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研究課題名（和文）Role of intra and extracellular miRNAs in stem cell-based bone regeneration

研究課題名（英文）Role of intra and extracellular miRNAs in stem cell-based bone regeneration

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研究成果の概要（和文）：非コーディング領域におけるマイクロRNA（miRNA）による細胞制御が近年注目されている。本研究では骨芽細胞分化を制御するmiRNAの同定を目的とし，骨髄由来間葉系幹細胞の骨芽細胞分化過程におけるmiRNAの発現を解析した。今回の解析では回収したmiRNAの発現量が低く，信頼に値するプロファイリングを行うことが不可能であった。miRNAの精製法と適正な正規化方法の選択が重要であることが示唆されたことから，現在継続して解析を行っている。

研究成果の学術的意義や社会的意義
miRNAs have an important role in cell metabolism, understanding the contribution of miRNAs in the osteoblastic differentiation of MSCs could provide novel and more efficient clinical protocols for patients that require bone augmentation without recurring to the current invasive standard methods.

研究成果の概要（英文）：Cellular regulation by microRNAs (miRNAs) in non-coding regions has recently attracted attention. miRNAs are small non-coding RNA molecules that regulates post-transcriptional gene expression. In this study, we aimed to identify miRNAs that regulate osteoblast differentiation, and analyzed miRNA expression during osteoblast differentiation of bone marrow-derived mesenchymal stem cells. In this analysis, the expression level of the miRNA was low, making it impossible to conduct reliable profiling. Since it is suggested that the purification method of miRNA and the selection of the proper normalization method are important, further analysis is ongoing now.

研究分野：Tissue engineering

キーワード：　miRNA　Mesenchymal Stem Cell　Bone

１．研究開始当初の背景

In the field of prosthodontics, the patient's growing demand of aesthetic and functional restoration has increased the popularity of dental implants as a choice for restoration of missing teeth. However, many patients lack the amount of bone required in order to proceed with this treatment. As consequence, new procedures and treatments have been developed to restore the alveolar bone ridges in order to fulfill the minimum bone requirements for dental implant placement. Since dental implant placement success primarily depends on the quality and quantity of alveolar bone available in all spatial dimensions, clinicians use several surgical techniques and osteogenesis inducible supplements to accomplish these requirements.

Bone augmentation through stem-cell-based therapies and especially the use of Mesenchymal stem/stromal cells (MSCs) has demonstrated to be a viable candidate for tissue regeneration.

In a previous report we compared the mineralization ability of MSCs derived from Bone Marrow (BMCs) with MSCs derived from other tissue sources. MSCs were induced to the osteoblastic lineage through osteo-inductor mediums in order to improve bone formation. Results showed a low amount of Alkaline Phosphatase production (a well-known marker of the osteoblastic lineage) and limited mineralization. Although MSCs from other sources had more proliferative ability, BMCs showed a wider mineralization area. From this previous data, we concluded that cell's fate and activity needed further regulation by other elements to fine tune differentiating phenotype and mineralization. Many experiments have used elements like Platelet derived growth factors (PDGF), Bone Mophogenetic Proteins (BMPs) and others to stimulate the differentiation of MSCs to the ostoblastic lineage.

Recently, a class of noncoding single-stranded RNA molecules, composed of 20-24 nucleotides called microRNAs, proved to be important in the regulation of cell phenotype. To date, numbers of miRNAs (2,588 in human and 1,915 in mice) have been identified and partially characterized. miRNA can negatively regulate the phenotypic control of cells by a relatively small number of clues. In MSCs, modifications in miRNAs expression can promote cells to different types of phenotypes including osteoblasts; thus, proper control of miRNA expression is essential for maintaining the cellular metabolism and homeostasis.

Expression of miRNAs start at the cell nucleus, where primary transcripts (pri-miRNAs) are transcribed as individual miRNAs genes from introns or polycistronic transcripts, Drosha process pri-miRNAs in to smaller precursors (pre-miRNA) which leaves the nucleus with help of Exportin 5 into the cytoplasm; there, Dicer cleaves it into a duplex miRNA:miRNA*, then couples with an Argonaut protein (AGO2) to unwind the miRNA releasing one strand. The remaining "guide strand" will interact with the RNA Induced Silencing Complex (RISC); which binds to its target at the 3' UTR site of mRNA and either induce a translational repression or degrade the mRNA. Fig.1

The contribution of miRNAs as an important regulatory factor in cell fate, suggest its possible application to a better regulation of MSCs cell differentiation toward the osteoblastic lineage.

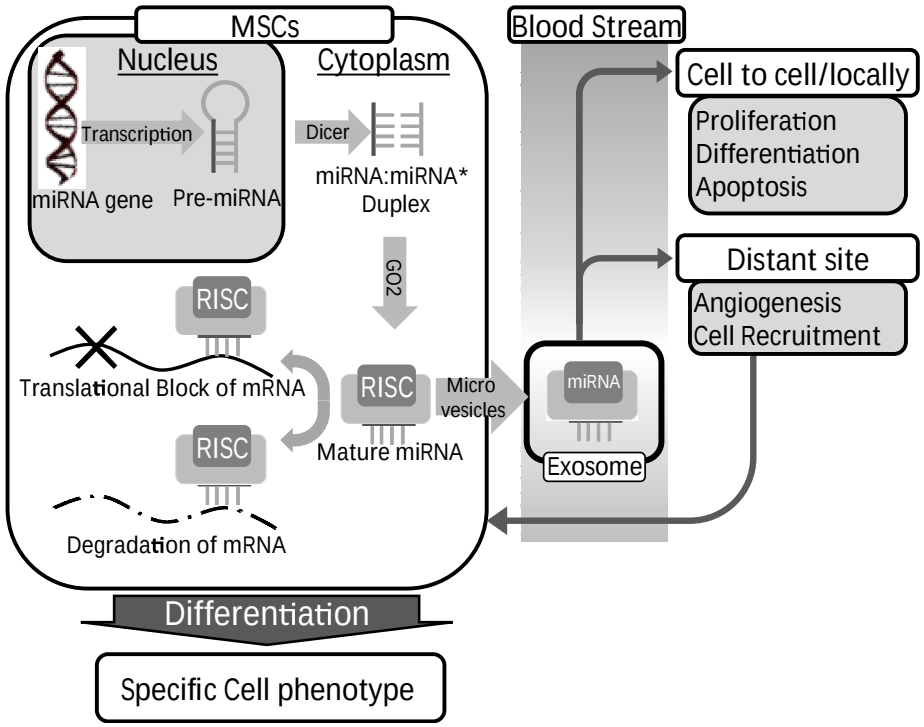

Fig.1 Biogenesis of miRNA and mechanism of action in cell differentiation. Extracellular transport through exosomes into blood stream and its role at distant sites.

２．研究の目的

Recently a new method for tissue regeneration involves the use of microRNAs (miRNAs) to regulate cell metabolism and differentiation by silencing a variety of genes. miRNAs do not only play an important role in regulating local cell to cell communication in situ, but also at distant sites moving throughout the bloodstream within small extracellular vesicles (exosomes). Although many studies have already demonstrated the important roles of miRNAs in many metabolic processes ranging from cell proliferation, differentiation, tumor oncogenesis; miRNAs related osteogenesis still remain largely uncertain. Since differentiation have a complex process involving numerous pathways, signals and interactions, there is only a few reports that determine the possible contribution of miRNAs expression in osteoblastic differentiation of MSCs and bone regeneration.
This research aims to identify the osteogenic miRNA in MSCs, toward the potential use of miRNA in bone regeneration through cell therapy as an alternative to the current more invasive methods.

３．研究の方法

(1) MSCs were isolated from 4-week-old Wistar rat's femur and tibia by flushing with ice-cold PBS. Cells were placed in a 100 mm plastic culture dish with alpha Minimal Essential Medium (alpha-MEM) containing 10% Fetal bovine Serum, and 1% Penicillin/Streptomycin and maintained in 5% CO2 at 37 C˚. Cells were then seeded in 35 mm dish in concentration of 1 x 10^5/ ml.

(2) Screening of osteogenic-related miRNAs in MSC. In this stage, profiling of the MSCs in normal conditions (Control) and under osteogenic induction (Induced) in order to determine the relevant miRNA associated with osteogenic differentiation of MSCs was performed. Cells were cultured in osteogenic media containing 2ml of alpha-MEM with 50 µg/ml of ascorbic acid (A.A), 2µM β-Glicerophosphate (ß-G); and 10nM of Dexamethasone (Dex). Osteogenic gene expression of induced and control was performed as well as miRNA Microarray. Results were analyzed and compared with each other to determine the differences in gene and miRNA expressions.

４．研究成果

Relevant miRNA associated with osteogenic differentiation of MSCs was performed using miRNA microarray. Fig. 2

In miRNA microarray data analysis, the point of interest is whether a specific miRNA or sets of miRNAs are differentially expressed. The concept of "differentially-expressed" is not well-defined, which makes it challenging to detect the differentially expressed miRNAs. To determine whether the miRNA was differentially expressed, the fold-change (FC), which is the ratio of miRNA expression in the samples were calculated. Usually, if FC = 1, the miRNA is not differentially expressed. If FC is much larger than 1.0 is upregulated; otherwise, if FC is smaller than 1.0, then miRNA is downregulated. Therefore both, the fold-change and the basal level of the miRNA were taken into consideration.

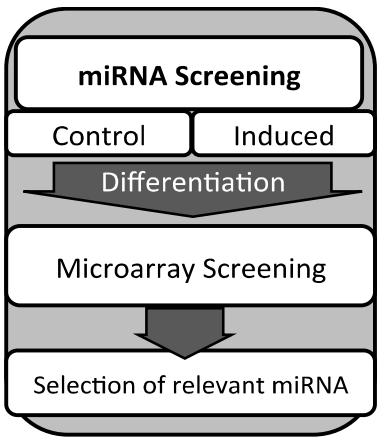

Fig. 2. Determination of the relevant miRNAs through a microarray screening of control and osteo-induced cell for further intracellular characterization.

In practice, the actual value of FC is unknown; and only an estimate is obtained. The majority of miRNAs had low signal to noise rations, and thus, the measurement errors played an important role in the intensity measures and severely influenced the estimate of the FC. Thus, measurement errors posed a high degree of uncertainty in identifying the differentially expressed miRNAs.

In the miRNA microarrays, the expressions of numerous miRNAs contained contradictory measurement, and the overall signal quality of the miRNA microarrays was deficient. Thus, the conclusions based on microarray data without proper normalization might be quite misleading.

Other difficulties relied on the source of microRNAs and the extraction method. The short sequence of microRNAs hindered an easy design of probes. The selection of reference genes is critical for miRNA expression analysis and for normalization of the signal. The diversity of miRNA microarray platforms and lack of reliable analytical methods made cross-platform miRNA microarray data comparison and integration challenging.

In summary, it is challenging, but necessary, to develop novel adaptive statistical methods to efficiently calibrate the measurement errors for normalization and for differentially-expressed miRNA detection.

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