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STEM-CELL BIOLOGY FOR TOOTH AND PERIODONTAL REGENERATION (M BARTOLD, SECTION EDITOR)

Translational Research and Therapeutic Applications of Neural Crest-Derived Stem Cells in Regenerative Periodontology

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Abstract Regeneration of periodontal tissues aims to utilize tissue engineering techniques to restore lost periodontal tissues including the cementum, periodontal ligament and alveolar bone. Regenerative dentistry and its special field regenerative periodontology represent relatively new and emerging branches of translational stem cell biology and regenerative medicine focusing on replacing and regenerating dental tissues to restore or re-establish their normal function lost during degenerative diseases or acute lesions. The regeneration itself can be achieved through transplantation of autologous or allogenic stem cells, or by improving the tissue self-repair mechanisms (e.g. by application of growth factors). In addition, a combination of stem cells or stem cell-containing tissue with bone implants can be used to improve tissue integration and the clinical outcome. As the oral cavity represents a complex system consisting of teeth, bone, soft tissues and sensory nerves, regenerative periodontology relies on the use of stem cells with relatively high developmental potential. Notably, the potential use of pluripotent stem cell types such as human embryonic stem cells or induced pluripotent stem cells is still aggravated by ethical and practical problems. Thus, other cellular sources such as those readily available in the postnatal craniofacial area and particularly in oral structures offer a much better and realistic alternative as cellular regenerative sources. In this review, we summarize current knowledge on the oral neural crest-derived stem cell populations (oNCSCs) and discuss their potential in regenerative periodontology.

Keywords Periodontal regeneration · Regenerative periodontology · Neural crest-derived stem cells · Oral stem cells · Osteogenic differentiation · Neuronal differentiation

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Introduction

Translational research involves application of basic scientific discoveries into clinical findings and, simultaneously, the generation of scientific questions based on experimental and clinical observations. Nearly 40 yeas after the discovery of stem cells within the human cord blood, the field of stem cell research is entering the translational phase. Being able to regenerate multiple cell types, stem cells offer hope to help patients in clinical situations, in which conventional methods have failed. According to studies from the United States Centers for Disease Control and Prevention, nearly 50 % of Americans over 30 develop at least one form of periodontal inflammation. Notably, current treatment options mostly fail to restore or maintain the natural periodontal structures. Fundamental research on oral stem cells provided strong evidences for their ability to regenerate bone cell and neurons in addition to periodontal structures. Thus, translational stem cell-based approach to the regeneration of periodontal structures represents an unique opportunity to improve the patient quality of life. Prior to the translation of stem cell research into the periodontal clinical practice, several ethical, practical and regulatory issues must be addressed, which we discuss in the following.

Chapter 1: Neural Crest-Derived Stem Cells as a Tool in Regenerative Periodontology

Neural Crest and Adult Neural Crest-Derived Stem Cells

The neural crest was first described as the *Zwischenstrang* (German, *zwischen*, between; *Strang*, cord) by the Swiss anatomist and cardiologist Wilhelm His. He described the neural crest as a transient embryonic structure appearing between the neural tube and epidermis during avian embryonic development. After neurulation, embryonic neural crest cells (NCCs) migrate out of their niche and engender ectodermal cell types (e.g. Schwann cells, peripheral neurons, melanocytes and keratinocytes) in addition to mesenchymal cells (cranial bones, cartilage and fat cells). Due to this intrinsic potential to give rise to cells of two germ layers (ectoderm and mesoderm), NCCs possess an extraordinarily high developmental potential surpassed only by totipotent cells of the zygote and pluripotent embryonic stem cells. Adult, tissue-resident neural crest-derived stem cells (NCSCs) were long term believed to be an in vitro phenomenon. During the last 15 years, however, an emerging line of evidence supported the hypothesis that at least a limited number of adult NCSCs may exist in the human body even in the adulthood. NCSCs can be found in different tissues and organs, especially within the craniofacial region [1–7]. Such adult human NCSCs are able to undergo self-renewal and possess a surprisingly high differentiation potential in vitro and in vivo (reviewed in [8]). Due to their easy accessibility and high plasticity, adult NCSCs represent an ideal stem cell type for the use in regenerative dentistry. Concerning the marker expression, most adult human NCSC populations are characterized by the expression of the intermediate filament nestin, the surface receptor $p75^{NTR}$ (CD271) and the carbohydrate HNK-1 in addition to the neural crest-specific transcription factors Snail and Twist in vitro and in vivo (see [8] for a full marker list).

Neural Crest Origin as Unifying Definition of Adult Dental Stem Cells

The human oral cavity contains various adult stem cell populations able to perform self-renewal and multi-lineage differentiation and to contribute to dental regeneration (Fig. 1).

Among others, stem cells have been described within the dental follicle (dental follicle stem cells (DFCs) [9]) and periodontal ligament (periodontal ligament stem cells (PDLSCs) [10]) and in exfoliated deciduous teeth (stem cells from human exfoliated deciduous teeth (SHED) [11]). However, the definition and classification of such dental stem cells are a matter of ongoing scientific debate. While some reports classify them as ectomesenchymal stem cells, others define them as mesenchymal or neural crest-derived. Notably, dental stem cell populations and cranial NCSCs show comparable odontogenic differentiation potential [12]. Further, the neural crest is embryonic origin of most dental cell types and surrounding tissues including the odontoblasts, cementoblasts in addition to the dental pulp, periodontium and alveolar bone [13]. The developmental origin of the tooth has been spectacularly demonstrated by Mitsiadis in 2003. In that study, xenogenic transplantation of mouse neural crest into a chicken embryo resulted in development of tooth-like structures [14]. Based on these developmental evidences, we propose a unifying classification of such dental stem cells that is based on their clearly demonstrated developmental origin and their broad multi-lineage differentiation potential (ectoderm and mesenchyme). Consequently, we suggest the term oral neural crest-derived stem cells (oNCSCs) as a new category that would include most adult dental stem cells described so far.

Animal Serum-Free Cultivation

Theoretically, separated NCSCs could be directly re-transplanted back into the patient. However, the number and density of endogenous human NCSCs in most niches within the craniofacial compartment are too low to achieve significant therapeutic effects without prior ex vivo expansion. Routinely, adult human stem cells are cultivated in a medium (e.g. DMEM) supplemented with foetal calf serum (FCS) that contains essential growth and differentiation factors and assures this way on optimal cell expansion. Notably, due to its animal origin, the use of FCS-containing medium harbours the risk of transmission of animal-borne pathogens in addition to potential immune response

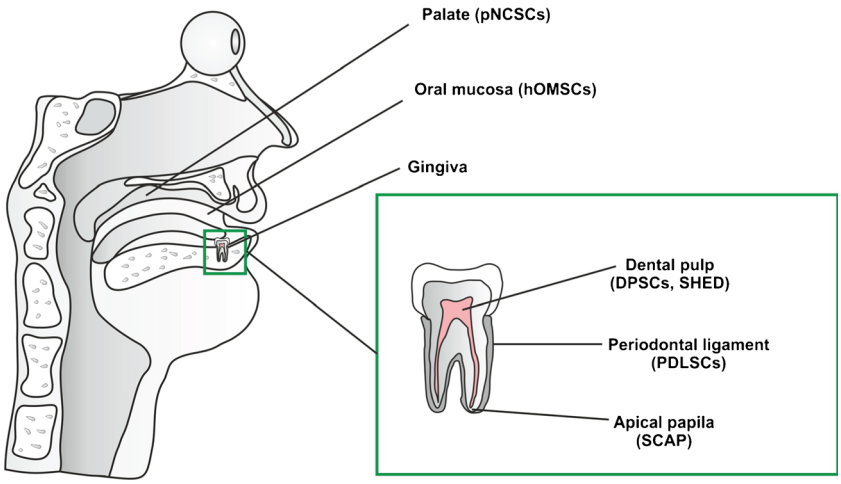

Fig. 1 Neural crest-derived human stem cell within the oral cavity

reactions in the human body [15–17]. Recently, various animal serum-free cultivation methods for different adult stem cells have been developed (e.g. the supplementation of medium with recombinant growth factors (such as FGF-2 or EGF).

The insufficient support with growth and differentiation factors in serum-free medium however can negatively influence the cellular viability [18]. Concurrently, the Food and Drug Administration (FDA) recommends that maximal in vitro expansion of human stem cell preparations should not exceed 5 weeks [19]. To address this challenge, we developed a novel cultivation method for NCSC-derived therapeutics based on the use of human blood plasma [20] (Fig. 2) and combined it with a closed, clinical Good Manufacturing Practices (GMP)-grade Afc-FEP bag system [21] (Fig. 3). Since human blood plasma can be isolated from the same patient, this approach can be personalized.

In particular, we showed that adult NCSCs can be grown embedded in a 3D blood plasma matrix within gas permissive closed bag system resulting in increased proliferation, unchanged ploidy and capability for self-renewal as well as remaining potential to differentiate into neuronal and osteogenic lineage. These findings emphasize the potential of this expansion method for application of NCSCs in complex regenerative approaches, including regenerative dentistry. Since NCSCs and human blood plasma can be theoretically obtained from the same patient, this approach is personalizable and potentially reduces the exposure of the graft to xenogenic ingredients to a minimum.

Osteogenic Properties of oNCSCs

Further underlining the neural crest origin of these cells, all oNCSCs described so far have the intrinsic ability to give rise to osteogenic cell types. In general, methods originally developed for osteogenic differentiation of mesenchymal derivatives (MSCs) can be transferred to oNCSCs. In particular, a combination of dexamethasone, β-glycerophosphate and L-ascorbic acid-2-phosphate in a FCS-containing medium has been widely used for osteogenic induction of various oNCSC populations including palatal NCSCs, oral mucosa NCSCs in addition to NCSCs derived from the periodontal ligament and dental pulp [22–27]. Osteogenic induction at early phase can be assessed by determination of the activity of alkaline phosphatase (ALP) or by RT-PCR- or qPCR-based measurement of marker gene expression (e.g. Osteopontin, Osterix, Osteonectin).

Neurogenic Properties of oNCSCs

Besides their ability to generate mesenchymal progeny, embryonic neural crests (NCs) can differentiate into ectodermal cell types, including sensory neurons and glial derivatives such as Schwann cells. In addition to embryonic NC, several promising attempts have been made to investigate the neurogenic potential of postnatal oNCSCs. In particular, cultivation of oNCSCs of periodontal and palatal origin under conditions leading to neuronal differentiation of neural stem cells (Fig. 4) leads to upregulation of neuronal marker genes at transcript and protein level [3, 4]. Importantly, this differentiation method leads to generation of neuronal cells exhibiting several features of functional neurons including the ability to generate KCl-induced Ca^{2+} spikes and integration into pre-existing neuronal networks in vitro and in vivo [28]. Alternatively, oNCSCs can be differentiated into neuronal lineage in a serum-free medium supplemented with neurotrophin-3 and brain-derived neurotrophic factor [29].

Chapter 2: Comparison of Human NCSCs Isolated From Pulp and Periodontal Ligament

The critical part of stem cell-based engineering of periodontal tissues is the selection of the appropriate cell type. Intraoral sources for human NCSCs are exfoliated deciduous tooth pulp, dental follicle, apical papilla, dental pulp (DP), periodontal ligament (PDL), gingiva, oral mucosa, palatal

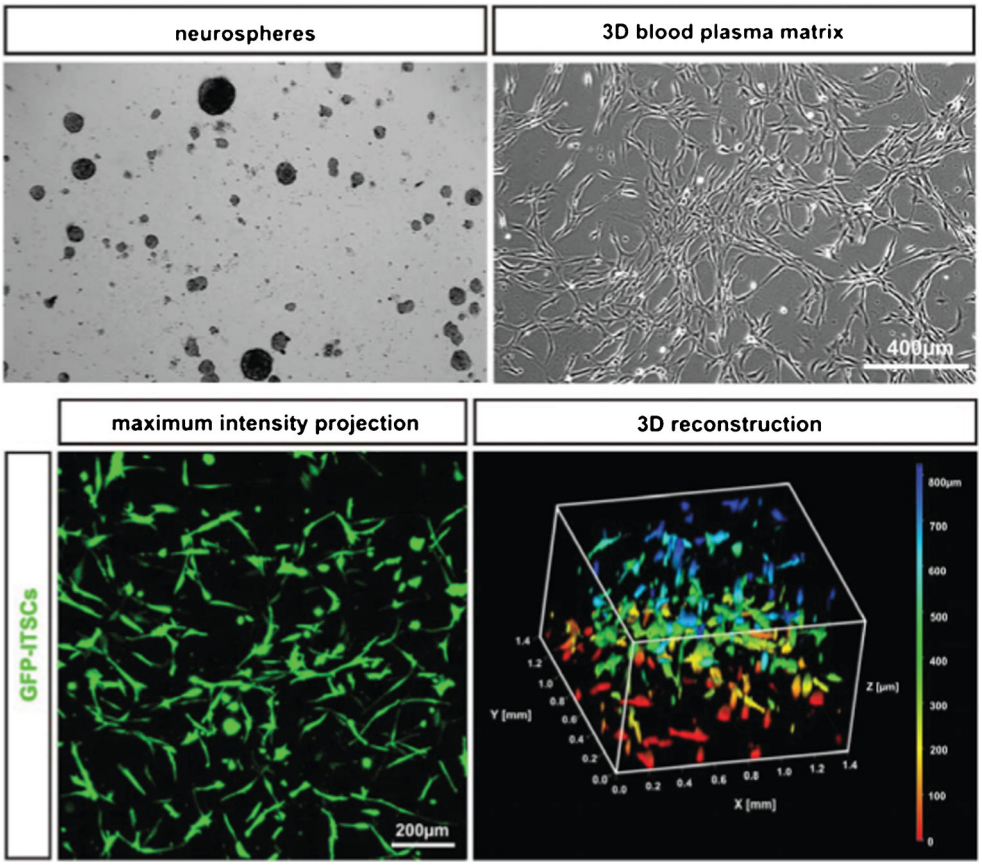

Fig. 2 Cultivation of human NCSCs within human blood plasma-derived matrix. Human NCSCs were transiently transfected with GFP and cultivated as neurospheres. After dissociation of the spheres (*top left*), NCSCs were expanded as 3D culture embedded in the matrix (*top right*) not only on the top or at the bottom of the matrix as visualized using confocal laser scanning microscopy. The figure is taken from the OA version of [20]

connective tissue and palatal adipose tissue (see overview by Hakki et al. 2015 [30]. The extra-oral sources for stem cells are mesenchymal derivatives (MSCs) isolated from long bone/iliac crest bone marrow and adipose tissues not covered by this chapter [30–44].

Implications for Human NCSC-Based Periodontal Tissue Engineering

A profound knowledge on the biological differences and shared characteristics between the dental NCSC populations

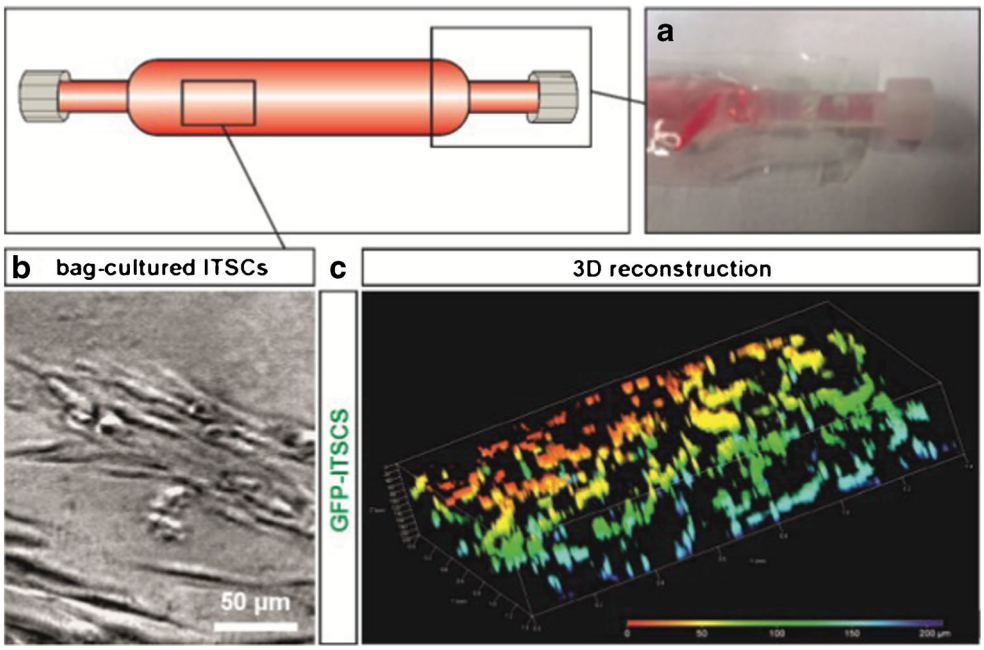

Fig. 3 Human NCSCs grow three-dimensionally in clinical grade culture bags. **a** Afc-FEP bag containing NCSCs in medium supplemented with human blood plasma. **b** Phase contrast microscopy image of bag-cultured NCSCs revealing characteristic morphology comprising long-shaped cell bodies. **c** Confocal laser scanning microscopy analyses (Z-sectioning) followed by three-dimensional reconstruction showed three-dimensional growth of bag-cultured NCSCs previously transfected with GFP. The figure and figure legend are taken from the open access version of [21], CC4.0 licence

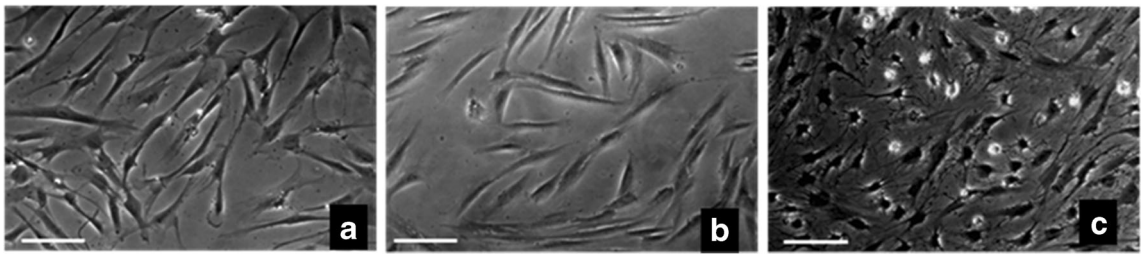

Fig. 4 oNCSCs have the intrinsic ability to give rise to neuronal cells. Morphology of human PDL-NCSC cultures viewed under a phase contrast microscope (**a**). When cells were subjected to our previously published three-step neurogenic differentiation protocol (Király et al. 2009 [68]), after the first phase of neuroinduction, cells changed morphology and became shorter and higher (**b**). By completion of the three-step treatment protocol, after 8 days, the majority of PDL-NCSC cells display multipolar neuronal morphology. (**c**) Confluent oNCSCs after 3 weeks. *Bars* indicate 100 μm

introduced above is critical for design and future application of regenerative periodontal therapies. Some studies [45–47] investigated the potential and limitations of human NCSCs regarding their ability to regenerate DP and PDL. Notably, both the dental pulp and the periodontal ligament represent unique and very complex tissues. In contrast to MSCs, palate neural crest-derived stem cells (P-NCSCs) have been shown to differentiate towards dentinogenic lineage, whereas PDL-NCSCs are able generate cementogenic progeny [48]. This unique plasticity encourages the use of these cells to reconstruct lost dental and periodontal tissues. Comparison of PD-NCSCs and PDL-NCSCs isolated from impacted third molar revealed that both cell types express the ESC markers Oct-4 and Nanog (weak in PDL-NCSCs), the mesodermal marker vimentin and strong expression of MSC markers (CD73 and CD90) at a RNA level [49]. In sum, this study concluded that P-NCSCs represent a more primitive stem cell (SC) population in comparison to PDL-NCSCs. In contrast, we and other groups reported similar expression of stem cell-associated cell surface antigens in both NCSC populations [50–52]. These contradictory results could be explained by the different origin of the investigated cells. In particular, Ponnaiyan et al. [49] used impacted third molars for NCSC isolation, while Hakki et al. [30] isolated NCSCs from premolar tooth. Notably, besides the different source, the age of the stem cell donors was higher in the case of the impacted third molars [49]. Thus, age-associated differences cannot be excluded. In our hands, DP-NCSCs express higher levels of cytokeratins 18 and 19 (CK-18 and CK-19). In addition, they seem to possess odontoblast differentiation potential, but not to be able to generate functional neurons or muscle cells [30]. Further, DP-NCSCs showed faster proliferation rate and a higher telomerase activity. We also showed that DP-NCSCs express higher level of BMP-2 and BMP-6 mRNA. While there was no significant difference in BSP and RunX2 transcripts in both cell types, the DP-NCSCs expressed higher OCN and lower COL I levels when compared to the PDL-NCSCs. The expression of anti-inflammatory cytokines was higher in the PDL-NCSCs compared to that in the P-NCSCs. Our data also suggested that PDL-NCSCs can be a good candidate for allogeneic SC-based therapies due to their superior immunomodulatory properties. These properties of PDL-NCSCs can be beneficial for wound healing process of periodontal tissues. While premolar DP-NCSCs showed significantly higher proliferative behaviour compared to premolar PDL-NCSCs, molar DP-NCSCs show proliferation rates that are comparable with molar PDL-NCSCs. Thus, further comparison studies should be performed and interpreted with caution as the methodology and donor age of the tissues may affect the results. In in vivo conditions, it was concluded that P-NCSCs and PDL-NCSCs may maintain their NCSC characteristics after implantation [32]. Notably, P-NCSCs seem to be more stable when compared to PDL-NCSCs [32]. However, the reason for reduced lineage-specific differentiation potential in case of PDL-NCSCs remains unclear. This could be explained by lower proliferation rate and telomerase activity of PDL-NCSCs as shown by the Hakki et al. study [30]. Differences in the potential of these NCSCs should be considered for cell-based bioengineering of periodontal tissues (Fig. 5).

Immunologic Considerations: Allogenic Use and Immunomodulation

Studies demonstrating that bone marrow MSCs appear to be immunoprivileged in allogeneic settings by modulating lymphocyte activity have paved the path for exploration into the potential use of allogeneic MSCs for tissue regeneration [17, 53].

The immune system recognizes tissue compatibility and can raise an effective immune response against pathogens or incompatible allogenic tissues. Immune mechanisms confer immediate protection against foreign organisms (innate immunity) and specific immune responses to neutralize pathogens (adaptive immunity). Immuno-regulatory functions of P- and PDL-NCSCs were reported in the literature [54, 55]. Tissue compatibility or incompatibility is determined from allelic similarities or disparities at genetic loci that encode the major histocompatibility complex (MHC) antigens, also called the human leukocyte antigen (HLA) system. In our study, we were able to demonstrate that PDL-NCSCs expressed higher level of HLA-G compared to DP-NCSCs. Thus, we suggest that PDL-NCSCs could represent a more immunoprivileged stem cell type.

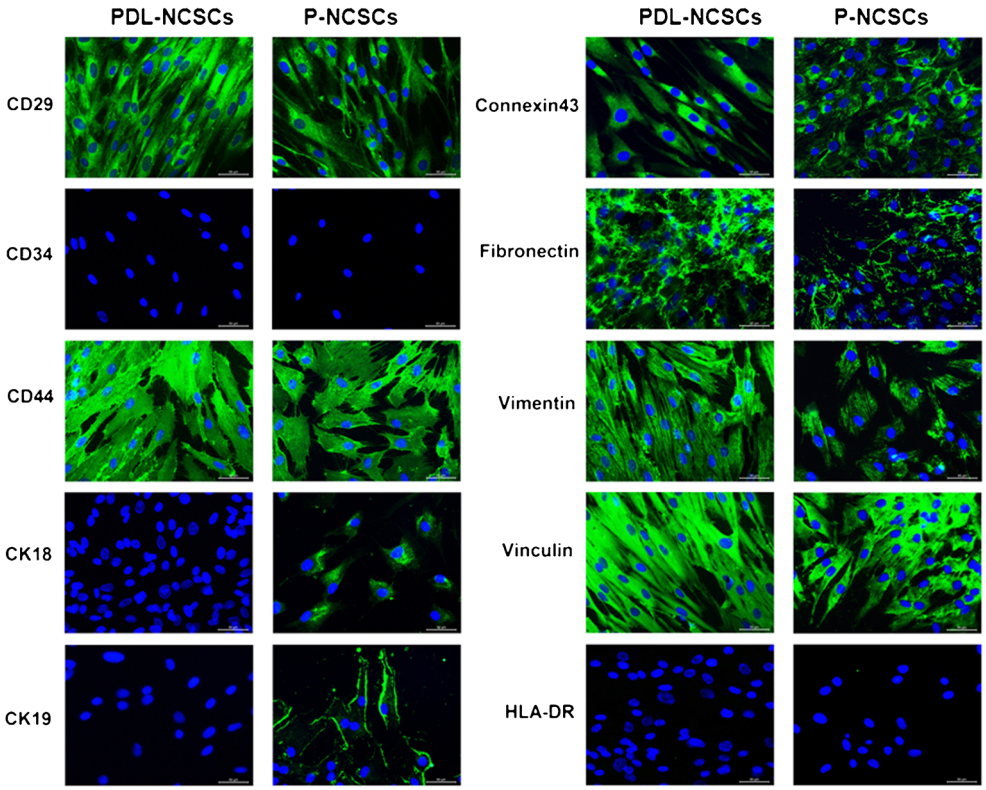

Fig. 5 In order to compare, the newly propagated population of P-NCSCs and the PDL-NCSCs was assayed using antibodies. The RNA expression of the lineage markers using RT-PCR was also been assessed (not shown). Modified from [30]

Chapter 3: Delivery of Stem Cells to the Periodontium

Human NCSC Biobanking

The term "biobank" describes facilities that store biological samples, from small tissue collections to wide repositories featuring a variety of tissues and biological sample types [55, 56]. Biobanking has been defined as a structured resource for genetic and medical research and their therapeutic applications. It includes human biological material and extensive associated information [57–59].

For future clinical application, oNCSCs could be easily adapted to such set-up, since at least in our hands; these cells tolerate cryogenic storage remarkably well without any negative effects of their proliferative behaviour or multipotency. In particular, human NCSCs were successfully cryopreserved and stored for up to 6 months without changes of their crucial properties including marker expression, differentiation potential and proliferative behaviour [20, 21]. In the future, oNCSCs could be propagated in unstirred or stirred cultures in a large-scale bioreactor culture set-up suitable for controlled production of high numbers of periodontal stem cells for therapy. After this expansion step, oNCSC with no immediate plans for expansion and use could be processed to biobanking and stored until needed.

Transplantation of oNCSCs into Rodent and Large Animal Models

We investigated the in vivo regenerative capacity of human adult NCSCs in 10-week-old athymic nude rats as described early [60]. In particular, we investigated whether NCSCs could be differentiated into the osteogenic lineage and, if so, whether these cells are able to regenerate periodontal tissue in vivo in an athymic rat model. In sum, our data clearly demonstrated that human adult NCSCs are capable to regenerate elements of bone and collagen fibres in vivo. Light microscopic sections taken from the experimental rats clearly revealed all histological features related to a normal periodontium including the periodontal ligament, the supporting bone and the dentin covered with cementum (Fig. 6). Within the periodontal ligament itself, we detected typical collagen fibres in addition to blood vessels.

In addition to our study, the efficacy of implanting autologous oNCSCs into a variety of surgically created periodontal defects in animal models in order to improve the restoration of damaged periodontal tissues has been documented by other groups [45, 55]. Also, in these experimental set-ups, oNCSCs revealed their beneficial effects in terms of improved periodontal regeneration. Besides the rodent models, stem cell-mediated regeneration of the periodontium has been demonstrated in a large animal model (reviewed in [44]). Ultimately, being more comparable to the human system, large animal

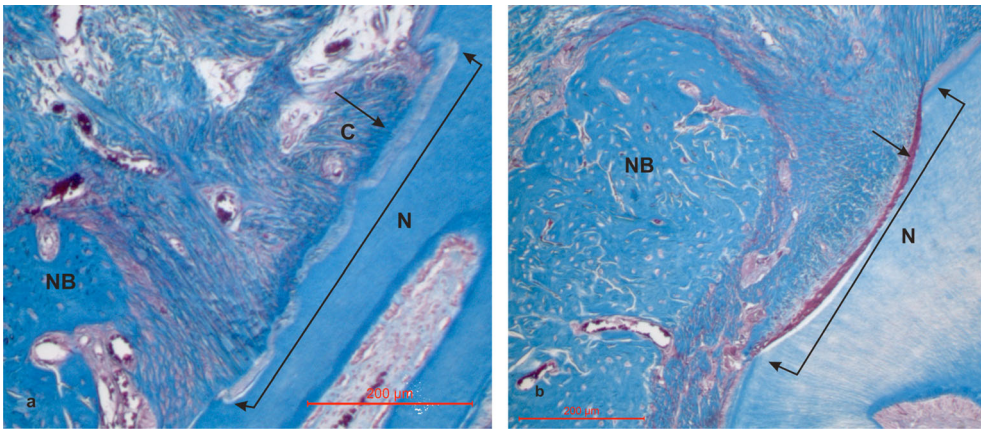

Fig. 6 Overview of a histologic section from a test site (rat model). Note the formation of new bone and the irregular periodontal-like ligament (Alcian blue staining)

model in combination with well-characterized oNCSCs may pave the way for the development of novel, efficient and safe therapies for patients with severe periodontitis or following craniofacial surgery.

Methods Development and Good Manufacturing Practices

The Food and Drug Administration (FDA), in the USA, and the European Medicines Agency (EMA), in Europe, are responsible for creating and enforcing these regulations. In Europe, stem cells for clinical therapies are classified as advanced therapy medicinal products (ATMPs) if they are only minimally manipulated and intended for autologous use [61]. A Committee for Advanced Therapies (CAT) has been set up to evaluate cell production marketing by assessing the quality, safety and efficacy of ATMPs, in accordance with the regulatory framework. EMA regulation defines the current Good Manufacturing Practices (cGMP) guidelines to manufacture ATMPs, even though clinical grade production of NCSCs needs to be implemented [62]. As introduced in the chapters above, NCSCs can be isolated, stored and expanded by applying rational modifications to the commonly used methods in order to continue complying with good manufacturing practices from the donor site to the scale-up procedures [63].

Legal and Practical Issues

The use of live cell-based therapies in medicine is a well-established therapeutic concept. Notably, the first successful allogeneic human stem cell transplant (haematopoietic stem cells) took place in 1968 and is now a routine clinical procedure for bone marrow regeneration [64]. Up to date [65], a total of 1342 active cell-based therapy clinical trials have been conducted with different target indications and trial phases. Translation of NCSC research into clinical periodontal regeneration relies on abundant in vitro and in vivo preclinical data.

In 2008, the International Society for Stem Cell Research (ISSCR) introduced stringent guidelines for translational stem cell research, suggesting rigorous standards and a thorough informed consent process for the patients. Notably, these guidelines should be taken into account prior to translation of oNCSCs into the clinical practice. Most importantly, it should be strictly avoided to offer NCSC-based treatment to patients without clinical trial results. Thus, there is a high need of development of regulatory mechanisms ensuring maximal and evidence-based safety. Moreover, practical barriers can appear concerning potential therapeutic applications of NCSCs in periodontal medicine. The risks of not validating reagents prior to experimentation and the destabilization of partnerships arranged with commercial partners are very high. Strict quality control is mandatory for all steps of the process including isolation, expansion and storage of NCSCs. The importance of such QC mechanisms is even more obvious under consideration that an estimated 15–20 % of cell lines used worldwide in the fundamental research are misidentified [66].

Conclusions

In sum, although very promising, stem cell-based therapies in periodontology are still in very early stages of translation into clinical practice. Nevertheless, human adult oNCSC populations represent a great opportunity to bring the periodontal regeneration to a next level. Having a similar mesenchymal differentiation capacity than bone marrow MSCs, oNCSCs seem to surpass them in terms of the ability to give rise to dental and periodontal tissue in addition to neural crest derivatives including neuronal cells. Consequently, oNCSCs do have potential applications not only in periodontology but also in neurodegenerative and ischemic diseases, diabetes research or bone repair [67].

Authors' Contribution W.-D. Grimm and S. Hakki are responsible for the experimental and clinical works, study design, text writing and editing

and literature references. B. Giesenhagen, I. Schau, S.V. Sirak and A. Sletov are responsible for the clinical works, text editing and literature references. G. Varga and D. Widera are responsible for the experimental works, study design, text writing and editing and literature references.

Compliance with Ethics Guidelines

Conflict of Interest W.-D. Grimm, B. Giesenhagen, S. Schau, S.V. Sirak and A. Sletov have no conflict of interests.

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