

## Cryopreservation and Banking of Dental Stem Cells

Peer-reviewed author version

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Annelies & LAMBRICHTS, Ivo (2016) Cryopreservation and Banking of Dental Stem  
Cells. In: Advances in Experimental Medicine and Biology, 951, p. 199-235.

DOI: 10.1007/978-3-319-45457-3\_17

Handle: <http://hdl.handle.net/1942/23997>

## **Cryopreservation and banking of dental stem cells**

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**Key words:**

Dental stem cells, Mesenchymal stem cells, Paracrine effects, Multilineage differentiation, Cryopreservation, Dental stem cell banking, Good Manufacturing Practice.

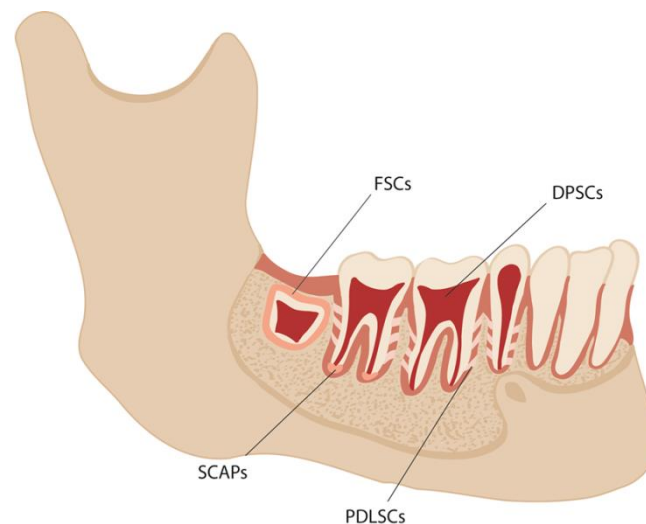
**Abstract**

Over the past decade, dental tissues have become an attractive source of mesenchymal stem cells (MSCs). Dental stem cells (DSCs) are not only able to differentiate into osteogenic, chondrogenic and osteogenic lineages, but an increasing amount of research also pointed out their potential applicability in numerous clinical disorders, such as myocardial infarction, neurodegenerative diseases and diabetes. Together with their multilineage differentiation capacity, their easy availability from extracted third molars makes these stem cells a suitable alternative for bone marrow-derived MSCs. More importantly, DSCs appear to retain their stem cell properties following cryopreservation, a key aspect in their long-term preservation and upscale production. However, the vast number of different cryopreservation protocols makes it difficult to draw definite conclusions regarding the behavior of these stem cells. The routine application and banking of DSCs is also associated with some other pitfalls, such as interdonor variability, cell culture-induced changes and the use of animal-derived culture medium additives. Only thorough assessment of these challenges and the implementation of standardized, GMP procedures will successfully lead to better treatment options for patients who no longer benefit from current stem cell therapies.

## 1. Introduction

Mesenchymal stem cells (MSCs) are derived from adult tissues and have been extensively studied as therapeutic application for tissue regeneration in a wide range of diseases. This is due to their multipotent ability to transdifferentiate into other cell types such as chondrocytes, osteocytes and cardiac myocytes, which have high potential to replace damaged tissue. Sources for MSC isolation include the bone marrow (BM-MSCs) [1], adipose tissue (ASCs) [2], Wharton's Jelly in the umbilical cord (UMSCs) [3], umbilical cord blood (UCBC) [4] and as discussed in this chapter, dental tissues.

In 2000, Gronthos et al. discovered an alternative source of MSCs, i.e. odontogenic progenitor cells, which were retrieved from the dental pulp. This finding counted as a starting point for the discovery of a heterogeneous population of dental stem cells (DSCs) which are defined by their localization in the developing tooth and its associated tissues (Figure 1). These populations comprise dental pulp stem cells (DPSCs) [5], stem cells from the apical papilla (SCAPs) [6], periodontal ligament stem cells (PDLSCs) [7] and dental follicle precursor cells (FSCs) [8]. The periodontal ligament (PDL) is believed to contain another source of DSCs residing in the epithelial cell rests of Malassez (ERM) which are present in the periodontal ligament matrix [9]. Full-grown or developing teeth are therefore a valuable source of DSCs. In addition, several studies reported that deciduous teeth can be utilized to isolate stem cells from the pulp of human exfoliated deciduous teeth (SHEDs) [10] and deciduous periodontal ligament (DePDL) [11]. DSCs originate from the neural crest [12,13] however, a large fraction of these cells are of glial origin [14].



**Figure 1:** Schematic representation of the different sources of DSCs. FSCs: dental follicle precursor cells; DPSCs: dental pulp stem cells; SCAPs: stem cells from the apical papilla; PDLSCs: periodontal ligament stem cells. Figure was adapted with permission from [15].

The application of DSCs offers several advantages over more conventionally used MSC populations, such as BM-MSCs. Not only are these cells easily obtained with minimally invasive surgery but once in culture they display a high proliferative capacity, making them a suitable candidate for cell banking. The heterogeneity of DSCs encouraged numerous researchers to explore their multilineage differentiation potential, which resulted in a broad range of differentiation protocols for various cell types. Emerging therapeutic strategies are focusing on the integration of pre-differentiated and preconditioned DSCs in the diseased host tissue exerting a beneficial effect through either intercellular contacts or via paracrine mediated interactions. Autologous as well as allogeneic transplantation of DSCs in clinical studies thus requires the need for a quality control system which provides a detailed assessment of the specific DSC phenotype and its matching paracrine profile. Upscaling of DSCs and the establishment of a dental stem cell banking system should follow standard Good Manufacturing Practice (GMP) regulations. In this chapter we will provide in-depth knowledge on the heterogeneity of DSCs and their concomitant differentiation potential *in vitro* and *in vivo*. We will focus specifically on the current strategies of cryopreservation to maintain phenotypic stability and to prevent risks of cell death and contamination. An overview of the pitfalls which could arise in creating a dental stem cell bank is also given as well as practical guidelines and prospects for application in clinical studies.

## **2. Dental stem cells**

In order to be classified as MSCs, several requirements need to be fulfilled. DSCs are characterized by the expression of the surface markers CD73, CD90 and CD105 but lack the expression of CD34, CD14, CD45 and human leukocyte antigen (HLA)-D. Moreover, they adhere to plastic and are capable of multilineage differentiation into bone-, cartilage- and fat producing cells [6,10,5,7,8,16] which classifies them as MSCs as proposed by the International Society for Cellular Therapy [17].

### **2.1 Dental stem cells: a heterogenous pool of stem cells with multilineage (trans)differentiation potential**

DSCs form a very heterogenous cell population in culture. High interdonor variability exists in terms of protein marker expression including CD31, CD117 (c-kit), Stro-1 and the low affinity growth factor receptor p75 (LANGFRp75) [18-21]. Remarkably, DSC variants have different stem cell characteristics as shown by studies that used subsets of DSCs for differentiation experiments. For example, Stro-1<sup>+</sup>/CD146<sup>+</sup> SCAPs show an enhanced colony forming unit capacity and osteo/odontogenic mineralization potential [22], a feature which was previously shown to enhance the cementoblastic differentiation of PDLSCs [7]. Stro-1<sup>+</sup> DPSCs, but also CD34<sup>+</sup>/CD117<sup>+</sup> DPSCs have been shown to enhance bone formation [23].

In addition to multilineage differentiation towards classical mesodermal lineages, DSCs have also been subjected to myogenic-, neural- and Schwann cell differentiation experiments to provide an exogenous alternative to these slowly or not regenerating cells or to aid endogenous repair. Additionally, DSCs have been differentiated towards endothelial cells (ECs) to stimulate revascularization directly or indirectly, which will be discussed in the following sections.

### ***2.1.1 Myogenic differentiation of DSCs***

Studies reporting and supporting the differentiation potential of DSCs towards myogenic cells are scarce and are mainly focused on the expression of myogenic proteins such as myosin heavy chain, titin, desmin and  $\alpha$ -smooth muscle actin. Zhang et al. were one of the first to investigate the differentiation potential of DPSCs towards myogenic lineages *in vitro* and *in vivo* [24,25]. To induce myogenic differentiation, DPSCs were kept in culture with 0.1  $\mu$ M dexamethasone, 50  $\mu$ M hydrocortisone, 50  $\mu$ g/ml gentamycin and with both fetal calf and horse serum. The differentiated cells showed immunoreactivity for myosin heavy chain *in vitro* as well as in DPSC/collagen scaffolds after transplantation into subcutaneous tissue of immunocompromised mice. Additional research by Nakatsuka et al. showed that administration of 0.5 mM 5-Aza-20-deoxycytidine induced myotube formation and myosin heavy chain expression in mouse DPSCs [26]. A similar protocol promoted myogenic differentiation in PDLSCs, which expressed desmin [27]. While DPSCs were investigated first, the myogenic differentiation potential of SHEDs was studied into more detail. Kerkis et al. reported the expression of myogenic proteins in SHEDs such as titin and actin but they also observed the formation of Z-discs [28]. Unfortunately, none of these studies performed a functional assessment of the myogenic differentiated cells and experiments were solely restricted to evaluate expression levels of myogenic protein markers.

### ***2.1.2 Endothelial differentiation of DSCs***

Due to the need for new clinically applicable revascularization strategies, the differentiation potential of DSCs towards ECs was investigated by several research groups as other MSC sources showed great promise in endothelial differentiation experiments (i.e. [29-32], reviewed in [33]). An early report described the differentiation of DPSCs towards EC-like cells through incubation of DPSCs for 40 days in high serum conditions [34]. The outcome of the study was that the majority of DPSCs differentiated towards bone-producing cells. Surprisingly, a fraction of the cells differentiated towards vascular endothelial growth factor receptor 1 (VEGFR1)<sup>+</sup>/CD44<sup>+</sup>/CD54<sup>+</sup> EC-like cells which also expressed von Willebrand factor (vWF), CD31 and angiotensin-converting enzyme. By following the protocol described in [29], Marchionni et al. were able to obtain EC-like cells from DPSCs which expressed CD54, CD34 and vWF. Furthermore, these cells were able to form tubes *in vitro* [35]. Remarkably, a CD31<sup>-</sup>/CD146<sup>-</sup> subset of DPSCs highly expressed CD34 and VEGFR2 and was able to

form extensive networks and had a high proliferative and migratory capacity. Moreover, these cells displayed functional endothelial activity as they gained capacity to take up acetylated low density lipoprotein (Ac-LDL) and release vWF upon histamine stimulation after being cultured in 10 ng/ml vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) for 14 days [36]. Similar to DPSCs, SHEDs formed tubes *in vitro* and expressed VEGFR2, CD31 and VE-cadherin after exposure to 50 ng/ml VEGF in endothelial growth medium [37]. Amorim et al. recently showed that differentiation of PDLSCs in endothelial cells resulted in expression of VEGFR2 and lectin and the formation of tube-like structures [38]. A report by Bakopoulou et al. demonstrated the endothelial-like differentiation of SCAPs under serum and oxygen/glucose deprivation as evidenced by their capillary forming capacity [39]. To date, only limited evidence for the *in vivo* endothelial differentiation potential of DSCs is available. A recent study of Zhang et al., for example, reported the presence of human CD31<sup>+</sup> blood vessels following the transplantation of DPSCs and SHED in a tooth slice model [40]. However, transplanted DSCs mainly appear to act as pericytes as they are often found in juxtaposition to host endothelium [36,41,42].

### **2.1.3 Neural differentiation potential of DSCs**

The neuroectodermal and/or glial origin of DSCs makes these cells interesting candidates to study their neurogenic differentiation potential. Although DPSCs are the most meticulously studied subtype of DSCs, the neurogenic differentiation potential of SCAPs, SHEDs, FSCs and PDLSCs has also been a focus of investigation. Remarkably, DSCs are able to differentiate both towards neuronal-like cells and peripheral glial cells with Schwann cell-like properties. Multiple approaches have been envisaged to differentiate DSCs towards neuronal cell lineages. The two most often used methods *in vitro* are either the application of neuro-inductive medium which contains specific neuronal inducing chemicals and/or cytokines or through neurosphere formation. Arthur et al. were one of the first to demonstrate that exposure of DPSCs to epidermal growth factor (EGF) and bFGF, increased the expression of neuronal-related markers such as neural cell adhesion molecule (NCAM), neurofilament-M (NF-M) and NF-H. Contrarily, the expression of early neuronal markers i.e. nestin and beta-III tubulin were markedly decreased. Moreover, patch-clamp recordings revealed that differentiated DPSCs acquired functional neuronal characteristics such as inward sodium currents but lacked action potential firing [43]. Follow-up research aimed to optimize the differentiation protocol of Arthur et al. to evoke generation of action potentials. Király et al. enhanced neurogenic induction via epigenetic reprogramming whereas Gervois et al. used neurosphere formation in the presence of EGF and bFGF signaling. Both procedures were followed by a maturation period based on cyclic AMP and growth factor signaling [44,45]. The outcome of these studies was similar to the study of Arthur et al. showing decreased expression of nestin and a marked increase in expression of neuronal markers. In addition, these studies revealed the existence of tetrodotoxin- and tetraethylammonium-sensitive sodium and potassium currents. The study of Gervois et al. was also able to demonstrate the

generation of a single action potential. Other studies which focused on the neurogenic differentiation potential of DPSCs used neurosphere formation [46] or commercially available differentiation media [47].

Neurogenic differentiation of SHEDs is based on neuronal induction with EGF and bFGF [48], sonic hedgehog, bFGF and FGF-8 [49,50] and/or neurosphere formation [51,50]. Jarmalaviciute et al. were able to differentiate SHEDs towards peripheral sensory neurons after neuronal maturation by exposing the neuronally induced SHEDs to elevated cyclic AMP levels, glial derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). PDLSCs were also differentiated towards neuronal cells with commercially available medium [47], EGF and bFGF [52] supplemented with glucose, progesterone, insulin, transferrin and selenite [53] or neurosphere formation [54]. SCAPs were differentiated towards neuronal cells by Lee et al. [47] with commercially available medium while both SCAPs and FSCs could be differentiated by neurosphere formation [55,51]. In addition to directed differentiation towards neuronal cells, DPSCs were successfully differentiated towards Schwann cells with a multistep protocol. The differentiated cells expressed the glial markers CD104, GFAP, p75 and laminin but lacked nestin expression. Moreover, these cells were able to myelinate and guide neurites of dorsal root ganglia *in vitro* [56]. PDLSCs from dogs were differentiated towards Schwann cell-like cells by Li et al. using different protocols [57]. These differentiated cells expressed GFAP and S100, but also nestin. SHEDs were differentiated towards Schwann cells after exposure to a mixture of growth factors such as bFGF, EGF, BDNF, GDNF, NGF and cyclic AMP.

## **2.2 DSCs as an ambulant growth factor delivery system: paracrine mediated actions of DSCs**

In addition to direct cell replacement strategies, DSCs have the potential to stimulate endogenous repair mechanisms through paracrine interactions. Current research is focusing on the pro-angiogenic, neuroregenerative and neuroprotective properties of DSCs which are all attributed to their rich secretome. To induce angiogenesis, pro-angiogenic growth factors promote ECs to migrate, proliferate and form hollow tubes. Each of these prerequisites are evaluated *in vitro* on commercially available EC-lines such as human umbilical cord vein endothelial cells (HUVECs) or human microvascular endothelial cells (HMECs). The secretome of DSCs contains a significant amount of pro-angiogenic growth factors including platelet derived growth factor, angiopoietin, bFGF, colony stimulating factors, VEGF and endothelin-1 [58-61]. On the other hand, DSCs also secrete anti-angiogenic proteins such as plasminogen activator inhibitor-1, endostatin, thrombospondin-1, tissue inhibitor of matrix metalloproteinase -1/4 and pentraxin-3 [58,59]. Iohara et al. used the secretome of CD31<sup>+</sup>/CD146<sup>+</sup> DPSCs to investigate the proliferative effect on HUVECs and they showed a significant increase in HUVEC proliferation [36]. However, when HMECs were used, the same effect



could not be repeated with the secretome of either DPSCs, SCAPs or FSCs [58]. The second most commonly investigated paracrine effect of DSCs on EC function is the migration of ECs along a gradient of chemotactic proteins. Endothelial migration, evaluated by means of a transwell assay, was found to be significantly stimulated by DPSCs and SCAPs, whereas FSCs had no apparent effect [58,39]. The tube formation assay is a third assay which evaluates the influence of angiogenesis-promoting substances. Tube-promoting effects have been observed for all DSCs, excluding FSCs, when they were in direct co-culture with ECs [41,60,62]. Although these results support the pericyte-like role for DSCs in angiogenesis, paracrine mediated tube formation has also been observed. An indirect co-culture of DPSCs and HUVECs demonstrated tubular network formation [63]. Similar results were achieved when HMECs were incubated with the secretome of DPSCs [58] or HUVECs with the secretome of SCAPs [39] or SHEDS [64].

Preconditioning of DSCs with hypoxia-mimicking agents such as deferoxamine (DFO) or oxygen/glucose deprivation are current strategies to enhance the *in vitro* angiogenic properties of DSCs. Hypoxic preconditioning enhanced VEGF expression but did not affect DPSC proliferation. By applying the secretome of hypoxic preconditioned DPSCs to HMEC cultures, proliferation and sprouting of HMECs was evident [65]. Bakopoulou et al. demonstrated that deprivation of SCAPs from nutrients and oxygen induced the secretion of pro-angiogenic growth factors, which had an additional positive influence on EC migration and tube formation [39]. Moreover, oxygen and nutrient deprivation also seemed to enhance SCAP differentiation towards EC [39,66]. Hypoxic preconditioning of PDLSCs increased VEGF and IL-6 secretion. Hypoxia-mimetic agents have also been used to precondition DSCs. PHD inhibitors such as cobalt chloride (CoCl<sub>2</sub>), DFO, L-mimosine and dimethyloxalglycine promoted the secretion of VEGF and increased the expression of HIF-1 $\alpha$  in DPSCs [67], PDLSCs [68] and SCAPs [62]. When SCAPs and HUVECs were treated with CoCl<sub>2</sub>, increased tube formation was observed [62]. Similar to PHD inhibitors, the iron chelator hinokitiol upregulated HIF-1 $\alpha$  expression and VEGF secretion in DPSCs. Moreover, the conditioned medium of these hinokitiol-exposed DPSCs enhanced angiogenesis *in vitro* and *in vivo* [69]. Together these data indicate a promising future for the use of hypoxia and hypoxia-mimicking agents to increase the angiogenic properties of DSCs.

Similar to the paracrine mediated effect of DSCs on angiogenesis, the paracrine effects of DSCs on neuroprotection, neurogenesis and neuritogenesis have been evaluated. Human DSCs display a rich secretome including BDNF, NGF, neurotrophin-3 (NT-3) and GDNF, which are considered hallmark factors involved in neurotrophic and neuroregulatory signaling pathways [45,56,70,64,61]. Moreover, DPSCs and SCAPs secrete a significant higher amount of these factors *in vitro* compared to bone-marrow and adipose-derived MSCs [71,61]. The DPSC-secretome has been shown to enhance the survival of axotomized retinal ganglion cells and stimulate their neurite outgrowth [71]. DPSCs themselves and their secretome were able to protect astrocytes from ischemia [72]. Apel et al.

observed a neuroprotective effect of DPSCs when co-cultured with amyloid beta peptide 1–42 and 6-hydroxy-dopamine (6-OHDA) treated neurons which are *in vitro* models for Alzheimer's and Parkinson's disease respectively [73]. Furthermore, neuroprotection by DPSCs was observed in cultures of dopaminergic neurons treated with either 6-OHDA [74] or a combination of MPP<sup>+</sup> and rotenone [75]. Finally, the secretome of DPSCs and SHEDs enhanced Schwann cell survival, proliferation and migration *in vitro* [76,64].

## **2.3 Immunomodulatory properties of DSCs**

DPSCs have the ability to modulate the immune response [77,78]. Demircan et al. showed that the suppressive actions of DPSCs on T-cells were mediated via paracrine effects by means of a transwell and mixed lymphocyte reaction (MLR) assay. Increased levels of hepatocyte growth factor (HGF), HLA-G, transforming growth factor beta (TGF- $\beta$ ), CD54, IL-6, IL-10, CD106, and VEGF were found in DPSC/T-cell co-cultures. Moreover, in the transwell system, the expression of pro-inflammatory cytokines by T-cells such as interferon-gamma (IFN- $\gamma$ ), IL-2, IL-6 receptor, IL-12, IL-17A and tumor necrosis factor- alpha (TNF- $\alpha$ ) were decreased whereas the expression of the anti-inflammatory cytokine inducible protein-10 was upregulated. Interestingly, DPSCs induced the expression of the regulatory T-cell markers CD4, CD25 and Foxp3. Apoptosis of T-cells was increased after 24h incubation with DPSCs [79]. A similar paracrine mediated immunosuppression was exerted by the secretome of porcine and human DPSCs [21,80,81]. Additional insight into the immunomodulatory properties of DSCs has arisen from a study by Wada et al. who showed that PDLSCs suppressed peripheral blood mononuclear cell (PBMCs) proliferation after stimulation with a mitogen or in a MLR. Moreover, they were able to demonstrate that IFN- $\gamma$ , produced by activated PBMCs, was partially responsible for the immunomodulatory effects of PDLSCs. PDLSCs cultured in the presence of activated PBMCs increased the expression of HGF, TGF- $\beta$  and indoleamine 2,3-dioxygenase (IDO) [82]. In addition to these factors, Fas ligand (FasL) also appears to play a role in DSC-mediated immunosuppressive effects, as knockdown of FasL reduced the capacity of DPSCs to induce T-cell apoptosis [83]. Similarly, Toll-like receptor 3 (TLR3) agonists improved the immunosuppressive effect of DPSCs and FSCs, by upregulating TGF- $\beta$  and IL-6 production. Interestingly, TLR4 agonists only improved the immunosuppressive effect of FSCs and completely abolished the effect of DPSCs by decreasing TG- $\beta$  and IDO production [84]. Ding et al. confirmed that TGF- $\beta$  is a key regulatory molecule in the immunomodulatory properties of DPSCs [85]. SCAPs were also found to secrete immunomodulatory proteins although in lower levels compared to bone-marrow derived MSCs [61].

## **3. Applications in regenerative medicine**

Increasing evidence indicates that stem cells represent a potential therapeutic tool for tissue engineering and regeneration in a wide variety of diseases and disorders [86,87]. The main goal of

stem cell-based therapies in regenerative medicine is to replace, repair or to enhance endogenous repair processes of injured tissues and organs [88,89]. Until now, only limited stem cell-based therapies have been established as a clinical standard, including hematopoietic stem cell transplantation for leukaemia and epithelial stem cell-based interventions for burn wounds and corneal disorders [90,91]. DSCs have gained great interest during the last decade because of their accessibility, (trans)differentiation potential and high proliferative capacity. Elaborate preclinical research has highlighted the underlying biological and cellular properties of different DSCs, as well as their potential application in the treatment of medical conditions [92-97], such as myocardial infarction [98], ischemic disease [99], nerve injury and other neurodegenerative disorders [70,47,100-105,50], inflammatory diseases [106,107], diabetes [108-112], muscular dystrophy [113,26,114], bone/cartilage defects [115-119], hair follicle loss [120], skin injuries [121], salivary gland defects [122], corneal epithelial defects [123-126], and the regeneration of dental tissues [127-130,15,131,132].

### **3.1 Dentin, pulp and periodontal tissue regeneration**

The main applications for DSCs are in the regeneration of damaged tooth-related structures such as dentin, pulp and periodontal ligament [128-130,15,131-135]. A significant number of studies in animal models have accomplished regeneration of dentin/pulp tissue and of the cementum/periodontal complex by applying DSCs in combination with an appropriate scaffold or supporting matrix [5,7,136,134,137]. DPSCs have a potent dentinogenic potential and they promote repair and reconstruction of a dentin-pulp-like complex when they are transplanted alone or in combination with growth factors (e.g. BMP-2) in the pulp cavity [138,139]. Several growth factors such as TGF- $\beta$ , bFGF and dentin matrix protein 1 (DMP1) can induce odontoblast differentiation of DPSCs [140], and Almushayt et al. demonstrated that these differentiated DPSCs form reparative dentin that covers pulp tissue [141]. Prescott et al. transplanted dentin slices containing DPSCs, collagen and dentin matrix protein 1 subcutaneously in nude mice, which resulted in the formation of a well-organized matrix similar to pulp tissue [142]. Tran and Doan recently achieved regeneration of dentin-like tissue in vivo by culturing human DPSCs onto dentin-derived scaffolds [143]. In line with these findings, Huang et al. reported that a vascularized dentin-pulp-like complex can be regenerated de novo in an emptied root canal space by DPSCs and SCAPs after subcutaneous transplantation in mice [132]. Additionally, also SHEDs combined with a scaffold formed dental pulp-like structures within human tooth slices [144]. Furthermore, an ongoing phase 1 clinical trial aims to repair immature permanent teeth with necrotic pulps by using SHEDs (ClinicalTrials.gov NCT01814436).

New therapeutic strategies for periodontal tissue diseases (e.g. periodontitis) focus on the addition of exogenous growth factors (e.g. bone morphogenetic proteins, platelet derived growth factor) and stem cell therapy [128,145]. Extensive research studies have explored the use of PDLSCs to improve periodontal tissue regeneration. PDLSCs showed the best capacity to regenerate periodontium compared to other DSC populations [146,147]. Transplantation of autologous PDLSCs

combined with tricalcium phosphate/hydroxyapatite (TCP-HA) into periodontal defects improved bone formation and regeneration of cementum and periodontal ligament [148,149]. Seo et al. also transplanted PDLSCs loaded onto TCP-HA into immunocompromised rodents, which resulted in the formation of a cementum/periodontal ligament-like structure and contributed to periodontal tissue repair [7]. Moreover, PDLSCs applied together with titanium dental implants improved periodontal ligament regeneration in a rat molar implant model [150], but also in humans [151]. In addition to the abovementioned animal models, a retrospective pilot study performed in humans provided evidence that autologous transplantation of periodontal ligament stem/progenitor cells might provide therapeutic improvement for periodontal defects without adverse effects [152]. Apart from PDLSCs, also implantation of DPSCs into periodontal defects of dogs results in bone formation with neovascularization [153,154]. A clinical study is currently ongoing in chronic periodontal disease patients which receive a local injection of allogeneic human DPSCs in order to improve periodontal tissue regeneration (ClinicalTrials.gov NCT02523651).

The ultimate goal in dentistry is the regeneration of a functional and living tooth. Recent advances in DSC biotechnology explored the use of stem cells and scaffolds to engineer teeth with the appropriate functional properties. This approach represents a promising therapeutic strategy for the replacement of a diseased or damaged tooth [155,156,133]. Sonoyama et al. transplanted both human SCAPs and PDLSCs with TCP-HA as a carrier in a minipig model, resulting in the formation of a viable root/periodontal complex formation that is capable of supporting a porcelain crown [155]. Moreover, Khorsand et al. succeeded to regenerate periodontium using autologous DPSCs loaded onto Bio-Oss scaffolds in dogs that received an experimental defect [157]. Furthermore, Nakashima and coworkers were able to induce whole-pulp regeneration after pulpectomy in a dog model using a combination of autologous DPSCs and stromal cell derived factor-1 loaded onto a 3D collagen scaffold [158,130]. Implantation of FSCs combined with a treated dentin matrix in the alveolar fossa of rats also generated root-like tissues with a pulp–dentin complex and a periodontal ligament that connected the cementum-like layer to host alveolar bone [159,160].

### **3.2 Osseous regeneration**

Tooth-derived stem cells have also been extensively used in several preclinical studies of osseous regeneration, such as craniofacial and alveolar bone healing [161,92]. DSCs represent a promising cell-based therapy for repair of bone-related diseases and orthopedic surgeries due to their osteoregenerative capacities. Especially DPSCs are considered to be a potent cell source to enhance bone regeneration [115]. Because of their ectomesenchymal origin, DPSCs express bone-specific markers and they exhibit an osteogenic differentiation profile [34,162]. Moreover, DPSCs can differentiate into (pre)osteoblasts and they deposit extracellular matrix that forms mineralized woven bone [163-166]. Graziano et al. showed that CD34<sup>+</sup> DPSCs transplanted subcutaneously in rats forms several bone nodules [167]. Another study in rats demonstrated that human DPSCs promote repair of a

large-scale cranial bone defects [115-117]. Clinical studies performed by d'Aquino et al. demonstrated that a construct composed of DPSCs and a collagen sponge scaffold promotes bone repair following oro-maxillofacial defects [168]. Several studies have indicated that DPSCs gain osteogenic differentiation potential when combined with different biomaterials, such as collagen and titanium [169-173]. Another important factor that stimulates the osteogenic properties of DSCs is bone morphogenetic protein-2 (BMP-2) [174,175,23]. Liu et al. showed that DPSCs expressing BMP-2 have better mineralization capacities, and they generate more bone after alveolar bone defects in a rabbit model [176]. Other DSC populations, such as PDLSCs and SHEDs also represent a promising tool for bone regeneration [177-180]. As reported by Seo et al., SHEDs combined with TCP-HA can repair critical-sized calvarial defects in mice with substantial new bone formation [180]. In line with these findings, Zheng et al. demonstrated that stem cells from miniature pig deciduous teeth were able to engraft and induce bone to repair critical-size mandibular defects [181]. A clinical trial is currently ongoing that uses SHEDs for alveolar bone tissue engineering for cleft lip and palate patients (ClinicalTrials.gov - NCT01932164). Another study showed that human PDLSCs expanded *ex vivo* and seeded in three-dimensional scaffolds (e.g. fibrin sponge) also generated new bone [147]. When SCAPs are combined with HA scaffolds and implanted subcutaneously in immunocompromised rats, both bone- and dentin-like mineralized tissues are formed [182].

### **3.3 Neural tissue regeneration**

Several DSC populations such as DPSCs and PDLSCs originate from ectomesenchyme, indicating its interaction with the neural crest during embryonic development [183-186]. This implicates that DSCs may possess distinct functions compared to other MSC populations (e.g. bone marrow-derived stem cells) [183,187]. As already mentioned, DSCs also have potent neural characteristics besides their classical MSC-properties. This indicates that DSCs may have a therapeutic benefit for the treatment of neurological diseases and disorders. Studies describing the effect of DSCs in animal models of central- or peripheral nervous system pathology are scarce and mainly focused on DPSCs. Arthur et al., pioneers in the use of DPSCs for neuronal regeneration, showed that DPSCs acquired a neuronal morphology, expressed neuronal markers and were able to attract trigeminal neurons after transplantation into chicken embryos [43,188]. Moreover, neurogenic pre-differentiated DPSCs were able to migrate to the host brain after injection in the cerebrospinal fluid where they integrated in the host circuitry [189]. The therapeutic effect of DPSCs is also studied in various animal models of neurological dysfunction. These animal models include hypoxic ischemic encephalopathy [100], spinal cord injury [101] and ischemic stroke [190], in which DPSCs ameliorated the outcome of the disease. Preclinical evidence in a rodent model of middle cerebral artery ischemic stroke indicates improvement in neurobehavioral function with adult human DSC therapy [190-192]. Recently, the “first-in-human” autologous DPSC therapy clinical trial, TOOTH – The Open study Of dental pulp stem cell Therapy in Humans, was initiated and it evaluates the safety

and feasibility of autologous human adult DPSC therapy in patients with chronic disability after stroke [193]. SHEDs have also been successfully applied in heatstroke animals [194], cerebral ischemia [102], perinatal hypoxia/ischemia [103], spinal cord injury [104,105] and Parkinson's disease [50] with an improved disease outcome after transplantation. Research of PDLSCs in *in vivo* models is lacking, but Bueno et al. were able to observe engraftment of PDLSCs in the host brain after transplantation [53]. FSCs were used in a spinal cord injury model after being seeded on aligned poly( $\epsilon$ -caprolactone)/ poly-DL-lactide-co-glycolide (PCL/PLGA) fibers which supported nerve fiber outgrowth. Unfortunately, no functional improvement was observed after transplantation of these constructs [195]. In addition, SCAPs were transplanted in a hydrogel or in the apical pad of a rat SCI model [196]. Remarkably, functional improvement was only observed in the experimental group which received the entire tissue graft [197]. The proposed mechanism of disease amelioration by the transplanted cells in these studies was mainly based on paracrine effects of the transplanted cells which stimulated proliferation and differentiation of endogenous neural stem cells [198,102] or stimulated host repair mechanisms [101,100,190,102].

*In vivo* studies that apply DSCs for peripheral nerve injury were previously limited to reports that used artificial tubes seeded with DSCs in animal models of facial nerve injury. Sasaki et al. indicated that rat DPSCs promoted remyelination, blood vessel formation and normal nerve regeneration when applied in combination with silicon or PLGA tubes as nerve conduits. Paracrine factors secreted by the DPSCs were believed to be responsible for this effect [199,200]. Later, Sasaki et al. showed that a silicon tube containing a DPSCs/type I collagen gel mixture improved the electrophysiological and functional characteristics of the facial nerve, with a similar outcome than nerve autografts [201]. More recently, the paracrine effects of DPSCs and SHEDs on Schwann cells and peripheral nerve function were evaluated in a sciatic nerve model [76,64]. The study by Yamamoto et al. showed that transplanted DPSCs promoted regeneration of myelinated fibers with a larger axon-to fiber ratio (G-ratio) and revascularization. Direct differentiation into Schwann cells was not observed, but endogenous Schwann cells showed reduced apoptosis and increased proliferation [76]. Similarly, Sugimura-Wakayama demonstrated that silicon conduits containing the SHED-secretome significantly increased the number of myelinated axons and G ratio. Moreover, the sciatic functional index and gastrocnemius wet weight ratio suggested reinnervation of the target muscle with functional recovery [64].

### 3.4 Diabetes

Another important research area in which DSCs may be successful is diabetes, a chronic endocrinal disease that is associated with pancreatic islet dysfunction. Transplantation of insulin-producing pancreatic islet cells represents an important therapeutic strategy for this condition. Several preclinical studies indicate that DSCs can differentiate towards a pancreatic cell lineage [108-112]. Govindasamy et al. showed that differentiated DPSCs resemble islet-like cell aggregates, which

release insulin and C-peptide in a glucose-dependent manner [108]. Other studies confirmed these findings, and transplantation of islet-like cell clusters derived from human DPSCs and SHEDs restored normoglycemia in diabetic mice [109,112,111]. As shown by Lee et al., PDLSCs can also transdifferentiate into functional pancreatic islet-like cells and therefore represent an alternative stem cell population for pancreatic repair [110]. These preclinical studies implicate that DSCs offer a source of human tissue that can be used as an autologous stem cell therapy for diabetes in order to improve islet regeneration.

### **3.5 Angiogenesis - a role in myocardial infarction and ischemic disease**

As DSCs display a pronounced angiogenic potential, they are also utilized in clinically relevant disease models, such as myocardial infarction (MI), hindlimb ischemia and a pulpectomized tooth model. Intracardial injection of GFP-positive DPSCs in a rat model of MI resulted in increased capillary density, a reduction in infarct size and a reduced thickening of the anterior ventricular wall. Interestingly, these effects were thought to be caused by paracrine effects of the transplanted DPSCs, as no GFP-positive cells were observed in the tissue [98]. The study by Iohara et al. in which a subfraction of CD31<sup>+</sup>/CD146<sup>+</sup> porcine DPSCs was injected in a mouse model of hindlimb ischemia, demonstrated a higher capillary density. The transplanted cells were found in proximity to the newly formed vessels, supporting the pericyte and paracrine function of DPSCs [36]. Interestingly, the same subpopulation of DPSCs was capable of increasing vasculogenesis and neurogenesis after transplantation in a rat model of focal cerebral ischemia [21,192]. Finally, vasculogenesis was enhanced in a pulpectomized tooth model after activation of chemotaxis in DPSCs by granulocyte-colony stimulating factor [80,202].

## **4. Cryopreservation of dental stem cells**

As stated above, DSCs are an attractive source of mesenchymal stem cells for multilineage differentiation and regenerative medicine. However, long-term culture of these stem cells may be accompanied with deleterious effects such as phenotypic instability, cell death, senescence or contamination [203]. Therefore, adequate long term storage procedures are required that not only keep the cells viable, but also safeguard the phenotypic stability and differentiation capacity. The advances made in rapid growing fields such as (stem) cell therapy, personalized medicine and cancer research, further drive the need to overcome this hurdle in order to move forward towards clinical applications [204,205]. For allogeneic patients, cryopreservation allows the transport of cellular products and provides a time window to screen the cells prior to transplantation. For autologous patients, cells can simply be stored for later clinical use [206]. Currently a vast number of protocols are being tested for the cryopreservation of DSCs, each with their own criteria to evaluate the potential effect on the intrinsic behavior of these stem cells (Table 1).

During cryopreservation, the ultra-low temperatures bring an indefinite halt to the cell's metabolism. However, cryopreservation represents a physical insult on the cells and is currently only effective for single cell suspensions and a few simple tissues [205,204]. Since cryopreservation aims at preserving living cells, it is essential to design protocols that minimize the injuries that are associated with the freeze-thaw process in order to ensure maximum recovery of viable and functional cells [206]. One of the most important aspects of freezing cells is the rate of cooling. When cells are cooled too slowly they will dehydrate and shrink due to osmotic stress, whereas rapid cooling results in intracellular ice formation [206,207]. However, while intracellular ice formation is lethal to cells in suspension, it could be innocuous to cell monolayers due to its propagation via gap junctions. When exposed to 100% intracellular ice formation, DPSC monolayers have been shown to retain membrane integrity, however they lost the ability to proliferate [208]. Despite the fact that different cell types have a different membrane permeability, cooling rates of 1°C/min are often applied for several mammalian cell types [206,205]. Besides carefully controlling cooling rates, media can also be supplemented with cryoprotectant agents in order to minimize cryoinjury. Cryopreservation solutions for MSCs are most commonly supplemented with 10% dimethyl sulfoxide (DMSO) [209,205]. DMSO is a permeating cryoprotectant as it is able to penetrate the cell membrane, thereby preventing cell rupture. Several DMSO-based cryopreservation protocols have been tested for the preservation of DPSCs [203,210,211,204,212], SCAPs [213] and PDLSCs [214] (Table 1). Woods et al. reported that 7.8 to 11.6% DMSO was optimal for DPSCs cooled at -1°C/min in an isopropanol bath to -85°C for 24 h followed by storage in liquid nitrogen [212]. DPSCs have been shown to retain their stem cell markers [203,210,204,212] as well as their multilineage differentiation potential post thawing [203,25]. Ding et al. reported no difference in cell viability, colony forming efficiency, proliferation rate, multilineage differentiation potential, MSC marker expression, karyotype anomalies and immunomodulatory capacities between freshly isolated SCAPs and cryopreserved (10% DMSO + 90% fetal bovine serum (FBS)) SCAPs [213]. Cryopreservation of PDLSCs using 10% DMSO did not affect their proliferative capacity [214].

Despite the widespread use of DMSO as a cryoprotectant, it is known to be potentially cytotoxic. DMSO-preserved bone marrow cells have been shown to cause adverse effects post transplantation [215-217]. Since MSCs have shown great resilience during cryopreservation many other strategies have been explored to reduce cryoprotectant toxicity, minimize cryoinjuries and to develop xeno-free freezing solutions [206]. Hoping to lower or even completely remove DMSO from the freezing solutions, several other compounds have been investigated. Boron is a micro-mineral that is involved in membrane integrity and is important for membrane structure and function and could therefore minimize/inhibit intracellular ice formation. Demirci and colleagues reported an increased viability when tooth germ derived stem cells derived from a single donor were preserved in a solution containing 20 µg/mL borate and 5% DMSO. Furthermore, this solution did not affect surface antigen



expression nor did it alter the osteogenic and chondrogenic differentiation potential of these cells [218]. Umemura et al. developed a DMSO-, serum- and xeno-free cryopreservation method by encapsulating DPSCs in an alginate gel. Cryopreserved DPSCs displayed a normal morphology, showed high proliferative capacity and maintained MSC marker expression [219]. Furthermore, Ducret and colleagues recently described a medicinal manufacturing approach for producing DPSCs. In this study, DPSCs were cultured under xeno-free conditions and were cryopreserved for 510 days. Cryopreserved DPSCs were reported to be free of karyotype abnormalities and to have stable doubling times, comparable to those of fresh DPSCs [220].

Using a freezing protocol involving a magnetic field, DPSCs can be frozen in a serum-free freezing solution containing only 3% DMSO [221]. Magnetic cryopreservation (Cells Alive System, CAS) was originally designed by the ABI Corporation for the preservation of food, but has now found its way into the scientific world as well. By using magnetically induced non-thermal vibrations this freezing method prevents the formation of ice crystals [222] (Table 1). Within the field of dentistry, tooth auto-transplantation is a useful technique for replacing missing teeth with several advantages such as maintaining bone volume and PDL regeneration capacity as well as allowing dentofacial development [223]. It occurs that patients no longer possess a suitable donor tooth, due to prior extractions. In these cases tooth banking could greatly expand the usage of auto-transplantation [224] (Table 1). The survival and regeneration of the PDL is crucial for the success of the transplantation and the prognosis. Kaku et al. reported an increased survival rate of PDL cells after they were frozen with a magnetic field compared to those without a magnetic field. A magnetic field of 0.01 mT, hold time of 15 min and plunging temperature of -30°C were determined as the optimal cryopreservation parameters for PDL cells [224]. Furthermore, no difference in mRNA expression or collagen type I was demonstrated and only a minor decrease in alkaline phosphatase was reported between cryopreserved PDL cells and control groups [222]. More importantly, there was no progressive root resorption after re-implantation of cryopreserved teeth into Wistar rats, in contrast to the widespread root resorption and ankyloses in dried teeth [222]. When intact teeth were frozen for five years using the CAS freezer, Abedini et al. showed no difference in collagen type I, alkaline phosphatase and VEGF both at mRNA level as well as at protein level [225]. Moreover, a clinical case illustrated PDL regeneration one month after the transplantation of a third molar which was cryopreserved for 3 months. Sixteen months post-surgery there was sufficient bone regeneration and complete apexogenesis was achieved [225]. Abedini and colleagues also investigated dental pulp cells isolated from either mature or immature fresh third molars or mature or immature 3-month-cryopreserved molars. Cryopreservation resulted in a delay in cellular outgrowth and pulp cells from cryopreserved molars with complete root formation could not survive properly until confluence was reached. These effects could probably be ascribed to the difficulty of the cryoprotectant to reach the pulp chamber

through the narrow tooth apex. Dental pulp cells from cryopreserved immature teeth showed a decreased protein expression of VEGF and NGF compared to their fresh immature counterparts [225].

As demonstrated by the case study of Abedini et al., the field of dentistry would greatly benefit from the ability to cryopreserve intact teeth or at least dental tissues prior to stem cell isolation (Table 1). Studies investigating cryopreservation of teeth have mainly focused on the transplantation of permanent teeth and therefore questioned the survival of the periodontal ligament and pulp tissue of the cryopreserved teeth [225-228]. Oh and colleagues reported no significant difference in the viability and osteogenic differentiation capacity of PDL cells from 1-week-cryopreserved teeth [226]. Min et al. found a decrease in bFGF receptor expression in PDL cells isolated from 1-week-cryopreserved teeth [227]. However, after cryopreservation of intact teeth, Woods et al. could only establish DPSC cultures from 20% of the frozen teeth [212]. The low yield could be correlated to the dimensions of the apical foramen, as Temmerman et al. obtained maximal pulp viability after cryopreservation of teeth with an apical opening of at least 9.42 mm<sup>2</sup> [229]. For this reason, cryopreservation of pulp tissue could offer a suitable alternative. DPSCs isolated from cryopreserved pulp tissues showed no difference in CD marker expression and no loss in osteogenic and adipogenic differentiation potential compared to their fresh counterparts [230]. Gioventu and colleagues tried to circumvent the issue of a limited apical foramen by laser piercing deciduous teeth prior to cryopreservation and found that DPSCs from cryopreserved teeth showed similar growth rates and marker expression than DPSCs isolated from fresh tissue [228]. Since deciduous teeth cannot be replanted, the ability to cryopreserve them for future stem cell isolation would greatly decrease costs and efforts associated with stem cell banking from deciduous teeth [231]. Lindemann et al. only reported a 30% isolation success rate of 1-week-cryopreserved teeth. Despite the lower culture rates and morphological aberrations, no changes in immunophenotype and differentiation potential were seen [232]. Increasing serum levels could possibly improve the culture rate after cryopreservation as Bressan and colleagues found that increasing serum concentrations from 10% to 20% resulted in a more than 50% increase in culture rate [233]. The abovementioned studies only cryopreserved teeth for a short period of time and according to Ji et al. the period of cryopreservation also plays a crucial role in the viability of the resulting pulp cells. They reported a reduction in proliferation rate and increased apoptosis when teeth were cryopreserved for over 3 months [231]. Perry et al. reported a 70% success rate of isolation from 1-month cryopreserved whole teeth [211]. Dental follicle tissue has also been shown to yield viable DSCs after 3 months of tissue storage. Tissue samples were stored in phosphate-buffered saline (PBS) supplemented with glucose, sucrose and ethylene glycol for cryoprotection. DSCs isolated from cryopreserved dental follicle showed no difference in the expression of stem cell markers or the ability for multilineage differentiation. However, cells from cryopreserved tissues did show a higher intensity of p53 protein, indicating damage from the freeze-thaw process [234]. Furthermore, upon transplantation into mandibular defects in miniature pigs, both FSCs from both fresh and

cryopreserved dental follicle significantly enhance new bone formation after 8 weeks [235]. Freezing of the intact PDL, only yielded 40% of single-colony derived PDLSCs compared to the number of PDLSC colonies recovered from fresh periodontal ligament tissue. These cells showed a high proliferation rate and maintained their multilineage differentiation potential. Moreover, upon transplantation, PDLSC derived from cryopreserved PDL generated a typical cementum/periodontal ligament-like structure [236]. The possibility of storing tissue samples for longer periods of time without significant damage to the inherent stem cell population offers great perspectives for regenerative medicine and stem cell banking.

## **5. From bench to bedside**

Stem cell research holds great promise for the development of treatment strategies for various injuries and diseases. The scope of potential stem cell therapies is expanding due to advances in stem cell research. The emerging demand of stem cell therapeutics necessitates the establishment and collaboration of centralised biobanks at an international level. Initiatives like the International Stem Cell Banking Initiative (ISCBI) and the International Society for Stem Cell Research (ISSCR) strive for the creation of such centralized biobanks to provide high-quality stem cells for therapeutic purposes in a standardized approach [237,238]. Though, this seems challenging given the heterogeneous ethical, regulatory and legal setting. In order to provide a standardized stem cell-based therapy, strict rules have to be followed regarding the different processes that are performed from isolation to transplantation [239,240]. These processes vary highly depending on the stem cell source, type and clinical application.

Standardized procedures are essential to ensure optimal reproducibility, safety and efficacy when DSCs are used for clinical applications (Figure 2). However, current manufacturing procedures for DSC-based products are not fully in line with the international guidelines for GMP for stem cell therapies. GMP requires quality control regarding donor eligibility, isolation procedure, labeling, transportation, processing, storage, lab equipment, reagents, distribution to the patient and documentation [241-243]. Briefly, the manufacturing steps of DSC-based products can be generally defined as (1) tooth extraction and pulp tissue collection, (2) DSC isolation, (3) cell culture and expansion, (4) cryobanking (5) safety control and quality testing, (6) transplantation [244]. Extraction of third molars for the isolation of DSCs is being performed in donors of different ages and with third molar in different stages of development [211,245,246,72,190]. This impairs the comparison of the experimental results and makes it difficult to provide a standardized therapy. There are no guidelines that specify the developmental stage of the tooth for the isolation of DSCs and DPSCs in particular. However, it has been suggested to use impacted third molars between Nolla developmental stage 5 (almost completed dental crown) and stage 7 (root completed for one third) in young donors, since the use of impacted molars in this developmental stage reduces the risk for contamination and avoids the

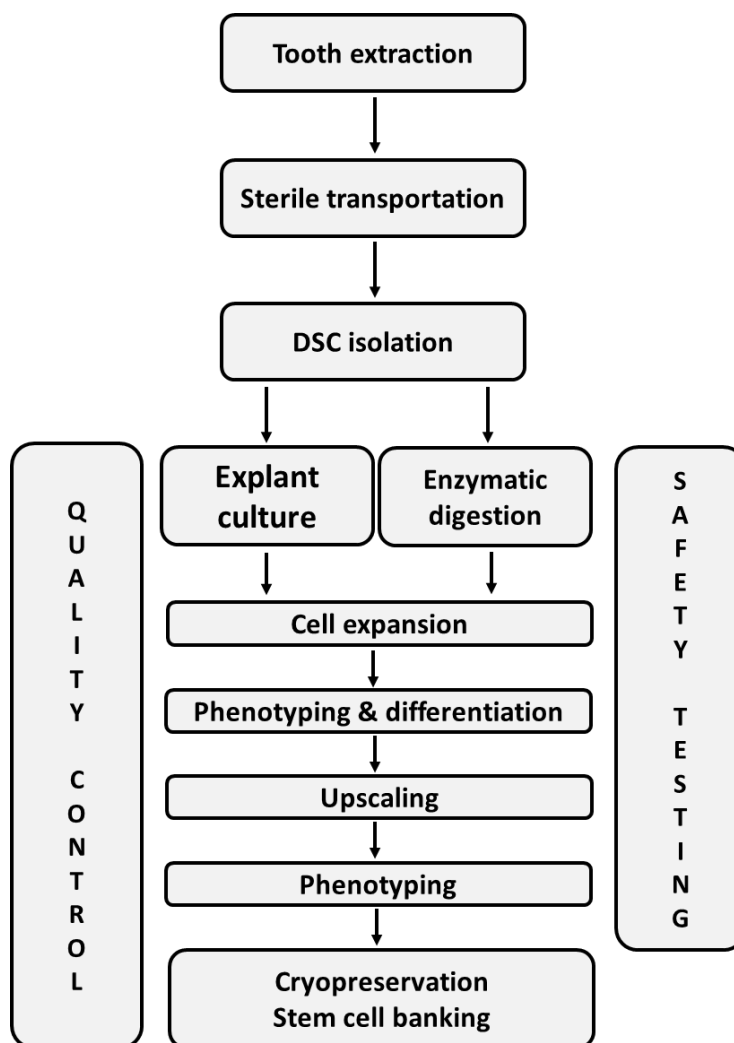
use of mechanical separation [244]. Dental pulp tissue, for example, can be collected in PBS for transportation to the lab, as DPSCs remain viable for up to 5 days in this medium [212]. For the isolation of DSCs two widely used methods are available, namely enzymatic digestion and explant culture. Considering translation to clinical applications the explant method seems to be the preferred approach. Explant culture is easier, safer, less expensive and more compliant with the GMP guidelines [246]. Cell culture and expansion is arguably the most important process in providing a cell-based therapy, as it is essential for the stem cell products to be free of any microbiological contamination. A major pitfall of standard DSC culture is the continuing use of animal culture medium additives, which will be elaborately discussed in the following section. Therefore, the development of xeno-free, serum-free media is a critical objective for standardized manufacturing of DSC-based products. Additionally, the use of xeno-free dissociation buffers such as TrypLe® or Accutase® is highly recommended for passaging the cells. Nevertheless, further research is necessary to optimize the culture properties and to ensure the phenotype of the cells is not negatively altered.

To provide an adequate amount of cells for clinical trials and commercialization of the cell-based product, efficient expansion or ‘upscaling’ is crucial for the establishment of a novel successful therapy. For autologous applications, the goal is to produce an appropriate number of cells for the treatment of one patient, mostly in the range of 1 to 5 billion cells per manufacturing process [239]. In this context, the use of conventional cell culture using culture flasks or small bioreactors is feasible. However, in the case of allogenic applications where multiple patients are targeted, the upscaling has to be performed to a much higher extent. Here, the use of cell factories with large scale bioreactors becomes inevitable. Providing a cell-based therapy often involves the storage of the cell product or an intermediate form of the cell product for future use. Cryopreservation and long-term storage of cells permits completion of safety control, quality testing and stable transportation to the clinical site [216]. Optimal cryopreservation conditions are necessary to maximize cell stability, recovery and function, as extensively discussed in the previous section. Furthermore, it is required to phenotypically characterize the cells and perform additional tests to ensure the safety of the cell-based product. Flow cytometry is an ideal technique to analyse various phenotypic markers simultaneously on the cellular level. It is advised to use a panel of markers that provides a high level of specificity for DSCs. However, this seems to be challenging since there is no unique marker for DSCs and many markers that were proposed by the ISCT to identify MSCs are also expressed in other cell types, including mature fibroblasts [247,248]. Supplementary testing of the trilineage differentiation capacity should be performed to guarantee the stem cell properties of the isolated cells.

Despite the current lack of standardization in the upscale production and long-term storage of DSCs, there are already several commercial tooth banking services available. In Japan, for example, both the Hiroshima University and Nagoya University have founded their own tooth bank. In 2008, a collaboration between the Norwegian Institute of Public Health and the University of Bergen led to the

first European tooth bank [249]. Commercial tooth banking companies can also be found in the United States, such as BioEden (Austin, Texas, USA), Store-A-Tooth (Provia Laboratories, Littleton, Massachusetts, USA) and StemSave (Stemsave Inc, New York, USA).

It has to be emphasized that all variables in the manufacturing process of DSC products have to be optimized for the specific application before continuing into clinical trials, since these can affect the properties of the cell-based product. Consequently, if the product successfully passes clinical trials, it can be produced without altering the manufacturing process.



**Figure 2:** From bench to bedside. Standardized procedure for the cryopreservation and manufacturing of dental stem cell-based products.

## 6. Pitfalls associated with dental stem cell banking

When contemplating the long-term cryopreservation as well as the upscale production of (dental) stem cells, one certainly needs to take into account certain pitfalls associated with these procedures. Although considered to be common nutritional components, the use of animal-derived culture medium additives, for example, has been the subject of debate for quite some time. Next to

animal welfare concerns and the high costs associated with the use of FBS and other animal-derived proteins, the addition of these cell nutrients to culture media has some other drawbacks as well as they are considered to be potentially hazardous [250-253]. More specifically, not only are these additives a potential source of endotoxins and other infectious agents such as bacteria, viruses, fungi and prions, but the xenogeneic antigens could also evoke a severe immunological response within the host [250-252]. Together with the high batch-to-batch variety in protein content, these disadvantages urged the need for alternative, serum-free cell culture systems [250,252,254].

With regard to the culturing of DSCs in serum-free conditions, Tarle et al. developed a chemically defined serum-free culture medium for SHEDs and PDLSCs. There were no significant differences in comparison to the stem cells cultured in FBS-containing medium; both stem cell populations were able to expand and maintained their multipotent capacity [255]. Comparable observations were made by Hirata et al. and others, showing both (sub-optimal) proliferation and expression of stem cell markers by DPSCs, PDLSCs and FSCs when cultured in serum-free media containing a range of different growth factors [256-259]. Depending on the specific growth factor supplement, however, stem cell differentiation can also be induced. Bonnamain et al., for example, indicated the expression of neuronal and oligodendrocyte markers when incubating DPSCs with EGF and bFGF [260]. Neurogenic differentiation of DPSCs was also reported by Xiao and Tsutsui, showing the expression of neural markers after using a commercially available serum replacement [261]. In addition, a CD117<sup>+</sup> subset of DPSCs differentiated into pancreatic cells following incubation with a specific combination of growth factors and chemical substances [111]. An increase in the expression of endothelial markers was also mentioned by Karbanova et al., after incubating a serum-free DPSC culture with VEGF and insulin-transferrin-sodium selenite (ITS) [262]. In the search for appropriate alternatives for animal-derived culture medium additives, numerous publications have reported on the use of human serum or other blood-derived products in stem cell cultures [263-266,252,267,254,268-271,257]. With respect to DSCs in particular, a recent study of Pisciolaro et al. indicated a significantly improved proliferation rate and mineralization potential when incubating DPSCs with human autologous serum [268]. A more prominent osteogenic and adipogenic differentiation of DPSCs following incubation with allogenic human serum was also found by Khanna-Jain et al., while Govindasamy et al. demonstrated the pronounced expansion of DPSCs in medium containing human platelet lysate [266,257]. However, the use of human blood derivatives still holds several disadvantages. As with any donor-derived product, inherent differences between samples and batch-to-batch variability cannot be excluded. Besides the extensive amount that would be needed for the upscale production of (dental) stem cells, the current lack of consistent study set-ups demands for further analysis of the precise composition as well as the potential impact of these human-derived blood products on the intrinsic properties and behavior of the stem cells [264,265,252]. Further

research is thus required before any decision can be taken by scientists as well as regulatory agencies regarding the future application of alternative culture medium additives.

Another important aspect one definitely has to keep in mind when considering the application of autologous and/or allogenic DSCs is potential donor-related variability, as the inherent behavior of these cells can be affected by a range of different factors. The pressure and tension generated during the application of orthodontic force, for example, causes the release of various growth factors and cytokines and, subsequently, the creation of a supportive microenvironment for bone remodeling, root resorption and differentiation of residing (stem) cells [272-275]. With regard to the effect of the age of the donor at the time of stem cell isolation, contrasting results are described in literature. Iohora et al., for instance, reported an age-related decline of stem cell-mediated dental pulp regeneration in dogs [276]. Similar observations were made by Feng et al. and others, showing an age-dependent decrease in both proliferation, migration and osteogenic differentiation potential of DPSCs and PDLSCs [277-280]. Atari et al., on the other hand, successfully isolated DPSCs from 14 to 60-year-old donors, with no significant differences in gene expression [281]. These results were also confirmed by Kellner et al. and others, stating that age is not a determining factor for the stem cell properties and regenerative potential of DPSCs [127,282]. Next to orthodontic tooth movement and age, the (oral) health of the patient also seems to significantly influence the biological properties of DSCs. Nicotine, for example, does not only influence their osteogenic differentiation potential, but also alters the viability, growth and migration rate of PDLSCs in particular [283-286]. In 2010, Cooper et al., already described the complex interaction between inflammation and the natural regenerative potential of dental pulp, as inflammatory mediators can modulate the repair processes within the tissue [287]. Since then, a number of papers have been published reporting on the effects of caries and/or inflammatory processes on DSCs. Alongi et al., for example, showed a decreased expression of MSC markers as well as a diminished proliferation rate and osteogenic differentiation potential *in vitro* by DPSCs originating from inflamed dental pulps. However, no notable differences were observed when comparing the formation of pulp/dentin complexes in immunocompromised mice [288]. Similar results were found by Liu et al. and others, indicating an altered proliferation and migration rate as well as a differential protein expression profile and immunomodulatory properties for carious and/or inflamed DPSCs and MSCs from periapical lesions [289-293]. In contrast, several papers mentioned no significantly different behavior of DPSCs isolated from carious deciduous and/or permanent teeth, thereby suggesting their potential use in cell-based therapies [294-296]. When considering the therapeutic application of autologous stem cells in cancer patients, the potential detrimental effects caused by previous treatment with radiotherapy certainly need to be taken into account. With regard to the effects of ionizing radiation (IR) on the behavior of DSCs, a couple of studies reported the induction of cell cycle arrest, premature senescence and differentiation of DPSCs following exposure to different dosages of IR [297,298]. In contrast, Abe et al., demonstrated a radio-resistant phenotype for SCAPs,

albeit with a lower hard tissue forming capacity in vivo [299]. Before any therapeutic application is possible, the implementation of a dental stem cell banking system not only demands extensive evaluation at scientific as well as regulatory level but also requires additional characterization of its associated pitfalls such as culture medium additives and donor-related confounding factors.

## **7. Conclusion and future perspectives**

Taken together, DSCs seem to hold great promise for various applications. One of the major advances made in the field of regenerative medicine is 3D-printing. This technique evolved from a simple 3D- printed construct towards the fabrication of a complex structure containing multiple cell types and biological constituents/components [300-302]. These constructs can ultimately serve as a 3D-scaffold in which DSCs can be studied for multilineage differentiation or more specifically for dental pulp regeneration. However, to ensure future therapeutic applications, one should strive to maintain a cost-effective DSC cell banking system which guarantees phenotypic stability of the heterogeneous populations of DSCs. Some hurdles have to be overcome to establish clinical translation of DSC-based products, since current manufacturing methods are not in line with the GMP guidelines for stem cell therapy. Developing a more standardized and compliant manufacturing protocol will result in an increased efficacy, reproducibility and safety.



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