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Effects of Cellular Senescence on Dental Follicle Cells

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Abstract
The dental follicle is part of the tooth germ, and isolated stem cells from this tissue (dental follicle cells; DFCs) are considered, for example, for regenerative medicine and immunotherapies. However somatic stem cells can also improve pharmaceutical research. Cell proliferation is limited by the induction of senescence, which, while reducing the therapeutic potential of DFCs for cell therapy, can also be used to study aging processes at the cellular level that can be used to test anti-aging pharmaceuticals. Unfortunately, very little is known about cellular senescence in DFCs. This review presents current knowledge about cellular senescence in DFCs.

Introduction

Various types of tooth stem cells have been isolated from human mesodermal tooth tissue in the past 2 decades [1–4]. Dental stem cells were isolated from the dental pulp and the periodontal ligament from permanent teeth, wisdom teeth, and deciduous teeth [5–7]. However, some human dental stem cells can only be isolated from impacted wisdom teeth such as dental follicle cells (DFCs), which are actually tooth germ cells [1, 8]. Somatic stem cells such as dental stem cells are discussed for a number of applications in regenerative medicine, for example, for biological implants in dentistry or for bone defects of critical size [9, 10]. Unfortunately, actual dental stem cell treatments are rare because induction of senescence is a major problem for cell proliferation in order to achieve a sufficient cell count for treatments. While this problem needs to be addressed in advance for stem cell-based therapies, the induction of senescence in somatic stem cells could also be an ideal cell model for in vitro aging studies to further investigate the molecular effects of pharmacological products. The induction of senescence in dental stem cells is, therefore, an opportunity for a new test method and of great importance for research. This article focuses on our current knowledge of senescence in a particular type of dental stem cell that can be isolated from the dental follicle.

Isolation of Undifferentiated Cells from the DFCs

The dental follicle can be easily separated from extracted wisdom teeth [8, 11]. This tooth germ tissue mainly consists of a collagen-like connective tissue-like stroma, which can

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be made visible by trichrome staining [12–14]. This connective tissue contains small vessels and around these vessels, some cells express Nestin or Notch-1, which are typical markers for cells of the neural crest or of neural precursor [8, 13, 15, 16]. A single-cell suspension was yielded from the dental follicle after mechanical treatment and protease treatment, and undifferentiated DFCs can be obtained from single cells as colony-forming unit fibroblasts (CFU-F). A colony contains at least 50 cells with a fibroblast-like morphology, and DFCs not only express Nestin and Notch-1 but also other typical mesenchymal stem cell markers such as CD105 [8, 17]. DFCs can be propagated in cell culture for at least 6 passages and differentiated into functional tissue cells such as fibroblasts, osteo-/cementoblasts, and neural cells (including glial cells) [8, 18, 19].

The multipotency of DFCs make them suitable for use in regenerative medicine, and they can be considered for a number of regenerative therapies of oral tissues, including hard and soft tooth tissues such as dentin, cementum, alveolar bone, gingiva, and the periodontium. The origin of the DFCs in cells of the neural crest also makes them interesting for the regeneration of nerve cells (tissue cells), including Schwann cells. In addition, it is also possible that DFCs could be used for immunotherapy in the near future [20].

Before dental stem cells can be applied, for example, in regenerative dentistry, however, extensive work in basic research is required. In addition, to some general questions regarding the assessment of differentiation potentials, the elucidation of differentiation mechanisms, and a thorough test of suitability in immunotherapy, an optimal protocol for the cultivation and multiplication of DFCs in cell culture is required [20–22]. Another important issue is how senescence can be induced in undifferentiated DFCs.

Induction of Senescence in DFCs

During cell culture, more and more DFCs acquire senescence, which is noticed, for example, in cell culture due to a reduced possibility of cell proliferation [23]. Senescence is generally induced by a number of factors in cell culture, for example, DNA damage, telomere erosion, or oxidative stress [24–26]. Senescent stem cells are of limited value for regenerative medicine because they can, for example, secrete senescence-associated secretory phenotype (SASP), which is an important mediator of the pathophysiological functions of senescent cells (fibrosis, wound healing, etc.) [27, 28].

Previous observations have shown that DFCs can easily be cultivated for more than 6 passages [8]. Here, the cell morphology and the population doubling time did not change significantly. After passage 10 and in later stages of cell culture, however, cell proliferation decreased significantly and finally stopped [23, 29]. The appearance of longer population doubling times is associated with the induction of β-galactosidase activity in senescent cells. This is an important and reliable marker of cellular senescence, and the enzyme catalyzes the hydrolysis of β-galactosides [27]. DFCs exhibit a high β-galactosidase activity at later stages of cell culture [23]. In earlier times of DFC cultures – under 10 passages – almost none of the cells examined showed any visible β-galactosidase activity. The observed induction of β-galactosidase activity between passages 10 and 16 indicates the most susceptible point in time for the induction of cellular senescence in DFCs [29, 30]. Another feature for the upcoming cellular senescence is the increasing cell size. An increased size of DFCs is visible, at least after passage 14 in cell culture, but the heterogeneity of the cell size is very high [23]. Interestingly, we have shown in preliminary data that the expression of dental stem cell markers such as CD105 did not change significantly after induction of cellular senescence. Only minor changes for the markers CD146 and Nestin were found between cell passages 7 and 18. We do not know why the expression of stem cell markers did not decrease, but – as I will discuss later – the differentiation potential in senescent DFCs is decreasing [23].

Telomere Length and the Induction of Cellular Senescence in DFCs

Since the publication of the Hayflick limit on the relation of the limited ability of cell division and the shortening of telomeres in the 1960s, a lot of publications have shown that the limit ability of cell division (senescence) depends highly on the telomere length [31, 32]. In DFCs, the shortening of the telomeres is small and probably only plays a subordinate role for the induction of senescence, since it hardly or only slightly decreases [23]. However, cellular senescence induction is still associated with telomere length. Recent works with dental pulp stem cells have shown that significant heterogeneity in stem cell expansion and regeneration capacity is associated with different telomere lengths and an associated susceptibility to replicative senescence [33, 34]. However, the original telomeric length of DFCs could be critical for induction of cellular senescence. A recent study compared telomer-

ic length of 6 different isolations of DFCs [29]. Telomeric lengths of 5 DFC cell lines were almost similar, but 1 cell line had shorter telomeres. This cell line (DFC_F) with short telomeres was compared with another cell line with longer "standard" telomeres (DFC_S) [29]. Although we expect cells with shorter telomeres to have higher β-galactosidase activity than cells with longer telomeres, the opposite was observed in this study. The DFC_Fs with shorter telomeres had lower β-galactosidase activity than the DFC_S with longer telomeres in an early phase of cell culture. Contrary to the relationship between DFC_F and DFC_S at high passages, here the relationship was reversed, indicating a higher susceptibility to senescence in cells with short telomeres. Previous studies on telomere length in dental pulp stem cells and induction of cellular senescence have shown similar results [33]. Interestingly, the telomeric erosion did not occur differently in the 2 cell lines during cell culture since almost no erosion was found for DFC_F and DFC_S in senescent DFCs [29]. Genomically higher rates of DNA damage and chromosome reorganization or aberration in DFC_F was the reason for the accelerated induction of cellular senescence. DFC_F became aneuploid after long-term cell cultures, while the number of aneuploid cells in DFC_S was almost negligible. The same applies if the expression of genes that are associated with DNA damage and cellular senescence was compared [29]. In conclusion, telomeric erosion probably plays a minor role in the induction of cellular senescence, but short telomeres favor induction.

The Cell Cycle Regulator Protein P16 Supports Induction of Senescence

In a very simplified model, 2 signaling pathways are involved in the induction of cellular senescence in mammalian cells [35–38]. These 2 pathways control the progression of cell cycle in the G1 phase by inhibition of cyclin-dependent kinases (CDKs) [37, 39–41]. Each of these 2 pathways can be represented by proteins that are unique to that pathway. One pathway, for example, uses protein p21, which is activated by protein p53 (TP53), and the other pathway uses protein p16. Both pathways are indirectly or directly induced by oxidative stress, telomerase inhibition, oncogenes, and enzymes that are involved in DNA repair [42–44]. Both pathways are also involved in additional biological processes such as autophagy and apoptosis, which can alternatively be induced [39]. However, it must be elucidated individually which of the pathways is responsible for the induction of cellular senescence.

A previous study, therefore, examined both pathways by investigating the expression of genes and proteins, which are involved in DNA damage and by the inhibition of telomerase activity and cellular senescence [41]. DFCs were selected at different stages of cell culture: before the induction of senescence (passage 7), at an early stage of senescence induction (passage 12), at a later stage of senescence induction (passage 16), and from a cell culture with almost only senescent DFCs (passage 21). PCR array analyses containing gene expression data of the genes from p21, p53, and p16 showed that only the gene of the p16 protein (CDKN2A) was induced in senescent DFCs [41]. In contrast, p21 and p53 proteins were downregulated or constitutively expressed in senescent DFCs [41, 45]. During cellular senescence, p21 gene expression resembles genes associated with cell cycle progression and senescence inhibition, such as CDK2 or E2F1 [41]. Western blot analyses confirmed that p16 protein expression was induced after induction of cellular senescence, while p21 protein expression was downregulated. The protein expression of p21 was again comparable to the expression of CDKs [41]. The relationship between p16 expression and induction of cellular senescence confirmed that inhibition of p16 significantly inhibited induction of senescence. These experiments supported the main assumption that p16 induced senescence in DFCs.

It is already known that typical stem cell characteristics such as the osteogenic differentiation in dental stem cells are affected after the induction of cellular senescence [33, 34, 44, 46, 47]. In senescent dental stem cells, for example, the expression of osteogenic markers is repressed and the biomineralization is greatly reduced, but the molecular mechanisms for this phenomenon are unknown. Induction of p16 in senescent DFCs does not inhibit osteogenic differentiation because downregulation of p16 inhibited the expression of osteogenic differentiation markers and apparently had no influence on the alkaline phosphatase activity of DFCs, which is an important marker for osteogenic differentiation [41]. These data provide some evidence that p16 supports but cannot inhibit osteogenic differentiation in senescent DFCs. The molecular mechanism that inhibits differentiation in senescent DFCs remained unclear after this study.

Role of WNT5A in Senescent DFCs

While p16 plays an important role in the induction of senescence, its expression does not cause a reduced osteogenic differentiation potential of senescent cells. Good

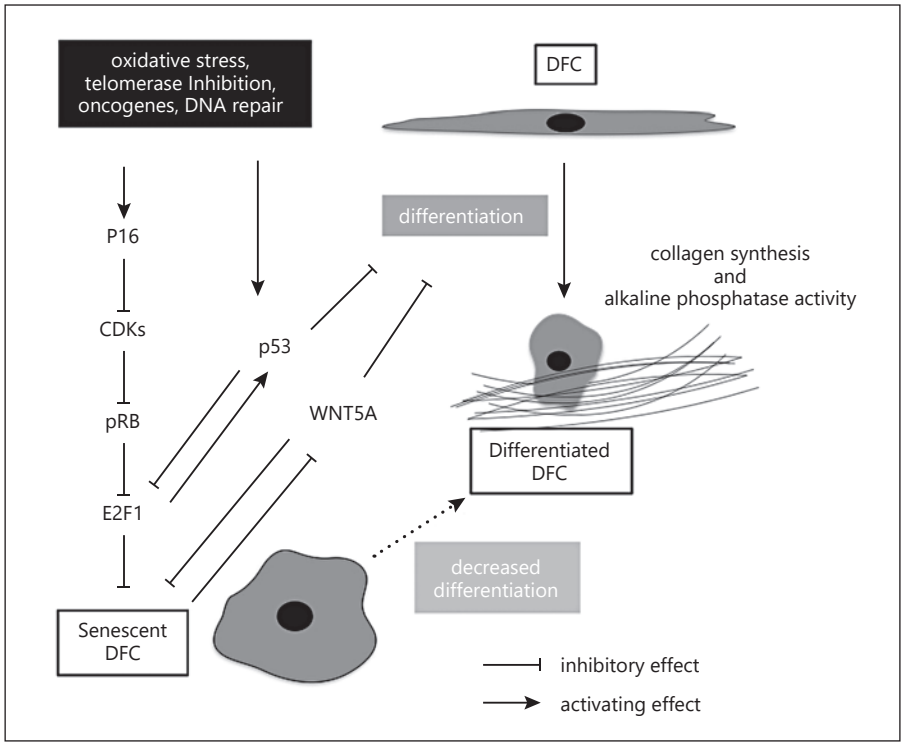

Fig. 1. Putative mechanisms of cellular senescence in DFCs. This figure also contains proteins CDK and pRB, which are generally involved in this biological process. ALP, alkaline phosphatase; DFCs, dental follicle cells; CDK, cyclin-dependent kinase; pRB, retinoblastoma protein.

opportunities to find key factors for the reduced osteogenic differentiation potential are investigations on the role of typical proteins, which are generally involved in the induction of cellular senescence and/or the osteogenic differentiation of stem/progenitor cells. One protein is WNT5A, which is involved in the non-canonical WNT pathway [30]. Previous studies have shown that WNT5A is involved in both osteogenic differentiation and induction of cellular senescence [48–51]. Studies with DFCs from rats, for example, showed that WNT5A supports bone morphogenetic protein (BMP)2-induced differentiation, WNT5A specifically inducing JNK [49]. Interestingly, WNT5A is also associated with an alternative activation of β-catenin [52]. A published study by Nemoto and co-workers suggests a feedback mechanism between the canonical and the non-canonical WNT signaling pathways that could regulate the specific activation of β-catenin in DFCs during osteogenic differentiation [53]. The studies by Nemoto and co-workers [53, 54] and by Ling and co-workers [49] on WNT5A and osteogenic differentiation were made with murine DFCs, however, and these 2 groups showed very different effects of WNT5a on osteogenic differentiation. In contrast to the study by Ling and co-workers, WNT5A inhibited the alkaline phosphatase activity in the study by Nemoto and colleagues [53]. Studies with human DFCs showed that both the canonical WNT signaling pathway and BMP signaling pathway are involved in the osteogenic differentiation of DFCs [55, 56]. However, the roles that some members of the canonical WNT pathways such as β-catenin or APCDD1, a known inhibitor of the WNT pathway, play are not clear [57].

WNT5A is likely to play a role in human DFCs during senescence induction. It is gradually downregulated after the induction of cellular senescence; similar to osteogenic differentiation markers [30]. After induction of cellular senescence, inhibition of WNT5A affects osteogenic differentiation. However, WNT5A had no significant influence on the expression of osteogenic differentiation markers in DFC before induction of senescence. The effect of WNT5A is, therefore, not directly related to pathways or genes that are directly involved in the expression of osteogenic differentiation markers. Since WNT5A strongly affects cell proliferation, induction of senescence, and the number of apoptotic and viable cells, it is important for the viability of DFCs after induction of osteogenic differentiation [30]. Interestingly, also another factor related to cell viability and cellular senescence is involved in a reduced osteogenic differentiation potential of senescent DFCs. The TP53 transcription factors are al-

most always involved in cell division and cell viability, but also in other biological processes such as ostoegenic differentiation [35, 40, 58–62]. A recent study showed that TP53 inhibits the osteogenic differentiation in senescent DFCs, but is not involved in senescence induction [45].

Conclusion

Cellular senescence affects the use of DFCs in stem cell therapies and needs to be regulated. Basic research on cellular senescence is aimed at this problem, and its current progress can be summarized by the following points (Fig. 1):

- The shortening of the telomeres is not strongly related to the senescence induction of DFCs.
- Shorter telomere length of DFCs accelerates senescence induction and favor DNA damage (aneuploidy).
- The p16 signaling pathways drives senescence induction.
- Senescence inhibits osteogenic differentiation of DFCs.
- Reduced expression of WNT5A in senescent DFCs decreases cell viability and osteogenic differentiation of DFCs.
- P53 is unlikely to be involved in senescence induction but affects osteogenic differentiation of DFCs.

Future studies will need to evaluate new cell culture protocols that reduce senescence induction in DFC and/or improve osteogenic differentiation in senescent cells. It will be of great importance to manipulate certain targets such as WNT5A to directly obtain DFCs that are suitable for therapies. The type of manipulation is still unclear. However, the summarized studies in this article show that a DFC-based cell culture model can already be used for in vitro tests, for example, to investigate the molecular and cellular consequences of drugs in aging cells.

Statement of Ethics

The author has no ethical conflicts to disclose.

Conflict of Interest Statement

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Author Contributions

Christian Morsczeck had the idea and is the only author of this article.

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