

Protocols for Dental-Related Stem Cells Isolation, Amplification and Differentiation

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*If I have seen further, it is by standing on the shoulders
of giants. Isaac Newton*

Election of Research Material

To elect research material which would appropriately accommodate individual dental-related stem cell experiments it is essential to understand both the genesis of specific tissue-stem cell lineages and their consequent inherent properties such as population curve in time and behavior under various environmental conditions in vivo (in an intact organism) as well as in vivo modified (transplanted as modified autologous or heterologous material) and in vitro (outside of an organism) [1]. The below chapters provide comprehensive overview of the up-to-date scientific knowledge in this field of study concerning all the tissues of tooth and the tissues immediately adjoined.

Firstly, we outline dental-related tissues genesis and anatomical location, and thereafter the quality and quantity of their stem cell populations. We consider the complete range of postnatal dental-related tissues-stem cell populations, starting from the incident predeciduous dentition [2] to the primary and secondary dentition [3].

Specifically, the feasible dental-related postnatal sources of stem cells are all the mature tissues of all dentitions (natal/neonatal dental pulp [4], immature dental pulp [5], deciduous teeth dental pulp [6], adult dental pulp [7]; apical papilla [8], periodontal ligament [9] and gingiva [10] plus the immature tissues of the secondary dentition dental follicle [11] and dental papilla).

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Dental-Related Tissues' Genesis

Dental-related tissues—lifecycle spans from early stages on intrauterine life and depending on tissue type can last till the host's organism demise. These tissues histogenesis and odontogenesis start when the cells of the ectoderm and mesoderm germ layers interact, differentiate and form increasingly specialised tissues in the area of mouth cavity. The process does not stop until the tooth organ maturation and eruption by when all the dental-related tissues are mature. Seeing that different dentitions (predeciduous, primary, secondary) start forming at different times from tissues of different level of maturation, it is sensible that their dental-related tissues quality differs as well. The following overview of human teeth histogenesis and odontogenesis describes the developments in the dental-related tissues-quality over time.

Histogenesis

All the cells which form dental-related tissues originate in adjoined leaf-like layers of ectoderm and mesoderm. These germ layer-cells move around and create tissues which take on different morphological characteristics and purpose throughout the process of odontogenesis and regeneration.

While the tissues originating from mesoderm germ layer gives rise to oral mesenchyme (the source of all connective and supporting soft tissues and vessels composing the tooth and tooth socket), the ectoderm germ layer gives rise to two original types of tissues: the stomodeal ectoderm (oral epithelium) and the cranial neural-crest-derived ectomesenchyme (neural-crest cells which have undergone an epithelial to mesenchymal transition and manifest mesenchymal phenotype [12]); which together construct all the hard tissues of the tooth.

Organogenesis/Odontogenesis

The complex process of tooth organogenesis/odontogenesis starts at the 6th week of the human embryogenesis and, depending on the type of a tooth, lasts up to 18 years. Odontogenesis is divided into four stages: (1) *the initiation stage*, (2) *the bud stage*, (3) *the cap stage*, and (4) *the bell stage*.

The initiation stage starts with the migration of oral epithelium cells into the underlying mesenchyme and ectomesenchyme of the stomodeum where they form the primary (general) dental lamina, the first signaling centre of tooth development which gives rise to all the teeth buds of primary dentition (20 deciduous teeth) and 12 molars of secondary dentition. Subsequently, around 12th week, the secondary (successional) dental lamina evolves, as a lingual extension of the primary lamina. Finally, at 14th week of embryonic development, the secondary dental lamina gives rise to the first germs of permanent teeth with predecessors (20 teeth)/at the primary dentition bell stage.

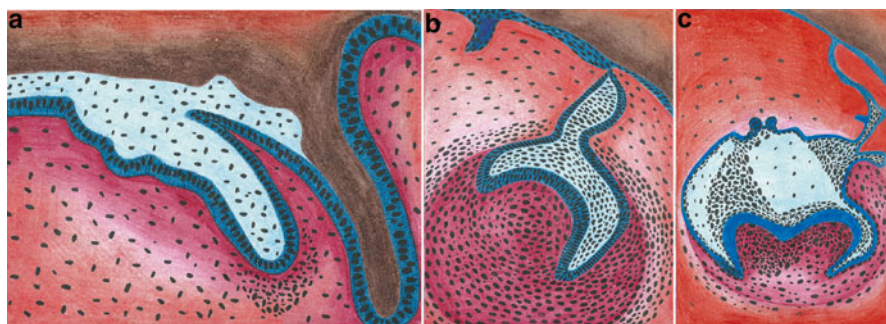


Fig.1 (a) developing tooth in a bud stage, (b) developing tooth in a cap stage, (c) developing tooth in a bell stage

The bud stage (Fig. 1a) is the first stage of the actual tooth germ development (at about 8th week for deciduous teeth). The ectodermal (epithelial) solid bud induces the proliferation and condensation of the underlying mesenchymal and ectomesenchymal cells to form the dental papilla which inductive capacity is essential for further development of ectodermal epithelial bud.

During *the cap stage* (Fig. 1b) (at about 9th–10th week), 3 basic structures develop, i.e. the enamel organ, dental papilla and dental follicle.

The bell stage (Fig. 1c) (approx. 11th–12th week) is primarily characterized by the activation of the enamel organ and the formation of the dentino-enamel junction. The enamel organ is composed of 4 epithelial layers—the outer enamel epithelium (OEE), reticular epithelium, intermediate stratum and inner enamel epithelium (IEE). The IEE starts to differentiate into preameloblasts which induce the proliferation of ectomesenchymal cells in the outer layer of dental papilla and their differentiation to (pre)odontoblasts. Odontoblasts start to secrete predentin which induces the process of preameloblasts maturation into ameloblasts, and so the secretion of the enamel commences. When the amelogenesis (i.e. the production and full mineralization of the enamel) is finished, the ameloblasts degenerate and disappear.

Later in the bell stage, the cervical loop (an area of the enamel organ located on the future crown-root border, composed of the IEE and the OEE) elongates apically through proliferation towards the epithelial diaphragm and eventually becomes Hertwig's epithelial root sheath (HERS). The HERS then initiates the tooth root formation and activates the production of root dentin (in the same manner as in the crown region). After the activation of the local odontoblasts the root sheath disintegrates, this way the newly generated dentin becomes exposed to the ectomesenchymal cells in the inner layer of dental follicle, which eventually induces the differentiation of these follicular cells into cementoblasts and so the production of precementum. The exposed dentin further induces the differentiation of fibroblasts from the middle layer of dental follicle and so eventually the creation of periodontal ligament, and the differentiation of osteoblasts of alveolar socket leading to the creation of the outer layer of the dental follicle.

Eventually, the mature soft dental-related tissues are dental pulp, periodontal ligament, gingiva and apical papilla, and the mature hard dental-related tissues are enamel (oral epithelium), dentine and cementum (neural-crest-derived ectomesenchyme).

Dental-Related Tissues' Stem. Cell Lineages Differentiation Potential and Anatomic Location

Tissues' stem cell populations quality and quantity vary with their origin and stage of maturation. At maturation, the hard dental tissues possess no stem cell populations, while the soft dental-related tissues do and facilitate both self-regeneration as well as regeneration of them adjoined hard tissues [13, 14]. The only hard dental tissue which lacks the possibility to repair is enamel for the absence of communication with any soft tissue.

For the summary of up-to-date differentiation potential of dental-related stem cell lineages see Table 1.

Tooth Organ-Stem Cell Lineages

Immature Tooth Organ-Stem Cell Lineages

Dental Papilla Stem Cells: iTDPSC

Immature Tooth Dental Papilla is an engineering tissue of mesenchymal origin enclosed by the enamel organ on the top and by the dental follicle on the bottom, which eventually converts to pulp tissue. It plays a central role in epithelial-mesenchymal interactions responsible for tooth morphogenesis. It is firstly responsible for the crown morphogenesis and later, when it becomes apical to dental pulp (a converted top part of dental papilla, after the onset of dentinogenesis/bell stage) it becomes secondly also responsible for the morphogenesis of the tooth roots (in the form of Apical Papilla) [54–56]. The most suitable teeth for obtaining dental papilla are third molars where the roots are not developed at all, this condition is fulfilled before the 14 years of age (Figs. 2 and 3a).

The up-to-date knowledge of the differentiation potential of the Dental Papilla Stem Cells is minimal and calls for further research. Most recently, in 2014, Vishwakarma et al. have stated that the dental papilla stem cells “can induce both enamel-dentine and dentine-cementum production”, but only under certain specific conditions and combined with the appropriate cell populations.

Apical Papilla Stem Cells/Immature Root Papilla Stem Cells: SCAP/iRPSCs

The root formation of developing tooth starts when the cervical loop-epithelial cells proliferate apically and induce the differentiation of dental follicle mesenchymal cells into odontoblasts and cementoblasts [57] This apically extending epithelial

Table 1 Differentiation potential of dental-related tissues stem cells lineages

Differentiation capacity	DPSC	iRPSC (SCAP)	iTDPSC(germ)	NDP-SCs	SHED	DFSCs	PDLSCs	GMSCs
Adipogenic	Struys [15]	Sonoyama [16]	Yalvac [17]	Akpınar [18]	Miura et al. [6]	Kémoun [19]	Xu [20]	Gao et al. [21]
Chondrogenic	Koyama [22]	Wang [23]	Demerci [24]	Akpınar [18]	Kerkis [5]	Kémoun [19]	Xu [20]	El-Sayed [25]
Osteogenic	Mori [26]	Bakopoulou et al. [27]	Yalvac [17]	Akpınar [18]	Miura et al. [6]	Morsezeck [11]	Xu [20]	Gao et al. [21]
Neurogenic	Osathanon [28]	Sonoyama [29]	Yalvac [17]	Karaöz [30]	Miura et al. [6]	Morsezeck [11]	Shi [31]	Zhang et al. [32]
Myogenic	Zhang [33]	Abe et al. [34]	Tasli [35]	Karaöz [30]	Kerkis [5]	d' Aquino [36]	Shi [31]	Ansari [37]
Endothelial cells	Hilkens [38]	Bakopoulou [39]	Yalvac [17]		Cordeiro [40]	Dögan [41]		Zhang et al. [32]
Odontogenic	Huang [42]	Sonoyama [29]	Demerci [24]		Miura et al. [6]	Chen [43]	Shi [31]	Gao et al. [21]
Hepatogenic	Ishkitiev [44]	Patil [45]	Ikeda [46]		Ishkitiev [47]	Patil [45]	Kawanabe [48]	
Cementogenic	Kim [49]	Kim [49]				Kémoun [19]	Seo [9]	
Melanocytes	Paino [50]							
Cardiomyocytes	Armiñán [51]							
Epithelial cells			Dögan [41]					
Endodermal cells								Zhang et al. [10]
Periodontal ligaments						Morsezeck [11]		
Pancreatic islets	Ishkitiev [52]							
Cornea cells	Gomes [53]							

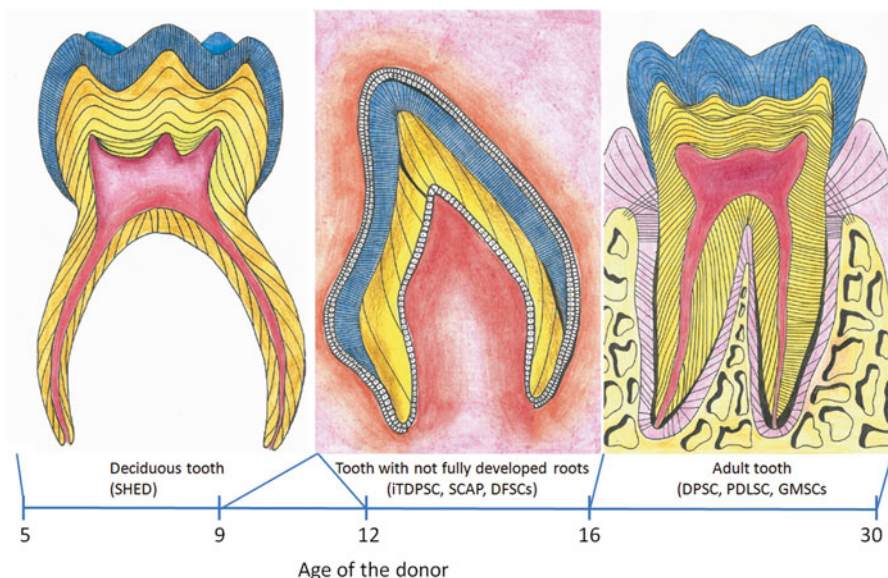


Fig. 2 Dental related stem cells lineages and the most common age of the donors of the different stem cells populations

wall forms Hertwig's epithelial root sheath (HERS) which guides the shape of the tooth roots. It is at this time dental papilla becomes apical to dental pulp tissue (hence apical papilla) and forms apical cell-rich zone as a divide between the two [55]. Overall, apical papilla is histologically different from dental pulp of immature tooth in multiple ways, according to Sonoyama et al., its stem cells proliferate 2 to 3 times quicker than those of the adjoined immature dental pulp, it contains less cellular and vascular components and it further contains unique potent MSCs. As apical papilla is a tissue derived from the neural crest, its stem cells express numerous neurogenic markers [58].

As SCAP are isolated from the apical papilla of a tooth with not yet fully developed roots, the most suitable teeth to extract are the first premolars of up to 12 years old patients and the third molars of 12–16 years old patients (Figs. 2 and 3b–d).

Mature Tooth Organ-Stem Cell Lineages

Dental Pulp

Dental pulp originates in dental papilla and is made up of connective tissue and four distinct cell-zones. The dental pulp connective tissue consist of an extracellular matrix (ECM) composed of ground substance with relatively high content of glycosaminoglycans, proteoglycans and other adhesion molecules (fibronectin, laminin, etc.) and the sparsely distributed type III collagen fibres which form a rich network only around vessels and nerves. The outmost cell-zone of the dental pulp is the odontoblast layer-zone (gives rise to the primary “predentine”, secondary and tertiary

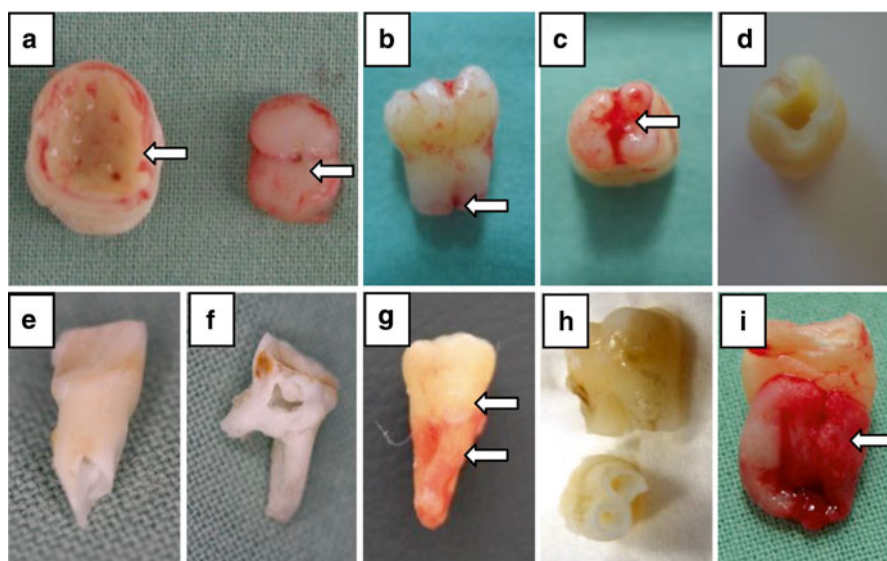


Fig. 3 (a) Extracted 3rd lower molar in a bell stage (germectomy) on the left side and its pulp on the right side. (b) Extracted 3rd lower molar with not fully developed roots. (c) Extracted 3rd lower molar with not fully developed roots with the apical papilla covering the root orificium. (d) Extracted 3rd lower molar with not fully developed roots from the (c) after extraction of the pulp tissue. (e) Extracted upper deciduous first incisor where the root apex is reabsorbed and the root canal widely opened. (f) Extracted second lower deciduous molar after splitting the crown using the bur to open the pulp chamber. (g) Extracted upper 3rd molar, the lower arrow indicates the area which is covered by the periodontal ligaments and presents the suitable source of the periodontal ligament stem cells, the *upper arrow* indicates the cement-enamel junction. (h) Two extracted upper first premolars after the isolation of the dental pulp. (i) Extracted lower 3rd molar with dental follicle tissue marked with the *arrow*

“reparative” dentine), followed inwards by a cell-free-zone called the Weil basal layer (contains dense capillary and nerve network), then cell-rich-zone, and finally arriving at the pupal core. The last two zones share similar structure as they are composed of larger vessels, nerves and great amount of cells, specifically: fibroblasts, cells belonging to the defense system, and undifferentiated mesenchymal stem cells.

Dental pulp stem cells lineages’ quality and characteristics differ with dentition (predeciduous, primary, secondary) and level of their maturation.

Predeciduous Dentition Dental Pulp Stem Cells/Natal, Neo-Natal Dental Pulp Stem Cells NDP-SCs

The natal (erupted before birth) and neonatal (erupted within the days after birth) teeth are rarely found in some newborn babies within the first few days after birth. Natal teeth are more frequent than neonatal teeth, with the ratio being approximately 3:1 [59]. These teeth are usually indicated for extraction due to the danger connected to their inhalation, the risk that they could cause deformity and mutilation of tongue, dehydration, malnutrition, growth retardation of the following dentitions and due to

the health risks they can cause to the mother during breastfeeding. Histologically, the natal teeth have only thin or completely absent layer of both enamel and cementum. The predentin layer is often of various thickness compiled of irregular dentinal tubules. Furthermore, unlike dental pulp of teeth of the primary and secondary dentitions, the natal dental pulp has extra inflammatory areas and does not have the Weil basal layer and cell-rich-zone. These teeth tend to have a wide pulp chamber and underdeveloped roots which makes isolation of their dental pulp easier.

Primary Dentition Dental Pulp Stem Cells: SHED

The amount of stem cells from human exfoliated deciduous teeth decreases with the receding dental pulp (which is gradually replaced by gum). This implies that the deciduous teeth that fall out spontaneously are likely to have little to no pulp, therefore little to no SHED population. To ensure a sufficient amount of stem cells will be isolated, the rule of thumb is to focus on teeth which have at least 1/3 of the original root length (after the onset of primary dentition root resorption), and for the multi-rooted teeth it is best to isolate teeth with the furcation area still present (Fig. 3e, f).

The ideal time to extract the deciduous teeth for their dental pulp stem cells is between the ages of 5 and 9. Beware; dental pulp of teeth affected by dental carries is not fitting for use in research.

Secondary Dentition Dental Pulp Stem Cells: DPSCs

The teeth most commonly used for isolation of the dental pulp stem cells are the first premolars and the third molars (Fig. 3g, h), as they frequently attract preliminary extraction at early age of the dental tissue. The first premolars are usually extracted shortly after eruption, due to the orthodontic reasons, around the age of 12. The “youth” of their soft dental-tissues, not fully developed root, and very low probability of carries makes these teeth the ideal stem cell source. The third molars are often extracted to prevent health complication such as the surrounding tissue inflammation, as well as due to the orthodontic reasons. While the extraction for orthodontic reasons comes around the age of 16, the extraction due to tissue inflammation occurs later, usually between the ages of 16–30, hence involves at least partially-erupted and often fully developed tooth.

Tooth-Supportive Tissues-Stem Cell Lineages

Immature Tooth-Supportive Tissues-Stem Cell Lineages

Dental Follicle Stem/Progenitor Cells: DFSCs

The dental follicle is a loose connective tissue that surrounds the developing tooth and is separated from dentin by an epithelial layer (Hertwing's sheet). Once this epithelial layer disintegrates and dental follicle touches the dentin, it is prompt to differentiate into periodontium including the alveolar bone, cementum, and

periodontal ligament [60]. According to Vishwakarma et al. [61], the tissue contains at least three distinct stem cell populations (hDF1, hDF2, hDF3) with distinct morphologies, gene expressions and differentiation potentials.

Most commonly, the unerupted third molars are used for isolation of these stem cells, usually before 14 years of age (Fig. 3i).

Mature Tooth-Supportive Tissues-Stem Cell Lineages

Periodontal Ligament Stem Cells: PDLSCs

The periodontal ligament connects alveolar bone and cementum to support teeth in situ and preserve the hard tissues homeostasis [9]. Periodontal ligament stem cells are commonly isolated from the soft tissue adjoined to the root below its enamel-cementum border (Fig. 3g); they form adherent clonogenic population of fibroblastic-like cells, forming flat and loose aggregates [62]. In 2010, Kawanabe et al. [62] have shown that PDLSCs express embryonic stem cell-associated antigen SSEA-4, implying these stem cells are great source of building material for regenerative medicine, being capable of differentiating into cells of all three germ layers.

The periodontal ligament tissue is usually isolated from around the first premolars and third molars from donors who have no history of periodontal disease and healthy periodontium.

Overall, PDLSCs were demonstrated to be able to differentiate into adipocytes, collagen-forming cells, cementoblast-like cells [9, 63], chondrocytes, neurons, hepatocytes [62], and osteoblasts [32] in vitro; and into cementum- and periodontal ligament-like tissues in vivo modified [9].

Gingiva-Derived Mesenchymal Stem Cells: GMSCs

The gingiva is a mucosal soft tissue which forms a shield-like physical protection of the adjoined dental-related tissues by sealing the gap between tooth's exposed enamel and periodontal ligament protected cementum; it further exhibits both immunomodulatory and anti-inflammatory capacities, which allow it to help the adjoined tissues to regenerate [10].

The GMSCs are usually derived from the spinous layer of human gingiva [64]. This highly homogenous population of stem cells carries not only the usual mesenchymal stem cell markers but also a positive expression of extracellular matrix proteins [10]. GMSCs have been successfully differentiated into osteoblasts, chondrocytes, adipocytes.

Donor

Theoretically any vital tooth and its enveloping tissues provide possible source of dental-related stem cells. However, it is important to narrow the pool of possible donors to those who are most likely to donate viable and research feasible stem cell

populations, will be the least impacted by the extraction procedure, populations, while undergo the least impactful extraction procedure. Eventually, electing the right research material is directly related to choosing a fitting donor.

The three basic recommendations in choosing the right donor:

1. *Dental-related tissues to be extracted*

- Different stem cells lineages can only be isolated from donors of certain age/development status

2. *Health status of the donor*

- General medical history—as certain illnesses and diseases (e.g. genetic predispositions) can cause researcher bias as they may influence the qualities of the research material
- Possible impact of tissue extraction on the donor's health

3. *Clinical status of the tissue*

- The general condition of the tissue and its immediate environment represent yet another research bias (e.g. caries lesions represent risk of bacterial transmission, inflammation of the soft tissue can adversely impact the research material, erupted tooth is contaminated by the oral micro microflora, fully developed roots complicate the process of the dental pulp isolation)

Election of Research Method

Bioethics, Legislation and Regulation of Human Stem Cell Research

The principal goal of human biomedical stem cell research is to relieve and avert suffering caused by both genetic and acquired impediments to health condition [65].

Due to the interdisciplinary character of the knowledge base and the worldwide network of research which aims to build upon each other, while oftentimes towards different goals, it is eminently important to unify the standards for information integrity, full disclosure, and respect for research participants—their health and freedoms [65]. While the research focused on the unipotent, multipotent and pluripotent stem cells avoids the major ethical and legal issues connected to the totipotent embryonic stem cells and oocytes, there are other concerns which remain standing.

The international consensus over the stem cell research guidelines is conveyed by the ISSCR—*International Society for Stem Cell Research*, established independent non-profit organisation founded in 2002, which releases yearly updated guidelines for stem cell research and clinical trials and provides templates of legal documents connected to biomaterial donation and transfer [65]. The ICSCN—*International Consortium of Stem Cell Networks* then provides the contacts and means to bridge varying national stem cell legislation and regulation to foster best practice international research collaborations. The consortium is managed by a

secretariat comprising of nationally funded representatives from Australia, Canada, Germany and Scotland and meets annually in conjunction with ISSCR.

Beyond the international organisations, the research ethics and regulations are further narrowed by supra-national consortia such as the European *EuroStemCell* and the American USFDA—*Food and Drug Administration of the United States of America*; national legislation and regulations issued by National Academies; and eventually by the institutional SCRO—*Stem Cell Oversight Committee* [66]. The SCRO must respectively consist of proxies of both scientific and ethical communities (also referred to as: IRE—Institutional Review Board/IEC—Independent Ethics Committee/ERB—Ethical Review Board or REB—Research Ethics Board), from which a part is non-affiliated. The ethical community further demands membership of representatives of ethical expertise and those representing the lay public [67]. Finally, the text summarising the institutional professional standards and self-regulations, issued by the institutional SCRO, deals with all the above issues in two separate parts: Part 1: *Respect for research participants* and Part 2: *Integrity of the research enterprise* [67]. The SCRO standards for non-totipotent stem cells research should, according to the ISSCR 2015 Draft Guidelines [65] and Hyun [68], address the following:

1. *Respect for Research Participants*

The respect for research participants remains accompanied with the issue of donor/patient consent to share genetic information and the issue of donor/patient mental and physical state now and in the future; which prohibits scientists to treat the biomaterial freely and subject it to downstream research which has not been approved by the SRCO and the donor in advance [68]. The above issues require written and legally valid informed consent to donate (if the donor is not *sui juris*, their legal representative has to subscribe the informed consent instead), which according to the ISSCR Draft 2015, should address both the donor and research organisations' rights and obligations towards each other, specifically:

- (a) The provision of accurate information on risks to donor health connected to tissue retrieval.
- (b) The provision of accurate information on risks, limitations, possible benefits, and available alternatives to patient treatment.
- (c) The donated tissue treatment and storage—genetic modification, recipient profile, discard protocol, *ad cetera*.
- (d) The genetic and disease screening of the donated material.
- (e) The full disclosure of the derived information.
- (f) The donor/patient private information (identification, genomic information, *ad cetera*) treatment and discard protocol.
- (g) The future accessibility of the donor/patient to obtain additional consent, additional material or information.
- (h) The commercial potential of the donated material and intellectual property rights of the researchers/donor.
- (i) The treatment of incidental findings.
- (j) The treatment and legal liability of the researchers and medical staff in case of unforeseeable impacts on patient health [65].

2. Integrity of the Research Enterprise

The treatment of the biological material founded upon valid, timely, reliable and reproducible protocol; executed following the GLP—Good Laboratory Practices; and reported in transparent, reliable manner and accessible format ensures integrity of research enterprise. All of the above is pertinent for independent peer review and downstream research.

The US FDA provides a step-by-step toolkit to institutional SCRO organisation, operation, administration and reporting which can be used as the building blocks of the institutional standards [65].

Dental-Related Tissue Extraction and Transport

Dental-Related Tissues Extraction

The extraction of tissues for stem cell research is performed under the same condition as an ordinary prescribed extraction, while we are interested in preserving the biological material as intact as possible, the health of the donor/patient comes first. Fundamentally, there are two types of extraction, the simple extraction and the surgical extraction; where both are performed under local anesthesia.

The simple extraction is adopted in the case of fully erupted tooth and is executed using various dental tools including elevator and forceps to break the tooth' bonds to periodontal ligament and to widen its alveolar bone socket, and so allow for smooth removal of intact tooth.

The surgical extraction is adopted in case of an impacted or not yet fully erupted tooth (the most common source of dental-related stem cell material) (Fig. 4).

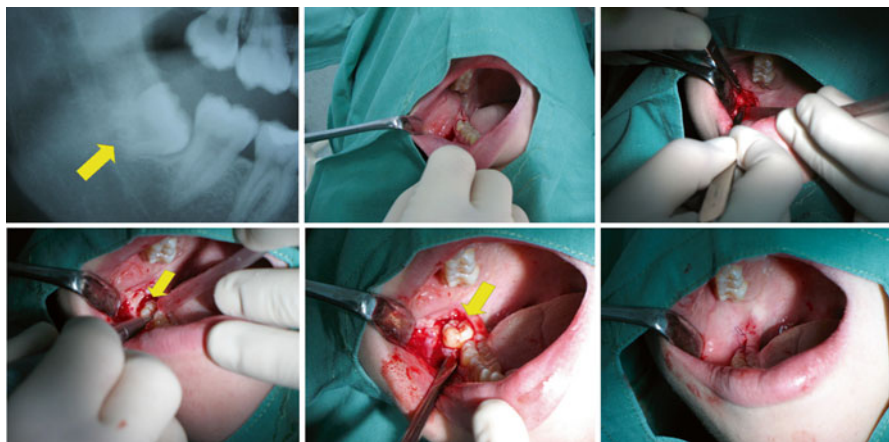


Fig. 4 The procedure of complicated extraction of lower 3rd molar. First picture represent the cut of the ortopantomogram. Second and third pictures show the moving of the mucoperiosteal flap. On the fourth picture the bone covering the tooth is removed and on the fifth picture we can see the extraction using the levers

This procedure involves rising of mucoperiosteal flap/tissue flap on the intra-oral side of gums covering the tooth (for esthetic reasons) and trimming the exposed bone present around the tooth roots to allow for smooth root removal (here the prime consideration is to limit the amount of bone being removed). At this point, depending on situation, the surgeon either retains the tooth as a whole, which is preferable, or sections it to further ease its extraction. Once sectioned, however; the dental-related tissues are compromised together with their value in research.

Immediately after the extraction it is recommended to disinfect the tooth using any disinfectant solution commonly used in the dental cavity, to decrease the possible amount of bacterial presence. Then, the tooth needs to be fully immersed in the Hanks balanced salt solution enhanced by antibiotics and antifungals (composed of 1 ml of Hanks balanced salt solution, 9 ml water for inj., 200 µl/10 ml gentamycin, 200 µl/10 ml streptomycin, 200 µl/10 ml and 200 µl/10 ml penicillin) to fully eliminate any remaining bacterial presence.

Dental-Related Tissues Transport

The temperature of the solution during transport is highly recommended to be kept at 4 °C. Even though stem cells are theoretically resistant to hypoxia and survive longer than somatic cells, it is absolutely essential they are isolated from the extracted tissues as soon as possible (no later than 24 h after extraction). For this reason it is necessary to invest in proper preliminary planning to avoid any wastage.

Isolation of the Dental-Related Tissues

Tooth-Organ Tissues Extraction

Immature Tooth-Organ Tissues Extraction

Dental Papilla and Apical Papilla Extraction

Dental papilla tissue is extracted from tooth without developed roots, hence sourced from very young donors/patients. Such extraction is called germectomy (Fig. 3a) (an identical procedure is used to obtain dental follicle tissue (Fig. 3i). The most common source of the dental papilla tissue for stem cell research are third molars of donors/patients aged 10–14 years, as those teeth are commonly indicated for extraction. After the onset of root dentine formation and root development, the dental papilla splits into the soft tissues of dental pulp and apical papilla.

Apical papilla is localized at the area of root growth, below dental pulp (Fig. 3b–d). The dental pulp and apical papilla are clearly separated by apical papilla adjoined apical cell-rich zone which however does not stick to dental pulp and so allows smooth separation of the two tissues.

Whilst dental and apical papilla tissues can be obtained from immature teeth of both dentitions, it is rare to find immature deciduous teeth indicated for extraction which have no caries lesion; therefore the SCAP and iTDPSC are widely considered as stem cell lineages of secondary dentition.

Mature Tooth-Organ Tissues Extraction

Dental Pulp Extraction

Dental pulp consists of connective tissue and odontoblasts locked together inside the dental pulp chamber. During the process of tooth maturation, dental pulp tissue becomes increasingly enclosed in hard dental-related tissue, until only a tiny opening at the roots' apex (physiological apical foramen) is left (0.25–0.35 mm wide at maturation) to allow the nervous connections and blood vessels. To ensure the extracted dental pulp tissue is as intact as possible, we aim to use the least destructive method which varies with the tooth level of maturation (including all postnatal immature and mature dental pulps of all dentitions).

The three most commonly used methods of dental pulp extraction are: (1) Extraction of the dental pulp through the physiological apical foramen, (2) Splitting the tooth using forceps and (3) Splitting the tooth using burr (rotary file).

The first method is regarded as the easiest, fastest and the most qualitatively efficient of all; however, it can only be performed on teeth with wide apical foramen (approx. $x > 2$ mm), therefore reserved exclusively for teeth with roots which are not yet developed (isolating immature dental pulp tissue of primary and secondary dentition) or for teeth which roots are resorbing (isolating primary dentition dental pulp tissue). The first step of this extraction process is to gently detach the dental pulp from the adjoined dentine (commonly performed using a needle). The following step is to pull the pulp out downwards using tweezers (commonly performed using anatomical stainless steel hard tweezers, model ADSON) (Fig. 5a).

When the physiological foramen is too narrow to use as the extraction canal, we are left with other methods which involve mechanical disintegration of the hard dental tissue surrounding the pulp, opening the dental chamber from the top. To access the soft dental pulp, we tend to aim to split the tooth at the enamel-cementum junction (cervical area) using either forceps (crashing the junction) (Fig. 5b) or a burr (employing a diamond burr on a fast micromotor handpiece—red stripe, about 200,000 rpm; cooling the tooth with water spray; performing horizontal cut at the junction (Fig. 5c) (it is also possible to employing a disc shaped burr and performing vertical cut, though this approach is regarded as very risky as far as the pulp integrity and quality is concerned (Fig. 5d)). It is also important to mention that the burr method has potentially detrimental impact on the dental pulp due to the heat emitted during filing. If successful the dental pulp can be pulled out upwards using the same method as described above. While these mechanical methods are relatively complicated, slow and less efficient in the amount of tissue harvested (in comparison with the first method), they allow researchers access to much greater number of donors/donated tissues.

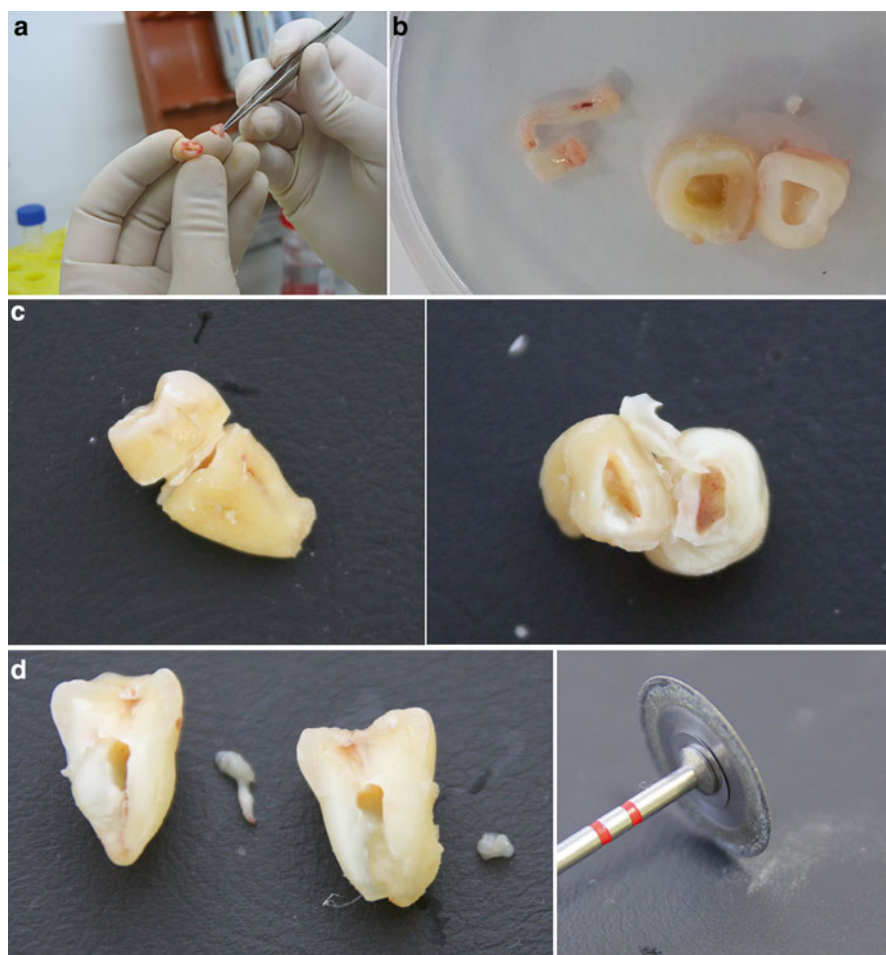


Fig. 5 (a) Extraction of the pulp tissue from the tooth with not fully developed roots, where the pulp chamber is widely opened. (b) Extracted lower 3rd molar where the crown was separated from the root in the using the forceps and the isolated dental pulp tissue. (c) Extracted upper 3rd molar where the crown was separated from the root in the using the diamond bur on the *left side* and the same tooth still containing the pulp tissue on the *right side*. (d) Extracted upper 3rd molar where the crown was separated from the root in the using the forceps soflex discs (the bur is on the *right picture*)

Tooth-Supportive Soft Tissues Extraction

Immature Tooth-Supportive Tissues Extraction

Dental Follicle Extraction

Dental follicle is loose connective tissue surrounding the developing tooth germ (Fig. 3i) which degenerates after the tooth erupts and finishes its development. It's

harvest after tooth organ extraction is relatively easy as it is not firmly adjunct to the tooth. Due to this timing issue, the most frequent source of this tissue are the third molars extracted from the young donors (around 10–14 years old). These teeth are frequently extracted for orthodontic reasons and are commonly completely embedded in the jaw bone.

Mature Tooth-Supportive Tissues Extraction

Periodontal Ligament and Gingiva Extraction

Periodontal ligament is specialized connective tissue localized between the alveolar bone and root surface, and is hidden under gingiva. While not all extracted teeth carry a research-sufficient amount of gingiva tissue, as the gingiva is commonly raised from the tooth surface preceding the tooth organ extraction, it commonly carries enough periodontal ligament tissue. Both the tissues are harvested off the tooth root surface by scraping.

Dental-Related Stem Cells Isolation

In general, dental-related stem cells are isolated from their maternal tissue using one of the two following approaches: (1) Enzymatic Digestion (ED) of tissues or (2) Spontaneous Outgrowth (OG) from the maternal tissue mince [64]. Recent scientific research suggests that isolation method most probably impacts the qualities of the isolated stem cell lineages [27, 69]. For example, while some researchers, comparing the impact of the above isolation methods on the resulting dental-related stem cell populations, concluded that OG isolated stem cell population had weaker stem cell-markers expression (SHED-ED $\times$ SHED-OG [70]) and lower proliferation rate (DPSC-ED $\times$ DPSC-OG [69], other studies concerning non-dental-related stem cells report contradicting results, mostly arriving to the decision to opt out from using the collagenase enzyme as it was believed to alter the stem cell phenotype [38, 71]. Ergo, it is important to keep an eye on the upcoming research in this field to help us narrate the most probably bias-free methodology.

Enzymatic Digestion/Dissociation: ED

During enzymatic digestion the extracted tissue is submerged in digestive enzymes solution (most commonly Hank's balanced Salt Solution (HBSS) carrying diluted collagenase I (3 mg/ml), dispase (4 mg/ml) and phosphate-buffered saline (PBS) (buffer solution) in a ratio of 1:1:1:1) for about 30–60 min, depending on the size of pieces of the tissue, at 37 °C. The result of ED is a single-cell suspension which contains many different cell types and remnants of vessels and extracellular matrix (Fig. 6). While it is not imperative to remove the remnants from the suspension, it

Fig. 6 Dental pulp stem cells in the prime culture 6 days after seeding with the remnants of the soft tissue and the erythrocytes

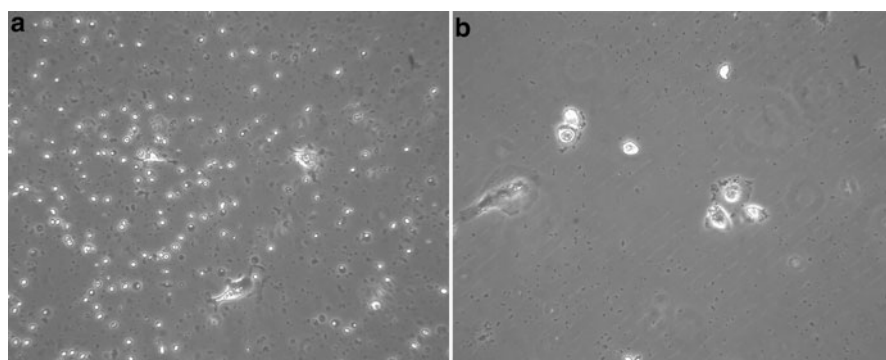
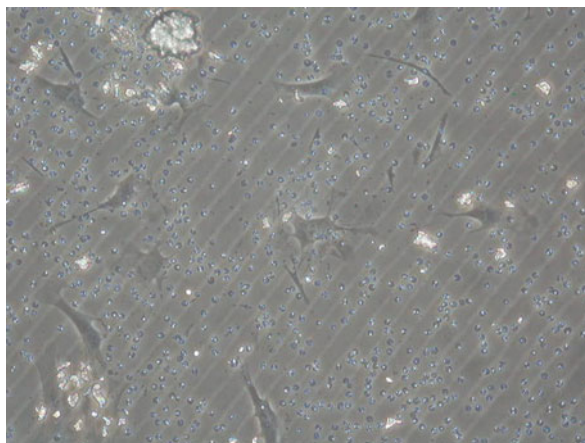


Fig. 7 (a, b) The same lineage of dental pulp stem cells before and after changing the media

can be done by either sieving through a strainer (3 μm followed by 20 μm), by magnetic-activated cell sorting (MACS) or by stem cell colony cultivation [64]. Whether researchers decide to work with suspension containing remnants or not, the single-cell suspension is centrifuged (600 g (g-force), 2000 rpm for 5 min) and the cell pellet is finally seeded into cultivation dishes where it is resuspended in amplification media. After 7 days of amplification the stem cells adhere to the surface of culture dish. At this point all the other material is washed away and new amplification media is added (Fig. 7a, b).

Spontaneous Outgrowth/Explant Culture: OG

The outgrowth method is technically easier and faster than ED. The soft tissue is minced to pieces smaller than 2 mm (explants) and planted on cultivation dish, fully immersed in amplification media. After 3 to 5 days of amplification we can observe stem cells adherent to the culture dish everywhere under and around the minced tissue (Fig. 8) [64].

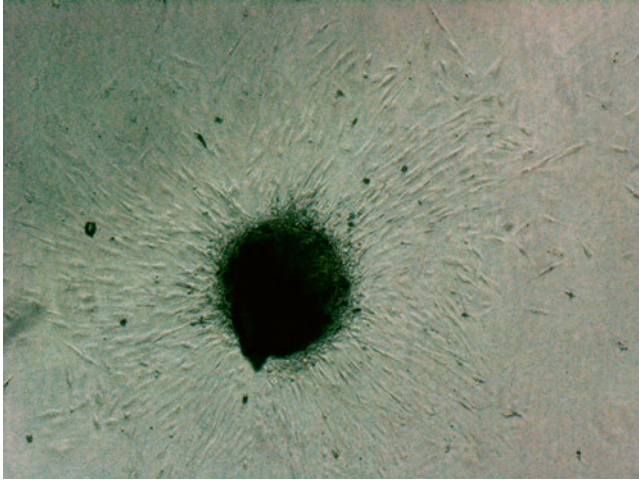


Fig. 8 Part of the dental pulp tissue with outgrowing stem cells. The photo was taken 12 days after seeding

Dental-Related Stem Cells Amplification and Differentiation Culture Systems

After we have successfully isolated our stem-cell lineages we aim to expand their population to research-relevant amounts (their amount at isolation varies by maternal tissue and method of extraction [72]). Depending on the future use of the amplified population it is important to choose the right amplification conditions and environment (physical characteristics of the culture system (adherent, non-adherent/sphere-forming), amplification media sera (xenogeneic, autologous serum, serum-free) and co-culture (epithelial/ectodermal-mesenchymal interaction) [64]) as they do affect the quality of the resulting stem cell population [73]. Especially, when human regenerative medicine is concerned, the researchers should avoid using xenogeneic sera as their use carries multiple legal, ethical and medical issues ranging from research results bias, to transfer of zoonoses (interspecies infectious agents); viral, bacterial and fungal contamination; and permanent alteration to the stem cell population genetic code [74].

Amplification and Differentiation Culture Systems

Generally, dental-related stem cells are amplified and differentiated at 37 °C at 5 % CO₂ atmosphere. To maintain high proliferation rate, cultivated stem cells should be passaged when the population reaches 70 % confluence (confluence >70 % leads to decrease in proliferation due to the physiological contact inhibition). The scientific

community recognises two basic physical cultivation environments for stem cell amplification and differentiation: the (1) *Adherent Culture Systems* and the (2) *Non-adherent (Suspension/Sphere-Forming) Culture Systems*; plus one extra type of differentiation environment, the (3) *Co-Culture Systems*. Overall, while the non-adherent culture systems are easier for passaging and the growth of cells is not limited by the surface area of the cultivation dishes, the adherent culture systems allow for easier visual inspection and provide less opportunities for cell heterogeneity [75]. The co-culture systems then provide “site-specific” lineages interaction which leads to differentiation of specific progenitor/adult cells [64].

Adherent Culture Systems

The adherent system consists of a dish which allows the cultured stem cells to adhere to it. At the end of cultivation, the dish is washed with phosphate-buffered saline, which safely removes all cultivation media. Once the stem cells are the only content of the dish, it's whole surface is covered by trypsin which detaches the stem cell off the surface of the dish. The trypsin is neutralised after 5 min by solution of α -MEM and serum (in concentration of 20%). The resulting suspension with cultivated cells is centrifuge (600 g, at 2000 rpm for 5 min). After centrifugation, the supernatant is removed and the resulting cell pellet is resuspended in new cultivation media and placed on new culture dish, in concentration of 2500 cells per cm². This culture system is the default system in stem cell research, except for cultivation and differentiation of non-adhesive cell lines (e.g. neural and hematopoietic cells).

Non-adherent/Suspension/Sphere-Forming Culture Systems

Spheres are free-floating cultures of stem cells. The non-adherent sphere-forming culture is generally serum-free and contains epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) and rests upon either super-hydrophilic (non-adherent) plates or in a culture flask [76, 77]. While this system has been widely applied for assaying neural stem cells up to neurogenesis [78] it has been also studied an effective system for generation of mineralized tissues (from SCAP) [34].

Jensen and Parmar's report on the applicability of this system raises a few important issues, they argue that the cultured cell types properties appeared to be sensitive to the culturing method used, variation in cell density in the system, as well as the concentrations of factors in the media, method and frequency of passaging and the number of passages after isolation [78]. Furthermore, multiple researchers report that the sphere-forming cells become increasingly heterogeneous with further passage as the growing spheres contain cells at various stages of differentiation, from stem cells to progenitor cells and specific cell types [75, 79].

Co-culture Systems

Co-culturing of various dental-related stem cells is not used for their amplification as much as it is used for their differentiation. These co-cultivation systems applied in the dental-related stem cell research are based on our knowledge of the dental-tissue genesis (epithelial-mesenchymal co-culture/ectodermal-mesenchymal co-culture). For example, Bai et al. [80] have demonstrated that co-cultivation of DFSCs and SCAP (rat) can lead to formation of bone-like particles and Xiao and Tsutsui [81] demonstrated that co-cultivation of oral epithelial cells (OECs) and DPSCs can build an epithelium invagination like model. These systems are very promising tool for future research as they replicate the original in vivo environment more accurately than the above single-cell systems.

Amplification Media

The cultivation media, as well as any component of extracellular environment affects the properties of cultivated cells. Due to the vast heterogeneity of media composition used through dental-related stem cell studies, it becomes challenging to make any generally applicable judgements about the media on the amplified or differentiated cell populations. Therefore it is imperative that when outlining a research method, one should critically and attentively evaluate the validity of preliminary research' methods and their results to eventually arrive at scientifically credible conclusion and results in their work, results which can be build upon. The three general groups of media are: (1) *Serum-Free Medium*, (2) *Allogeneic-Serum Medium*, and (3) *Xenogeneic-Serum Medium*. The serum-free media are currently the most preferable solution due to absence of risks associated with the xenogeneic and allogeneic media (e.g. transfer of viruses and zoonoses).

Serum-Free Media

The serum-free medium, most frequently used in dental-related stem cell research, is a combination of the following additives: Dulbecco's modified Eagle's medium, insulin, transferrin, sodium selenite supplement, Embryotrophic Factor [82]. While the serum-free media are most possibly the future of stem cell research, they are not yet universally effective when used for various types of stem cell lineages, and their efficiency in boosting stem cell proliferation and accessibility remains low [83]. Today, the serum-free media are used as a complementary media to amplification and differentiation processes powered primarily by either allogeneic or xenogeneic media.

According to a study, conducted by Tarle et al. [84], on use of serum-free media for amplification of PDLSCs and SHED; the stem cells cultivated partially under serum-free condition express higher positivity of stem cells markers then when amplified in xenogeneic medium alone. They also manifested similar differentiation capacity, while their proliferation rate was significantly slower [84].

Allogeneic Serum-Containing Amplification Media

The most commonly used allogeneic sera are human plasma (HP) or platelet rich plasma (PRP) [85–89]. While HP does deliver on its promise, it is proven that PRP, due to its boosted contents of platelets/platelet lysate, is more potent in maintaining the cultured dental-related stem cell properties and its use results in greater cells' proliferation rate, immunosuppressive activity and migration activity [87, 90, 91].

The disadvantages of these sera are the hazards involved in transferring heterologous material which can carry diseases (e.g. HIV, Hepatitis), drug metabolites, and depending on the donor also irregular concentration of growth factors and other components which vary within physiological borders. Nonetheless, the disease and drug metabolite hazards can be managed and the concentrations of the components can be successfully averaged by pooling material sources from multiple donors (>4) [92].

Xenogeneic Serum-Containing Amplification Media

The xenogeneic media contain around 2–20% of fetal calf serum/bovine serum, where concentrations of >10% are the most common. The advantages of this serum include its high content of growth factors and low concentration of inhibitory factors. The disadvantage is; however, that the concentration of those, together with the serum nutrients, enzymes and proteins, is not stable and varies sample to sample, within the species physiological borders. In general, the higher concentration of the bovine serum in media the better cell adherence, the more colony forming units, and the greater proliferation potential. Nevertheless, these research beneficial outcomes are offset by research detrimental impacts on the cultivated cells such as chromosomal instability, phenotype amalgamation, malignant transformations, and at concentrations >20% even toxicity.

When attempting to minimise the above mentioned detrimental effects of high concentrations of xenogeneic serum on cultured cells while retaining the high proliferation rate and good adherence, it is recommended to use media with <10% bovine serum and to make up for the higher concentrations by adding growth factors like platelet derived growth factor and epidermal growth factor to boost its performance. According to some studies the addition of insulin-transferrin-sodium selenite solution results in higher proliferation rate as well [92].

Nevertheless, there exists no solution for one of its major shortcomings, its xenogeneic origin, which brings a whole lot of possible hazards to the table. Eventually, its application can lead to internalization of bovine serum proteins by the cultured stem cells and finally stimulation of the recipient immune system and unwanted immunological reaction [93–95], or to transmission of the zoonoses [96, 97], and induction of metabolic and morphological changes in the cultured cells [98, 99]. Overall, while this serum is easily obtainable and relatively cheap, it is recommended it is phased out off the stem cell scientific research as it provides biased results which are not applicable for human medicine [100, 101].

Dental-Related Stem Cells Differentiation and Evaluation

According to their origin dental-related stem cells are pre-programmed to differentiate into certain mature cell lineages and it is harder, although not impossible, to induce them to differentiate into foreign lineages. To prove that the amplified dental-related stem cells are indeed dental-related stem cells we would test their capacity to differentiate into osteoblasts, chondroblasts and adipocytes, which capacity is characteristic for all mesenchymal stem cells. Yet again, the results of the differentiation process are highly dependent on the artificial extracellular environment and hence this step attracts good planning and thorough preliminary research. Besides choosing the basic culture system, as per chapter *i. Amplification and Differentiation Culture Systems*, we have to choose the right differentiation method (there are multiple approaches to the same end) and follow its evaluation process to produce valid results. *(Interesting new fact, in 2015, Zhang et al. have proven that LMHF mechanical vibration promotes PDLSCs osteogenic differentiation. This discovery implies that vibration may be one of the mechanisms of external environment which impacts the biological processes, hence should be considered as a variable when planning and reporting research results.)*

The below overview lists the most commonly used differentiation protocols in dental-related stem cell research:

Osteo-Differentiation Protocol and Evaluation

The dental-stem cells undergoing osteogenic differentiation are cultivated in adherent cultivation dish in a monolayer and are supposed to become almost 100 % confluent. The differentiation is induced by osteogenic differentiation media composed of α -MEM, 0.2 mM ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM β -glycerophosphate, 0.1 μ M dexamethasone and 10 % fetal calf serum and the process takes >4 weeks.

To prove a successful differentiation we use standard histological methods like Masson's blue trichrome and Von Kossa stain, to identify calcium deposits and collagen I fibres; or immunodetection of osteonectin, osteopontin, or procollagen I. Karbanová et al. [102] states that, by the week 3–4, we should be able to visually identify small extracellular aggregates of bone tissue.

Chondro-Differentiation Protocol and Evaluation

The dental-related stem cell chondro-differentiation can be successfully performed in either an adherent system (monolayer cultivation dish) and a non-adherent system (sphere-forming system). The chondrogenic differentiation medium is composed of high glucose DMEM, 0.2 mM ascorbic acid 2-phosphate, 0.1 μ M dexamethasone, 1 % ITS+1 supplement, 100 U/ml penicillin; 100 μ g/ml

streptomycin, 40 µg/ml proline, 100 µg/ml sodium pyruvate, 10 ng/ml TGF-β3. Four weeks into the chondro-differentiation the acid mucins within the extracellular matrix can be visualized by alcian blue staining [102], proving the success of the process.

Adipo-Differentiation Protocol and Evaluation

Nowadays, adipogenic differentiation is most often induced using copyrighted adipogenic media. While the precise formula is not public, it is usually some mixture of dexamethasone, 3-isobutyl-1-methyl-xanthine, insulin, and indomethacin and standard cultivation media [103]. The results can be evaluated 4 weeks after the onset of the differentiation and the proof is provided by 0.18% Oil red O staining of the intracellular lipid droplets in mature adipocytes with.

Neuro-Differentiation Protocol and Evaluation

The dental-related stem cells differentiation into neuro-like cells can be done, alike the chondro-differentiation, in both adherent and non-adherent systems using neurogenic differentiation medium composed of 50 ml DMEM, 50 ml F12 HAM, 2 ml B27 Supplement, 1 ml N2 Supplement, 10 ng/ml bFGF, 20 ng/ml EGF, 1 ml L-Glutamine, 1 ml Penicilin. The positive differentiation is proven using immunodetection methods to detect the markers typical for neural tissue—β-III tubulin, FORSE-1, GFAP, Nestin and PanNF.

Myogenic-Differentiation Protocol and Evaluation

Myogenic differentiation can be induced by treating the dental-related stem cell population with 3 µM 5-azacytidine in serum-free medium DMEM for the duration of 24 h. After this treatment, cells are washed with PBS and maintained in DMEM containing 10% bovine serum [104]. To prove a successful myogenic-differentiation we should be able to visually identify myocyte markers using immunofluorescence-labeled antibodies against human cardiac actin, cardiac actinin, cardiac troponin C, cardiac troponin T, connexin 43, sarcomeric actin).

Hepatogenic-Differentiation Protocol and Evaluation

The dental-related stem cells undergo hepato-differentiation in media composed of 20 ng/ml recombinant human hepatocyte growth factor and 2% bovine serum, for the first 5 days, and then a mixture of 10 ng/ml Oncostatin M, 10 nM Dexamethasone and 1% Insulin-Transferrin-Selenium-X (ITS-X), for another 15 days [47].

The success of the differentiation is evaluated by either using immunohistochemistry detection of albumin, α -fetoprotein, hepatic nuclear factor 4 α and insulin like growth factor, or periodic-acid-shift staining for visualising the stored glycogen.

Dental-Related Stem Cells Cryopreservation

SHED and third molar DPSCs represent the majority of dental-related stem cells material which is cryopreserved for either research or future personal-medical use. There is no clear winner amongst the cryopreservation methods available today as there are important questions pending to be answered: (1) What is the optimal cryoprotective agent and in which concentration should it be used to reduce or eliminate the side effect of cryopreservation?; (2) How many cells should be cryopreserved to obtain viable lineage after thawing?; and (3) What is the optimal process of cryopreservation, storage time and storage temperature to maintain maximum amount of viable cells?

Preceding cryopreservation the stem cell population should be harvested from the cultivation system. After centrifugation the cell pellet is resuspended using the chosen cultivation media and the amount of viable cells is measured. It is recommended to cryopreserve stem cell populations composed of at least one million cells. To avoid damage to the cells inflicted by the cryopreservation itself we expose the cells to cryoprotectants (e.g. glycerol or dimethyl sulfide—DMSO) in the concentration of maximum 10 % (as DMSO is cytotoxic) at room temperature for about 30 min, to allow the protectant to enter the cells but not damage them. The DMSO concentrate should be premixed before it touches the cells as it releases heat during the process of dilution. Finally, the treated cells should be fastly frozen using liquid nitrogen.

Good luck researchers!

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