

A Paradigm Shift in Bone Regeneration Therapy: Using Mesenchymal Stem Cells and the CRISPR-Cas9 Technology

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ABSTRACT

The current mainstream approach to bone regeneration treatment is autologous bone grafting and artificial bone/bone substitute materials. However, satisfactory treatment results have not been achieved. There is no doubt that Mesenchymal Stem Cells (MSCs) are a useful "tool" for bone regeneration therapy and use of MSCs has been desired. However, MSCs cells are still not widely used in bone regeneration treatments due to their limited osteogenic differentiation ability and efficiency at the transplanted site. Thus, dissection and control of osteogenic differentiation in molecular and cellular level are keys to overcome this issue. To this end, cellular engineering is one of promising approach for it, and various valuable tools for cellular engineering have been developed in recent years. In particular, Clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) has revolutionized genome editing techniques. By utilizing genetically modified MSCs or osteogenic cells derived from such modified MSCs, osteogenic differentiation process should be more understandable and controllable. In other words, these technologies may have a potential to standardize and optimize bone regeneration treatment outcomes that so far wide individual differences are observed. Then, as a result, reduced surgical invasion, stable treatment results, and shorter treatment may be achieved during bone regeneration treatments. In this review, we discuss the potential therapeutic and clinical application of this mesenchymal stem cell and its development by means of genome editing tool, CRISPR-Cas9 technology in particular, in bone regeneration therapy.

Keywords: CRISPR-Cas9; Mesenchymal stem cells; Osteogenic differentiation; Bone regeneration therapy; Bone gamma-carboxylglutamate protein; Enhanced green fluorescence protein

Abbreviations: MSCs: Mesenchymal Stem Cells; ES: Embryonic Stem; iPS: induced Pluripotent Stem; BGLAP: Bone Gamma-Carboxylglutamate Protein; hiMSC: Human Immortalized MSCs; EGFP: Enhanced Green Fluorescence Protein.

INTRODUCTION

We demand high-quality bone regeneration treatment for diseases of the oral and maxillofacial region, such as cleft lip and palate [1-3] facial trauma [4-6], and bone reconstruction after

tumor resection [7-9]. Facial bones are not only essential for aesthetics but also indispensable for maintaining individuals' dignity, masticatory function, and conversation abilities. Therefore, bone regeneration always should be a reliable and

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efficient treatment method. The current mainstream approaches to bone regeneration treatment are autologous bone grafting [10] and artificial bone grafting with bone substitute materials [11,12]. However, these 'grafting' approaches have not been achieved satisfactory treatment results: There are multiple surgical sites for harvesting donor bone fragments for autologous bone grafting, but this entails unavoidable surgical invasion, and postoperative infection and rejection are likely to occur when using artificial bones. In such bone regeneration treatments, undifferentiated cells, transplanted with or within an appropriate support system, that is, in a suitable microenvironment, are expected to differentiate into osteoblasts. However, it takes time to induce bone differentiation in the graft or implantation site when using current bone regenerative approaches, such as autologous bone grafting. As a result, the bone regenerative ability is insufficient, leading to low treatment success. A Grafting approach with either autologous, artificial bone, or in combination may remain as one of standard treatment options in future. But, to improve current clinical results, elucidation of the bone regeneration mechanism at the cellular level is extremely important for establishing new therapeutic strategies for the highly sought-after bone regenerative medicine.

In recent years, a methodology for enhancing the osteogenic differentiation at the transplantation site by utilizing the bone differentiation ability of Mesenchymal Stem Cells (MSCs) has attracted attention as a solution to the conventional bone regeneration treatment problems. Basic [13-15] and clinical [16-18] research is being actively pursued, aiming to assess the utility of *in vitro*-cultured MSC transplantation to treat bone defects. Indeed, it is easier and faster to collect MSCs from the living body than it is to collect bone fragments used for autologous bone grafts. The ethical hurdles for clinical application of MSCs are lower than those for Embryonic Stem (ES) cells and induced Pluripotent Stem (iPS) cells. Currently, many of the recent attempts to use MSCs for bone defect treatments focus on origins or properties of cells to be introduced; such as the tissue from which the MSCs are derived, the donor's age, and the cell culture history and so on. These conditions are important for successful optimization in treatment, however, it is still not an critical improvement measures focusing on the differentiation potential of stem cells within. Thus, it is necessary to realize a condition or method to efficiently induce osteogenic differentiation in MSCs not only 'before', but 'after' transplantation. To this end, it is necessary to establish cellular experimental system, ideally real-time techniques, to track the osteogenic differentiation process of viable MSCs and clarify the molecular mechanism that follows.

Tissue engineering is generally defined as technology which applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [19]. Among general strategies in tissue engineering, an isolation of genetically modified cells with improved function is widely performed in the course of development in regenerative medicine. Historically, genetic modification was mainly performed in ES cells so that researchers could get insight for specific function of modified genes not only through *in vitro* differentiation experiment, but

through *in vivo* production of genetically modified ES cell derived animals. Due to low efficiency in genetic modification such as homologous DNA recombination in somatic cells, ES cells and its derived animals are valuable. Because either from *in vitro* differentiation model or from produced animals, genetically modified somatic cells can be isolated. Thus, a method that can genetically modify DNA in somatic cells, especially in human cells, was long been required.

The situation has changed with the advent of transcription activator-like effector nuclease and zinc finger nuclease, technologies that efficiently modify (edit) genomic DNA, even in somatic cells. More decisively, genome editing by CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9) transformed this field. It was first reported *in vitro* in 2012 [20]. After that, genome editing results in human cells were reported in succession [21-23]. Accordingly, successful application in mammalian genome editing accelerated cell engineering, and theoretically any kind of cells from soma can be modified including MSCs *via* CRISPR-Cas9 technology.

We would like to discuss in this review how cell or tissue engineering combined with genome editing technologies such as CRISPR-Cas9 can be utilized or adopted for MSCs application in the field of a bone regeneration therapy. We will also discuss how the results could contribute to creating new bone regeneration therapies and their therapeutic and clinical application potential.

POTENTIAL AND LIMITATIONS OF MSCs IN BONE REGENERATION THERAPY

MSCs have a self-renewal ability and can differentiate into various cell types such as osteoblasts, adipocytes, and chondrocytes [24]. MSCs can be collected from multiple sites, including bone marrow, adipose tissue, and cord blood [25]. Thus, as a cell source for bone regenerative medicine, they have the advantage in collection techniques that are less surgically invasive than those used for autologous bone grafts. Furthermore, when using the patient's MSCs, safety issues, such as the risk of tumorigenesis when using genetically manipulated iPSCs or ethical issues, as those associated with ES cells, are lower. Thus, MSCs have many useful aspects when considering them for clinical applications, comparing with using pluripotent stem cells such as ES cells or iPS cells [26].

The collected MSCs can be cultured *in vitro* in preparation for use in bone regeneration treatment. The required number of cells are determined based on the size of bone defect and the patient's status. One treatment strategy being attempted is to promote bone regeneration by transplanting the cultured MSCs to their proper place [5,17,18]. Positive results have been obtained when MSCs were administrated with scaffolding materials and/or osteogenic differentiation-inducing factors to treat relatively small bone defects [17,18]. However, these strategies could be insufficiently effective for moderate or large bone defects because large numbers of cultured MSCs are required to fill such defects. Moreover, as long term culture may cause genetic instabilities, to obtain large number of MSCs to be administrated for relatively large bone defect is still challenging issue to be overcome [27]. In addition to the cell number issue

described above, self-renewal and differentiation ability of multipotent MSCs may be declined during longer period of culture [28]. MSCs are thought to be heterogeneous population and, indeed, differentiation-induction and proliferative ability differ even with MSCs, depending on the tissue of origin and the donor's state, and a differentiation-inducing protocol suitable for each collected cells is required [29-32]. Thus, to utilize MSCs as a source for bone regenerative medicine, it is essential to examine the primary conditions in various stages, such as MSCs collection, culture condition, co-transplanting materials and/or safety management. Additionally, since MSCs are multipotent, it is challenging to predict or control which lineage they would differentiate into or the differentiation efficiently at the transplantation site. A major issue in bone regenerative medicine is that osteogenic differentiation at the bone graft site cannot be controlled and that only part of the MSCs after transplantation seem to undergo bone differentiation. The only way to solve these problems is to dissect the MSCs differentiation process at the molecular level and identify cell-dependent and environment-dependent elements that control it. However, elucidating this information for native MSCs is highly challenging due to the diversity of lineages and not unlimited stabilities even under appropriate culture condition. Therefore, constructing a system that can analyze the cell properties in live cell is highly desirable. To this end, efficient and accurate tissue engineering of MSCs are required including human MSCs.

A FUSION OF MSCs AND THE CRISPR-CAS9 TECHNOLOGY IN BONE REGENERATION THERAPY

When considering using MSCs for bone regenerative medicine, one of current problems is the absence of an evaluation method to assess the process of osteogenic differentiation performance of transplanted MSCs and/or osteogenic-directed cells. As discussed above, molecular dissection of MSCs osteogenic differentiation is a key to overcome this issue. Two directions may be considered: Isolation of an absolute osteogenic differentiation marker (s), and isolation of live and pure osteoblast population. Only when these are available will it be possible to search for conditions that efficiently and reliably differentiate MSCs into the osteoblastic lineage. Currently, however, a real-time screening system to find compounds, molecules, or microenvironment that would promote or inhibit osteogenic differentiation has not yet been developed.

Osteogenic differentiation markers isolated to date include alkaline phosphatase, Bone Gamma-Carboxylglutamate Protein (BGLAP), Bone Sialoprotein I (BSP-1), and Dentin Matrix Protein 1 (DMP1). However, these are all endogenous markers, including transcription factors, and monitoring their expression in live cells is challenging. Therefore, the introduction of genetic markers by cell engineering is required. Among the wide variety of genome editing tools so far developed, the advent of the CRISPR-Cas9 technology has evolutionized genome editing strategy for mammalian cell engineering [21-23]. Accordingly, these advantages have led to the development of research related to genome editing in stem cells including human MSCs [33-35]. Attempts have been made to construct a system that could monitor the induction of osteogenic differentiation in living

cells using CRISPR-Cas9 by other [35] and by our [36] group recently. In these studies, by introducing fluorescent protein which express during osteogenic differentiated cells, it was possible to monitor osteogenic differentiation induction in viable modified cells by observing fluorescent protein expression. Although next essential steps will be a detailed molecular confirmation of the properties of cells which express these fluorescent markers toward identification of bona fide osteogenic differentiation markers. Different from endogenous markers described above, these markers are preferably cell surface markers, as these will be valuable to develop a system to recover osteogenic cells derived from native, non-genetically modified MSCs in future. Our study used human immortalized MSCs (hiMSCs) to establish osteogenic monitoring cells [36]. One of the main reason for this choice is long term process of cloning. The hiMSCs we used are multi-potent as the original MSCs and, once osteogenic differentiation is induced, express the osteogenic differentiation markers known to date [37,38]. However, in the case of native MSCs, there was a possibility that their properties including osteogenic differentiation potential may be declined in the course of establishing of genetically modified MSCs. Thus, we used hiMSCs and successfully knocked-in the Enhanced Green Fluorescence Protein (EGFP) reporter gene under the promotor of the BGLAP gene, a differentiation marker for mature osteoblasts, by using CRISPR-Cas9 system. The expression of BGLAP could be monitored by EGFP signals in the established hiMSC lines under live culture, indicating the induction of osteogenic differentiation in these cells. Moreover, we conducted cell sorting experiment to purify EGFP-positive cells to evaluate the relationship between EGFP signal and osteogenic properties. As a result, we demonstrated that BGLAP expression was increased in these EGFP positive population, compared with expression level in EGFP-negative population and with parental (non-genetically modified) hiMSCs. This indicated that EGFP expression reflect osteogenic differentiation induction. Furthermore, we confirmed that EGFP expression were maintained at least 5 weeks of osteogenic differentiation induction (unpublished results) and that from two weeks of induction EGFP positive cells became positive for Arizarin-red S staining, a hallmark of calcification, indicating functionality of this population as osteoblast-directed cells. By using DMP1 gene as knock-in target gene, Fahimeh et al. also reported monitoring osteoblast differentiation in live cells. Although MSCs used were different (hiMSCs vs. native), CRISPR-Cas9 tissue engineering successfully visualized osteogenic differentiation in live cells. These monitoring system and differentiated monitoring cells will be valuable for molecularly dissecting osteoblast differentiation and/or elucidating molecular properties of osteoblast cells by applying an omics analysis in future. In regard to this, we demonstrated that live and pure osteoblasts can be enriched and this would enable us to conduct focused and detailed analysis of the purified osteoblasts. These results cannot contribute to bone regenerative medicine unless they lead to the identification of the osteoblast-specific cell surface markers. And next, these results should be validated using unmodified patient MSCs cells for clinical application, hoping to create an efficient and effective new bone regeneration cell therapy that could replace autologous bone grafting.

Finally, our work may provide a system that can reassess current bone regenerative medicine methodologies. As discussed in this review, basal problem of MSC transplantation for bone regenerative medicine may due to poor or declining osteogenic differentiation efficiency in transplanted site. There are several potential strategies to tackle this challenge. First, prepare undifferentiated MSCs to be transplanted destined for osteogenic differentiation in *in vitro* culture. Second, prepare or direct a transplant microenvironment in which introduced MSCs are able to differentiate into bone efficiently. Third, transplant osteogenic cells differentiated in *in vitro* culture system. Although additional components such as artificial substances and/or factors to drive osteogenic induction may be required, manipulating or directing cell fate of MSCs to osteogenic lineage *in vitro* may be challenging, but, promising prerequisite for successful transplantation. In one reports, good results in human fracture treatments were achieved using osteoblasts obtained by inducing osteogenic differentiation of MSCs *in vitro* [39]. This suggested that transplantation of purified osteoblast might be give better results in osteogenic differentiation efficiency and treatment outcomes than the case of transplanting MSCs itself directly. Then, if this is true, our established monitoring cells or derivatives in future by other groups will be valuable to evaluate the advantage in using differentiated osteoblast for bone regenerative transplantation.

CONCLUSION

We recognized the possibility of a paradigm shift in bone regenerative medicine by applying the CRISPR-Cas9 genome editing technology to MSCs. Genome editing technology using CRISPR-Cas9 in particular has realized the production of modified MSCs useful for bone regenerative medicine more efficiently and easily than before. This fact provided a new insight into the process of osteogenic differentiation of MSCs *in vitro* and isolation of purified osteoblast from native MSCs are necessary to continue developing a bone regenerative medicine. In future, gap between outcome in basic research and requirement in clinical setting should be filled to promote bone regeneration treatment. To this end, genetic modification technologies such as CRISPR-Cas9 system will be powerful solution to bring the gospel of bone regeneration treatment for both doctors and patients.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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