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UNDERSTANDING STEM CELL HETEROGENEITY – A PREREQUISITE FOR SUCCESSFUL (DENTAL) REGENERATION

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Summary

Advancements in regenerative dentistry could soon equip dentists with tools to regenerate dental pulp and other tissues. Developing effective regenerative therapies has proven to be a challenging task due to the complexity of tooth structure, functional and aesthetic requirements, safety and patient factors. However, recent studies looking at behaviour of adult mesenchymal stem cells in murine teeth *in vivo* offer new insights for development of translational approaches for tissue regeneration. Molecular heterogeneity within the mesenchymal stem cell (MSC) niche has recently been studied *in vivo* using genetic lineage tracing and single-cell RNA sequencing. The role of an established, Thy1 (CD90)-marked sub-population is studied in homeostasis and following stimulation of increased growth of mouse incisors. Recent results suggest that this sub-population plays a specific role in rapid growth, during development as well as in stimulated increased growth and can be replenished by activation of dormant stem cells. These findings have implications for development of new, targeted regenerative therapies harnessing the potential of tissue-resident stem cells in a surgical intervention or non-surgical, molecular signal delivery approach.

***Keywords*:** mesenchymal stem cells; adult stem cells; regenerative dental medicine; dental pulp.

INTRODUCTION

The field of regenerative dentistry has steadily progressed within recent years aiming to improve patient care with new solutions for common dental pathologies. Developing safe and efficient therapies has proven to be a challenging task due to the complexity of tooth structure, functional and aesthetic require-

ments, safety and patient factors. A prerequisite for development of successful therapies is an understanding of the environment in which regeneration takes place and identification of appropriate cells and signals required to induce tissue regeneration. A number of basic science and translational studies have already contributed to the field by elucidating mechanisms of tooth development, dissecting cellular and molecular components of stem cell niches and trialled protocols for dental pulp regeneration and dentine repair. Here we summarize the current knowledge on the role of mesenchymal stem cells in tooth regeneration and repair and implications for development of regenerative clinical therapies.

BIOLOGIC CHARACTERISTICS OF MSCS

Mesenchymal stem cells (MSCs), also known as multipotent mesenchymal stromal cells (1) and skeletal stem cells (2), were first isolated from bone marrow (3) but can also be found in niches in all stromal tissues. Their properties such as proliferative potential, multipotency, immunomodulation, accessibility and absence of tumorigenicity make them an appealing candidate for cell-based therapies. Multiple dental sources of MSCs have been identified, such as dental pulp, periodontal ligament and apical papilla (4–7) holding promise for regeneration of teeth and supporting structures. Numbers of stem cells in adult tissues are low so current regenerative approaches use *in vitro* expansion under good manufacturing practice (GMP) conditions (8). Mesenchymal stem cells are typically isolated using surface markers and while most of the markers are known from *in vitro* studies they do not reflect properties of MSCs *in vivo*. Transgenic animal models are thus increasingly used to study behaviour of stem cells and their progeny during normal turnover and under challenging conditions, e.g. tissue injury. These *in vivo* models better predict dynamics and behaviour of stem and progenitor cells in a clinical scenario by giving insights into functional heterogeneity of MSCs. Studies to date suggest similarities with the hematopoietic stem cell (HSC) compartment which consists of several HSC subpopulations with pre-programmed behaviour, contrary to the initial idea of homogeneous population of cells with a high degree of differentiation flexibility (9).

DENTAL REGENERATION – PROSPECTS AND CHALLENGES

Enamel, the highest mineralised substance in the mammalian body, is not likely to be successfully regenerated and replaced using cell-based approach-

es as ameloblasts do not persist beyond tooth eruption (10). Instead, efforts are directed towards generation of synthetic enamel utilizing bioengineering approaches (11). Underlying dentine, however, is secreted by odontoblasts during dentine formation *in utero* and maintained throughout life. Odontoblasts are able to respond to low grade stimuli, such as in tooth wear and early caries, by secreting reactionary dentine (12). If external stimuli are severe, involving pulp exposure, dental pulp stem/progenitor cells are recruited and differentiate into reparative odontoblast-like cells that then secrete less structured reparative dentine (13,14). Lineage tracing studies in mice, whose odontoblasts also have limited regenerative capacity, suggest that sources of those stem/progenitor cells are pericytes - cells present on blood capillaries (15) and peripheral-nerve associated glia (16), conveniently located for efficient local repair. In a common clinical setting, where the capacity to regenerate and repair the tissue is insufficient, dentine is most commonly replaced by glass ionomer and calcium-silicate based cements (17). A recent murine study has shown that delivery of small molecule inhibitors of glycogen synthase kinase 3 activity (Wnt/β-catenin signaling antagonists) directly to exposed pulps promotes the production of reparative dentine (18). The study used clinically-approved collagen sponges and low doses of tideglusib, a novel GSK-3β inhibitor currently used in multiple clinical trials. A related study found that pulp exposure results in upregulation of axis inhibition protein 2 (Axin2) and that Axin2-expressing cells differentiate into odontoblasts-like cells secreting reparative dentine (19). On the dental pulp front, a small clinical study on pulpectomised teeth in five patients with irreversible pulpitis showed promising results for total pulp regeneration. Mobilized dental pulp stem cells (MDPSCs) transplanted with granulocyte colony-stimulating factor (G-CSF) in atelocollagen resulted in no adverse effects and toxicity. During a relatively short follow-up period of 24 weeks, there was a positive response on electric pulp testing in four patients while radiological evaluation detected widened periodontal ligament space in two patients and periapical radiolucency in one patient. Several points for improvement in future studies were listed including microleakage of restorations covering the pulp and the need for better infection control during root canal treatment (8). Preclinical models that better mimic infected environment and translational research on microbial modulation (20) could perhaps inform more predictable outcomes in future clinical trials.

MOUSE INCISOR AS A MODEL FOR DENTAL REGENARATION

An important model in regenerative biology, the continuously-growing mouse incisor, has been used in recent studies for characterising dental pulp MSCs. Several populations have been studied including Gli1, Sox10, Thy1 (16), however, their functions within the niche are still not completely understood.

Thymocyte differentiation antigen 1 (Thy1) or CD90 is an evolutionally conserved GPI-anchored glycoprotein. It is used as a mesenchymal and hematopoietic stem cell marker but also found on cell surface of murine thymocytes, T lymphocytes, neurons, mature glia, endothelial cells, fibroblasts and cancer cells (21,22). Studied for over five decades but still not fully understood, Thy-1 plays a role in immune response, cell adhesion and in modulation of fibrogenetic potential of fibroblasts (23). Using lineage tracing we showed in our recent study (24) that MSCs expressing Thy1, an archetypal surface marker known from *in vitro* studies, contribute to only about a third of dental pulp cells and odontoblasts during development *in vivo.* Multicolour clonal analysis revealed a previously described niche position-dependent distribution pattern (16) in which pulpal fate vs. odontoblast fate varies and is influenced by the MSC position - MSCs closer to the epithelial cervical loop predominantly gave rise to clusters of odontoblasts while those further were likely to have pulpal fate. In 2-3 month old animals with fully erupted, homeostatic incisors, the numbers of Thy1-derived cells decrease significantly and do not contribute to differentiated progeny. In a simulation of growth experiment, two days post incisor clipping, the number of proliferating cells had doubled compared to intact incisors. For the first time, mitotic cells were found in the most proximal, quiescent-cell residing area of the incisor, resulting in expansion of Thy1+ MSCs and increased contribution to cell differentiation. Flow cytometry revealed that Thy1+ proliferating cells are the main contributor to the re-establishment of homeostasis suggesting that Thy1+ MSCs are a sub-population specific for rapid growth. Immunohistochemistry analyses further suggested that the depleted pool of Thy1+ MSCs can be replenished by mobilisation of a quiescent cell population. These quiescent cells, observed in the most proximal mesenchyme as EdU-labelled cells chased for a year, are marked by Celsr1, a marker known from the hematopoietic stem cell niche (25). This small Celsr1+ population is clearly distinct from Thy1+ cells as single cell RNA sequencing confirmed that Celsr1 expressing cells do not express Thy1 in unclipped incisors (unpublished data). Preliminary results suggest that Thy1 also plays a key role following murine molar pulp exposure. Similarly, a clinical

study reported upregulation of THY1 in intracanal blood during regenerative endodontic procedures in mature teeth, THY1 values being significantly higher than other tested MSC marker transcripts (26), suggesting that murine studies could predict human pulp regeneration. Taken together, these results provide the basis for understanding functional heterogeneity in dental pulp and similar tissues and have implications for developing regenerative therapies targeting specific stem cell populations to accelerate repair.

TOOLBOX FOR FURTHER UNDERSTANDING OF MSC HETEROGENEITY

Recent developments in RNA sequencing and analysis technology have enabled studies looking at MSC heterogeneity at single cell resolution (27). Several experimental methods and analysis tools have been developed in the recent years (28–39) and enabled identification of new and rare cells types, studies of stem cell and tumour heterogeneity, reconstruction of cell hierarchies and insights into cell fate determinants (40–43). Single-cell RNA sequencing technologies have proven to be useful in dissection of complex cell niches and their rapid development has already contributed significantly to the field of stem cell biology. Challenges of the approach include computational analysis and validation. A limitation of current methods is that they do not preserve environmental context and they capture cell populations at one point in time (44). Additional single-cell transcriptomic tools are therefore being developed such as an imaging-based multiplexed error-robust FISH (MERFISH) (45). It is expected that we will see more advanced and cost-efficient experimental solutions in the upcoming years as we continue to understand mechanisms involved in tissue maintenance and repair.

CONCLUSION

In conclusion, genetic lineage tracing has enabled insights into dynamics within the dental pulp mesenchymal stem cell niche. Key MSC populations, including a discrete CD90/Thy1-expressing sub-population of mesenchymal stem cells have been identified and studied in organ growth and regeneration informing development of new regenerative therapies. More studies are needed to investigate molecular signals that could be delivered locally to stimulate endogenous stem cells and induce tissue regeneration. Challenges to be considered

and overcome in future studies include safety, efficiency and patient morbidity, including infection in treated regeneration sites.

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Saţetak

Razumijevanje heterogenosti matičnih stanica – preduvjet za uspješnu (dentalnu) regeneraciju

Napredak u regenerativnoj dentalnoj medicini uskoro bi mogao opskrbiti doktore dentalne medicine alatima za regeneraciju zubne pulpe i drugih tkiva. Razvoj djelotvornih regenerativnih terapija pokazao se izazovom zbog složenosti zubne strukture i funkcije, estetskih zahtjeva, sigurnosti primjene i faktora rizika pojedinačnih pacijenata. Ipak, nove studije koje prate ponašanje mezenhimskih stanica *in vivo* u zubima odraslih miševa nude nove spoznaje za razvoj translacijskih pristupa za regeneraciju tkiva. U zadnje vrijeme dolazi se do novih spoznaja o molekularnoj heterogenosti unutar niše mezenhimskih matičnih stanica koristeći genetsko praćenje stanične loze *in vivo* i RNK sekvenciranjem pojedinačnih stanica. Uloga subpopulacije obilježene dobro poznatim biljegom Thy1 (CD90) proučavana je u homeostazi i nakon stimulacije ubrzanog rasta sjekutića. Posljednji rezultati sugeriraju da ova subpopulacija igra ulogu u fazama ubrzanog rasta, tijekom razvoja i nakon stimulacije rasta te da se može dodatno producirati aktivacijom stanica u fazi mirovanja. Ovi rezultati imaju implikacije za razvoj novih, ciljanih regenerativnih terapija koje bi koristile potencijal tkivnih matičnih stanica u kirurškoj intervenciji ili u nekirurškom pristupu primjenom molekularnih signala.

Ključne riječi: mezenhimske matične stanice; tkivne matične stanice; regenerativna medicina; zubna pulpa.

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