The Involvement of Mir-210 in Unrestricted Somatic Stem Cells Differentiation into Osteoblasts

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Introduction: Bone surgery as a current bone treatment method is not always successful to fulfil bone repair in bone degenerative diseases or extensive injuries. Due to the limited capacity of bone remodeling, the demand for alternative approaches remains to be met. Thus, efforts in *ex vivo* generation of bone forming cells, osteoblasts, and their further application in cell therapy as a promising approach are of vital prominence from a scientific perspective. Though several studies have focused on microRNA roles in osteoblast differentiation in various cell recourses, yet none has reported miR-210 enhancing role in human mesynchymal stem cells (MSCs) so far. **Materials and Methods**: Hence, we wished to examine the nature of the relationship between osteoblast differentiation and miR-210 in unique human mesynchymal stem cells, unrestricted somatic stem cells (USSCs). Osteoblast markers at gene level namely, Runx2, col I in addition to osteocalcin were assessed using qRT-PCR, and Alizarin Red S staining was also carried out to observe histochemical changes 7 days following miR-210 transduction. **Results**: The conclusion that follows from our findings represents a marked increase in osteoblast differentiation markers. Interestingly, for the first time, human USSCs differentiation into osteoblasts was performed in our research. **Conclusion**: our study may provide helpful insights into surmounting bone related issues by combination of both gene and cell therapy.

Keywords: Differentiation; miR-210; Osteoblasts; USSC

Introduction

The structure of bone has now been fully understood. Bone is resorbed and rebuilt by two major abundant bone residing cell types of osteoclasts and osteoblasts respectively, with distinct origins to maintain tissue hemostasis. From a scientific viewpoint, osteoblasts are of high importance due to their bone forming nature, hence these cells if generated *ex vivo* could be prime candidates to serve useful future bone treatment purposes(1). To attain the desired goal, molecular and genetic research has been constantly attempting to unravel the mysteries of osteoblast differentiation regulatory layers. The first and most well-known regulatory layer of osteoblast differentiation is the specific central transcription factors, Runt-related transcription factor 2 (Runx 2) and Osterix, which initiate and tightly regulate osteoblast differentiation. They target osteoblast-related genes such as osteocalcin, bone sialoprotein, osteopontin and collagen type Iα1. Other transcription factors such as Twist, Distal-Less Homeobox 5 (DLX5) inhibits Runx2 transcriptional activity(2).More recently, it has been explained that the multi-layered regulation of osteoblasts is not solely restricted to transcription factors at gene level, but also is contributed and controlled by post transcriptional modulation epigenetically at protein level. Amongst the post transcriptional modulators, microRNAs are considered to mediate rapid fine-tuning of gene expression in specific tissues such as bone(3) MicroRNAs are classed as short noncoding single stranded RNAs and major epigenetic modulators of protein levels. These modulators exert their influence either by accelerating the target mRNAs degradation or blocking their translation. Biological significance of microRNAs has been understood in various multicellular organisms' processes ranging from metabolism, migration, proliferation, and apoptosis to differentiation. Among the investigated microRNAs, miR-133 has been reported to regulate muscle differentiation (4). In addition to muscle differentiation, a growing number of microRNAs

regulating bone differentiation have been elucidated over the past recent years (5). Accordingly, miR-223 role has been demonstrated to control osteoclastogenesis in RAW 264.7 cells (6). Also, miR-26 regulates osteogenic differentiation in human adipose tissue-derived stem cells. Another research showed that miR-125b inhibits bone morphogenic protein-4 (BMP-4)-induced osteoblast differentiation in mouse mesenchymal ST2 stem cells (7). The inhibitory role of miR-214 in bone formation has been revealed in transgenic mice as well (8). MiR-206 connexin 43 ,which is a gap junction protein in osteoblasts, has been shown to be down regulated when miR-206 is overexpressed and conversely, its knockdown expression promoted osteoblast differentiation in C2C12 cells (2). miR-26a also inhibits osteoblast differentiation by targeting Smad1 in human adipose tissue derived stem cells (9).

Yet, the role of miRNAs in the differentiation and recruitment of mature cells derived from mesenchymal stem cells (MSCs) or hematopoietic stem cells still remains to be established(1). Although miR-210 has already been claimed to be over-expressed during osteoblastic differentiation in mouse ST2 cell line, as far as we are concerned, no data to date have been reported on osteoblastic differentiation in human umbilical cord stem cell source by means of microRNAs. Thus, we aimed to assess the likely osteoblastic differentiation potential of miR-210 in a unique human derived cell source named unrestricted somatic stem cells (USSCs) cells by means of Lentivirus delivery. The newly emerged human umbilical cord blood stem cells known as USSCs have lately gained popularity due to their multitude favorable intrinsic properties in comparison with other stem cell resources (10). In principle, these cells are mainly renowned for their non-invasive isolation compared to bone marrow derived mesenchymal stem cells. Furthermore, no ethical issues has been addressed regarding their usage since they are discarded right after child birth and could be applied in research with maternal consent afterwards. In addition, they have been found to be highly potential of the three germ layer differentiation while remaining undifferentiated. Lastly, it is also worth mentioning that their long-term *ex vivo* expansion or maintenance would cause no transformation. Put simply, USSC characteristics have made them a promising cell source that can offer a new perspective on cell-based therapies and regenerative medicines (11-13).

Materials and Methods

Cell culture

USSC was obtained from the Stem Cell Technology Research center (Tehran, Iran). Previously characterized USSCs were expanded in high DMEM medium (Gibco), supplemented with 10 % FBS (Gibco).

Transfection

In order to achieve the entire Lentivirus production, initially, the vital plasmids, namely, packaging plasmid psPAX2, envelope plasmid pMD2.G as well as pLenti-III-GFP (scramble vector), and pLeX Turbo-GFP (miR-210 vector) were separately extracted from the 16 hour cultured bacteria in LB, according to the plasmid extraction kit procedure (Ferments). For the Lipofectamine™ 2000 transfection purposes, nearly 4×10^4 HEK293T cells were first cultured 24 hours earlier in a 10 mm plate nourished by DMEM and 10 % FBS to reach 70 % of confluency in time for the transfection. Subsequently, the previously extracted plasmids and Lipofectamine™ 2000 were each diluted in Opti-MEM a serum free medium and combined to form the oligomer-Lipofectamine complexes. The prepared complexes were added to the HEK293T plates and incubated at 37°C in a CO_2 incubator thereafter in accordance with the manufacturer's instructions.

Lentivirus production and harvesting

16 hrs following the transfection day, HEK293T supernatant medium containing the newly formed complete Lentiviral particles were collected in falcons once per every 24 hrs for three days long and stored at -70°C for long term use.

Transduction

30% confluent adherent USSCs previously seeded in 16-well culture plates were transduced with filtered as well as concentrated Lentiviral particles. The efficiency of USSC transduction was enhanced with the addition of 8 µl polybren.

Total RNA isolation

Having harvested USSC samples at day 7th, the transduction effect of miR-210 was qualitatively and quantitatively assessed by GFP tracking via fluorescence microscopy and RNA extraction for further analysis by Real Time PCR respectively. The total RNA isolation was carried out with the aid of RNA X-plus lysis reagent in accordance with our provided RNA isolation protocol and followed by the RNA concentration quantification using AG bio photometer.

qRT-PCR

To meet the miR-210 detection, the total isolated RNA was reverse transcribed by RT enzyme provided by 1st cDNA synthesis kit (Thermo Scientific) and with an exclusively designed forward stem loop primer for miR-210 in addition to a forward primer of Snord 47 used as an endogenous control and a universal reverse primer in TAKARA Thermo cycler. The primer sequences are respectively for miR-210: 5'- TGATTAGCCCCTGCCCAC -3' and for the internal control: 5' ATCACTGTAAAACCGTTCCA 3'. Using 1 µl of the consequent 1st cDNA as templates and SYBR Green/ROX qPCR Master Mix (TAKARA), the miR-210 expression level was measured utilizing the quantitative Real Time PCR (Applied biosystem), with the total cycle of 41.

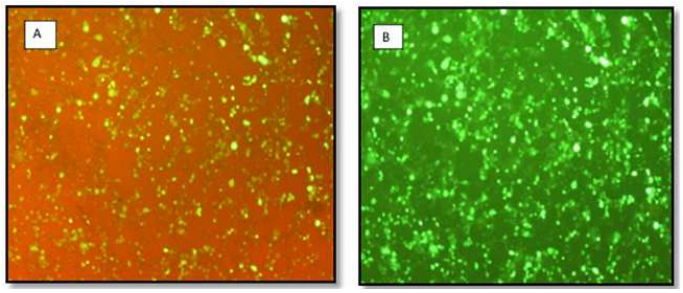

Figure 1. HEK293Ttransfection fluorescence microscopy images after 72 hrs

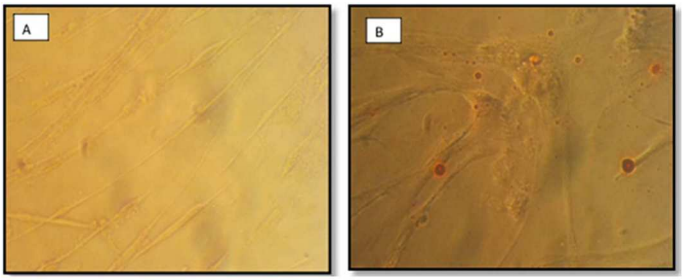

Figure 2. *A)* Alizirin red staining of USSCs as control; *B)* Alizarin red staining of transduced cells after 7 d

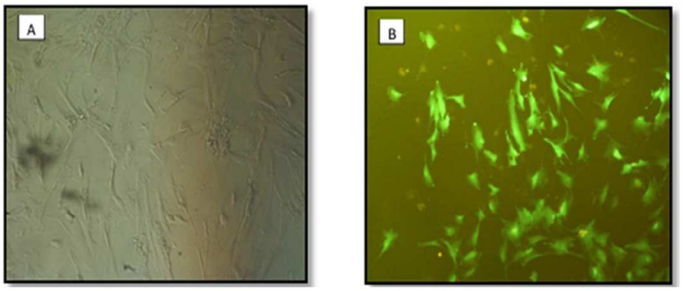

Figure 3. *A)* Fibroblastic morphology of USSCs under optical microscope; *B)* 72 hrs double transduced USSCs under fluorescence microscope

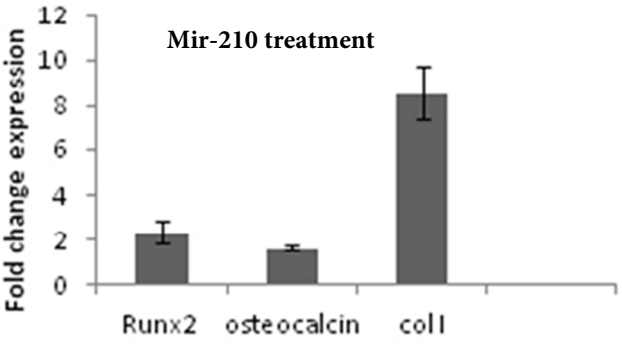

Figure 4. Real Time PCR shows 2.23, 1.6, 8.6-fold change in expression of Runx2, Osteocalcin and col I, respectively in miR-210 treated USSCs at day 7th compared to the control

For the purpose of qRT-PCR analysis in other words, quantify the difference between mRNA levels of osteogenic markers; the total RNA was isolated from all cell samples, using RNA-X plus reagent. After the RNA concentration measurements, cDNAs were synthetized by M MuL V Reverse Transcriptase and random hexamer primers in a sequential two-step process using TAKARA Thermo cycler. cDNA amplification in q-RT PCR begins with a cycle of the initial secondary structures denaturation for 30 seconds at 95°C, followed by 40 cycles circulating 5 seconds of 95°C denaturation and 30 seconds of annealing/extension. All reactions were performed in a minimum of duplicates and normalized to the internal control gene. Relative expression was quantified using ΔΔCt method and changes in microRNA and mRNA expressions were normalized to the relevant internal control. The applied Biosystem was used for quantitative miRNA and mRNA transcript expressions.

Alizarin red staining

To assess the histochemical changes for osteoblastic differentiation, the Alizarin Red S staining (Sigma) was performed on USSC control cells in comparison with miR-210 treated USSC cells following 7 days. Briefly, cells were first once washed with cold PBS and then fixed 20 minutes with 4 % Paraformaldehyde at 4°C. Immediately after fixation, the second wash with PBS and a 5-minute incubation at room temperature were carried out. Subsequently, the fixed cells were stained with prepared 2 % Alizarin red S with a pH of 7.2. Having added the stain slowly and drop-wise to the cells, cells were then incubated at room temperature. Not long after the incubation, stain was discarded and the final cell wash was done for optical microscopic examination and imaging (Figure 2).

Results

Transfection and packaging plasmids

Transfection and Lentivirus production of pLEX TurboGFP vector of miR-210 as well as helping plasmids of psPAX, PMD2.G were packaged successfully by means of HEKT 293 and eventually miR-210 Lentivirus production was readily detectible due to GFP marker presence in pLEX TurboGFP vector of miR-210. Therefore, GFP expression was detected via fluorescence microscopy 72 h after transfection in HEKT 293 cell line. Fluorescence microscopy imaging results are illustrated in Figure 1.

Transduction

USSC cells were twice transduced with newly produced miR-210 Lentivirus. 72 hrs following transduction, GFP was detected. In this regard, expression of GFP in USSCs is presented in Figure 3.

Alizarin Red Staining

Alizarin Red S staining (Sigma) was performed on USSC control cells in comparison with miR-210 treated USSCs following 7 days. As depicted in Figure 2B, USSCs treated with miR-210 lentiviral vector reveals presence of redish-brown color staining that could be interpreted as calcium nodules while Figure 2A represents Alizirin Red staining of USSCs as control cells. The absence of redish-brown color staining of mentioned cells that equates with no calcium nodules is noticed.

MicroRNA and gene expression analysis

Transduction results demonstrated a remarkable up regulation of miR-210 in USSCs after duration of 7 days. The expression of microRNA was compared with a control. Gene expression analyzed by Real Time PCR revealed that there was an increase in osteoblast markers namely, Runx2, col I as well as osteocalcin in comparison with the untreated USSCs. Gene expression analysis is illustrated in Figure 4.

Discussion

Significance of bone and the mounting concern for its associated defects today have resulted into attempts to differentiate distinct cell resources into bone generating cells, osteoblasts (14). In this regard, fine- tuning molecules known as microRNAs are gaining ground against the conventional methods of costly growth factor or osteogenic reagent applications in osteoblast differentiation. miR-210 cellular functions, that have been clarified in recent years, is a case in point. miR-210 is believed to be involved in cell proliferation inhibition by directly targeting E2F3 in a wide variety of cells (15). Studies dissecting mechanistic insight revealed that miR-210 promotes stem cell survival via targeting caspase-8 associated protein 2 (CASP8AP2), or its human homologue FLICE-associated protein homolog (FLASH), a protein that facilitates Fas-induced apoptosis (16). miR-210 can, in addition, act as an angiogenesis inducer in myocardial infarction as well as a potent mitochondrial repressor(17).

Based on recent data, miR-210 has been claimed to be overexpressed in ST2 mouse bone marrow derived stem cells during osteoblast differentiation via targeting Activin receptor type 1 B when induced by BMP4. Despite the extensive research in microRNAs, their cellular functions, specifically, their role in the differentiation of mesynchymal stem cells is still in need of elucidation (18), as a result we took the initiative to examine the nature of the relationship between miR-210 and a newly emerged human mesynchymal stem cells, unrestricted somatic stem cells (USSCs). USSCs are recent emerged potent stem cells which hold great promise as cell-based therapeutics. Bearing these in mind, we evaluated the effect of miR-210 on human USSCs. The relative expression of osteoblast markers such as Runx2, col I and osteocalcin were assessed at molecular level following treatment of USSCs with miR-210. Our data indicated that there were an 8.6 -fold and 2.23-fold increase in col I and Runx2 expression respectively. Similarly, the well characterized marker of osteoblasts, osteocalcin, experienced a 1.6-fold overexpression of in comparison with the untreated cells as control. We further confirmed the osteoblast differentiation by detecting calcium in USSCs treated with miR-210, using Alizarin red staining. Although miR-210 effect has been previously reported, to the best of our knowledge, no data has focused on the human stem cell recourse yet. Simply put, USSCs were selected due to their great proved potential of multipotency. Last of all, our data confirms that microRNA approach to osteoblast differentiation can have an outstanding merit in the cell therapy future.

Conclusion

On the whole, we demonstrated osteoblastic potential of miR-210 in USSCs. In fact, here, we presented that miR-210 is capable of enhancing USSC differentiation into osteoblasts. This research may provide helpful insights into stem cell therapy for bone degenerative diseases not in the too distant future.

Conflict of Interest: 'None declared'.

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