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ORIGINAL RESEARCH

Candidate markers, culture strategies and DPSC perspectives used as cellular therapy in dentistry

Marcadores candidatos, estrategias de cultivo y perspectivas de las DPSCs como terapia celular en odontología

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ABSTRACT

Tissue engineering based on dental pulp stem cells is considered as a promising approach for regenerative dentistry. It purports the final target of morphologically and functionally replacing periodontal tissues and/or lost teeth by means of the *in vitro* synthesis of tissue-analog substitutes, or even a human tooth (called bio-tooth). Dental pulp stem cells represent a colony of adult cells which have the ability to auto-renovate and differentiate in different lineages. Dental pulp stem cells exact origin has yet to be fully determined; these stem cells seem to be the source of odontoblasts, which contribute to the formation of the dentin-pulp complex. Recently, achievements obtained through research conducted on stem cells, have allowed us to contemplate the possible therapeutic applications of dental pulp stem cells. Some studies have shown that dental pulp stem cells are able to produce *in vivo* dental tissues, including dental pulp and crown structures. Other research has demonstrated that these stem cells differentiate *in vivo* and *in vitro* into osteoblasts, neuroblasts, chondrocytes fibroblasts, and endothelium. In theory, a bio-tooth synthesized from autogenic dental pulp stem cells should be the best option to recover the whole structure and function of a human tooth. The aim of the present review article was to undertake a brief description of the location, origin, isolation and candidate markers of dental pulp stem cells in order to thus present application perspectives to be used in the dental clinic.

RESUMEN

La ingeniería tisular basada en las células troncales de pulpa dental se considera como un enfoque prometedor para la odontología regenerativa, con el objetivo final de reemplazar morfológica y funcionalmente los tejidos periodontales y/o los dientes perdidos a través de la síntesis *in vitro* de sustitutos análogos tisulares o, incluso, de un diente humano denominado biodiente. Las células troncales de la pulpa dental representan una colonia de células adultas que tienen la capacidad de autorrenovación y diferenciación en diferentes linajes. El origen exacto de las células troncales de la pulpa dental no ha sido completamente determinado y estas células troncales parecen ser la fuente de los odontoblastos que contribuyen a la formación del complejo dentinopulpar. Recientemente, los logros obtenidos a partir de la investigación de las células troncales nos han permitido contemplar las posibles aplicaciones terapéuticas de las células troncales de la pulpa dental. Algunos estudios han demostrado que las células troncales de la pulpa dental son capaces de producir tejidos dentales *in vivo*, incluyendo la dentina, la pulpa dental y las estructuras de la corona. Mientras que otras investigaciones han demostrado que estas células troncales se diferencian *in vitro* e *in vivo* en osteoblastos, neuroblastos, condrocitos, fibroblastos y endotelio por ejemplo. En teoría, un biodiente sintetizado a partir de las células troncales de la pulpa dental debe ser la mejor opción para recuperar la totalidad de la estructura y función de un diente humano. El objetivo de este artículo de revisión es hacer una breve descripción de la localización, origen, aislamiento y marcadores candidatos de células troncales de pulpa dental, para así plantear las perspectivas de aplicación en la clínica odontológica.

Key words: Dental stem cells, tissue engineering, CD105+, regenerative medicine.
Palabras clave: Células troncales dentales, ingeniería tisular, CD105+, medicina regenerativa.

INTRODUCTION

Tissue engineering based on dental stem cells opens new doors, which seem to be a promising pathway in order to be able to regenerate not only organs compromised by systemic diseases or irreversible conditions of the periapical and pulp-dentin complex, but also to be able to regenerate the complete structure of a lost tooth, that is to say,

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to synthesize *in vitro* a functional morphological substitute of a tooth or «bio-tooth».

Dental pulp stem cells (DPSC) represent a niche of mesenchymal stem cells harvested from an adult tissue. They have the ability to self-renovate and differentiate into multiple lineages whenever required. Self-renovation involves mitosis which can be symmetrical and asymmetrical; symmetrical mitosis occurs when two daughter stem cells totally identical to the parent cell are the result of cellular division, they exhibit high differentiation and self-renovation potential. Asymmetric mitosis occurs when cellular division elicits one stem cell identical to the parent cell and another stem cell compromised in a cellular line with limited ability to renovate and differentiate.

Another important aspect is the fact that these cells possess high therapeutic potential whenever there is a tissue lesion in different human organs, such as the central nervous system, bone tissue, cartilage and liver. Dr Huang *et al.*[1] conducted studies on DPSC differentiation and proliferation in neurons in the hippocampus of mice. Ikeda *et al.*[2] conducted research where they found that these cells effectively prevented drug-induced fibroses in the liver of rats, increasing thus hepatic function. In addition to their potential applications in the treatment of systemic diseases, DPSC have been considerably explored with the aim of achieving *in vivo* regeneration of the pulp-dentin complex of dental tissue. Therefore, currently, they are considered a promising strategy in the field of dental tissue engineering, due not only to the fact of avoiding ethical issues when obtaining the tissue source of the stem cells, but also due to the essentially easy surgical access during the process of harvesting the sample, preservation of cellular viability, and low morbidity after pulp extraction, since DPSC can generate more typical dentin within a short period of time when compared to non-dental stem cells. All the aforementioned renders them competent when the time comes to create a bio-tooth *in vitro.*

The aim or the present review article was to achieve a short description of location, origin, isolation and candidate markers of dental pulp stem cells, so as to be able to pose perspectives of future applications in the dental clinic.

DPSC ORIGIN AND LOCATION

The dental pulp is basically composed of four layers: the odontoblastic zone, a cell-free zone, a cell-rich zone and a central zone. Dr Shi *et al.*[3,4] have manifested that the expression of markers CD146+ and STRO-1+ of these stem cells in the dental pulp is restricted at the wall of the blood vessels, and absent in areas surrounding fibrous tissue and perineurium. This would tend to indicate that DPSC are located in the peri-vascular region of the pulp.

Even though DPSC have been isolated in recently exfoliated primary teeth, even in supernumerary teeth, the origin of these stem cells is not yet clearly known.

Analysis of DNA microarrays shows that human DPSC share a genetic expression profile similar to that of bone marrow mesenchymal stem cells. Expression of a variety of common mesenchymal markers would then imply that DPSC would possess a probable origin at the cranial neural crest, since embryologically, just like bone marrow, most oral tissues are originated at the neural crest.[5-7] In fact, DPSC could be originated from the neural crest, since they share certain similar genetic activity as well as biological behaviors. Additionally, it is believed that after histo-differentiation and morphogenesis of the dental germ the pulp experiences several mesenchymal odontogenic development stages in a certain order (neural crest stem cells, dental papilla ecto-mesenchymal stem cells, dental pulp stem cells, dental pulp precursor cells, pre-odontoblasts and odontoblasts).[8]

PREPARATION OF MEDIA AND REAGENTS

Sterilization measures

All media must be sterilized through filtration through a 0.22 µm membrane filter, to be then stored at 4 °C.

Detailed protocols for cultures

Security measures

Knowledge of health circumstances of donors is a requirement that must be fulfilled, since they could alter the sample's viability and endanger the success of possible treatments. Therefore, special care must be given to instruments and materials used in order to prevent disease transmission. Additionally, all ethical protocols must be observed, including signing an informed consent release which must be approved by an ethics institutional committee.

Culture medium

Mensenchymal stem cell medium (MSC medium)

Preparation of α- medium modified essential minimum (α- MEM) supplemented with glutamine 2 mM, fetal bovine serum 15% (FBS), 100 µg/mL

penicillin and 100 g/mL streptomycin. After this, the medium is supplemented with specific factors which will induce differentiation in desired cellular lineages (odontogenic, adipogenic neural, osseous).

Odontoblast differentiation medium

0.01 mM dexamethasone sodium phosphate and 1.8 monopotassium phosphate (KH_2PO_4) must be added into the MSC medium.

Adipogenic differentiation medium (adipocytes)

0.5 mM methylxanthine isobutyl, 60 mM indomethacin, 0.5 hydrocortisone and 10 insulin g/mL must be added to the MSC medium.

Neuroblast differentiation medium

A neurobasal medium should be prepared with B27 supplement, 20 ng/mL epidermal growth factor (EGF), 40 ng/mL basic fibroblast growth factor (bFGF), 100 µg/mL penicillin and 100 µg/mL streptomycin.

Tissue manipulation

Immediately after extraction, the tooth must be submerged into a sterile solution, normal saline solution or phosphate buffer saline (PBS), 7.4 pH. Addition of double concentration antibiotics, such as penicillin and streptomycin is optional, nevertheless advisable in all cases. In cases when a primary tooth unexpectedly exfoliates and cannot be kept in a sterile solution as previously described, it must be discarded due to the very high possibility of it becoming contaminated, afterwards it must be stored at 4 °C in order to preserve cellular viability. The faster the sample is harvested, with isolation of the cellular population in the laboratory, greater will be the probability of obtaining dental mesenchymal stem cells.

Pulp tissue isolation

In order to preserve cellular viability, it is important to keep the pulp tissue at a low temperature, in humid circumstances and under bacterial contamination control. This measure must be observed even during the tooth's sectioning procedure. The tooth could become contaminated due to the great amount of bacteria present in the oral cavity, even though the pulp is surrounded by hard dental tissues (enamel, dentin and cement) in the mouth. Nevertheless, during isolation of stem cells of exfoliated decidous teeth (SC-EDT) the dental pulp of the primary tooth is exposed to the environment of the mouth as well as the external milieu. Therefore, disinfection of teeth with hypochlorous acid, 250 ppm, pH 5.2 before extraction of human dental pulp tissue is a critical procedure in order to avoid bacterial contamination during sample processing and successful isolation of stem cells.

Primary culture of DPSCs/SC-EDT

Isolation protocol is based upon the ability of stem cells to adhere to culture plates and form cellular colonies.[9,10] Colonies derived from each of the cells can be obtained by two methods:

1) Culture by explants: in this procedure the pulp is cut into 3-5 mm segments, which are then taken to a polypropylene box, after this, medium is applied so that cells migrate from the tissue and perform confluence. It is very important to change the medium every 3 days.
2) Performance of enzymatic disintegration: this procedure consists on applying collagenase-dispase in a polypropylene box with the whole pulp, after this, it is taken to incubation during 2 hours at 37 °C; pipetting will be performed (with pipettes of successively smaller size) in order to achieve complete mechanical dissociation.
   It is recommended to change the medium one day after cell isolation procedure, this minimizes contamination incidence. Nevertheless, when there is high risk of contamination, the medium can be changed 5 hours after isolation.

In vitro cellular expansion

Colonies are easily recognizable in culture recipients, generally one week after cell isolation. When cells reach approximately 80% confluence (generally after 2-3 weeks) the following cell passage must be followed: in theory, each colony derives from a single Fibroblast-Colony Forming Unit (F-CFU).[9-11] It can be isolated through the use of cloning rings. Moreover, individual colonies can be harvested through a series of passages after a dilution process, or through classification of Fluorescence Activated Cells (FAC) using STRO-1 and CD146 antibodies.[12]

Cryopreservation and recovery

The most important points of the procedure are the following:

When cultured cells reach 80-90% confluence, they become candidates for cryopreservation. The number of cells should be around 1-2 x 106/vial which contains 1.5 mL of freezing medium. When the number of cells is either too high or too low recovery rate might decrease.

The cryopreservation agent dimethylsulphoxide (DMSO) must be gradually incorporated until reaching 10%, at low temperature along with a high serum concentration (90% FBS and 10% DMSO) which must be used to assist cell survival.

Common mesenchymal markers

Dr Lin H *et al*. in 2009 and Dr Beyer Nardi *et al*. in 2008 reported that phenotypically, mesenchymal stem cells express the following markers: CD49a/CD29+, CD44+, STRO-1+, CD90+, CD1-105+, CD-106+, CD-146+, CD-140b+, CD-166+, CD34/45-, and CD-271+. The expression of these markers help to suitably classify within the group of human mesenchymal stem cells (HMSC).

CD24: human CD24 cellular surface antigen is a sialoglycoprotein which is anchored to the cellular surface by means of a glycosylphosphatidylinositol considered a specific marker for apical papilla stem cells (APSTC).

CD90: CD90 is a protein belonging to the immunoglobulin superfamily, its main ligand is CD45,[13] it is expressed in 10-40% in CD34+ cells. It is involved in cell-cell interaction. It is considered that this marker is expressed in early mesenchymal precursors which have the capacity of differentiate in osteoblasts. CD90+ can be expressed in neurons, umbilical cord cells, bone marrow cells and in the stroma of mensenchymal cells.

CD105: also called endoglin it is an homodimeric membrane glycoprotein mainly associated to human vascular endothelium. It is also found in bone marrow pre-erythroblasts, activated monocytes and lymphoblasts in child leukemia. CD105 is a component of the receptor complex of beta transforming growth factor (TGF-β) and it bonds with great affinity to TGF-B1. It is expressed in activated monocytes, activated macrophages, erythroid precursors, fibroblasts, dendritic follicular cells, melanocytes, cardiac cells, smooth muscle vascular cells and endothelial cells.[14]

STRO-1: this is a marker expressed in the early development of mesenchymal stem cells, its expression declines when genes associated to osteogenic expansion and differentiation such as the Core Bonding Factor A1 (CBF-A1) interacts with osteopontin and osteocalcin. This molecule identifies bone marrow stromal precursors and it expresses in deciduous tissue cells (the maternal component of the maternal-fetal interface mainly composed by stromal type cells such as glandular cells and leukocytes) as well as placenta, adipose tissue and dental pulp. STRO-1 was identified as a specific antigen for bone marrow isolated mesenchymal stem cells.[15]

CD146: this is a glycoprotein with MUC18 cellular surface. It is a member of the immunoglobulin super-family, it is homologous to several cellular adhesion molecules and is associated to tumor progression and metastasis development in human malignant melanoma. It is expressed in several endothelial cell lines such as bone marrow stroma and some T lymphocytes sub-types.

CD 34: it is an antigen to hematopoietic system precursor cells. It is a trans-membrane protein which was initially detected in cells of the lymphohematopoietic system, precursors of the myeloid series and present in the bone marrow. This antigen is also observed in the vascular endothelium in dendritic cells of the upper dermis, in the endonerium as well as several soft tissue tumors. Like the CD34 marker, it is not expressed by hMSCs, but is used for their immunophenotypic characterization.

CD44: it is a cellular membrane glycoprotein. CD44 is a marker of several cancer stem cell types, it is also expressed in mesodermal cells, hepatocytes and fibroblasts.

CD271: it is a nervous growth factor receptor (NGFR) It is also known as p75 due to its molecular mass. It can be found in the central and peripheral nervous system, in Schwann cells as well as in bone marrow.

DENTIN-PULP COMPLEX REGENERATION WITH DPSC

Several studies have demonstrated the fact that DPSC play an essential role in the process of pulp-dentin tissue regeneration.[16-22] Dr Gronthos *et al*. have recombined human DPSC with hydroxyapatite tricalcium phosphate (HA-TCP), and transplanted them sub-cutaneously in immunodeficient mice. In this study, tissue recovered contained dentin-typical structures surrounded by cells such as odontoblasts, with long cytoplasmic processes in the mineralized matrix. Certain dental pulps, as well as structures contained in blood vessels can be observed around the dentin matrix.[16,19-21] Similar dentin formations can be detected *in vivo* in SHEDS recombined with HA-TCP. Moreover, DPSC are able to generate an outline of pulp-dentin complex and reparative dentin on a matrix.[22,23]

BIO-TOOTH ORIGINATING FROM DPSC

Tissue engineering based on dental pulp stem cells is considered a promising approach for dental regeneration with the aim of replacing lost teeth by means of an analogous substitute of the human tooth called bio-tooth. Dental pulp stem cells (DPSC) represent a niche of mesenchymal stem cells obtained from an adult tissue which has the ability to self-renovate and differentiate in different lineages. The exact origin of DPSC has not yet been fully determined, and these stem cells seem to be the source of odontoblasts which contribute in turn to the formation of the dentin-pulp complex. Recently, achievements obtained based on stem cell research, have permitted to contemplate possible therapeutic applications of DPSC. Some studies have demonstrated that DPSC are capable of producing dental tissues *in vivo*, including dentin, pulp and crown structures. Other research projects have proposed that these stem cells can give rise to tissue formation, such as bones. In theory, a bio-tooth originated from autogenous DPSC should be the best option in order to recuperate the whole structure and function of a human tooth.

Loss of a tooth is a common disease, and is a common occurrence in individuals of all populations. This negatively affects masticatory efficiency, language function, facial esthetics, and patients self-esteem. Current therapeutic approaches are mainly centered in conventional prosthetic rehabilitation, or implant supported rehabilitation, which inevitably may fulfill, or not, the patients esthetic and functional expectations.

A bio-tooth is designed as an autologous substitute for a human tooth which could be re-integrated into the mandible and perform all normal functions of a natural tooth, including regeneration ability in case of a lesion. Several studies have demonstrated that a bio tooth could be reconstructed on a matrix from dental stem cells.[18,20,24] Dr Nakao *et al*., by means of their bio-engineering studies, showed that incisor teeth could be completely reconstituted using dental mesenchymal and epithelial cells in a three-dimension collagen gel.[25] These tooth buds can replicate dental embryonic organogenesis. Moreover, Dr Ikeda *et al*.[26] demonstrated the fact that these bio-teeth in the alveolar bone can perform functions of a normal tooth, including eruption, occlusion, and mastication. This presents a new and thrilling perspective on the future applications of bio-teeth.

Other studies have indicated that proportion of mesenchymal and epithelial cells is much more important than regulation of normal morphogenesis of the crown.[18,27,28] Nevertheless, it has been observed that no root structure has been formed during a process of regeneration based on DSPC; this could be due to the complex mechanism involved in root development. Many types of cells, including DPSC (dental pulp stem cells), SCED (stem cells of exfoliated deciduous teeth) and SCAP (stem cells of apical papilla) have recently been successfully re-programmed into induced pluripotential cells (IPC), which represent a great promise for regenerative medicine.[29,30]

Finally, the greatest challenge for bio-engineering is the formation of a bio-tooth which can be transplanted in the jaws of the patient and achieve continuous growth and timely eruption.

DPSC APPLICATION PERSPECTIVES IN THE DENTAL CLINIC

For many years, dentistry has devoted itself to traditionally or conventionally restore teeth, whereby the tooth is restored with synthetic materials. We have also observed how, through time, the generation of a bio-tooth from stem cells extracted from the patients´ third molars has been mentioned. Research in this subject has advanced in leaps and bounds, obstacles to obtain this type of bio-tooth have been overcome. Nevertheless, technical obstacles still need to be addressed. Generation of a tooth from DPSC will be an unpredictable opportunity for tooth loss treatment as well as treatment for other dental diseases. DPSC, as multi-potential cells, play a fundamental role in the regeneration of different human tissues. Nevertheless, the greater challenge when dealing with these dental cells is found in the tooth regeneration area where it can find immediate application in dental practice. Dental pulp stem cells can be used *in situ* in different types of autologous treatments. Future strategies will undoubtedly concentrate on *ex vivo* and *in situ* differentiation of these dental stem cells, matrix optimization and exploration of necessary odontogenic micro-environment for odontoblast differentiation.

During the last years of dental stem cells study, it is evident they have provided an important basis upon which we could begin to explore their therapeutic potential in the clinical environment. Nevertheless, the process of synthesizing a bio-tooth with masticatory, neurovascular and sensory functions as well as support tissues, based on dental stem cells, could be a much more complex procedure than expected.

The most challenging aspect of tissue engineering might be nervous, vascular regeneration as well as functional regeneration. Several issues are involved in the making of a bio-tooth, among others, we can mention:

- **Identification and «primordial undifferentiated state»** of stem cells: the understanding of self-renovation mechanisms will allow *in vitro* regulation of adult stem cells in order to generate sufficient number of cells necessary to the different applications. An alternative could be embryonic stem cells (ESC) obtained by nuclear transfer technologies. Nevertheless, this process implies the use of donated fertilized ovum and discarded embryos. Another approach would be the *in vitro* manipulation of stem cells in order to allow the preservation of their undifferentiated primordial state, known as «stemness». Recent experimental methods for reprogramming somatic cells are conducted through the introduction of inducing factors; this provides the possibility to manipulate stem cells into multi-potent cells to be the used in a variety of applications.[31-33]
- **Dental morphogenesis and tooth type determination.**
- **Controllable growth of bio-tooth and eruption:** control and prevention of abnormal expansion must be conducted under supervision. Careful observation of this possibility is of the utmost importance, since evidence has proven that MSC lose genetic stability with time, and are prone to tumor formation.[34]
- **Re-vascularization:** vascularization can be difficult for teeth which might have small-sized entrance of the blood vessels´ apical channel (< 1 mm). The size of the apical orifice would affect the internal growth of the blood vessels in the processed pulp tissue. The larger the opening, more probabilities will arise of angiogenesis occurrence. Therefore, immature teeth with open apexes are the best candidates for pulp tissue regeneration. It was considered that use of angiogenic inducing factors such as vascular endothelial growth factor (VEGF) and/or derived from platelet development growth factor (PDGF) should improve and accelerate pulp angiogenesis. Moreover, there is now the possibility of manufacturing synthetic matrixes impregnated with these growth factors.[35,36]
- **Neuronal regeneration:** with respect to innervation, it is probable that regenerated pulp might contain nervous fibers of adjacent tissues. DPSC have shown they are able to produce neurotrophic agents or possess the potential of neuronal differentiation.[37] Nevertheless the development of *in vivo* or *in vitro* techniques to achieve innervations of the pulp-dentin complex is a difficult subject. The reason why dentin is so sensitive to several irritations lies within the hydrodynamic activities of the dentin tubules in association with A-delta sensorial fibers which extend within the dentin tubules in the pre-dentin layer. Since recently generated dentin seems to lack suitable organization of the dentin tubules, A-delta regenerated fibers which reach the pulp-dentin junction, would not elicit dentin normal sensitivity as would be the case for natural teeth.
- **Ability to regenerate new odontoblasts which coat the surface of existing dentin and produce new dentin:** understanding the regulation of stem cells during differentiation and production of specific tissue require the production of extra-cellular specialized materials such as bone, dentin, cartilages and tendons. Production of extra-cellular matrix and its maturation in specialized tissues imply an sequential activation of signaling cascades. Control and availability of these artificial signals in a given stage can facilitate regeneration of the desired tissue.[38] It must be equally borne in mind that with a better blood supply, blood density might be optimal, resulting thus in an extracellular matrix of suitable quality. Dr Huang G *et al*. showed that new cells, such as odontoblasts, formed in the existing dentin wall which has been chemically disinfected.[39] Another problem encountered in the process of functional regeneration of pulp/dentin is enamel regeneration. Intact dentin is required in order to superpose enamel or repair the damaged tooth. Enamel cannot self-regenerate, nevertheless, enamel regeneration has been proven when using methods involving enamel proteins such as amelogenin or the use of chemical agents such as hydroxiapatite.[40,41] Nevertheless, these approaches seem to be difficult to apply clinically; or they can only produce a minimal amount of regenerated enamel on the existing natural enamel. Thus, the enamel is the final challenge of the biological restoration of teeth after pulp and dentin regeneration, even though use of artificial materials will probably still be required.
- **Host rejection:** the understanding of interactions taking place between stem cells and immunological system is essential to the use of cells in the clinic. DPSC are not immunogenic. Moreover, they modulate the immune response during graft of cellular transplant procedure. Nevertheless, more extensive research is needed in order to determine whether allogeneic DPSC might exhibit short-or long-term graft rejection.

  As was mentioned before, availability of cell sources for the regeneration of tissues based on cells is a clear difficulty. A stem cell and teeth bank is an essential infra-structure that needs to be implemented with the aim of developing therapies based on dental pulp stem cells, both for autologous or allogenic applications.

Ultimately, current scarcity of post-natal stem cells could be solved with generation of induced pluripotent cells (IPC) based on somatic cells or dental stem cells.

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