mutations cause congenital heart defects such as atrial or ventricle septal defects, abnormalities of outflow tract and etc. (Bao et al. 1999). Similarly, various pathological lifestyle factors like smoking, low physical activity, toxic and noxious agents and other environmental factors might also negatively affect cardiovascular function and promote heart failure (O'Toole et al. 2008; Nayor and Vasan

2015). Since it is impossible to exactly pinpoint how certain gene mutations influence development of human heart at the earliest stages, different mouse models have been created to better understand regulation of human heart development and its relation to various diseases (Camacho et al. 2016). Many of the genes studies in mouse models have similar vital roles in the development and function of human heart (Xu and Baldini 2007). Therefore, investigation of human disease and cues from mouse heart development models have revealed an important role of homeobox genes, including those that encode transcription factors.

Aside from already known homeobox genes, there are more homeobox genes that are essential for the formation of human and/or mouse myocardium. Some of these homeobox genes code transcription factors (TF), whereas other form a tight network regulating heart development and fate of heart progenitors. Several review articles have explored individual families of homeobox gene and their roles in embryo development. However, knowledge concerning the involvement of homeobox genes and homeodomain TF in the development of human heart referring mouse models are still lacking. Therefore, in this review we describe the role of more than 20 homeobox genes that are mainly involved in heart development and around 15 homeobox genes that are known to play minor or less investigated, but nonetheless important roles in cardiac development. Data summarized in this review will help to broaden the possible future applications of homeobox genes and their coded TF in targeted therapeutic strategies for cardiac regeneration and therapy.

2 Development of the Human Heart

Starting from the day first of fertilization, the zygote undergoes multiple cell divisions leading to the formation of third germ layer, known as the mesoderm (Moorman et al. 2003). Later mesodermal cells migrate towards anterior part of embryo to form a distinct crescent-shaped epithelium, named the cardiac crescent (Buckingham et al. 2005). Cells situated in the distinct anterior-lateral territory within the cardiac crescent contribute to the formation of first heart field (FHF), distinguished by the expression of hyperpolarization-activated cyclic nucleotide-gated channel 4 gene (Hcn4) (Liang et al. 2013). Cardiac progenitor cells also develop into second heart field (SHF), which is located medially to the cardiac crescent and extend posteriorly (Cai et al. 2003). Formation of SHF is marked by the expression of LIM-homeodomain transcription factor islet 1 (ISL1) (Cai et al. 2003). Sometimes progenitors of FHF and SHF are called cardiogenic or cardiac mesoderm (Dupays et al. 2015; Liu et al. 2014; Kitajima et al. 2000). These distinct heart fields fuse to form heart tube, which eventually develops into functional heart (Fig. 1) (Moorman et al. 2003; Nemer 2008). During this time, the primitive cardiac conduction system, including sinoatrial node (SAN), ventricular conduction system and other, starts to form (van Weerd and Christoffels 2016). The FHF cells develop into the left ventricle, as well as into the atrioventricular canal and part of the atria, whereas SHF cells develop into the right ventricle and outflow tract, with contribution to the formation of atria and inflow vessels (Buckingham et al. 2005). Once the heart fields are formed, they fuse into heart tube and undergo process called heart looping. During this phase the whole heart tube twists in the rightward direction eventually forming clearly visible, but still primitive, heart chambers (Santini et al. 2016). Later on, the heart undergoes septation to fully separated left and right sides of the heart.

There are many factors regulating human and mouse heart development, however only some of them may be considered to be core regulators of cardiogenesis. One of the most important TF is GATA4 which orchestrates expression of multiple transcriptions including other major determinants of cardiomyogenesis like Nkx2-5, T-box transcription factor 5 (Tbx5), heart- and

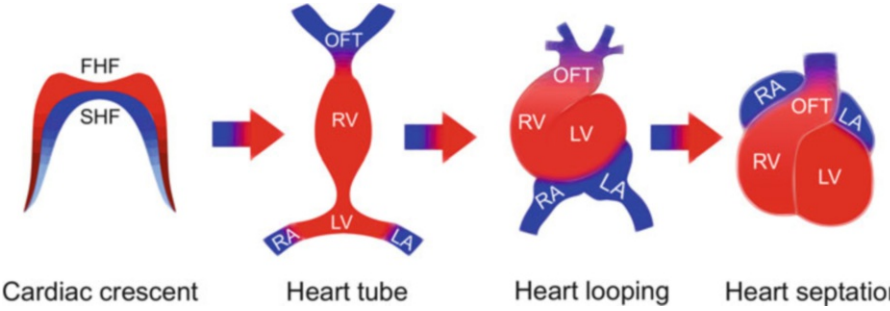

Fig. 1 Schematic representation of heart development in human and mouse. *FGF* first heart field, *SHF* second heart field, *RV* right ventricle, *LV* left ventricle, *RA* right atrium, *LA* left atrium. (Scheme adapted from (Nemer, 2008))

neural crest derivatives-expressed protein 1 (HAND1/2) and others (Bruneau et al. 2001a; Belaguli et al. 2000; Sepulveda et al. 1998). GATA4 integrates bone morphogenetic proteins (BMP) and SMAD, the main signal transducers for receptors of the transforming growth factor beta (TGF-β) superfamily, to ensure cardiac cell survival and stable lineage during cardiac development (Benchabane and Wrana 2003). Of course, there are other factors that promote development of various structures within the heart. For example, it is know that Tbx5 controls atrial gene expression, whereas myocyte-specific enhancer factor 2C (MEF2C) promotes development of ventricle and vasculogenesis (Bruneau et al. 2001a; Lin et al. 1997). Altogether, the development of human heart is a carefully controlled multistep process involving many genes, intracellular and extracellular signalling factors leading to proper cardiac function. The miss-controlled heart development process leads to various inherited or acquired cardiac disorders. This review focuses mostly on the homeodomain proteins, as one of the most important group of transcription factors regulating heart development, function and impairment.

3 Homeobox Genes

Homeodomain proteins are one of the most important group of proteins/transcription factors regulating plan of body structure and organogenesis in eukaryotes including heart development and disorders. DNA binding proteins have been extensively studied, but even today there are no established rules for predicting the specificity of DNA sequence based upon the amino acid sequence of the proteins. Homeodomain proteins are characterized by specific 60 amino acid long helix-turn-helix DNA binding homeodomain motif (Seifert et al. 2015). The homeodomain is a very highly conserved structure and consists of three helical regions folded into a tight globular structure that binds a 5′-TAAT-3′ core motif. The high degree of conservation of homeodomain proteins is an ideal model to study specific protein-DNA interactions. The DNA sequence that encodes the homeodomain is called the “homeobox” and homeobox-containing genes are known as “hox” genes.

Most of the transcription factors belonging to this group are not only structurally but also evolutionary conserved and play crucial roles in embryonic patterning and differentiation (Pearson et al. 2005). The main role of homeodomain proteins *in vivo* is to control the genetic determination of development and implementation of the genetic body plan. There are 102 homeobox gene families that represent 235 active human homeodomain proteins, but only some homeodomain classes have close association with cardiac development and/or diseases (Bürglin and Affolter 2016). This review covers description of around 20 homeobox genes that up today are known to have a major

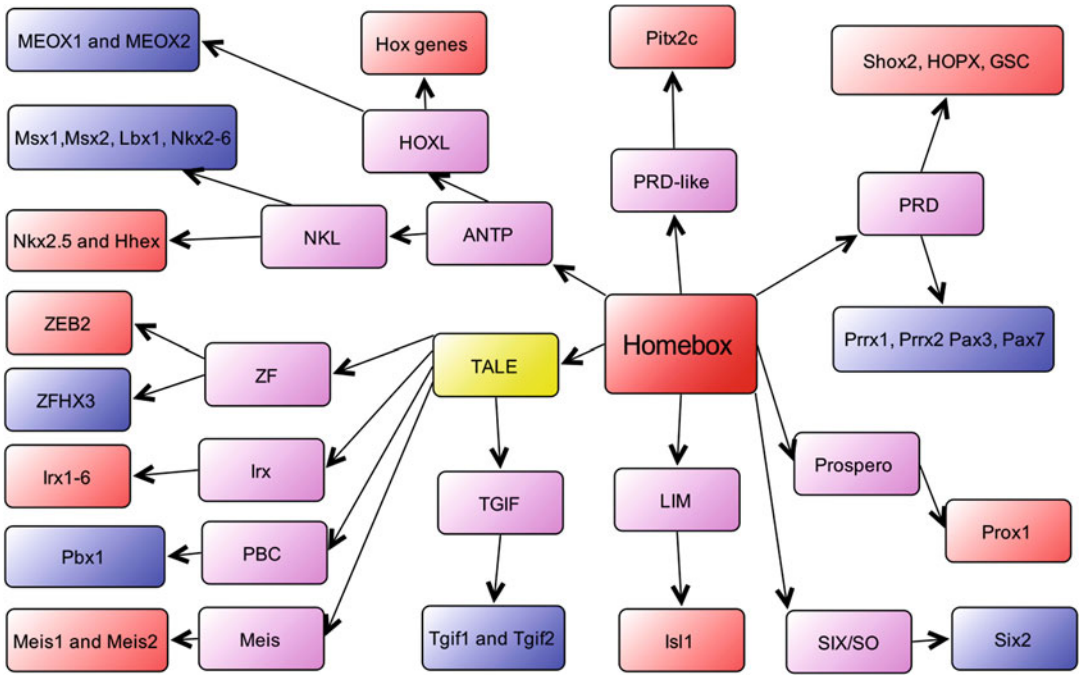

Fig. 2 Hierarchy of homeobox genes involved in heart development. Pink boxes indicate homeobox gene classes, yellow box indicates TALE gene superclass. Red boxes indicate genes with major involvement in heart development and diseases. Blue boxes indicate genes having less important role in heart development and diseases

impact in the regulation of heart development and functioning (Fig. 2).

4 Homeobox Genes in Mouse Heart Development and Human Disease

Humans have more than 67 genes that are important for cardiac hypertrophy and over 92 genes that control cardiovascular system, therefore it is reasonable to assume that some of the genes might be responsible for cardiac development and disease (van der Harst et al. 2016; Smith and Newton-Cheh 2015). Congenital heart disease (CHD) have structural heart anomaly, including atrial or ventricle septal defects, overriding aorta, right atrium isomerism and other structural changes in new born heart. Patients do usually display multiple symptoms, like, rapid breathing, bluish skin, poor weight gain and feeling tired in general (Sun et al. 2015). The main cause of these abnormalities is reduced blood oxygen levels in whole organism, which is a result of improper heart septation leading to the mixture of oxygenated and deoxygenated blood in systemic blood circulation. Additionally, CHD could be caused by genetical or environmental factors like infections during pregnancy such as Rubella, drugs and maternal illness (Sun et al. 2015). Since homeobox genes are important for the heart development, some mutation of the homeobox genes including *MEIS2*, *Nkx2-5* and others can potentially cause CHD (Zakariyah et al. 2017; Johansson et al. 2014). Of course, homeobox gene mutations is not a sole cause of heart defect present at human birth. For example, mutations in *GATA4* and *Tbx5* can also affect integrity of heart tissue (McCulley and Black 2012). In addition, impaired gene functioning might be also related to the other heart diseases like cardiomyopathy, hypertrophy,

defects of the heart rhythm and other abnormalities (Kathiresan and Srivastava 2012). However, the impact of homeobox genes in heart disorders is still not clear and needs further investigation to clarify their role not only in the cardiac development but also in the physiological and pathophysiological conditions.

5 ANTP Class of Homeobox Genes

The Antennapedia (ANTP)-class of homeobox genes are involved in the determination of pattern formation along the anterior-posterior axis of the animal embryo. NK-like (NKL) and homeobox transcription factor Hox-like (HOXL) are an ancient subclasses of homeobox genes that belong to an ANTP homeobox gene class (Holland et al. 2007). It is likely that HOXL gene clusters originate from NKL, since NKL genes are widespread throughout the genome as tight clustered Hox genes (Bürglin and Affolter 2016). Both subclasses of the genes (NKL and HOXL) are evolutionarily conserved and play predetermined roles in heart patterning and disease.

6 Hox Gene Families of HOXL Subclass

Hox gene families belong to the HOXL subclass of ANTP class of homeobox. Hox genes code transcription factors which are important for whole body patterning and development (Pearson et al. 2005). It total, there are 39 human Hox genes grouped in HOXA, HOXB, HOXC and HOXD gene clusters. Hox genes are highly conserved, because they play a vital role in anterior-posterior formation of body axis (Pearson et al. 2005). The precise function of these genes is achieved by their specific temporal and spatial expression over the life course. During early mouse cardiac development, the retinoic acid (RA) might be responsible for the anterior-posterior patterning in SHF (Bertrand et al. 2011). Hoxb1, Hoxa1, and Hoxa3 act as downstream targets of RA and participate in forming outflow tract (OFT) and normal SHF development (Bertrand et al. 2011). *Hoxb1−/−* or *Hoxa1−/−*, *Hoxb1+/−* mouse embryos develop shortened OFT and display abnormal proliferation and premature differentiation of cardiac progenitors (Bertrand et al. 2011). This is probably related to the altered fibroblast growth factor (FGF) and BMP signaling pathways in developing mouse embryo (Roux et al. 2015). Clinical studies have revealed that Hoxa1 mutations might cause congenital human heart defects and other abnormalities like, mental retardation, deafness, horizontal gaze restriction and etc. (Bosley et al. 2008). Several other studies have shown that Hox genes might be also related to the human heart diseases, however more research is needed to unveil exact functions of these genes in human heart development (Gong et al. 2005; Haas et al. 2013).

7 Nk4 Gene Family of NKL Subclass

There are multiple NKL genes in mouse and humans regulating various developmental processes, however only some of them contribute to the development of the heart (Larroux et al. 2007). The *Nkx2-5* and *Nkx2-6* genes are the members of the NK4 homeobox gene family of NKL subclass and are closely related to the Drosophila *tinman* gene (Bürglin and Affolter 2016; Harvey 1996). To our knowledge, only *Nkx2-6* and *Nkx2-5* relate to the mouse and human heart development and disease, whereas *Nkx2-3*, *Nkx2-7*, *Nkx2-8* and *Nkx2-10* might be important for the heart development of zebrafish, frog or chicken (Newman and Krieg 1998; Wang et al. 2014; Tu et al. 2009; Allen et al. 2006; Brand et al. 1997).

During the early stages of embryogenesis, Nkx2-5 is expressed in myocardium and pharyngeal endoderm, whereas Nkx2-6 can be found in sinus venosus, pharyngeal endoderm and myocardium of the outflow tract (Lints et al. 1993).

Moreover, during normal heart development, Nkx2-5 expression is essential for the looping of vertebrate embryonic heart, heart septation and formation of cardiac conduction system, whereas most of the *Nkx2-5* mutations are related to human congenital heart disease and conduction defects (Tanaka et al. 1999). Inactivation of *Nkx2-5* arrested heart formation at the looping stage revealing its critical role in cardiac development (Lyons et al. 1995). However, targeted disruption of *Nkx-2.6* did not cause any abnormalities in the heart suggesting a possible compensatory function of Nkx-2.5 (Tanaka et al. 2000).

It is important to note that *Nkx2-5* mutations lead to an altered spatiotemporal development of human heart, improper heart septation and formation of cardiac conduction system (Dupays et al. 2015; McCulley and Black 2012; McElhinney et al. 2003). Analysis of human *Nkx2-5* mutants and gene truncations showed that most of the mutations affected *Nkx2-5* binding to DNA or its localization but not protein-protein interactions (McCulley and Black 2012; Reamon-Buettner et al. 2004). Several different studies of mice *Nkx2-5* knockout and human embryonic stem cells (ESC) revealed that *Nkx2-5* mutations might alter gene expression of specific transcription factors like SP1, SRY, JUND, STAT6, *MYCN*, *PRDM16*, *HEY2* and others (Anderson et al. 2018; Li et al. 2015). Also some studies support an idea that, *Nkx2-5* mutant proteins might alter space and time specific human cardiac development by dysregulating BMP, Notch and Wnt signalling pathways (Anderson et al. 2018; Wang et al. 2011; Luxán et al. 2016; Cambier et al. 2014). There is a possibility that Nkx2-5 modulates these pathways by interacting with multiple transcription factors in time-dependent mode. For example, in mouse heart Nkx2-5 interacts with Hand2 transcriptions factor to activate Irx4, which is necessary for the ventricular identity (Yamagishi et al. 2001). Conversely, Nkx2-5 expression is also timely regulated since Nkx2-5 overexpression leads to an improper SAN formation in early mouse development (Roux et al. 2015). Mammalian heart development is also regulated by the combination of cardiac transcription factors having specific DNA motifs in their centrally located DNA binding domains. It was also shown that Nkx2-5, GATA4 and Tbx5 can physically interact and synergistically regulate targeted genes (Hiroi et al. 2001; Pradhan et al. 2016). Since these genes are the master regulators of heart development, functional mutations in these genes are linked to various types of congenital heart diseases (Benson 2002; Hatcher et al. 2003). Taken together, Nkx2-5 and other transcription factors like Isl1, GATA4, Tbx5, Hand2, MEF2C, Irx4 form a core of transcription factors essential for the heart development and congenital heart disease (Fig. 3) (McCulley and Black 2012).

8 HHEX Gene Family of NKL Subclass

Proline rich homeodomain protein or homeobox protein (PRH/HHEX) expressed by hematopoietic system is a transcription factor belonging to the family of NKL subclass gene (Bedford et al. 1993). As the name implies, it is important for the development of hematopoietic cell, but not less is essential for the development of other systems, including heart (Bedford et al. 1993). Mouse double *HHex* mutants have multiple developmental issues, including defective vasculogenesis, hypoplasia of the right ventricle, aberrant development of the compact myocardium and other complications related to forebrain, thyroid and liver developmental disorders (Hallaq et al. 1998). Additional studies have revealed that HHex plays distinct role in mouse cardiac mesoderm specification and development. HHex expression is controlled by Sox17 transcription factor, which is known to be essential for the formation of mouse cardiac mesoderm (Liu et al. 2014). Several studies of human population have shown that common variants of *HHex* gene (rs7923837 and rs1111875) may also be associated with diabetes (Karns et al. 2013; Kelliny et al. 2009; Pechlivanis et al. 2010).

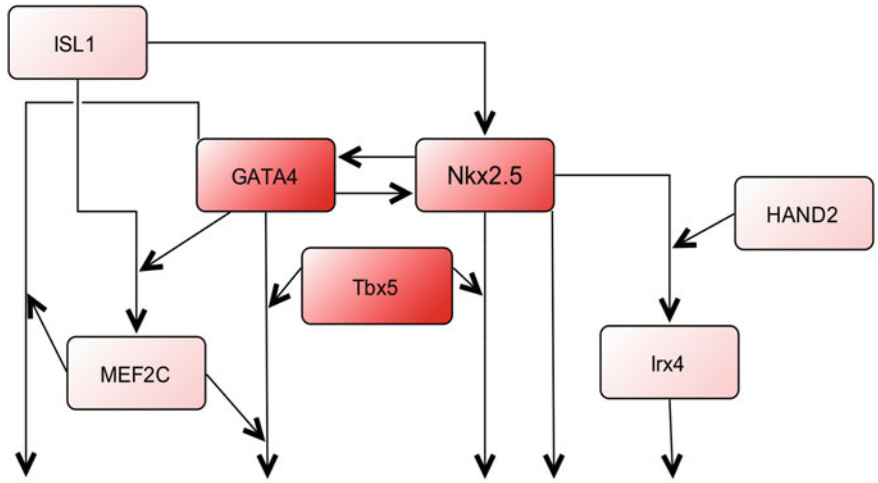

Fig. 3 Core transcription factors important for the heart development and congenital heart disease. (Scheme adapted from (McCulley and Black, 2012))

9 PRD and PRD-Like Homeobox Class

The PRD class is the second largest of the homeobox gene classes in animal genomes and, like the ANTP class, these genes have been found only in animals. The PRD class derives its name from the *Paired* (*Prd*) gene of *Drosophila*. Multiple gene families belong to the PRD homeobox class, including *Shox2*, *Hopx*, *GSC*, *Pitx2* and others, which are important for the heart development and disease.

10 Shox Gene Family of PRD Class

Short stature homeobox 2 (Shox2) is a homeobox gene belonging to the PRD class of homeobox. Shox2 is an essential for the development of limb and cardiac conduction systems, including formation of sinoatrial node (SAN) in mice and humans (Gu et al. 2008; Blaschke et al. 2007; Liu et al. 2011). Studies of Shox2 function during the mouse development revealed several cues how this homeodomain transcription factor in particular controls formation of SAN (Blaschke et al. 2007). Shox2 mice null mutants displayed severe cardiac conduction defects, such as low heart rhythm rate and drastically reduced cell proliferation (Espinoza-Lewis et al. 2009). This phenotype is probably related to the downregulation of *HCN4*, *Tbx3* and the upregulation of natriuretic peptide A (*Nppa)*, gap junction protein alpha 5 (*GJA5)* and *Nkx2-5* gene expressions (Espinoza-Lewis et al. 2009). It is also known that HCN channels play a vital role in autonomic control of heart rate, so it is no surprise why Shox2 null mutants do not develop SAN (Alig et al. 2009). During the normal development of mouse cardiac expression of Shox2 is also tightly controlled by several transcription factors. For example, transcription factor Tbx5 activate Shox2 expression, however transcription factors like Pitx2c and NKX2-5 potentially silence Shox2 expression (Espinoza-Lewis et al. 2011; Puskaric et al. 2010). Paired-like homeodomain transcription factor 2 (Pitx2c) also can potentially inhibit left-sided pacemaker specification by suppressing Shox2 expression in left atrium, therefore SAN develops only in the region of right atrium (Wang et al. 2010). All these results indicate that Shox2 is essential for the maintaining pacemaker cell program during the heart development. On the other hand, in adulthood Nkx2-5

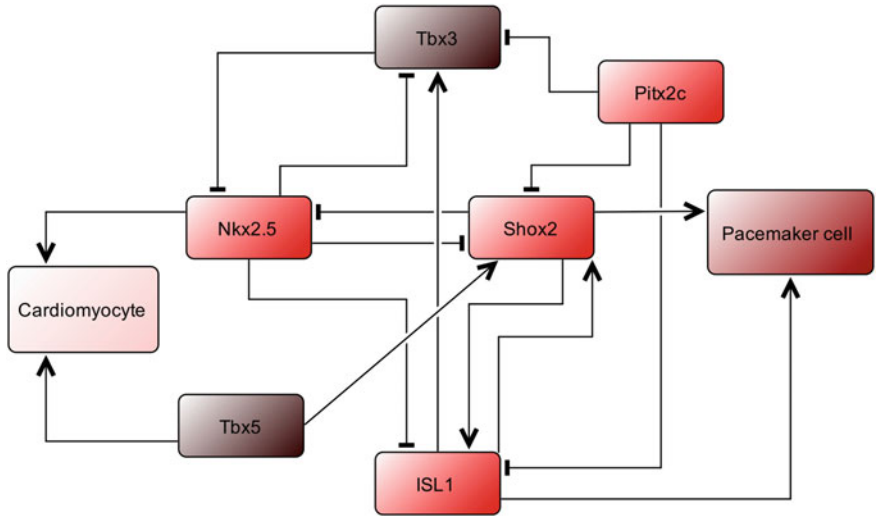

Fig. 4 Signaling networks governing pacemaker and atrial differentiation of cardiomyocyte in developing mouse heart. (Scheme adapted from (Liang et al. 2017))

antagonizes Shox2 and promotes cardiomyocyte formation (Fig. 4) (Liang et al. 2017).

Taken together, these studies indicate that Shox2 might be a good candidate to develop biological pacemaker. Results from mouse ESCs and canine mesenchymal stem cells (MSCs) have shown that cells overexpressing Shox2 induce expression of SAN markers such as HCN4, Cx45 and Tbx3 (Ionta et al. 2015; Feng et al. 2016). In addition, mouse embryonic bodies overexpressing Shox2 showed better contractile phenotype compared to the control group of embryonic bodies (Ionta et al. 2015). Moreover, human patients with an early-onset atrial fibrillation had significantly downregulated expression of *Shox2* gene (Hoffmann et al. 2016). These results are promising for patients suffering from heart rhythm defects, however, more studies are needed to test functions of biological pacemaker in order to treat human arrhythmias.

11 HOPX Gene Family of PRD Class

HOPX is another PRD-class homeobox gene family important for the multiple organ development and tissue homeostasis in adults (Chen et al. 2015; Schneider et al. 2015; Mariotto et al. 2016). Since it lacks a DNA binding domain, HOPX can only modulate gene expression by forming complexes with other regulatory proteins (Kook et al. 2006). In general HOPX acts as a cell proliferation inhibitor in humans cancer cells, however its function in mouse cardiac cell differentiation is not entirely clear (Chen et al. 2015; Waraya et al. 2012; Yap et al. 2016). Studies of mouse development have shown that HOPX plays a critical function in early formation of cardiomyocyte progenitors. HOPX integrates BMP and WNT signalling in developing mouse heart by interacting with SMAD proteins and inhibiting WNT signalling pathway leading to the formation and differentiation of cardiomyocyte progenitors (Jain et al. 2015).

On the other hand, there are some cues that HOPX might act as a negative regulator of cardiac differentiation in mice. HOPX interacts with HDAC2, thus reducing GATA4 transcriptional activity by deacetylation (Trivedi et al. 2010). These findings are consistent with previous reports that overexpression of HDAC2 inhibits the development of cardiomyocytes by downregulating the expression of *GATA4* and *Nkx2-5*

genes (Kawamura et al. 2005; Karamboulas et al. 2006). All these results highlight the complex nature of HOPX and its partners in heart development. HOPX undeniably plays a critical role in early heart development, because some mouse mutants cannot develop a functional myocardium and display cardiac conduction defects (Chen et al. 2002; Ismat et al. 2005). HOPX also might be related to the human heart failure, since HOPX is downregulated in patients having cardiac hypertrophy (Güleç et al. 2014; Trivedi et al. 2011).

12 Goosecoid Gene Family of PRD Class

Goosecoid (GSC) is another protein that belongs to the bicoid related paired (PRD) homeobox class of genes. Goosecoid is often associated with limb, skeletal and craniofacial development, although, it might be important for cardiac mesoderm formation, since its expression is controlled by Mesp1 (Zhu et al. 1998). Mesoderm posterior BHLH transcription factor 1 (MESP1) preferentially binds to two variations of E-box sequences and activates critical mesoderm modulators, including Gata4, mix paired-like homeobox (Mixl1) and GSC homeobox (Soibam et al. 2015). In addition, mesoderm formation can be induced with l-proline and trans-4-hydroxy-l-proline resulting in increased expression of Mixl1 and GSC (Date et al. 2013). GSC also is important for the cell migration in early embryonic development, therefore the overexpression of goosecoid enhances oncogenic cell growth and metastasis (Kang et al. 2014).

13 Pitx Gene Family of PRD-Like Class

Paired like homeodomain 2 *(Pitx2)* is a PRD-like homeobox class gene which is important for the establishment of the left-right axis and for the asymmetrical development of the mouse and probably human heart, lung, and spleen, twisting of the gut and stomach, as well as the development of the eyes (Campione et al. 1999; Shiratori et al. 2006; Evans and Gage, 2005). There are several alternative *Pitx2* transcripts, however only Pitx2c isoform plays determined role in the asymmetric development of mouse heart (Liu et al. 2002). Higher vertebrates, at an early heart development stage and after the heart tube formation, undergo embryonic heart looping, which is the first visual evidence of embryo asymmetry (Harvey 2002). Transcription factors like nodal growth differentiation factor (Nodal) and Cbp/P300 interacting transactivator with Glu/Asp Rich Carboxy-Terminal Domain 2 (Cited2) activate Pitx2 transcription leading to the rightward twist of the heart tube and forming prospective embryonic atrial and ventricular chambers. Deletion of *Pitx2c* in mouse caused drastic alteration of looping process leading to various heart defects including the isomerism of right atrium and ventricle (Lin et al. 1999; Yu et al. 2001).

Humans with Pitx2c mutations develop various heart abnormalities, including an improper formation of ventricle and atrial chambers septa, atrial fibrillation and others. It is quite likely that septation defects are caused by the downregulation of transcription factors downstream of Pitx2, since certain *Pitx2* mutants displayed reduced cardiac transcriptional activity in human patients (Wang et al. 2013; Wei et al. 2014). Surprisingly, the overexpression of Pitx2c in mouse R1-embryonic stem cells results in elevated gene expression of essential cardiac transcription factors like GATA4, MEF2C, Nkx2-5 and others (Lozano-Velasco et al. 2011). Consequently, *Pitx2c* might be a good candidate for heart regeneration, since it positively regulates multiple transcription factors important for cardiac development. Recently it was shown that mouse embryonic stem cells overexpressing Pitx2c could restore mouse heart function after a myocardium infarct through the multiple mechanisms including efficient terminal

differentiation, regulation of action potentials of cardiomyocytes and positive paracrine effects (Guddati et al. 2009). However, more studies need to be done to determine the utility of Pitx2c in human heart regeneration strategies.

14 TALE Homeobox Superclass

Three-amino-acid loop extension (TALE) is another superclass of homeobox genes, which codes for highly conserved transcription regulators essential for various developmental programs. These genes encode proteins with atypical homeodomain structure, defined by having three additional amino acids in homeodomain. TALE homeobox gene superclass includes the main zinc finger (ZF), PBC and Meis homeobox 1 (Meis) classes. Out of 20 human homeodomains only Meis1, Meis2 and Iroquois homeobox proteins 1-6 (Irx1-6) have their clearly defined function in heart development and disease.

15 Meis Genes Family of Meis Class

Meis1 encodes the TALE superclass homeobox transcription factor implicated in cardiac, hematopoietic and neural development (Mariotto et al. 2013; Azcoitia et al. 2005; Hisa et al. 2004). *Meis1* deficient mice have malformed cardiac outflow tracts with overriding aorta and ventricular septal defect (Stankunas et al. 2008). Downregulation of Meis1 leads to cardiac hypertrophy in humans and mice. Meis1 binds poly (rC)-binding protein 2 (*PCBP2*) gene promoter and activates its expression in order to suppress human or mouse heart hypertrophy (Zhang et al. 2016). In turn, PCBP2 represses angiotensin II, which enhances hypertrophic human or mouse cardiac growth (Zhang et al. 2015). There are around 79 cardiac specific genes that have Meis1 and NKX2-5 binding sites in developing mouse heart, some of them are associated with cell signaling and cardiac progenitor differentiation, like Tbx20, myocardin, cadherin 2 (Cdh2), Wnt11, and Wnt2 (Dupays et al. 2015). Adult mouse cardiomyocytes with mutant *MEIS1* exhibit increased proliferation and progression of the cell cycle (Mariotto et al. 2013). This function is emphasized in adult mouse hearts since Meis1 activates inhibitors of cyclin-dependent kinases (CDK) like p15, p16 and p21 (Mariotto et al. 2013). In humans non-synonymous *Meis1* gene variants might be associated with congenital heart defects, whereas patients carrying 2p14 microdeletions show symptoms of deafness and cardiomyopathy (Mathieu et al. 2017; Arrington et al. 2012). It is likely that Meis1 is required for the control of spatiotemporal cell proliferation in early developing heart to prevent hypertrophy, however more studies need to be done to fully understand the role of Meis1 in cardiac development and disease.

Meis2 encodes TALE homeobox superclass transcription factor essential for the development of mouse cranial and cardiac neural crest (Machon et al. 2015). Recent findings indicate that Meis2 might be an important factor for the proliferation of fetal human cardiomyocyte cells (Wu et al. 2015). Reduction of *Meis2* gene expression by miR-134 results in slowed progression of human cardiomyocyte progenitor cell cycle (Wu et al. 2015). A clinical and genetic study also revealed that small *Meis2* deletion can negatively affect several developmental processes: human patients with small *Meis2* non-frame shift deletion (c.998_1000del:p.Arg333del) had serious cleft palate and cardiac septal defects (Louw et al. 2015). It is known that Meis2 interacts with DNA and forms multimeric complexes with Hox and Pbx proteins (Louw et al. 2015). Single deletion of arginine residue affects the ability of Meis2 to bind DNA leading to serious developmental problems of human heart (Louw et al. 2015). Clinical studies have shown that patients having only one functioning Meis2 gene copy survive, however they have similar phenotype such as clefting and ventricular septal defects leading to delayed

motor development and learning disability (Johansson et al. 2014).

16 Irx Gene Family of IRX Class

Iroquois homeobox genes and their coded homeodomain proteins are another class of transcription factors belonging to TALE superclass of homeobox genes. Iroquois-class homeodomain TF (Irx) defining feature is atypical homeodomain structure and specific Iroquois (IRO) homeodomain family sequence motif, which is important for the recognition of DNA sequence (Gómez-Skarmeta and Modolell 2002; Cavodeassi et al. 2001). Humans and mice have six Irx proteins, which are important for the development of lung, nervous system, eye, pancreas, female gonad, early limb and, of course, heart patterning (Cavodeassi et al. 2001; Cheng et al. 2005; Schwab et al. 2006; van Tuyl et al. 2006; Ragvin et al. 2010; Jorgensen and Gao 2005; McDonald et al. 2010). Irx1 and Irx2 are expressed in interventricular septum from E14.5 onward, however, mouse *Irx2* mutants are viable and display no notable phenotype defects in the developing heart (Christoffels et al. 2000; Lebel et al. 2003). *Irx1* gene variants might be related to the congenital heart disease in humans (Guo et al. 2017).

Irx3 gene in mice seems to be very important for the ventricular conduction system (VCS) (Christoffels et al. 2000). Various studies suggest that Irx3 is required to maintain rapid electric conduction through the VCS for proper ventricular activation, via antithetical regulation of Cx40 and Cx43 expression (Zhang et al. 2011; Kasahara et al. 2003). Clinical studies have revealed that defects of *Irx3* gene can cause lethal cardiac arrhythmias in human patients (Koizumi et al. 2016). Irx3 function appears to be evolutionary conserved, since expression of Ziro3a, a Irx3 homologue in zebrafish, is detected in developing fish heart (Zhang et al. 2011).

Irx4 is associated with the formation of ventricular myocardium in mouse and humans (Christoffels et al. 2000; Cheng et al. 2011). Data from mouse and chicken indicate that Irx4 suppresses atrial gene expression by down regulating atrial myosin heavy chain-1 (AMHC1) (Bao et al. 1999; Bruneau et al. 2001b). Several *Irx4* mutations have been identified that might be associated with human congenital heart disease, particularly ventricular septal defect (Cheng et al. 2011).

Irx5 is expressed in adult mouse heart and maintains proper action potentials, particularly regulates T-wave seen in ECG (Costantini et al. 2005). Mice lacking Irx5 develop properly without any structural abnormalities in the heart (Costantini et al. 2005). This indicates that *Irx5* is not required for cardiac development or that other *Irx* genes can compensate for the loss of Irx5.

Irx6 is detectable in mouse developing heart, however its expression is relatively weak compared to other *Irx* genes (Christoffels et al. 2000).

17 Zeb Gene Family of ZF Class

ZEB2 or zinc finger E-box binding homeobox 2 is a gene coding transcription factor belonging to class of ZF homeobox gene and homeodomain class of ZN proteins (Bürglin and Affolter, 2016). It has multiple functional domains (E-box, Zinc finger, homeobox), so naturally it can control gene expression with a variety of transcription factors (Gheldof et al. 2012). The complex nature of Zeb2 shows that it drives multiple processes including the development of heart and neural systems, however, it usually acts as a transcription repressor rather than activator (Hegarty et al. 2015). Systematic study of mouse and human ESC transcriptome differentiation profiles revealed that Zeb2 might play important role in cardiac specialization. Human ESC with silenced *Zeb2* gene proliferate more slowly and fail to differentiate into mature cardiomyocytes compared to the wild cells (Busser et al. 2015). In addition, cardiomyocytes with silenced *Zeb2* do not show any contractile properties, although cardiac differentiation program is activated. More

detailed analysis has revealed that silencing of *Zeb2* gene negatively affects human striated muscle contraction program, including genes related to calmodulin pathway, HCN and potassium channels (Busser et al. 2015). Targeted regulation of Zeb2 gene expression improves cardiomyogenic processes and heart regeneration.

Zeb2 mutation is also often associated with Mowat-Wilson syndrome (Garavelli and Mainardi 2007). Major signs of this disorder frequently include distinctive facial features, intellectual disability, delayed development, an intestinal disorder called Hirschsprung disease, Congenital Heart Disease and other types of birth defects (Garavelli and Mainardi 2007). All mentioned disorders are related to the improper heart development caused by the Zeb2 defective heart cells. In addition, Zeb2 repress epithelial genes (claudins, tight junction protein 3 (ZO-3), connexins, E-cadherin, plakophilin 2, desmoplakin, and crumbs3) in order to induce epithelial to mesenchymal transition (EMT), which is crucial for the developmental processes such as gastrulation, neural crest formation, heart morphogenesis, formation of the musculoskeletal system, and craniofacial structures (Vandewalle et al. 2009; Garavelli et al. 2017).

18 LIM Homeobox Class

LIM homeobox class genes encode two Lim domains and one homeodomain. Lim domain is a 50–60 amino acid length zinc finger motif, which is primarily involved in protein-protein interactions, so naturally LIM transcription factors can interact with multiple proteins in cell, thus regulating its phenotype.

19 Isl Gene Family of LIM Homeobox Class

Isl1 is a LIM homeobox class member that encodes a homeodomain transcription factor important for cell differentiation, fate determination and generation of cell diversity in multiple mouse and human tissues including central nerve system (CNS), pancreas and heart (Zhuang et al. 2013). During early cardiac development, Isl1, Nkx2-5 and fetal liver kinase 1 (Flk1) support the formation of SHF, which gives rise to the right ventricle, outflow tract and part of the atria (Dyer and Kirby, 2009). Isl1 promotes expansion, migration and proliferation of SHF progenitor cells during the development of the mouse heart (Witzel et al. 2012). Additionally, Isl1+ mouse heart cells have potential to differentiate into multiple cell types within the heart, including cardiomyocytes, smooth muscle, pacemaker and endothelial cells (Laugwitz et al. 2007).

There are multiple mechanisms explaining how Isl1 can promote expression of target genes, which suggests the expression of Isl1 is tightly controlled during the mouse heart development. For example, Nkx2-5 homeodomain transcription factor downregulates Isl1 expression in order to promote ventricular development in mouse heart (Witzel et al. 2012; Prall et al. 2007). The newest studies indicate, that Isl1 may repress development of mouse heart ventricle in order to promote the development of SAN (Dorn et al. 2015). Mouse embryos overexpressing Isl1 develop SAN-like cells instead of ventricle myocardium (Dorn et al. 2015). It is likely that the expression of Isl1 activates Nkx2-5 expression in SHF progenitor cells, however, in later staged of heart development Nkx2-5 shuts down ISL1 expression to promote ventricular development (Dorn et al. 2015). Isl1 orchestrates the expression of hundreds of potential genes implicated in cardiac differentiation, mainly through epigenetic mechanisms (Wang et al. 2016). Isl1 in mouse ESCs acts together with JmjC domain-containing protein 3 (JMJD3) histone demethylase to promote the demethylation or tri-methylation of core histone H3 on the amino (N) terminal tail (H3K27me3) at the enhancer's place of key downstream target genes, such as myocardin (*Myocd*), *MEF2C* and others (Wang et al. 2016). In addition, Isl1 may reduce histone methylation near *GATA4* and *Nkx2-5* genes after the expression of Isl1 lentiviral gene, and can also recruit p300 histone acetyltransferase to the promoter of

MEF2C gene in order to promote Mef2c expression in developing mouse embryo (Yu et al. 2013). Other data suggest that lentiviral-induced overexpression of *Isl1* gene promotes not only *MEF2C* gene acetylation, but also *GATA4* and *Nkx2-5* in C3H10T1/2 mouse cell line (Xu et al. 2016). The tight control of *Ils1* gene expression is required, since it acts as a positive cardiomyogenic gene regulator reducing methylation and increasing acetylation levels of genes and histones by direct and indirect methods. Most of the published data concerning Isl1 function have come from the studies of mouse development, however there are some studies that link *Isl1* gene expression with the susceptibility to human congenital heart disease (Luo et al. 2014; Stevens et al. 2010). Development of mechanisms that could control expression of Isl1 might be important target in further regulation of heart regeneration.

20 PROS Homeobox Class

Homeobox prospero (PROS) genes code atypical C terminal prospero domain and belongs to a distinctive class of Prospero homeodomain proteins (Yousef and Matthews, 2005). The PROS domain is a DNA binding domain of approximately 100 amino acids. In addition, PROS homeobox genes code additional three amino acids in their HD domain (Yousef and Matthews 2005).

21 PROX Gene Family of PROS Homeobox Class

Prospero homeobox 1 or Prox1 is a gene coding a transcription factor that plays important role in the development of mouse heart, CNS, eye, liver and lymphatic system (Elsir et al. 2012). Firstly, it was discovered in Drosophila as an important player in the development of central nervous system in insects. However, later Prox1 homologues were found in vertebrates and mammals (Elsir et al. 2012). In mouse heart development of Prox1 is important for the sarcomere formation and muscle contraction (Risebro et al. 2009). Mouse Prox1 conditional mutants show increased number of fast twitch fibers compared to slow twitch fibers. *Prox1* mutant mice develop fatal dilated cardiomyopathy and die around 7–14th week (Petchey et al. 2014). It was shown that in mice Prox1 acts as a transcriptional repressor of genes like *Tnnt3*, *Tnni2* and *Myl1* that are essential for the formation of fast twitch fibbers (Petchey et al. 2014). Prox1 might also be important for the maintenance of cardiac conduction system in adult mice. It was also shown that uncontrolled Nkx2-5 expression led to cardiac conduction defects, surprisingly suggesting that Prox1 might act as a direct upstream modifier of *Nkx2-5* gene expression (Risebro et al. 2012). In humans dysregulation of *Prox1* gene expression might also lead to congenital heart disease, like, hypoplastic left heart (Gill et al. 2009). Thus, the close connection of Prox1 with Nkx2-5 and other heart development and diseases regulating genes makes it an attractive target in cardiac regeneration field.

22 Role of Homeobox Genes in Cardiomyogenesis

The summarized and reviewed data of estimated involvement of homeobox genes in the heart development, diseases and/or regeneration processes suggest that some homeobox genes play more important role than the other. Data summarized in Table 1 show the homeobox genes that have been most commonly investigated with important roles in cardiomyogenesis.

It is quite evident that dozens of homeobox genes are required for early cardiomyogenesis, heart septation, formation of pacemaker cell, cardiomyocyte and etc. Some of the homeobox genes are directly related to the development of CHD, atrial fibrillations and other cardiac pathologies. However, there are much more

Table 1 Homeobox genes with major involvement in heart development and diseases

Gene	Development	Disease	Reference
Hox	Hoxa1, Hoxb2 and Hoxb2 is important for anterior-posterior patterning in SHF. Integrating FGF and BMP signalling.	Hoxa1 mutations might cause CHD.	Pearson et al. (2005), Bertrand et al. (2011), Bosley et al. (2008), Gong et al. (2005) and Haas et al. (2013)
		HOXB13, and HOXC5 mutations might be related to heart disease.	
Nkx2–5	Heart looping, heart septation and cardiac conduction system formation. Integrates BMP, notch and WNT signaling during development.	Multiple gene variants and truncations are related to CHD.	McCulley and Black (2012), Tanaka et al. (1999), McElhinney et al. (2003), Anderson et al. (2018), Wang et al. (2011), Luxán et al. (2016) and Cambier et al. (2014)
Hhex	Cardiac mesoderm specification.	HHex gene variants might be associated with diabetes.	Liu et al. (2014), Karns et al. (2013), Kelliny et al. (2009) and Pechlivanis et al. (2010)
Shox2	Cardiac conduction system development.	Downregulation during early-onset atrial fibrillation.	Blaschke et al. (2007) and Hoffmann et al. (2016)
Hopx	Cardiomyocyte progenitor formation in mouse early heart. Negative regulator of GATA4 expression.	Downregulated in patients having cardiac hypertrophy.	Jain et al. (2015), Trivedi et al. (2010) and Trivedi et al. (2011)
GSC	Cardiac mesoderm specification.		Zhu et al. (1998)
Pitx2c	Establishment of the left-right axis in heart development. Heart looping and chamber septation.	Mutations cause improper ventricle and atrial chambers septa formation, atrial fibrillation.	Liu et al. (2002), Wang et al. (2013) and Wei et al. (2014)
Meis1 and *Meis2*	Meis1 and Meis2 control of cell cycle progression during heart development.	Meis1 and Meis2 gene variants might be associated with CHD.	Mariotto et al. (2013), Arrington et al. (2012), Wu et al. (2015) and Louw et al. (2015)
Irx1-6	Irx3 very important for ventricular conduction system.	Irx1 gene variants might be associated with CHD.	Christoffels et al. (2000), Guo et al. (2017), Koizumi et al. (2016) and Cheng et al. (2011)
	Irx4 is associated with the formation of ventricular myocardium in mouse and humans.	Irx3 gene defects can cause lethal cardiac arrhythmias in human patients.	
ZEB2	Controls striated muscle development and contraction.	Gene variants cause Mowat-Wilson syndrome. Patients display CHD and other defects.	Busser et al. (2015) and Garavelli and Mainardi (2007)
Islet1	Cell expansion, migration and proliferation. Marks formation of SHF. Repress ventricular fate in order to promote sinoatrial node development. Positive gene regulator, which reduces gene and histone methylation levels and increase acetylation.	Gene variants might be related to CHD.	Witzel et al. (2012), Dorn et al. (2015), Wang et al. (2016) and Stevens et al. (2010)
Prox1	Important for sarcomere formation and muscle contraction.	Dysregulation of Prox1 might lead to CHD.	Elsir et al. (2012) and Petchey et al. (2014)

homeobox genes related to the heart development, that so far have been less investigated or in one or another model system showed less direct involvement in cardiomyogenic processes (Table 2). Data summarized in Table 2 also highlight the fact that many more studies are needed to understand regulation of homeobox genes and their role in cardiomyogenic processes.

Table 2 Homeobox genes having less important role in heart development and diseases

Class	Subclass	Gene	Development and disease	Reference
ANTP	HOXL	*MEOX1* and *MEOX2*	Control of vascular endothelial cells proliferation in mice. Dysregulation might be associated with heart disease in mouse.	Lu et al. (2018), Douville et al. (2011)
ANTP	NKL	*Msx1* and *Msx2*	Regulate survival of secondary heart field precursors and post-migratory proliferation of cardiac neural crest in the outflow tract	Chen et al. (2007)
ANTP	NKL	*Lbx1*	Specification of a subpopulation of cardiac neural crest necessary for normal heart development.	Schäfer et al. (2003)
ANTP	NKL	*Nkx2-6*	NKX2-6 mutation predisposes to familial atrial fibrillation.	Wang et al. (2014)
PRD		*Prrx1* and *Prrx2*	Formation of cardiovascular system and connective tissues of the heart and in the great arteries and veins.	Bergwerff et al. (2000)
PRD		*Pax3, Pax7*	Involved in neural crest and cardiac development	JA (1996)
ZF (TALE)		*ZFHX3*	Genetic polymorphisms in are associated with atrial fibrillation in a Chinese Han population.	Liu et al. (2014)
PBC (TALE)		*Pbx1*	Patterning of the great arteries and cardiac outflow tract.	Stankunas et al. (2008) and Arrington et al. (2012), Chang et al. (2008)
			Pbx acts with Hand2 in early myocardial differentiation in zebrafish. Non-synonymous variants in PBX genes are associated with congenital heart defects.	
TGIF (TALE)		*Tgif1* and *Tgif2*	Left-right asymmetry formation and embryonic heart looping.	Powers et al. (2010)
SIX/SO		*Six2*	Six2 marks a dynamic subset of second heart field progenitors.	Zhou et al. (2017)

23 Concluding Remarks

Heart development is a complex process requiring strict spatiotemporal development to form a healthy organ providing properly functioning organism. Multiple transcription factors, signalling pathways, morphogens and other stimuli govern the heart development process. However, it is possible to state that multiple homeobox genes and their coded transcription factors come into the heart developmental stages when their function is needed (Tables 1 and 2). None of these homeodomain transcription factors can be separated from each other, since their ability to bind DNA affects patterns of multiple gene thus resulting in changed transcriptome level of multiple cells.

Homeodomain proteins are not the only transcription factors important for cardiac development. The transcription factors of other gene families also significantly contribute heart development. For example, GATA, Tbx, HAND, Mef2c and other accompany homeodomain factors like Nkx2-5, Isl1, etc. (Hiroi et al. 2001; Gao et al. 2011; Maves et al. 2009; Skerjanc et al. 1998). Most of these homeodomain transcription factors are conserved and display coexistence and codependence in heart development of human as well as simple invertebrates like fruit fly or ascidians (Jensen et al. 2013a; Olson 2006). Only birds and mammals display fully separated heart, however reptilians still have no septum between right and left ventricles (Jensen et al. 2013b). Deeper further insights into septum formation of lower vertebrates like snakes, lizards and turtles could also help to understand signalling networks of human congenital heart diseases. Maybe in the future will be possible to engineer a reptile with four chambered heart, thus leading to better understanding of cardiac regeneration process and allowing to develop new therapeutic

strategies for human cardiac congenital and other types of diseases.

Acknowledgements The study is funded by the Lithuanian Research council, project No. S-MIP-17-13.

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication All authors agree to the publication of this manuscript.

Availability of Data and Material Not applicable.

Competing Interests The authors declare that they have no competing interests.

Authors' Contributions RM wrote the manuscript draft. DB revised the manuscript. AM read, corrected and approved the final manuscript.

Funding The study is funded by the Lithuanian Research council, project No. S-MIP-17-13.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 179–220
https://doi.org/10.1007/5584_2019_340

Published online: 26 April 2019

Generation of Human Stem Cell-Derived Pancreatic Organoids (POs) for Regenerative Medicine

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Abstract

Insulin-dependent diabetes mellitus or type 1 diabetes mellitus (T1DM) is an auto-immune condition characterized by the loss of pancreatic *β-cells*. The curative approach for highly selected patients is the pancreas or the pancreatic islet transplantation. Nevertheless, these options are limited by a growing shortage of donor organs and by the requirement of immunosuppression.

Xenotransplantation of porcine islets has been extensively investigated. Nevertheless, the strong xenoimmunity and the risk of transmission of porcine endogenous retroviruses, have limited their application in clinic. Generation of β-like cells from stem cells is one of the most promising strategies in regenerative medicine. Embryonic, and more recently, adult stem cells are currently the most promising cell sources exploited to generate functional *β-cells* in vitro. A number of studies demonstrated that stem cells could generate functional pancreatic organoids (POs), able to restore normoglycemia when implanted in different preclinical diabetic models. Nevertheless, a gradual loss of function and cell dead are commonly detected when POs are transplanted in immunocompetent animals. So far, the main issue to be solved is the post-transplanted islet loss, due to the host immune attack. To avoid this hurdle, nanotechnology has provided a number of polymers currently under investigation for islet micro and macro-encapsulation. These new approaches, besides conferring PO immune protection, are able to supply oxygen and nutrients and to preserve PO morphology and long-term viability.

Herein, we summarize the current knowledge on bioengineered POs and the stem cell differentiation platforms. We also discuss the in vitro strategies used to generate functional POs, and the protocols currently used to confer immune-protection against the host immune attack (micro- and macro-encapsulation). In addition, the most relevant ongoing clinical trials, and the most relevant hurdles met to move towards clinical application are revised.

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Keywords
Cell differentiation · Innovative therapies · Pancreatic organoids · Stem cells · Type 1 diabetes mellitus

Abbreviations

2D	Bi-dimensional
3D	Three-dimensional
A. ECM	Artificial ECM
AHFBRs	Alginate-filled hollow fiber bioreactors
ALDHhi	High aldehyde dehydrogenase activity
BAPs	Bioartificial pancreas
BCD	*β-cell*-derived cells
BM-MSC	Bone marrow MSC
CaO2	Calcium peroxide
Cas9	DNA endonuclease Cas9
CPO	Calcium peroxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Dex	Dexamethasone
ECM	Extracellular matrix
ED	Eudragit
EPCs	Endothelial progenitor cells
FBM	Functional *β cell* mass
FICCs	Fetal porcine islet-like cell clusters
FTY720	Fingolimod
GHRH	Growth hormone releasing hormone
Glut-2	Glucose transporter 2
HARV	High aspect ratio vessels
Hep-PEG	Glycosaminaglycans and heparin enriched capsules
hESCs	Human embryonic stem cells
hHPCs	Human hepatic progenitor cells
hiPSC	Human induced pluripotent stem cells
HLSC	Human liver stem cells
HLSC	Adult human liver stem-like cells
HMGB1	High mobility group box 1
huPI-MSC	Adult human pancreatic islet mesenchymal stromal cells
IBMIR	Instant blood-mediated inflammatory reaction
IL-2	Interleukine-2
IPCs	Insulin producing cells
iPSCs	Inducible pluripotent cells
KCl	Potassium chloride
LbL	Layer-by-layer
MGC	Methacrylated glycol chitosan
MMP	Matrix metalloproteinase
MP	Microparticles
mPEG	Methoxy polyethylene glycol
MSC	Mesenchymal stem cells
NeuroD1	Neuronal Differentiation 1 protein
NICC	Neonatal islet-like cell clusters
NICHE	Neovascularized implantable cell homing and encapsulation
NK	Natural Killer T cells
NPCCs	Neonatal pancreatic cell-clusters
NOD	Non-obese diabetic
PANC-1	Human pancreatic ductal-cells
PB	Probucol
PB-CDCA	PB-TCA alginate-microencapsulation
PCL/PVA	polyvinyl alcohol/polyvinyl alcohol
PDMS	Polydimethylsiloxane
PDX1	Pancreatic and duodenal homeobox 1
PEG	Poly(ethylene glycol)
PEGCol	Poly(ethylene glycol) hydrogels containing collagen type I
PEG-VS	Vinyl sulfone-terminated polyethylene glycol
PEOT/PBT	Poly(ethylene oxide terephthalate)-poly(butylene terephthalate)
PERV	Porcine retrovirus
PES/PVP	Polyethersulfone/polyvinylpyrrolidone
PFTBA	Perfluorotributylamine
PGA	Polyglycolides
PLA	Polylactic acid
PLGA	Poly(lactide-co-glycolide)
PLLA/PVA	Poly-L-lactic acid-polyvinyl alcohol
PLO	Poly-L-ornithine
PMAA-Na	Sodium polymethacrylate
POs	Pancreatic organoids
PSCs	Pancreatic stem cells
Ptf1a	Pancreas Associated Transcription Factor 1a
PTFE	Polytetra-fluoroethylene

PTX	Pentoxifylline
RWV	Rotating wall vessels
SF	Silk fibroin macroporous
SIPN	Semi-interpenetrating polymer network
SP	Saccharide-peptide
SPO	Sodium percarbonate
starPEG	Star-shaped polyethylene glycol
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Taurocholic acid (bile acid)
Th	T-helper
TRAFFIC	Thread-reinforced alginate fiber for islets encapsulation
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2

1 Introduction

Type 1 diabetes mellitus (T1DM) develops when the endocrine pancreas fails to produce proper insulin concentrations to maintain the glucose homeostasis. The current approach to treat T1DM patients consists in the administration of exogenous insulin to supply the deficiency of pancreas secretion. This implies that pancreas transplantation would represent the finest therapeutic approach in these patients.

The protocols applied to isolate and purificate adult human pancreatic islets have been standardized and considerably improved over the last years (Brennan et al. 2016). Advances in peritransplant anti-inflammatory strategies, immunomodulation, and immunosuppression, have also contributed to the improvement of current clinical results (Ricordi et al. 2016). Nevertheless, only ~50% of recipients remain insulin-independent 5 years after pancreas transplantation. This mainly depends on the related inflammatory reaction, hypoxia and hypoxia–reoxygenation injury (Pepper et al. 2018).

Transplant of pancreatic islets is currently considered an approach to be used in highly selected patients with severe hypoglycemia or instable T1DM (Liu et al. 2015). For decades, the clinical application of islet transplantation has been limited by the inadequacy of the immunosuppressive therapy and the number of donor tissues (Liu et al. 2015). Once transplanted, only nearby 60% of the T1DM patients become insulin independent and maintain the glycemic control (Liu et al. 2015). In addition, most of these patients gradually lost insulin independence 2 years after transplantation (Shapiro et al. 2006). Beneficial long-term outcomes consisting in a long-term insulin independence (≥5 years) depends on an efficient immunosuppressive therapy able to improve islet mass engraftment and prevent recurrent autoimmunity (Bellin et al. 2012). However, long-term immunosuppressive therapy is associated with a significant morbidity, including the increased risk of cancer and infections (Shapiro et al. 2017). Moreover, as extensively demonstrated, nutrients cannot effectively reach the core of large-size transplanted islets resulting in cell death and islet loss. Such diffusional problems could be solved by breaking up islets into single islet cells. However, dissociated cells are less efficient than intact islets. Based in this concept, Li et al. (2017) established a novel islet engineering approach by encapsulating dissociated cells from human islets to generate newly formed islet-like organoids, similar in size and gene expression profile (Isl-1, Gcg, and insulin-1) to native islets. Furthermore, by limiting the diameter of these engineered islet cell clusters, to a maximum of 100 μm, cell viability and insulin secretion were improved (Li et al. 2017). Nevertheless, this approach faced the same problem as pancreas and islet transplantation, the shortage of donor organs (Matsumoto 2010).

Additionally, the lost of post-transplanted islets, due to the adverse immune and non-immune reactions (Croon et al. 2003), is associated with the failure of long-term insulin independence after intrahepatic islet transplantation (Bruni et al. 2014). This implies that to increase the number of transplantable T1DM patients, it is mandatory to obtain reliable and standardized sources of human transplantable islets, and avoid immunosuppression. To cover these needs, several efforts have been devoted to develop new potential

strategies to produce functional pancreatic organoids (POs) (Zhou and Melton 2018). However, similar to pancreas or islet transplantation, transplanted POs activate the host immune response (Szot et al. 2015) which targets POs and leads to their loss of function (Szot et al. 2015).

Xenotransplantation using porcine islets, has been considered as an alternative source of human islets, and extensively investigated (Cardona et al. 2006; Hering et al. 2006). The easy and less expensive isolation procedure of fetal porcine islet-like cell clusters (FICCs) and neonatal islet-like cell clusters (NICCs) represents the main advantage at using xenotransplantation instead of allogenic/heterogenic allografts (Liu et al. 2017). Significant improvement in glycaemic control, has been provided by xenotransplantation (Liu et al. 2017). However, after transplantation, islet xenografts gradually lose efficacy (Safley et al. 2018a). Furhermore, the strong xenoimmunity and the risk of porcine endogenous retroviruses transmission, have limitated their application in clinic (Liu et al. 2017; Van Der Laan et al. 2000; Samy et al. 2014).

In the last decades the research has been focused on the identification of alternative cell sources from which functional insulin-secreting cells could be generated (Matsumoto et al. 2016). Cell lines are currently considered as potential sources able to generate new and mature insulin-secreting cells (Ravassard et al. 2011; Boss et al. 2017). For a long time, the introduction of the insulin gene along with components of the stimulus–secretion coupling pathways have been applied to induce the commitment of immature cells to insulin-producing cells. However, these cells failed to effectively store and release fully processed insulin (Ravassard et al. 2011; Clark et al. 1997; Scharfmann et al. 2014; Xie et al. 2016). Stem cells, isolated from embryonic and more recently, from adult tissues, are currently the most promising approaches (Balboa et al. 2018). Despites the great efforts and the promising results, so far, cell products covering all the morphological and functional profiles recapitulating the mature pancreatic islets are still missing.

Pancreatic islets are three-dimensional structures (3D) composed by different endocrine cells. The cellular ineractions resulting from this 3D conformation play an important role in the modulation of hormone secretion (Navarro-tableros et al. 2018; Kim et al. 2016a). Assembly of stem cells in 3D structures facilitates cellular interactions and promotes morphogenesis and tissue organization mimicking native pancreatic islets (Navarro-tableros et al. 2018; Kim et al. 2016a). Both natural and artificial materials can be used to create 3D synthetic organic and inorganic porosous scaffolds (Hinderer et al. 2016). Nanotechnology have provided new opportunities to design distinctive materials with specific physicochemical properties (Liu et al. 2018). Thus, generation of POs similar, in terms of morphology and function to native pancreatic islets, not only may enhance long-term transplant viability in clinical settings, but could also be applied for drug screening purposes (Scavuzzo et al. 2018). Hydrogels are extensively used for drug delivery and tissue regeneration due to their similarity in terms of structure and biocompatibility to the native extracellular matrix (ECM) (Dimatteo et al. 2018; Steffens et al. 2018). Biomaterials are classified in three major groups: (i) naturally-derived materials; (ii) synthetic polymers; and (iii) decellularized organ- or tissue-derived scaffolds.

The size of organoids should be also considered to generate encapsulated POs. In general, it is widely accepted that smaller capsules provide better *β-cell* oxygenation (Buchwald et al. 2018). However, concerns related to their removal still remains if compared to macroencapsulation devices (Scharp and Marchetti 2014). Preliminar studies have suggested that islet cell encapsulation within meter-long microfibers might overcomes this issue (Dolgin 2016). In this review, all 3D isolated structures (islets) or artificialy generated, either encapsulated or not, and independently of their origin (human and other species) will be referred as POs.

In summary, key challenges for human translation still include: (1) generation of biocompatible materials, (2) generation of 3D POs with functional insulin secreting cells, (3) improvemnt of PO survival, (4) protection of POs from the immune attack, and (5) the availability of nutrient supply (Tomei et al. 2015). Thererfore, the ideal encapsulation method, should allow long-term survival and functional capability of implanted POs (Ryan et al. 2017). Encapsulated islets or newly generated POs are typically delivered in extrahepatical sides such as intraperitoneally, subcutaneously or into the omentum (Berman et al. 2016). However, the host immune response and the activation of the fibrotic process occurring after transplantation, still remain a challenge (Safley et al. 2018a).

In this review, the new strategies to generate *in vitro* POs (stem cells-based, gene editing and alternative 3D culture methods), immunoisolation approaches, such as macro- and micro-encapsulation, and polymers used for these purposes will be discussed. Moreover, the ongoing clinical trials, and the hurdles to move towards their clinical application, will be also overviewed.

2 The Relevance of 3D Structure

Islet architecture, including arrangement and interaction between endocrine cells is relevant for their precise function (Orci et al. 1975; Kitasato et al. 1996; Kanno et al. 2002). More importantly, the homologous and heterologous intercellular contacts are relevant to provide a finest regulation of insulin secretion (Wojtusciszyn et al. 2008). Furthermore, the paracrine and autocrine interaction between α- and *β-cells*, contributes to the firm dynamic of hormone secretion, on which depends the effective control of glucose homeostasis (Jo et al. 2009). Additionally, gap-junction-mediated cell-cell interactions protect pancreatic cells against apoptosis (Klee et al. 2011), and correlate with a correct developmental acquisition of a mature insulin secretory profile (Santos-Silva et al. 2012). Therefore, the 3D architecture and the intercellular regulatory mechanisms involving ions (electrical coupling) and hormones, particularly involved in the cross-talk among α-, δ-, and *β-cells* within the islets, are crucial to ensure a correct response to paracrine signals (Rorsman and Ashcroft 2018). Modification or loss of these properties results in abnormal glucose control (Rutter and Hodson 2015; Kilimnik et al. 2011). Upon islet isolation, insulin-producing cells undergo multiple cell death processes including apoptosis, anoikis, and necrosis, which have been attributed to the loss of critical interactions between cells, ECM and the vasculature (Paraskevas et al. 2000; Irving-Rodgers et al. 2014; De Vos et al. 2016). Modification of the islet architecture also occurs during isolation and culture of cadaveric human islets, prior to transplantation (Lavallard et al. 2016) or after infusion into the portal vein (Henriksnäs et al. 2012). This results in the failure to obtain a fine glycemic metabolic control (Morini et al. 2006). Therefore, the preservation of 3D cellular architecture of human islets or POs, derived from different cell sources, have a functional relevance for islet engraftment (Navarro-tableros et al. 2018). Improved protocols/devices for PO *in vitro* culture may thus offer crucial indications for appropriate growth and differentiation of stem/progenitor cells (S/PCs), allowing safeguarding of specific characteristics, such as multi-lineage differentiation capabilities and paracrine activity.

3 Microencapsulation

Native pancreatic islets are heterogeneous structures formed by almost 5 different endocrine cells: α, β, γ, δ, ε, and pancreatic polypeptide (PP) cells which by interacting in a complex and synchronized manner allow a fine autocrine and paracrine regulation. Generation of POs, reminiscent of native pancreatic islets in structure and function, should be pursued to provide a more efficient treatment. Indeed, generation of pancreatic endoderm and endocrine cells in 3D microenvironment has been extensively documented. Wang et al. (2017) have generated human embryonic stem cells (hESCs)-derived POs expressing

high levels of *β-cell* markers such as Pdx1, Ngn3, Insulin, MafA, and the Glucose transporter 2 (Glut-2). More importantly, they demonstrated that these POs contained pancreatic α, β, δ, and PP producing cells. Insulin biosynthesis was confirmed by the high C-peptide expression and by the presence of insulin-secretory granules. The improvement of insulin secretion response to high glucose concentration has finally demonstrated their functional maturation (Wang et al. 2017).

3.1 Nanotopographies as Drivers of Cell Differentiation

A number of studies strongly provided evidences that engraftment, proliferation and differentiation of stem cells into a specialized phenotype are modulated by their structural organzation (Guilak et al. 2009). The interactions between extracellular binding sites and cytoskeletal elements strongly affect *in vitro* cell morphology, adhesion, and consequently gene expression (Bettinger et al. 2009).

Nanotopographies are involved in stem cell adhesion, and regulate their functions by mechanotransduction signals through the cell membrane (Yim et al. 2010). On the other hand, nanotopographies inducing lower cell adhesion cues, allow cells to form POs by promoting cell−cell interaction (Shen et al. 2015). Evidences have been also provided that differentiating hESCs and iPSCs are able to perceive nanotopographical signals, and efficiently differentiate into pancreatic cells (Kim et al. 2016b). It has been also described that compared to traditional 2D cultures, human iPSCs (hiPSCs) cultured in nanofibrous scaffolds and driven to differentiate into POs are formed by insulin-producing cells, and exhibit a morphological and functional profile similar to mature pancreatic *β-cells* (Nassiri Mansour et al. 2018).

The establishment and preservation of a functional *β-cell* mass (FBM) are replacement. FBM is dogged by a direct sensing of its key components, i.e. the number and function of *β-cells* (Pipeleers et al. 2008). Contrasting results have been obtained by De Mesmaeker et al. (2018). They demonstrated that a sustained FBM could be obtained using immature proliferative porcine *β-cells*, but not human adult islet cells. These observations suggest that differatiation depends not only on the employed methodology, but also on the cell of origin.

Thereby, cell-based therapy requires simple and safe encapsulation methods to produce POs of uniform size and shape. Indeed, encapsulation promotes not only a quick cell aggregation of mesenchymal stem cells (MSC) in dense cell clusters, but also increases the expression of insulin and Pdx-1 mRNA (Barati et al. 2018). These concepts are currently exploited and widely applied in biomedical areas (Lee et al. 2011).

Encapsulation has been also pursued to provide immuno-isolation biocompatibility, optimized nutrient diffusion and insulin release by POs (Orive et al. 2015). In line with these needs, a number of new polymers have been generated (Gálvez-Martín et al. 2017). Besides the characteristics and configuration of microcapsules, the nature of the cells should be also considered. A number of different cell sources has been investigated for cell therapy including stem cells (autologous and allogenic), mature somatic cells, modified human cells, xenogenic cells and others (Kang et al. 2014). Alginate-based microcapsules have been reported not only to enable the proliferation and differentiation of hESCs into definitive endoderm-derived cells, but also to enhance their viability and proliferation, and to promote their aggregation into POs (Chayosumrit et al. 2010).

Independently of the origin, single cell within microcapsules, tend to spontaneously self-aggregate to form POs (Chayosumrit et al. 2010). These POs tend to mimic the structure and the features of the original in vivo-like cytoarchitectures (Wang et al. 2006; Zhang et al. 2006). Moreover, it is important to keep in mind that spheroid size also influences microcapsule efficacy (Perignon et al. 2015). POs with small diameters and with a low cell content, could not provide suitable conditions for cell aggregation due to inadequate number of adhering cells,

while large POs can undergo oxygen depletion with consequent hypoxia (Huang et al. 2012).

One of the most commonly used islet micro-encapsulation biomaterial is the alginate. Microcapsules have an ideal surface/volume ratio, which allows a better exchange of nutrients, insulin and glucose. Furthermore, islets could individually be included in an single capsule (Ryan et al. 2017; Desai and Shea 2017). Alginate is an anionic polysaccharide composed by unbranched polymers of 1,4-linked β-D-mannuronic and α-L-guluronic acid residues which forms a gel in the presence of multivalent cations such as Ca2+ or Ba2+ and provides a 3D biomimetic environment to the cells that resembles the in vivo conditions (Datar et al. 2015). An important issue of first alginate products relied on their chemical instability during long-term implantation that hindered feasibility of cell therapy. It has been reported that alginate-Ca2+ hydrogels tend to degrade, thus resulting in the contact of incapsulated structures with the host's immune system (Scharp and Marchetti 2014). Clinical and pre-clinical studies have noted that reducing the volume of encapsulated materials and the corrisponding diffusional restrictions are critical for the engraftment (Scharp and Marchetti 2014). Experts in biomaterials have improved the stability of alginate hydrogels by modifying their biomechanical properties by a process called "click" crosslinking (Breger et al. 2015). These modifications confer to the capsules a superior stability, but a higher permeability to small size "diffusates". The alginate matrix can stabilize the cell cluster size and can allow a more homogeneous cell morphology, leading to a long-term culture of these clusters compared to non-encapsulated ones (Formo et al. 2015). Moreover, alginate-encapsulated human stem cell-derived POs could be effectively protected from the immune reaction when intraperitoneally implanted in mice (Vegas et al. 2016). In fact, micro-encapsulation is the most investigated approach (Zimmermann et al. 2007), and a number of pre-clinical studies in non-human primate models (Sun et al. 1996; Elliott et al. 2005), and human trials (Soon-Shiong et al. 1994; Calafiore et al. 2006; Tuch et al. 2009; Jacobs-Tulleneers-Thevissen et al. 2013) based on this technology are ongoing.

Polymers applied to produce microcapsules should also have chemo-mechanical stability, and an easy to handle and appropriate pore size to allow a bi-directional diffusion of molecules in the semipermeable membrane (Kang et al. 2014). Currently, microcapsules have been obtained in many different forms, sizes, compositions, and with different permeability (De Vos et al. 2014). Both synthetic and natural polymers have been used for encapsulation purposes. Nevertheless, only alginate has been largely studied and it is currently certified as safe for human application (De Vos et al. 2014). As other polymers, alginate tends to be largely contaminated by original and additional contaminants such as polyphenols, endotoxins, and proteins introduced during the industrial extraction processes (Vos et al. 2006). In fact, a number of medicated alginates has been generated to solve this issue.

In general, it is accepted that smaller capsules (0.5 mm) better engraft functional POs (Buchwald et al. 2018). However, recent investigations suggested that the biocompatibility of alginate-encapsulated POs can be significantly enhanced by the use of larger capsules. In particular, it has been reported that larger capsules (1–1.5 mm) could generate a reduced immune reaction and fibrosis and avoid altered pattern of glucose-induced insulin secretion than smaller spheres (Veiseh et al. 2015). In contrast, other studies indicated that delayed and blunted responses to glucose and KCl depolarization, are present in larger capsules. As a consequence, larger capsules provide a reduced insulin response and a sustained and slow release of insulin in response to glucose (Buchwald et al. 2018).

A number of new microcapsules enriched with different combination of factors, has been developed. Recently, new capsules generated by a combination of antihyperlipidemic drug (probucol; PB), bile acid (taurocholic acid; TCA) and alginate-microencapsulation (PB-Alginate) called PB-CDCA capsules have been described. They are able to improve PO function and to reduce cell apoptosis driven by the hyperglycemic setting (Mooranian et al. 2016). Additional effects include

improved viability under the hyperglycemic environment, increased insulin production, and reduced TNF-α release by pancreatic *β-cells* (Mooranian et al. 2018). Furthermore, the PB-CDCA capsules, offer the mechanical stability, the buoyancy, the PB release, the thermal stability, and contain antioxidants (Mooranian et al. 2018). Similar results were obtained when capsules were enriched with absorption-enhancer chenodeoxycholic acid and Eudragit (ED) polymers (Mooranian et al. 2018) or ECM proteins (Mooranian et al. 2018). Moreover, it has been demonstrated that the addition of ECM proteins or trophic factors to alginate-encapsulated POs stabilizes the cluster size, cell morphology, and improves the oxygen consumption rate and PO survival in long-term culture (Formo et al. 2015). A newly ECM-based encapsulation system, named meter-long core shell alginate-hydrogel microfibers, has been shown to allow the formation of a core of pancreatic islet cells surrounded by ECM proteins and is considered a promising approach which can facilitate PO implantation and removing (Onoe et al. 2013).

The microencapsulated POs composed by pig islets seeded in human decellularized collagen matrix has been recently implanted in non-human primates (Dufrane et al. 2010). Long-term PO survival and insulin release were associated with improved glycemic control for 6 months, in the absence of immunosuppressive medications (Dufrane et al. 2010). However, the risk of porcine retrovirus (PERV) transfer, even if reduced, was still detected (Dufrane et al. 2010), possibly due to the damage of the alginate barrier (Crossan et al. 2018). Thus, additional quality control is mandatory before moving toward their clinical application.

By investigating alginate-encapsulated adult porcine islet transplants, it has become evident that despite long-term glucose normalization, a massive fibrosis occurred in harvested capsules (Vaithilingam et al. 2011). Nevertheless, improvement in avoiding fibrosis was obtained by using microcapsules enriched in photo-crosslinked methacrylated glycol chitosan (MGC) (Hillberg et al. 2015) or Rampamycin-PEG (Park et al. 2017). Although relevant data have been obtained long-term and large preclinical studies are still required (Park et al. 2017).

3.2 Immunomodulation

Even if the donor and organ managements, the surgical procedure, and the recipient management have been improved, the immune rejection still remains the most relevant issue to be solved (Schuetz et al. 2018). In order to safeguard graft survival, robust immunosuppressive treatments are required, some of which are toxic for *β-cells* (Nir et al. 2007). It has been demonstrated that the addition of multilayers composed bypolymers, such as poly-L-ornithine (PLO) or activated methoxy polyethylene glycol (mPEG) to the surface of alginate microcapsules could allow immune protection and decrease interleukine-2 (IL-2) secretion (Nabavimanesh et al. 2015).

As above discussed, encapsulation is an effective approach to protect transplanted cells from rejection by the host immune response by using a semi-permeable artificial membrane. Microencapsulation has the advantage to allow implantation of allogeneic and xenogeneic cells, and free bidirectional diffusion of nutrients, oxygen, and other molecules like insulin. Moreover, it is also able to prevent the host immune response (Steele et al. 2014). Thus, effcient encapsulation approaches should be pursued to reduce or abolish the requirement of pharmacological immunosuppression. For example, double-layer alginate/dextran–spermine microcapsules combined with pentoxifylline (PTX) have been shown to be effective in preventing the immune attack over standard alginate-based encapsulation (Azadi et al. 2016). PTX could be easily released from the porous microcapsules, however, extended destruction of the membrane capsules has been documented (Azadi et al. 2016).

Alternative approaches aimed to improve immune-protection consist on the blockage of one of the most expressed proteins on the islets: the high mobility group box 1 (HMGB1) which is known to induce inflammation (Wang et al. 2016). Therefore, the regulation of HMGB1-mediated inflammation and particularly of the HMGB1 A

box (Itoh et al. 2011) is actually considered a new target for immnuno-protection (Sama et al. 2004). In fact, recently, an anti-HMGB1 receptor-enriched encapsulation approach has been developed (Jo et al. 2015). Jo et al. (2015) have demonstrated that HMGB1 A box offers a protective effect on islet transplantation by decreasing the amount of TNF-α secreted by macrophages. It was also demonstrated that the HMGB-enriched-encapsulated method strogly improves PO survival rate after intraperitoneally xenotransplants into diabetic mice (Jo et al. 2015).

3.3 Encapsulated Xenografts

The possibility to use encapsulated porcine-islets to treat diabetes has been also evaluated. Promising results indicate that encapsulated porcine islets are more efficient than non-encapsulated porcine islets in restoring normoglycemia in diabetic monkeys (Sun et al. 1996). Co-encapsulation of pancreatic islets with MSC futher improved vascularization and oxygenation of the PO graft (Vériter et al. 2014). However, despite a glycemic control was detected up to 32 weeks, not substantial improvement in the xenograft function was observed (Vériter et al. 2014).

A first phase I/IIa clinical trial aimed to test the microbiological safety of porcine islet xenotransplantation has been opened by recruiting nonimmunosuppressed subjects with unstable T1DM (Wynyard et al. 2014) transplanted with microencapsulated neonatal porcine islets (Wynyard et al. 2014). A reduction in unaware hypoglycemia events, the HbA1c levels, and a decrease of daily insulin requirement have been reported (Matsumoto et al. 2014). Pig-islet-based PO biosafety was further demonstrated in a phase IIa efficacy trial in humans (Morozov et al. 2017). Interestingly, plasma porcine C-peptide level has been applied to demonstrate the function of alginate-encapsulated porcine *β-cells* implanted in large and small animals (Montanucci et al. 2013; Chen et al. 2015). However, insight on the relationship between the initial *β-cell* dose, the viability of the implanted *β-cells* and their function in term of metabolic control within time, are still missing (Dufrane et al. 2006; Foster et al. 2007).

It is important to remind that notwithstanding the relevant progress in xenotransplantation, significant limitations to clinical application persist:

1. No clinical applicable immunosuppressive protocols for preventing xenograft rejection are avalaible (Samy et al. 2014).
2. High number of designated pathogen-free donor pancreata are required to manufacture neonatal porcine (Thompson et al. 2011) and adult pig islets for each patient (Rogers et al. 2011).
3. Manufacturing of NICC is still challenging and costly (Korbutt et al. 1996).

4 Macroencapsulation

Human pancreatic islets are usually transplanted in the intrahepatic site, via the portal vein. However, this environment directly exposes the islets to the blood flow, thus eliciting the "instant blood-mediated inflammatory reaction" (IBMIR) and leads to islet damage and loss (Moberg et al. 2002; Nilsson et al. 2011). In addition, the concomitant reduced oxygen tension and the high intrahepatic concentrations of immunosuppressants further interfere with survival and engraftment of islets (Carlsson et al. 2001; Olsson et al. 2011; Desai et al. 2003). Unlike liver, the subcutaneous space has been proposed as an ideal implantation site, since it possesses a high grade of vascularization and can offer mechanical protections to the implanted islets (Pepper et al. 2015).

Even if transplantation of pancreatic islets is currently considered a promising therapeutically approach to cure diabetes, islet availability is limited by the number of donors and a large number of transplanted patients still requires insulin therapy to obtain the glycemic control (Shapiro et al. 2006). Additionally, due to the high oxygen demand, a highly vascularization is necessary to allow the long-term survival and function of transplanted islets. Furthermore,

accessible and minimally invasive sites are fundamental to allow implantation, replenishment and graft retrieval (Pagliuca and Melton 2013).

Recent advances in regenerative medicine and tissue engineering have preferred new strategies for the generation of 3D scaffolds. Thus, a number of biodegradable and biocompatible synthetic polymers have been used to produce nanofibers with the aim to improve maturation and function of the newly generated POs (De Vos et al. 2014). These materials rapidly undergo adaptation to the human body and do not stimulate the immune system (Ellis et al. 2017). These platforms provide a 3D environment that improves stem cell differentiation into β-like cells (Nadri et al. 2017; Abazari et al. 2018). It appears that these 3D nanofiber scaffolds are able to support an efficient cell-ECM interaction and cell-cell contact, by mimicking the in vivo condition (Mahboudi et al. 2018).

Macroencapsulation is an old technology also applied in diabetes research to provide a better survival of POs after transplantation (Desai and Shea 2017). Macroencapsulation devices are heterogeneous in geometry and in materials (Song and Roy 2016). A number of studies has shown the ability of pancreatic progenitor cells or endocrine cells to persist and function within subcutaneously implanted devices (Agulnick et al. 2015).

4.1 Differentiation of Macroencapsulated Cells

Compared to 2D cultures, PCL/PVA 3D scaffolds could efficiently improve differentiation of iPSCs into β-like cells by inducing the expression of endocrine markers such as Glucagon, Insulin, Pdx1, Ngn3 and Glut-2 genes (Enderami et al. 2017). Differentiation properties of Poly(lactide-co-glycolide) (PLGA)-based microporous scaffolds have been also documented (Blomeier et al. 2006; Salvay et al. 2008; Mao et al. 2009). Poly(ethylene glycol) hydrogels containing collagen type I (PEGCol) have been also reported to promote aggregation and differentiation of hESC-derived progenitors in glucose-sensitive POs, also displaying enhancement of long-term viability and morphology preservation (Mason et al. 2009; Amer et al. 2015). One of the most promising materials currently used for the generation of encapsulated POs is the poly-L-lactic acid-polyvinyl alcohol (PLLA/PVA) polymer, which provides a better microenvironment than 2D cultures (Mobarra et al. 2018). It has been reported that PLLA/PVA polymer is able to promote differentiation of iPSCs into insulin producing cells (iPSCs-IPCs) by inducing the expression of pancreatic-specific transcription factors such as Pdx1, insulin, glucagon and Ngn3 (Mobarra et al. 2018).

4.2 Macroencapsulation and Immunoprotection

Bioscaffolds used as cell or drug carriers are usually constituted of non-degradable or degradable biomaterials. Current methodologies use ECM and ECM-like materials, or ECM-synthetic polymer hybrids (Hinderer et al. 2016). Hydrogels have been exploited for regenerative applications due to their biocompatibility and similarity in structure to the native extracellular matrix (Dimatteo et al. 2018). Several aliphatic polyesters, such as polymers of lactic acid [polylactides (PLA)], glycolic acid [polyglycolides (PGA)], and their copolymers [polylactoglycolides (PLGA)] have been extensivly selected as biocompatible and bioresorbable matrices to reconstitute soft and hard synthetic engineered tissues (Mironov et al. 2017).

Polycaprolactone and polyvinyl alcohol (PCL/PVA)-based scaffolds display biological characteristics which improve differentiation of pancreatic β-like cells and organization into islet-like structures. PCL is a hydrophobic polyester which is biocompatible and biodegradable and has high mechanical stability. It is therefore considered a good candidate to produce 3D culture (Zarekhalili et al. 2017). Recent studies have demonstrated that the low-immunogenic polyethylene glycol (PEG)-based hydrogels used for islet transplantation have the ability to support islet engraftment and function (Jeong et al. 2013; Rengifo et al. 2014) and allow long-term function and restoration of normoglycemia in diabetic mice

(Rios et al. 2016). Some studies demonstrated that using polymer films and electrospun meshes made with poly(ethylene oxide terephthalate)-poly (butylene terephthalate) (PEOT/PBT) blocks copolymer, provides a protective environment to preserve islet morphology by preventing their aggregation in implanted islets (Buitinga et al. 2013). However, even if these scaffolds can allow a higher nutrient diffusion (glucose flux), and maintain the insulin and glucagon expression, a decrease of *β-cell* density in the islet core was observed (Buitinga et al. 2013). Further studies demonstrated that cell death, resulting from non homogeneous vascularization whithin the core and the outer shell of the scaffold-seeded islets (Buitinga et al. 2013), could be avoided by obtimizing the pore size of the PEOT/PBT-based scaffolds (Buitinga et al. 2017).

In addition, it has been shown that microporous scaffolds, made with non-degradable polyethylene glycol (PEG)-based hydrogels, protect islets from the host immune response and show comparable results to the initial engraftment and function of non-encapsulated islets. Furthermore, unlike encapsulated islets which lose vascular connections to the host tissue, the microporous scaffolds allow islet revascularization after transplantation (Rios et al. 2018).

Recent progresses in nanotechnology and materials used for encapsulation, as well as in immunomodulatory strategies have significantly improved vascularization. Meanwhile, differentiation strategies of ESC and iPSC into islet-like structures have achieved an upscalable production for potential clinical applications. However, these cells can potentially drive tumour development (Päth et al. 2019). New strategies based on micro- and nano-materials (i.e., PEG, PLGA, chitosan, liposomes and silica) alone or enriched with trophic factors, have been recently developed to achieve a better stability of the capsules and, more importantly, to avoid loss of *β-cell* mass and PO function (Hinderer et al. 2016). Bioengineers are focusing on the development of new synthetic materials able to maintain tissue- and organ-specific differentiation and morphogenesis. Indeed, the newly generated bio-products have important advantages such as (1) a great surface area for oxygenation and nutrient/cathabolite transport, (2) a porous structure allowing infiltration of cells and blood vessels, (3) a satisfactory mechanical strength supporting cell attachment, (4) could be easly implanted, and (5) eventually, undergo degradation over time (Lutolf and Hubbell 2005).

To promote vascularization of transplantable devices housing pancreatic islets is the use of TheraCyte™ system. TheraCyte™ device is made by a bilayered polytetra-fluoroethylene (PTFE) where either free or microencapsulated islets are placed in the membrane to obtain a planar, bilaminar membranous pouch (Sörenby et al. 2008). A first clinical study has demonstrated that one-year after transplantation, TheraCyte device was biologically inert and lacked adverse effects, when transplanted in humans. Nevertehless, marked fibroblast overgrowth occurred and almost all the tissues were fibrotic (Tibell et al. 2001). A further study has reported that an immortalized cell line derived from human islets (betalox5), and induced to differentiate, formed long-term survival and functional POs also immune-protected when allocated in TheraCyte devices (Itkin-Ansari et al. 2003). Afterthat, some preclinical studies were carried on to test the TheraCyte device housing rat islets (Sörenby et al. 2008), human islets and human fetal pancreatic POs (Lee et al. 2009). Sörenby et al. (Sörenby et al. 2008) improved the efficacy of the TheraCyte system by trasplanting rat islets in preimplanted TheraCyte devices. Briefly, empty and capped devices were first subcutaneously pre-implantated on the back of athymic mice before diabetes induction. Subsequently, the islets were transplanted in the already implanted and newly pre-vascularized device. Following these modifications, an improvement of PO efficacy was obtained. Moreover, the preimplantation procedure significantly reduced the number of macroencapsulated islets required to restore normoglycemia (Sörenby et al. 2008), without a detectable T-cell response (Lee et al. 2009). It is important to remark that *β-cells* at this stage of maturation may avoid the immune T-cell response (Lee et al. 2009).

In vivo differentiation into predominantly *β-cells* was also obtained when huESC-derived endoderm cells were seeded into the TheraCyte

device (Motte et al. 2014). Biosafety was supported by the absence of increased biomass or hESC cell escape for up to 150 days (Kirk et al. 2014). However, an accumulation of $CD8^+$ T cells surrounding the membrane was observed in some of these devices (Boettler et al. 2016), indicating a certain grade of immune reaction against the graft.

In vitro PEGylation maintains viability and insulin secretory capabilities of transplanted islets (Lee et al. 2002), but also protects them from cytokines secreted by immune cells (Lee et al. 2004). Moreover, PEGylated-macroencapsulation allows a stable blood glucose level of the allotransplants after one year (Lee et al. 2006a). Since PEGylation makes transplanted POs protected against the recipient immune system, a reduction of cyclosporine A dosage was requested (Lee et al. 2006b), and a reduced graft immune infiltration was detected (Lee et al. 2006c). The use of a combination of immunossuppresive drugs was associated with better outcomes (Im et al. 2013). Coated thin PEG-layers on islet surface named "Layer-by-layer (LbL) PEGylation" have been shown to allow a further reduction of islet volume per unit, which facilitates the exploitment of the portal vein for transplantation (Wilson et al. 2008).

4.3 Drug-Charged Devices/Scaffolds

The use of dexamethasone-charged macroporous scaffolds has been proposed to inhibit the host immunoreaction. This original approach was developed to accelerate islet engraftment by promoting the expansion of the anti-inflammatory M2 macrophages (Jiang et al. 2017). To generate favorable host responses and to improve the overall outcomes of the transplant polydimethylsiloxane (PDMS)-based 3D scaffold platform, a local and controlled delivery of dexamethasone (Dex) was used (Jiang et al. 2017). In particular, Dex-scaffold accelerated islet engraftment in a diabetic mouse model, improving glucose control early after transplantation. Remarkably, it was demonstrated that lower doses of Dex (0.1% or 0.25%) were able to induce a M2 phenotype of macrophages interfering with inflammation during the first post-implantation week, whereas higher doses of Dex (0.5% and 1%) significantly delayed the engraftment and function of islets (Jiang et al. 2017).

4.4 Vascularization-Enhanced Macroencapsulation

The use of immune-isolated macrodevices designed to islet delivery into extrahepatic transplant site is not limited to synthetic PEG-based hydrogel macrodevices. In fact, a two-component synthetic PEG hydrogel macrodevice system has been generated. These PEG-devices consist in a hydrogel core cross-linked with a non-degradable PEG-dithiol and a proteolytically sensitive vasculogenic outer layer to allow matrix degradation and to enhance vessel infiltration (Weaver et al. 2018). These PEG-dithiol devices promoted engraftment and overall graft efficacy, resulting in enhanced vascular density and in the improvement of islet viability when transplanted in diabetic rats (Weaver et al. 2018).

Through 3D printing technology, Farine et al. (2017) designed an innovative and refillable encapsulation system transcutaneously implated. The PLA-system generated a prompt and efficient PO vasacularization, and supported the long-term graft survival (Farina et al. 2017). Nevertheless, in this first study the in vivo experiments were performed in immunodeficient mice, which did not allow the evaluation of their efficacy in preventing the host immune attack (Farina et al. 2017). More recently, the same group developed a new PLA encapsulation system named neovascularized implantable cell homing and encapsulation (NICHE) that seems to be a promising alternative approach due to their ability to maintain PO viability and a robust hormone secretion (Farina et al. 2018). Despite NICHE system has provided promises to improve long-term viability and function using Leydig cells (Farina et al. 2018), its feasibility in POs still requires validation.

The pro-angiogenic and immunomodulatory effects of the local delivery of the immunomodulating drug fingolimod (FTY720) allow a better oxygenated and tolerant environment for islet engrafment (Bowers et al. 2018). FTY720 is a small molecule that activates sphingosine-1-phosphate receptors and is involved in the regulation of immunomodulatory and pro-angiogenic signals. Based on this concept, Bowers et al. (Bowers et al. 2018) developed a new encapsulation membrane for islet transplantation outside the portal circulation. The FTY720-releasing nanofiber-based semi-permeable membrane increased PO revascularization, and was also able to block the immune response by reducing the number of macrophages and their released cytokines (Bowers et al. 2018).

A similar strategy which can facilitate PO transplantation and preserve their function in extrahepatic sites is represented by the encapsulation of pancreatic islets in synthetic saccharide-peptide (SP) hydrogels (Liao et al. 2013). The SP hydrogel is constituted by natural blocks of amino acids and saccharides, nontoxic and entirely biodegradable (Chawla et al. 2011). The SP hydrogel also displays several exclusive properties, such as ease of handling, crosslinkable at mild physiological conditions, biocompatibility, and *in situ* polymerization after injection (Chawla et al. 2011). The SP hydrogel allows PO survival and function of normal islet structure, but also promotes a rapid vascularization (Liao et al. 2013). Furthermore, it has been demonstrated that SP hydrogels are also able to induce minimal inflammatory cell infiltration. However, they tend to degenerate (Liao et al. 2013).

4.5 Removal/Retrievability

Despite the huge efforts in improving the encapsulation technology, the major problem still relies on the removal (Scharp and Marchetti 2014). This raises significant concerns since this can be associated with transplant failure and clinical complications (Desai and Shea 2017). Retrievability is also an important issue for the regulatory approval processes (Matsumoto et al. 2016). Thereby, efforts are currently focused to generate alternative encapsulation systems readily scalable and conveniently retrievable, also allowing the delivery of a sufficient cell mass. Innovative approaches so far developed to generate easy retrievable devices consist in a cytocompatible enzymatic approach based on the addition of MMP (Amer et al. 2015). Alternatively, a highly wettable and Ca2 + −releasing nanoporous polymer, possessing particular mechanical properties which facilitates handling and retrieval of transplanted islets has been generated (An et al. 2018). Innovative retrievable encapsulation system has been also recently developed by An et al. (An et al. 2018). In particular, the thread-reinforced alginate fiber for islets encapsulation (TRAFFIC) consists in a one-step *in situ* cross-linking alginate hydrogel around nanoporous wettable, and Ca2 + −releasing polymer thread. TRAFFIC has the advantage to provide the essential fisical space and biocompatibility for islet transplantation, similar to conventional hydrogel capsules, but displaying mechanical strength enabling the easy handling, implantation, and retrieval (An et al. 2018). Furthermore, due to their particular design, this device may be extended to clinic (An et al. 2018). The therapeutic potential of TRAFFIC has been provided by the restoration of glucose control and inmuno protection of transplanted human or rat islets in diabetic mice, rats and dogs (An et al. 2018). Furthermore, rapid retrievability can be obtained through a simple laparoscopic procedure (An et al. 2018). The fast and minimally invasive retrievability makes TRAFFIC a promising encapsulation system with a great scale-up potential. Nevertheless, the feasible application in clinic, and particularly in T1DM, has to be evaluated.

4.6 ECM-Enriched Macroencapsulation

Islets in the pancreatic tissue are naturally surrounded by a capsule mainly composed by collagen type I, IV and laminins (Stendahl et al. 2009). Llacua et al. (2016) demonstrated

the ability of specific ECM components (collagens or synthetic laminin peptides) to support survival and function of encapsulated human islets (Llacua et al. 2016). This technology was further applied to evaluate the immunoregulatory effects of capsules covered with a mix of artificial ECM (A. ECM) and Layer-by-Layer (LbL) Pegylation (A. ECM + PEGylation) in non-human primate xenografts (Andrades et al. 2008; Haque et al. 2017; Llacua et al. 2018). The authors demonstrated that ECM-enriched encapsulation system acts as an effective immune barrier, translating in the increase of xenograft survival in the absence of immmunosuppresive therapy (Llacua et al. 2018). Although the ECM-enriched encapsulation system is also able to protect POs against the host immune response and to prolonge islet cell survival, its ability to induce islet re-vascularisation remains to be addressed. According with this concept, further studies have been performed to improve PO vascularization. In particular, vascularization improvement was obtained by incorporating heparin in nanofilms containing star-shaped polyethylene glycol (starPEG) (Lou et al. 2017) or silk fibroin macroporous (SF)-scaffolds (Mao et al. 2017). Additional anti-inflammatory and anti-coagulant properties along with the improvement of intra-islet vascularisation have been obtained by enriching capsules with glycosaminaglycans and heparin (Hep-PEG) (Lou et al. 2017). Heparin-mediated vascular endothelial growth factor (VEGF) binding, resulting in the activation of the endogenous VEGF/VEGFR2 pathway, has been reported to mediate endothelial proliferation and re-vascularisation (Lou et al. 2017). These mechanisms have been also previuosly described for both non-encapsulated (Cabric et al. 2007; Cabric et al. 2010) and encapsulated human islets (Marchioli et al. 2016). Given the pro-angiogenic, pro-survival and minimal post-transplantation inflammatory reaction, as well as the possibility to introduce different trophic factors, Hep-PEG-based encapsulation system is considered a very promising approach for clinical implementation.

4.7 Silk Fibroin-Based Macroencapsulation

The fibrin matrix, due to its good biocompatibility, rapid biodegradability, and easy production, is the most widely investigated natural biopolymeric material (Park and Woo 2018). In particular, the bioactive molecules conjugated with fibrinogen, can promote tissue morphogenesis, cell migration, proliferation, differentiation or maturation after cell adhesion on fibrin matrices (Park and Woo 2018). Thereby, fibrin matrices have been exploited as therapeutic strategies for tissue engineering applications (Park and Woo 2018). These concepts were used for the developmet of new materials such as autologous platelet-and plasma-derived fibrin scaffolds (Anitua et al. 2019). Synthetic scaffolds based on 3D silk matrices with ECM-derived motifs were also used to promote the aggregation of mouse and human primary cells into functional POs (Shalaly et al. 2016). Afterthought, POs generated by co-encapsulation of islets with MSCs in a silk hydrogel have been shown to increase insulin I, insulin II, glucagon and PDX-1 gene expression. This was associated with a better glucose-induced insulin response (Davis et al. 2012). Diabetic mice implanted with Silk fibroin hydrogel–islet–MSC system showed a prompt return to euglycaemia with a significative reduction of T-helper (Th)1-derived cytokines (Hamilton et al. 2017). Thereby, co-culture of islets and MSCs in a silk-hydrogen-islet system has been proposed to avoid the host immune-attack. However, additional studies are required to demonstrate the stability, the function and the biocompatibility before transfer of this approach to humans.

5 Oxygenated Devices

It has been postulated that chronic effects of non-immunologic factors such as hypoxia and the hyperglycemic milieu could damage the encapsulated islets, resulting in a gradual and short-term loss of efficacy (Safley et al. 2018b).

This prompted researchers to propose new preventing approaches. In particular, major efforts have been focused on generating new methodologies able to increase oxygen supply to POs. Oxygen supply is a crucial issue for cell survival in bioartificial pancreas (BAPs), since poor oxygen supply causes PO central necrosis. Implantation and removal of macrocapsules actually imply minimal risks, but the transport of oxygen and nutrients is still limited. Therefore, new strategies must be developed to improve oxygenation (Hwa and Weir 2018). At this regard, bioengineering research in diabetes mainly focused on the development of new 3D scaffolds able to supply sufficient oxygen and nutrients to POs (Iwata et al. 2018).

The generation of an oxygenated chamber system, based on the incorporation of a refillable oxygen tank (βAir device) into the immune-isolating alginate poly-membrane was the first technology developed to improve oxygenation of the encapsulated POs (Ludwig et al. 2012). Indeed, the feasibility to increase the oxygen supply in small and large animal models was demonstrated (Barkai et al. 2013; Neufeld et al. 2013). Based on these promising results, a phase 1 clinical trial was started to demonstrate the efficacy of the βAir (Ludwig et al. 2013). The authors demonstrated that βAir inhibits inflammation of human islets implanted in the absence of immunosuppression. Additionally, an increased vascularization and the enhancement of the oxygen supply were reported (Ludwig et al. 2013). A second phase 1 clinical trial demonstrated that βAir device was safe and able to enhance survival of allogeneic islets (Carlsson et al. 2018). Nevertheless, the real benefits obtained by the βAir device were limited, due to the low increase of circulating C-peptide and the lack of a real impact on the metabolic control (Carlsson et al. 2018). Additionally, the formation of fibrotic tissue, the presence of immune cells, and the deposition of amyloid were also detected in the endocrine tissue (Carlsson et al. 2018).

A new generation of 3D scaffolds containing oxygen generators have been proposed as alternative technology to improve PO oxygenation. Different approaches have been proposed. The new devices included, (1) polydimethylsiloxane encapsulated solid calcium peroxide (PDMS-CaO2) (Pedraza et al. 2012), (2) active microorganisms such as Synechococcus lividus, which photosynthetically generated oxygen (Evron et al. 2015), and (3) SPO and CPO (Lee et al. 2018). These studies demonstrated a successful enhancement of oxygenation that translated into a reduced hypoxia-induced cell loss during the precarious vascularization period (Pedraza et al. 2012). These beneficial effects resulted from the capability to supply oxygen at high cell loading densities (Pedraza et al. 2012), with an average of 0.026 mM/day oxygen, for more than 6 weeks (Evron et al. 2015). This prevented the development of detrimental oxygen gradients in the core of large implants. The beneficial effects of oxygen-generating scaffolds were further supported by the studies of Lee et al. (2018) who used neonatal pancreatic cell-clusters (NPCCs). The application of oxygen-generating microparticles (MP) included in a fibrin-conjugated heparin/VEGF and implanted in streptozotocin-induced diabetic NOD mice was also investigated (Montazeri et al. 2016). The MP improved the POs engraftment and function, the blood glucose control, body weight, glucose tolerance, serum C-peptide, and graft revascularization, by reducing the islet mass necessary to obtain this goal (Montazeri et al. 2016). It became evident that oxygen-generating materials such as sodium percarbonate (SPO) and calcium peroxide (CPO) could be efficient in supplementing oxygen to transplantable naked and encapsulated islets in diabetic patients (McQuilling et al. 2017). Indeed, it has been shown that SPO and CPO can be used to improve islet viability and function. Nevertheless, additional studies are required to control the oxygen generation rate able to provide the suitable oxygen supply over an extended period of time avoiding tissue damage resulting from oxidative stress (McQuilling et al. 2017). Of note, it has been suggested that this technology could be also applied, as a new animal-free experimental scaffold platform, to study the interactions between pig islets and human blood components *in vitro* (Lee et al. 2018).

5.1 Perfluorocarbons (PFCs) as Oxygen Carriers in POs

PFCs are obtained by fluorine replacement of hydrogen atoms in hydrocarbons (Hosgood and Nicholson 2010). The PFCs have a higher density than water and a high capacity to dissolve oxygen. Carbon-fluorine bonds are very long and render the molecule biologically and chemically inert (Hosgood and Nicholson 2010). Due to their lipophilic nature and their high oxygen solubility, PFCs have been largely explored to improve cell and tissue oxygenation. They can be also adapted and used as blood substitutes for the treatment of cardiac ischemia, anemia, and organ preservation (Hosgood and Nicholson 2010). However, controversal results have been reported. Bergert and collaborators (Hosgood and Nicholson 2010) have demonstrated that, compared to control rat islets, PFC-cultured islets showed a significant increase of DNA fragmentation and a reduced glucose sensitivity. On the contrary, other studies indicated that, althought advantageous to transfer human harvested organs, the use of PFCs may be comparable to conventional islet culture protocols used for transplantation (Bergert et al. 2005). A different study demonstrated that perfluorotributylamine (PFTBA) emulsion co-encapsulated insulin secreting cells (βTC-tet insulinoma cells) did not have additional effects in terms of viability or function (Goh et al. 2010). Nevertheless, it was suggested that PFCs could be potentially applied as a culture method to improve islet preservation, in particular for pancreatic islets isolated from marginal pancreata (Ricordi et al. 2003). Additional studies are still required to demonstrate the biological benefits of PFCs on POs.

5.2 Generation of Hypoxia-Resistant Islets

It became evident that, while encapsulation clearly protects POs against the host immune attack, it impairs PO survival and long-term function. VEGF plays a crucial role in *β-cell* function and islet regeneration (Nikolova et al. 2006; Jabs et al. 2008). Moreover, it has been reported that a splice variant-1 of the GHRH receptor expressed in human pancreatic islets was able to generate antiapoptotic signals by modulating VEGF levels and by inducing angiogenesis (Ludwig et al. 2010). "Hypoxia-resistant islets" have been generated using implanted devices containing pancreatic islets and GHRH agonists (JI-36) (Ludwig et al. 2012). A significant hypervascularization and enhancement of the graft function, associated with a parallel improvement of glucose tolerance and increase of the *β-cell* insulin reserve were observed (Ludwig et al. 2012). This also allowed a reduction of the islet mass required for metabolic control (Ludwig et al. 2012). However, even if promising, the feasibility of this innovative technology as alternative xenotransplantation approach requires further validation.

5.3 Mathematical Models

As extensively documented, the main goal to improve biotechnological approaches is an efficient oxygenation, allowing a long-term survival and function of newly generated POs. One of the most relevant challenges to deal with relies on the the thickness of the cell layer (Wu et al. 1999). In encapsulated POs, the inner core mimics pancreatic islets while the peripheral shell acts as an inert defense. Oxygen consumption by the cells is a hurdle that follows two rules, (1) the limited diffusion, and (2) the limited consumption. Using mathematical models it has been suggested that hypoxia directly depends on the PO radius and would likely occur in high cell density conditions (King et al. 2019). According with this model, a better viability should be obtained with a PO radius around 142 μm or fewer, and with an encapsulating radius lower than 283 μm (King et al. 2019).

The effectiveness of supplementary oxygen suppliers in a *β-cell* 3D culture has been estimated by calculating the spatio-temporal distribution of oxygen concentrations inside the scaffolds (McReynolds et al. 2017). Two different types of simulations (constant cell density and cell growth simulation) used in this work, have lent

a hand to determine the effectiveness of oxygen-releasing microbeads (McReynolds et al. 2017). Briefly, hydrogen peroxide was encapsulated into nontoxic PDMS disks which provided oxygenation to *β-cell* cultures (McReynolds et al. 2017). Detection of the spatial-temporal distribution of oxygen tension inside a scaffold would be a fascinating approach to be pursued in the future. MacReynolds and collaborators (McReynolds et al. 2017) have also suggested that optimization of the cell culture conditions, including cell seeding density and time of culture, are critical issues.

As widely described, the new 3D devices are mostly based on oxygen diffusion. Nonetheless, mathematical models indicate that convective transport should be a more efficient approach for the oxygen transfer (Iwata et al. 2018). Theoretical studies have described how oxygen supply in BAPs could predict the necrosis area in the islets (Iwata et al. 2018). Thereby, according with these models, to correct the difference in pressure between the device and the site of implantation, the BAPs should be directly connected to the blood vessels (Iwata et al. 2018). Platelet deposition usually takes place in implants subjected to blood flow, and eventually leads to thrombus formation (Affeld et al. n.d.). Thus, BAP coated with blood compatible materials could prevent thrombus formation in long-term BAPs.

In summary, despite enormous efforts devoted to the encapsulation technology, oxygenation remains the main challenge in diabetes bioengineering. Mathematical models, established that an oxygen-enabled 3D culture system should effectively provide an improved oxygen distribution within the scaffold, allowing an enhanced secretion of insulin from cells. Nevertheless, the application in humans is still missing.

6 Vascular Improvement in Transplants

Pancreatic islets are extremely vascularized structures by a capillary network wich is critical for glucose sensing and for providing nutrient supply and rapid secretion of hormones into the blood stream (Richards et al. 2010). Islets with an altered vascularization do not properly regulate glicemia (Lammert et al. 2003), indicating that engineered islets should permit an efficient mass transport and well-organized vascularization. This requires the presence of a porous implantable construct allowing vessel infiltration. Recent data indicate that the range of pore size that maximizes vascularization is narrow and corresponds to 30–40 μm, (Madden et al. 2010). This suggests that an accurate control of the pore size would significantly favor vascularization of implantable scaffolds (Buitinga et al. 2017). It has been clearly demonstrated that the lack of an adequate revascularization (Smink et al. 2017), the reccurrence of autoimmunity (Piemonti et al. 2013), the prompt blood-mediated inflammatory response (Kourtzelis et al. 2016; Samy et al. 2018), the ischemic injury (Faleo et al. 2017), and the activation of Natural Killer (NK)T cells (Saeki et al. 2017) are the main factors contributing to the engraftment failure.

Approaches exploiting angiogenic factors have been used in tissue engineering. Thus, encapsulation devices enriched in pro-angiogenic molecules such as VEGF, to improve PO vascularization is strongly encouraged (De Rosa et al. 2018; Schweicher et al. 2014). Trivedi et al. (2000) have proposed an alternative approach to solve the deficiency of vascularization in devices using a double layer capsule formed by three different layers combined with a transcutaneous infusion of VEGF. This approach leads to a reduced delay of the diffusion time due to the development of a new vascular network through large pores. The authors also showed that small pores could protect POs from the immune attack (Trivedi et al. 2000). However, even if a reduction in glycemia was detected, normoglycemia was never achieved in transplanted diabetic mice (Sweet et al. 2008). The authors have suggested that this unexpected result, rather than to the device itself, may be related to the number of implanted islets, corresponding to the half of islets usually transplanted into T1DM patients (Sweet et al. 2008).

Alternative studies applied a semi-interpenetrating polymer network (SIPN) as subcutaneous implantable carrier to deliver cells and to allow vascularization (Mahou et al. 2017). SIPN was generated by reacting a blend of vinyl sulfone-terminated polyethylene glycol (PEG-VS) and PMAA-Na with dithiothreitol (Mahou et al. 2017). This combination allowed the formation of vessels in the surrounding tissues, which is 2–3 times superior to that obtained with PEG alone (Mahou et al. 2017). The biological activity of the SIPN in glucose control was demonstrated in diabetic mice (Mahou et al. 2017).

Re-endothelization represents an innovative approach designed to solve the issue of blood supply. Endothelial progenitor cells (EPCs) have been used to generate pre-vascularized scaffolds. The main advantage identified was the direct connection between the pre-vascularized host blood circulation and the newly formed blood vessels (Guo et al. 2018). Very recently, Skrzypek et al. (2018a) developed a functional and not degradable flat polyethersulfone/polyvinylpyrrolidone (PES/PVP) porous membrane to create an early microvascular network without the addition of hydrogels (Skrzypek et al. 2018a). Applying this technology, pre-vascularized devices could be obtained by co-culturing different cells (Skrzypek et al. 2018a, b; Groot Nibbelink et al. 2018). It was demonstrated that the particular surface topography obtained with this method enhanced the formation of a stable vascular network in the membranes. Furthermore, the *in vitro* pre-vascularization allowed a faster connection of POs with the host vasculature, impacting on the engraftment, and on the long-term implant survival and function (Skrzypek et al. 2018b).

Oligomers have been used for tissue engineering approaches in T1DM pre-clinical models (Stephens et al. 2018). The feasibility of collagen I oligomeric-based macroencapsulation was already demonstrated (Stephens et al. 2018). Collagen-based macroencapsulation was able to improve mouse islet function and longevity (Stephens et al. 2018). Cytoarchitecture, revascularization, and immnunetolerance were also demonstrated (Stephens et al. 2018). Furthermore, STZ-induced diabetic mice showed a rapid glycemic control and a sustained normoglycemia until 40 days post-transplantation (Stephens et al. 2018).

7 Natural Extracellular Matrices as Biological Scaffolds

Undoubtedly, scaffolds dysplaing features similar to native islet extracellular matrix (ECM) would most likely succeed. The ECM is synthesized by cells of each tissue and provides a physical niche for cell attachment. ECM composition includes polysaccharides and proteins, such as collagen and elastin (Theocharis et al. 2016). One of the most recent technologies enabling the isolation of native ECM is the organ decellularization (Badylak et al. 2011). ECM derived from decellularized organs has been recently used for tissue engineering (Guruswamy Damodaran and Vermette 2018a). Decellularized scaffolds have been proposed as a source of native ECM exploitable in a wide-ranging regenerative medicine applications (Celikkin et al. 2017). ECM offers structural and function support and acts as a substrate for cell migration. The ECM mechanical behavior depends on its physical properties such as insolubility, porosity, rigidity, and its topography (Celikkin et al. 2017). The ECM components are very conserved through different species, and include fibers, collagens, proteoglycans, glycoproteins, and mucins (Brown and Badylak 2015). ECM-scaffolds respond and release growth factors, that modulate the immune response, and allow the recruitment of progenitor cells (Swinehart and Badylak 2016).

Islets embedded in the acinar tissue of the pancreas represent only the 1%–2% of pancreatic mass (Jansson et al. 2016; Aamodt and Powers 2017). They are spherical clusters of cells highly innervated and vascularized, containing ECM which coordinate and support cellular survival, proliferation and differentiation (Irving-Rodgers

et al. 2014; Aamodt and Powers 2017; Cheng et al. 2011; Kuehn et al. 2014; Alismail and Jin 2014). In islets the ECM regulates multiple aspects of cell function, including insulin secretion, proliferation, survival, and participates to the preservation of spherical morphology (Stendahl et al. 2009). Pancreatic decellularized scaffolds play an essential role in regenerative medicine and represent a step toward the development of bioengineered pancreas. As other natural bioscaffolds, pancreatic decellularized scaffolds retain the native 3D architecture, the vasculature, the ductal channels, and the pancreatic ECM composition (Guruswamy Damodaran and Vermette 2018b). The interaction between cells and scaffolds improves pancreatic islet cell survival and insulin production potentially used for regenerative purposes (Goh et al. 2013).

Bioscaffold plasticity has been also demonstrated by using stem cells, which received and responded to the environmental signals. In this context mechanical forces play a central role in the regulation of multiple functions (Rana et al. 2017). Indeed, the adhesion of cells to ECM, the formation of cell-cell junctions, and of a mechanoresponsive cell cytoskeleton, are critical features in stem cell biology (Rana et al. 2017), particularly in cell fate determination (He et al. 2018). Plasticity and compatibility of ECM scaffolds derived from different species have been also demonstrated (Chaimov et al. 2017). Decellularized pancreatic scaffold biocompatibility in the absence of toxicity has been also confirmed (Chaimov et al. 2017). Particularly, Chaimov and collaborators (Chaimov et al. 2017) developed an innovative microencapsulation platform based on solubilized whole porcine pancreatic ECM mimicking the original decellularized ECM (Chaimov et al. 2017). This natural fibrous 3D niche was able to support the viability of pre-differentiated cells to promote differentiation and to improve insulin secretion (Chaimov et al. 2017). Moreover, these re-cellularized scaffolds were non-immunogenic and were able to improve the glycemic control in diabetic mice (Chaimov et al. 2017). It has been reported that growth factor-enriched decellularized pancreatic scaffolds induce differentiation of mouse pancreatic stem cells (PSCs) into pancreatic β-like cells (Wan et al. 2017). Bioscaffold-induced differentiation, proliferation and insulin secretion, occur independently of scaffold-derived tissue (Zhou et al. 2016). This suggests the existence of a dual interplay between the ECM and the cell.

Decellularized pancreata show the characteristic homogeneous porous structure (Hashemi et al. 2018). Thus, to solve the problem of PO blood supply after *in vivo* transplantation, bioengineering has taken advantage of this valuable feature to enhance scaffold vascularization by co-seeding POs with EPCs (Guo et al. 2018). Newly formed blood vessels (Guo et al. 2018) and improvement in functional preservation characterized the re-endothelized scaffolds (Chaimov et al. 2017). Despite these promising results, vascularization still remains a challenge to generate a functional equivalent ECM-based pancreata for transplantation.

Generation of acellular pancreatic scaffolds from small animals represents a relevant issue for future pre-clinical studies. The next and most closely need is represented by the large-scale production. Some recent studies demonstrated the feasibility (Berman et al. 2016; Katsuki et al. 2016). Nonetheless, despite the improvement of islet metabolic function and the significant preservation of the islet architecture and the intra-insular vascularization (Berman et al. 2016; Katsuki et al. 2016), long-term studies and implantation *in vivo* are still needed. Moreover, it was recently described that signals promoting cell differentiation could be heterogeneous in nature, depending on the type of organ, and on the type of tissue from which they derive (Brown and Badylak 2015). For example, it has been shown that HLSC seeded in liver decellularized scaffolds can differentiate into three different cell types (hepatocyte-, endothelial- and epithelial-like cells) (Navarro-Tableros et al. 2015). This indicates the high ECM plasticity. Therefore, the use of ECM bioscaffold-based technology could be exploitable for hetero-organ approaches. This possibility has been recently validated by Vishwakarma and collaborators (Vishwakarma et al. 2018). Indeed, they were able to induce

differentiation of human hepatic progenitor cells (hHPCs) into insulin-progenitor cells when hHCPs were seeded into xenogeneic rat acellular spleen in the presence of specific trophic factors and hyperglycemic environment (Vishwakarma et al. 2018). These data further support the hypothesis (Navarro-Tableros et al. 2015; Vishwakarma et al. 2018), that cell commitment is a complex process, modulated in part by the nature of the scaffold, but also by the type of hosted cells. In fact, the study of Vishwakarma et al. (2018) suggests that natural bioscaffold properties should be further investigated for regenerative approaches. Finally, pancreatic scaffolds, used in most if not all studies, have been generated from "healthy" tissues. Nevertheless, there are not definitive data on the feasibility to use "sick" organs for clinical applications. A very recent study, aimed to evaluate the feasibility at using diabetic pancreata to generate ECM for regenerative purposes (Huang et al. 2018a), demonstrated that similar to healthy scaffolds, those obtained from T1DM and T2DM pancreata preserved their ECM composition, 3D ultrastructure and released cytokines (Huang et al. 2018b). Nevertheless, the biological activity of this "diabetic scaffolds" has been not yet tested. These important results, even if preliminary, support the idea that marginal organs could serve as alternative sources of natural ECM scaffolds. Thereby, the application of marginal tissues could be implemented in future regenerative approaches in order to strongly support this hypothesis.

8 CRISPR/Cas9 Genome Editing Technology to Enhance Insulin-Producing Cell Function

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) combined with the DNA endonuclease Cas9 (CRISPR-Cas9) has been considered an innovative application which could completely change both medical and biotechnology approaches in our century. Using short guide target-specific sgRNAs, the Cas9 can be directed to any genomic location by inducing double strand breaks to allow non-homologous end joining or homologous recombination of the genome at specific sites (Kim et al. 2017). CRISPR works together with *Cas* gene to cleave genetic material, thus opening the possibility to use CRISPR-based approaches to induce *β-cell* differentiation (Gerace et al. 2017). Other approaches different from CRISPR, are mainly based on viral-mediated transfer of key pancreatic transcriptional factors such as *Pdx-1* (Karnieli et al. 2007), *NeuroD1* (Kojima et al. 2003) to either somatic or adult stem cells such as MSCs (Gerace et al. 2017). However, althought CRISPR-Cas9 system is widely applied in a number of research fields (Kim et al. 2017), its application to generate or to enhance the function of insulin-producing cells is still limited. At this regard, Giménez et al. (2016) applied the CRISPR-ON system to activate the endogenous human insulin gene (INS), obtaining a significant upregulation of the insulin mRNA expression when the dCas9-VP160 construct and four sgRNAs (targeting the proximal INS promoter) were co-transfected in T1DM patients-derived skin fibroblasts (Giménez et al. 2016). More recently, one homozygous ATG > ATA mutation at codon 1 of the insulin gene was reverted to wild-type ATG in hiPSC by using CRISPR/Cas9 technology (Ma et al. 2018). The insulin mRNA expression and hormone secretion confirmed the functional correction (Ma et al. 2018). Moreover, the modified *β-cells* were able to reverse diabetes in STZ-diabetic mice (Ma et al. 2018). This work has opened the possibility to combine gene and cell therapy to restore glucose homeostasis in non-immune-mediated diabetic setting. As a future perspective, the combination of CRISPR-Cas9 and encapsulation technology may contribute to improve current methodologies to generate long-term functional POs to be applied in diabetes.

9 3D Bioreactor Systems to Produce and Maintain Insulin-Producing Cells

Although stem cells hold the promise to generate endless insulin-producing cells, most of the current preclinical protocols have been performed in lab-scale (Navarro-tableros et al. 2018; Kim et al. 2016a; Wu et al. 2007). Hence, the implementation of alternative culture systems, able to generate a large number of functional insulin-producing cells, is still needed. Cell culture in 3D bioreactors has been used for several decades by the biopharmaceutical industry (Abu-Absi et al. 2014), since they allow a tight control of relevant parameters such as pH, temperature and gas supply, and garantee a more efficient cell expansion and a higher cell viability and function (Petry et al. 2018). In this chapter, we will provide a general overview of different 3D bioreactors that have been employed to generate and/or maintain viable and functional POs obtained from different sources.

9.1 3D Bioreactors Used for Insulin-Producing Cell Cultures

Spinner Flasks (SF) SF, which are currently considered the simplest form of 3D bioreactors, consist in a magnetic stirrer-integrated flasks that provide a turbulent fluid flow environment to partly alleviate limitations of nutrient and oxygen diffusion (Ratcliffe and Niklason 2002) (Fig. 1a). However, SF suffer from similar drawbacks as classical 2D culture systems in terms of requirements of individual handling and scale-up (Ratcliffe and Niklason 2002), and to the best of our knowledge despite these limits, SF are the most commonly used bioreactors in the field of *β-cell* research.

In the earlies 2000s, Blyszczuk and collaborators (Blyszczuk et al. 2003) developed a 10 days culture protocol in SF (30 rpm agitation at 37°C and 5% of CO_2) to induce differentiation of mouse embryonic-derived nestin-positive progenitors into immature insulin-positive POs (Blyszczuk et al. 2003). Trasplantation of these POs was able to ameliorate the glycemic control in immunocompromised STZ-diabetic mice (Blyszczuk et al. 2003). More recently, hESCs, initiatly maintained in 500 mL SF (70 rpm agitation at 37°C and 5% of CO_2) and subsequenly cultured in standard 2D plates in presence of specific growth factors, were found to successfully differentiate into POs able to restore normoglycemia in diabetic mice (Pagliuca et al. 2014). Long-term glycemic control was obtained in diabetic mice, when encapsulated hESCs-derived POs were pre-cultured for 24 h in SF, before their implantation (Vegas et al. 2016).

Chawla and collaborators (Chawla et al. 2006) developed a new SF system to generate POs in a large scale. To this end, porcine pancreatic neonatal endocrine tissues, at different densities, were seeded in SF (100 rpm agitation at 37°C and 5% of CO_2) in serum-free media with a specific differentiation-cocktail (Chawla et al. 2006). Using this approach, and after 9 days of culture, a large number of POs containing insulin-positive cells were produced. In addition, the POs responded to glucose and contained all islet cell types (Chawla et al. 2006). More recently, Lock et al. (2011) demonstrated the superiority of SF in the generation of mouse cell line (MIN6)-derived POs compared to the 2D standard culture. The POs generated in SF (60–100 rpm agitation at 37°C and 5% of CO_2) were associated with a 20% higher viability (64 vs 84%), a more regular shape morphology and the ability to grow for at least 2 weeks (Lock et al. 2011). In addition, the POs generated in SF, were more similar to native islets, in terms of ultrastructure, glucose-insulin secretion, incretin expression and were characterized by a lower necrosis grade (Lock et al. 2011).

Microgravity Bioreactor (MB) The original version of the MB was developed by NASA engineers and patented in 1990 under the form of rotating wall vessels (RWV), also known as high aspect ratio vessels (HARV) (Schwarz et al. 1992) (Fig. 1b). MB provided a continuous circular rotation of media and allowed cultured cells to

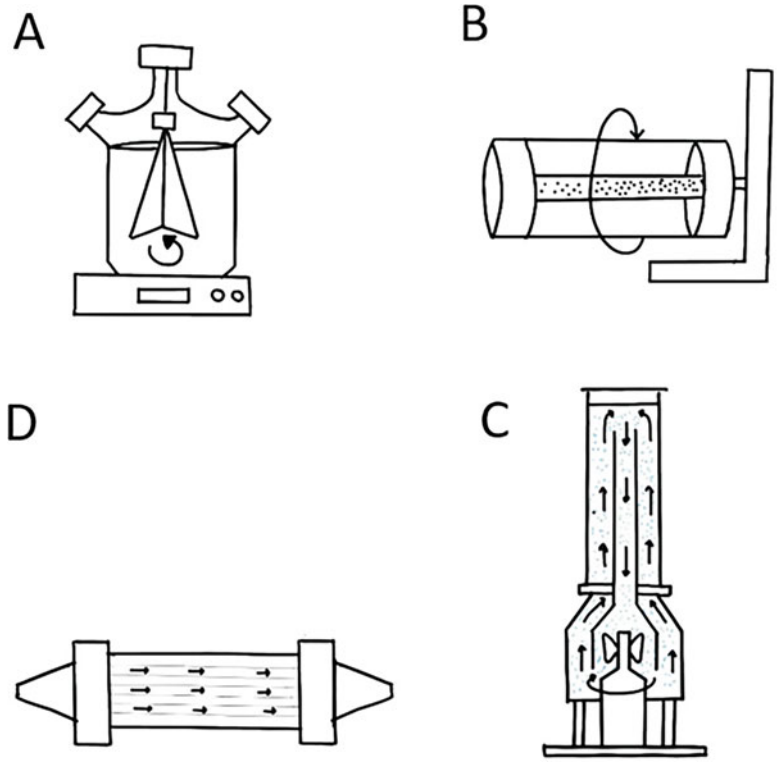

Fig. 1 Schematic representation of different bioreactor types used to generate and maintain insulin-producing cells. (**a**) Spinner Flasks (SF). (**b**) Rotating wall vessels (RWV). (**c**) Hollow fiber membrane bioreactor (HFB). (**d**) Fluidized-bed bioreactor (FBB)

be suspended in a low gravitational field (free fall), characterized by a low turbulence and hydrodynamic shear stress coupled with a high mass of oxygen transfer (Daoud et al. 2010). The first study involving MB and islets was carried out by Cameron and collaborators (Cameron et al. 2001) in 2001. To facilitate islet engraftment after transplantation, they co-cultured porcine neonatal islets with Sertoli cells (isolated from rat testis) in MB for up to 14 days. After 4 days in MB culture, large Sertoli-islet POs were formed (0.5–3 mm diameter). However, although cellular viability (>90%) and glucose responsiveness was maintained, islets underwent disaggregation into single cells. Primary mouse islets cultured in MB were significantly less immunogenic compared to standard cultured islets (Rutzky et al. 2002). Moreover, to maintain euglycemia throughout 100 days in diabetic animals the number of MB-cultured POs required were 50% less than the non-MB counterpart (Rutzky et al. 2002). Furthermore, ultrastructure studies revealed that islets appeared healthy and similar to fresh islets (Rutzky et al. 2002). Using MB (8 rpm at 37°C and 5% of CO_2) the improvement of human islet long-term function and viability was also demonstrated (Murray et al. 2005).

In an elegant work, Tanaka and collaborators (Tanaka et al. 2013) described the generation of mouse *β-cell* (MIN6 cell line)-derived POs. They loaded a two-compartment culture chamber onto the center of the 3D clinostat to generate microgravity-like conditions. Unlike the RWV, this new designed approach, allowed fresh media flow and gas exchange. In addition, POs had an average size of 250 μm and were glucose-responsive (Tanaka et al. 2013).

Hollow Fiber Membrane Bioreactor (HFB)

HFB utilizes specialized membranes to retain cells inside, and to allow an efficient exchange of nutrients and waste products by the flow of the medium through the lumen (Abu-Absi et al. 2014) (Fig. 1c). The main advantages of the

membrane-based-bioreactors relied on the possibility to obtain an high cell capacity, high volumetric efficiency, and low shear stress (Abu-Absi et al. 2014). However, disadvantages such as a reduced cell viability, membrane fouling and clogging, the lack of product homogeneity have limited their large-scale application (Abu-Absi et al. 2014). Hoesli and collaborators (Hoesli et al. 2009) developed a procedure for immobilization of large-scale cell batches in alginate-filled hollow fiber bioreactors (AHFBRs). This process improved insulin secretion and cell viability of porcine neonatal pancreatic cells (Tanaka et al. 2013) and enhanced the expansion of the insulinoma cell line, INS-1 (Sharp and Vermette 2017; Gundersen et al. 2010).

Fluidized-Bed Bioreactor (FBB) In the FBB system, the cells are seeded on microcarriers (e.g Cytopore or Cytoline) or polymer-microencapsulated (e.g alginate, collagen) and loaded in a stirred tank which allows the flow of the oxygenated nutrient through the cells (Fig. 1d). This design avoids the formation of local high shear stress and provides an interface suitable for large-scale production (Abu-Absi et al. 2014). The feasibility of FBB for production of encapsulated-POs was previously assessed (Buchi-395 encapsulator). Indeed, it was demonstrated their ability to enhance function, viability and integrity as well as insulin responsivness of 1% alginate-encapsulated POs (range 180–220 μm) (Nikravesh et al. 2017).

10 *In-vitro* Expansion of *β-Cells* from Adult Human Pancreatic and Hepatic Tissues

hPSCs are a promising source of cells for tissue regeneration due to their unlimited proliferative potential and their capability to differentiate into three germ layers including: ectoderm, endoderm and mesoderm (Zuber and Grikscheit 2018). However, concerns still remain regarding cell differentiation commitment and scalability required for human transplantation approaches (Jones and Zhang 2016). One of the major advantages of hPSCs compared to other stem cells, relies on their pancreatic origin. Indeed, they originated from the pancreatic islets, which could facilitate their commitment into mature POs (Nelson et al. 2009). Under physiological conditions, the endocrine pancreas (islets) has an extremely low turnover rate (Afelik and Rovira 2017), while under high metabolic demands (pregnancy or obesity), adaptation mechanisms such as cell hypertrophy, increased insulin synthesis and secretion, *β-cell* self-replication occur (Afelik and Rovira 2017). *In vivo*, the *β-cell* pool, is homogeneous (Brennand et al. 2007) and persist throughout the life as a "neogenic niche" within pancreatic islets (van der Meulen et al. 2017). Koop et al. (Kopp et al. 2011) demonstrated that after in vivo *β-cell* ablation, endocrine cells do not arise from the ducts, but from early pancreatic progenitors expressing Ptf1a, Nkx6.1, Pdx1 and Sox9 markers (Dor et al. 2004). Additionally, terminally differentiated adult *β-cells* retaining a significant proliferative capacity and accounting for pancreatic turnover and expansion throughout the life, have been identified (Wei et al. 2006). Pancreatic progenitors do not express hormones in basal conditions but can be induced to differentiate into hormone-expressing islet-like-cells (Dor et al. 2004). Although the hIPSC aggregation induces the expression of pancreatic endocrine markers such as Pdx1, MafA and hormones like insulin, glucagon or somatostatin, the mRNA and hormone levels are less expressed than in freshly isolated human islets. They fully differentiated only after implantation in mice (Kroon et al. 2008; Davani et al. 2007).

10.1 Pancreatic Islet-Derived Stem Cells

The presence of pancreatic precursor cells in adult human pancreas named pancreatic islet mesenchymal stromal cells (huPI-MSC) have been demonstrated and have been recently considered an alternative cell source to generate insulin-producing cells (Zhao et al. 2007; Joglekar and Hardikar 2012; Wang et al. 2013). These huPI-

MSCs exhibit all the characteristics of bone marrow MSC (BM-MSC), including the ability to suppress proliferation of lymphocyte stimulation. The number of harvested islet equivalents from a single donor varies from 300,000 to 600,000 (Kim et al. 2012). This implies that autologous huPI-MSC could be obtained from a small portion of adult pancreatic islets, including those which are discarded (Kim et al. 2012). These properties make huPI-MSC, an available and potentially exploitable source of adult human stem cells to generate *β-cell* substitutes for cell therapy in diabetes. From a therapeutic point of view, the use of adult stem cells from discarded human cadaveric islets represents an attractive perspective. It is well accepted that mature adult human *β-cells* could be significatively expanded *in vitro*, but complex de-differentiation and re-differentiation processes are required (Russ et al. 2008). Expanded human *β-cell*-derived (BCD) cells, constitute the ~40% of cells of the islet cell cultures, which can re-differentiated in response to a combination of soluble factors (Avnit-Sagi et al. 2009; Russ et al. 2011). However, only a part of these cells undergo re-differentiation (Russ et al. 2009). More recently, a different approach demonstrated that overexpression the miR-375, a miRNA highly expressed during human islet development, boosted adult pancreatic precursor cells to redifferentiate (Avnit-Sagi et al. 2009; Joglekar et al. 2009) into mature islets (Klein et al. 2013; Latreille et al. 2015).

Despite these promising results, further efforts are required for a successfully transability, and large scalability. In line with this concept, Carlotti and collaborators (Carlotti et al. 2010) have demonstrated the feasibility to use pancreatic-derived iPSCs in regenerative medicine. Recent studies, have demonstrated the presence of pancreatic stem/progenitor cell populations, named NIP/Nestin-positive Islet-derived Progenitors (Zulewski et al. 2001), PIDM/Pancreatic Islet-Derived Mesenchymal cells (Gallo et al. 2007) and PHID/Proliferating Human Islet-Derived cells (Ouziel-Yahalom et al. 2006) which are able to generate POs on tissue culture plates.

Very recently, Van der Meulen and collaborators (van der Meulen et al. 2017) identified a population of human immature *β-cells*, originating by trans-differentiation of *α-cells* on a specialized pancreatic "neogenic niche" located at the islet periphery (van der Meulen et al. 2017). These cells express insulin but not other *β-cell* markers. They are transcriptionally immature and do not sense glucose (van der Meulen et al. 2017). This cell population represents an intermediate stage of α-cell trans-differentiation into conventional *β-cell* (van der Meulen et al. 2017). These data suggest the possibility to use this cell population for generartion of functional POs. However, additional studies are crucial to demonstrate their potential application in clinic.

The presence of high aldehyde dehydrogenase activity (ALDHhi)-pancreatic stem cells, have been also reported by Loomans et al. (2018). The ALDHhi-derived POs contain insulin-producing cells that also express pancreatic progenitor markers such as PDX1, PTF1A, CPA1, and MYC. Nevertheless, these POs appeared immature and did not restore normoglycemia in diabetic mice (Loomans et al. 2018).

10.2 Human Pancreatic Ductal-Cells (PANC-1) and Non-endocrine Pancreatic Cells

It was recently demonstrated that de-differentiation of human pancreatic ductal-cells (PANC-1) promotes their differentiation into endocrine pancreatic cells (Donadel et al. 2017). Formation of POs expressing pancreatic markers such as C-peptide, insulin, pancreatic and duodenal homeobox 1 (PDX-1), Nkx2.2, Nkx6.1 was obtained from PANC-1 cells. The expression of glucagon, somatostatin, and Glut-2 was also observed. This was associated with a decrease in the expression of PANC-1 markers (Cytokeratin-19, MUC-1, CA19–9), supporting their pancreatic commitment (Donadel et al. 2017). However, additional studies are required to evaluate their potential use to generate functional and safe POs.

10.3 Biliary Tree-Derived Islet Progenitors

Recently, a new source of islet precursors has been identified in a niche located within the human biliary trees (Wang et al. 2013). This niche is characterized by a network of cells with overlapping commitment (Wang et al. 2013). The biliary tree-progenitor cells showed a high proliferation rate when cultured *in vitro*, under specific conditions (Wang et al. 2013). Moreover, these cells were able to aggregate in POs, resembling neo-islets generated from other cell sources (Wang et al. 2013). The POs generated from these progenitors showed ultrastructural, electrophysiological and functional characteristics, including the response to glucose, similar to other POs. Furthermore, the derived neo-islets were able to improve glucose control in immune-compromised diabetic mice (Wang et al. 2013).

10.4 Hepatic Stem Cell Derived Islet-Like Structures

We have recently found that adult human liver stem-like cells (HLSC, Fig. 2a), which are clonogenic and express several mesenchymal and embryonic markers, such as SSEA4, Oct4, SOX2 and nanog, were able to spontaneusly generate insulin-producing organoids (Navarro-tableros et al. 2018). This is not surprising since liver and pancreas share common embryonic origins. Charge-induced 3D aggregation of HLSC (Fig. 2b) promoted differentiation into viable insulin expressing cells (Fig. 2c and d) (Navarro-tableros et al. 2018) which contained heterogeneous secretory granules (Fig. 2e). These HLSC-derived POs were able to secrete human C-peptide in response to high glucose concentrations both in static (Fig. 2f) and dynamic conditions (Navarro-tableros et al. 2018). *In vitro* differentiated islet-like structures showed an immature endocrine gene profile but were able to further differentiate after *in vivo* implantation (Navarro-tableros et al. 2018). In fact, when transplanted in non-immunocompetent diabetic mice hyperglycemia was reverted and the human C-petide was detectable in mice (Navarro-tableros et al. 2018). As a proof of concept, after removal of implanted islet-like structures diabetes was restablished, suggesting that diabetes reversal was dependent on the transplanted islet-like structures generated by HLSC differentiation (Navarro-tableros et al. 2018).

11 Clinical Trials

MSC derived from different sources such as bone marrow umbilical cord and adipose tissue have been used in clinical trials. Although MSC did not displayed curative properties it is quite astonishing that a partial improvement of glycemia has been reported (Päth et al. 2019). To date CinicalTrials.gov listed a number of therapeutic approaches using different cell types. Among them over 850 have used MSC to target a broad variety of diseases and 60 of them for T1DM and T2DM. A trial investigating the islet graft survival and function of MSC co-transplantation has been closed (Päth et al. 2019). It has been demonstrated that MSC mediate immune tolerance enabling a partial recovery of residual *β-cell* mass and/or delaying the *β-cell* damage during the onset of T1DM (Päth et al. 2019). Some of these clinical trials using co-transplanted MSC were found to reduce the loss of islet graft, the HbA1c percentage and to increase the release of C-peptide compared to control patients during 2 years follow-up (Päth et al. 2019). However, currently the small number of patients recruited for the studies dictate the requirement of a more prolonged observational study to elucidate whether MSC could really delay the development of T1DM (Päth et al. 2019). Furthermore different strategies using ESC iPSC and MSCs are still under investigation (Welsch et al. 2018). Moreover, since cellular debris and fragments of proteins may be sufficient to prime the immune system using the encapsulation devices an effective encapsulation device should be generated to prevent activation of the immune response leading to graft rejection. It has been shown that Encaptra devices are able to normalize blood glucose in spontaneous NOD

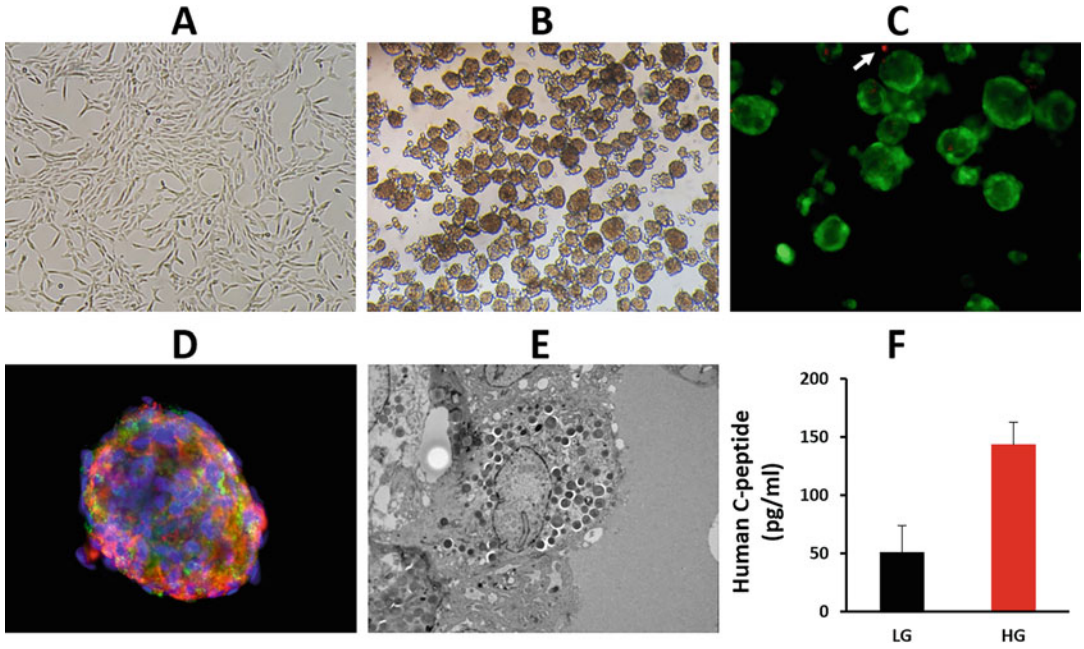

Fig. 2 Generation of POs from HLSC. (**a**) Non-differentiated HLSC showing their typical morphology. (**b**) Generation of HLSC-derived islet-like structures. (**c**) HLSC-ILS stained with fluorescein diacetate (FDA) and propidium iodide (PI) show viable (green) and dead cells (red, arrow). (**d**) Immnuofluorescence for human C-peptide (red) and Glucose transporter 2 (GLUT, green) in HLSC-ILS. (**e**) Transmission electron microscopy showing typical *β-cell* granules. (**f**) Glucose-stimulated C-peptide secretion in 2.8 mM glucose (LG) and 28 mM glucose (HG)

recipients (Faleo et al. 2016). In addition, in 2014 ViaCyte has started a phase I/II clinical trial (ClinicalTrials.gov identifier: Nbib2239354) currently ongoing. In 2017 a new Phase 1/2 clinical trial was started (STEP ONE) to test the PEC-Direct™ in patients with T1DM in San Diego (California USA) and Edmonton (Alberta Canada). More recently, the clinical trial was extended to Baltimore (Maryland USA) Minneapolis (Minnesota USA) Columbus (Ohio USA) and Vancouver (British Columbia Canada) to test the safety and efficacy of the ViaCyte's PEC-Encap (a.k.a. VC-01™) (ClinicalTrials.gov identifier: NCT03163511). The innovation consists in the use of a new open device that allows a direct vascularization of pancreatic progenitor cells (PEC-01) and avoids the use of immunosuppressive drugs. These studies are still ongoing but they could provide important information on safety and effectiveness for the use of stem cell-derived *β-cells* in humans. (Clinicaltrials.gov.)

The failure to generate feasible implantable devices to be applied in diabetic setting is still a challenge for many companies. This mainly depends on the fibrotic processes, the oxygenation and the diffusion of nutrients allowing a long-term PO viability.

12 Conclusions

In the last decades, the generation of insulin-secreting cells has gained particular attention. Different cell sources have been used and a number of studies have shown that functional insulin-secreting cells can be generated (Zhou and Melton 2018; Matsumoto et al. 2016; Ravassard et al. 2011; Boss et al. 2017; Clark et al. 1997; Scharfmann et al. 2014; Xie et al. 2016; Balboa et al. 2018; Wang et al. 2017; Russ et al. 2008; Avnit-Sagi et al. 2009; Russ et al. 2011; Russ et al. 2009; Joglekar et al. 2009; Klein et al. 2013;

Latreille et al. 2015; Carlotti et al. 2010; Zulewski et al. 2001; Gallo et al. 2007). Nevertheless, their clinical application is still missing.

Beeing organoids versatile for applications they have gained interest. In particular, the 3D conformation plays an important role in morphogenesis and tissue organization of pancreatic islets (Navarro-tableros et al. 2018; Kim et al. 2016a). Cell assembly in 3D structures facilitates cellular interactions, promotes morphogenesis and tissue organization, but also a fine modulation of hormone secretion (Orci et al. 1975; Kitasato et al. 1996; Kanno et al. 2002; Wojtusciszyn et al. 2008; Jo et al. 2009; Klee et al. 2011; Santos-Silva et al. 2012; Rorsman and Ashcroft 2018). Thereby, generation of POs, recapitulating the structure and function of native pancreatic islets, should be evaluated for long-term transplant viability. Moreover, organoid cultures are ideal to study stem cell-niche interactions in a 3D environment, and engineering POs could serve for drug studies (Scavuzzo et al. 2018).

Ideally, optimization would include feasible cell sources for a large production of functional POs. Thereby, generation of POs, mimicking the human native pancreatic islets in terms of morphology and function, is mandatory. Staminal cells isolated from a number of embryonic and adult tissues are currently used to generate functional POs (Zhou and Melton 2018; Balboa et al. 2018; Navarro-tableros et al. 2018; Kim et al. 2016a; Hinderer et al. 2016; Liu et al. 2018) (Fig. 3). Nevertheless, standardization of protocols for POs generation is still needed to achieve reproducibility and large scalability. Furthermore, effords should be directed to generate mature POs, similar to native pancreatic islets, in self-regulation of glucose metabolism. The recent gene editing technology is a challenge to generate POs from different cell types and to educate cells obtained from T1DM patients in order to generate functional POs for autologous transplantation (Giménez et al. 2016; Ma et al. 2018).

The 3D bioreactor-based culture systems have demonstrated to improve the production of functional stem cell-derived β-like cells *in vitro* (Abu-Absi et al. 2014; Hoesli et al. 2009; Sharp and Vermette 2017; Gundersen et al. 2010) (Fig. 3). Additionally, functional POs generated *in vitro*, were able to secrete insulin in response to glucose, and to restore the glycemic control when implanted in diabetic animals (Vaithilingam et al. 2011; Mason et al. 2009; Amer et al. 2015; Lee et al. 2006a; Jiang et al. 2017; An et al. 2018; Ludwig et al. 2012; Montazeri et al. 2016; Mahou et al. 2017; Faleo et al. 2016). Nevertheless, similarly to transplanted human pancreatic islets, oxygenation and activation of the host immune response are the main remaining hurdles.

To solve this problem, bioengineers and nanotechnologists have generated a number of new promising encapsulation approaches (Gálvez-Martín et al. 2017). Currently, nanotechnology research is focused on the generation of POs with a 3D morphology by using different polymers and natural ECM as encapsulating platforms (Gálvez-Martín et al. 2017) (Fig. 3). Pre-clinical studies have demonstrated their potential application to enhance PO vascularization (De Rosa et al. 2018; Schweicher et al. 2014; Trivedi et al. 2000; Sweet et al. 2008; Mahou et al. 2017; Guo et al. 2018; Skrzypek et al. 2018a, b; Groot Nibbelink et al. 2018; Stephens et al. 2018) and to improve the immune escape (Hinderer et al. 2016; Amer et al. 2015; Rios et al. 2016; Buitinga et al. 2013; Lee et al. 2004, 2006a, b, c, 2009; Jiang et al. 2017; Bowers et al. 2018; Liao et al. 2013; Andrades et al. 2008; Lou et al. 2017; Hamilton et al. 2017; Chaimov et al. 2017). Nevertheless, different clinical trials, many of which still ongoing, were not conclusive in confirming their safety for clinical application (CinicalTrials.gov).

A number of pre-clinical cell-based approaches have been also developed and each approach has contributed to solve the most relevant challenges. Indeed, the development of pre-vascularized or oxygenated POs represent the more promising and powerful tools for translational applications (Fig. 3). Finally, even though different clinical trials are currently ongoing to evaluate the potential application in diabetes, standardization of protocols for PO generation are still required to achieve reproducibility. Improvements should also include the feasibility and safety as well as the tuning of suitable protocols to obtain fully mature POs.

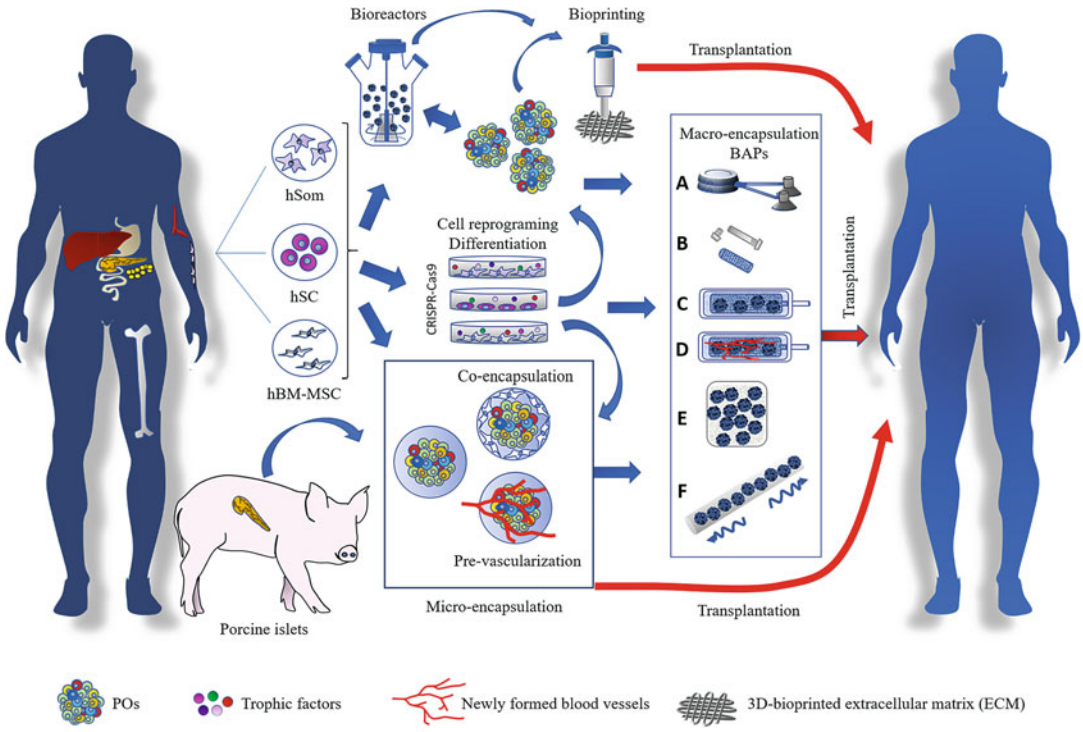

Fig. 3 Current strategies to generate 3D pancreatic organoids (POs)
A number of human cells including somatic (hSom), stem cells (hSC) and bone marrow-mesenchymal stem cells (hBM-MSC) as well as cells obtained from different embryonic or adult organs are currently used for their potential to generate *in vitro*, functional POs
In vitro cell commitment is promoted by the addition of specific trophic factors or by gene editing (CRISPR-Cas9)
Encapsulation allows oxygen, nutrients to pass through the porous membrane. Encapsulation also offers immuno-protection, promotes survival, differentiation, maturation and glucose-responsive insulin secretion. POs generated from human cells or from porcine islets (xenografts) can be macro-encapsulated and/or alginate-based micro-encapsulated to improve long-term survival.
Pre-vascularization or addition of oxygen generators are two potential approaches which could solve the reduced oxygen supply of transplanted POs
Bioartifical pancreas systems (BAPs) have been proposed to avoid the host immune attack against POs. The figure shows a schematic representation of some commercial BAP. βAir® Chamber System (**a**), Sernova's Cell Pouch System™ schematization (**b**), TheraCyte™ system schematization (**c**), VC-01TM (Pro-vascularized) System (**d**), Islets Sheet System (**e**) and Silk Fibroinc System (**f**)
3D bioprinting modalities are also used to generate ECM-containing POs. Natural or synthetic extracellular matrix (nECM, sECM) components are used for this purpose.
Bioreactors are proposed to large-scalability PO production.

Author Contributions V.N.T.: contributed to Manuscript writing and Figure preparation; Y.G.: contributed to manuscript writing and Figure preparation; M.F.B.: contributed to Manuscript editing; G.C.: contributed to Manuscript writing and editing.

Conflicts of Interest The Authors declare no conflict of interest. GC is a component of the Scientific Advisory Board of UNISYTE.

Funding This work has been supported by grants obtained by G.C. and M.F.B. from Unicyte AG.

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Adv Exp Med Biol – Cell Biology and Translational Medicine (2019) 6: 221–223
https://doi.org/10.1007/978-3-030-32823-8

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