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Probing Human Osteogenic Differentiation Using Double-Stranded Locked Nucleic Acid Biosensors

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Abstract— **Human mesenchymal stem cells (hMSCs) have great potential for tissue engineering and regenerative medicine due to their self-renewal and multi-differentiation properties. However, the cellular and molecular mechanisms that govern osteogenic differentiation are poorly understood due to a lack of effective tools to detect gene expression at single cell level. Here, we present a double-stranded locked nucleic acid biosensor to investigate gene expression analysis during hMSCs osteogenic differentiation. We first demonstrated this biosensor for gene expression analysis in single hMSCs. We next investigated the regulatory role of Notch1-Dll4 signaling in osteogenic differentiation. Our findings provide evidence that Notch1-Dll4 signaling is involved in hMSCs osteogenic differentiation. Inhibition of Notch1-Dll4 signaling significantly decreased osteogenic differentiation and Dll4 expression.**

I. Introduction

Human mesenchymal stem cells (hMSCs) are adult stem cells isolated from adult tissues such as bone marrow and adipose tissue. hMSCs have great potential in cell-based therapies for tissue engineering and regenerative medicine due to their multipotency and easy accessibility. It has been shown that hMSCs have the potential to differentiate into different lineages, including osteoblasts (bone), neuroblasts (neural tissue), adipoblasts (fat), myoblasts (muscle), and chondroblasts (cartilage) (1,2). In particular, osteogenic induction of hMSCs differentiation in conjunction with tissue engineering approaches has been exploited to provide an alternative method besides autologous bone graft to replace or restore damaged bone tissues. This evidence demonstrates the application of hMSCs as a promising cell source in bone tissue engineering, giving their regenerative properties. Although the differentiation capacity of hMSCs' has been studied extensively, the mechanisms that control their plasticity, especially how hMSCs can be differentiated into osteoblasts and make bones, is hindered by the availability of technologies for detecting behaviors of cells with single-cell resolution. Despite conceptual advancements in stem cell differentiation, the cellular and molecular mechanisms that govern hMSCs during osteogenic differentiation remain poorly understood.

Here, we established a double-stranded locked nucleic acid (LNA) /DNA (dsLNA/DNA) biosensor for gene expression analysis in single hMSCs. By incorporating this biosensor with live hMSCs imaging during osteogenic induction, we performed dynamic tracking of hMSCs differentiation efficiency and gene expression profiles of individual hMSC during osteogenic differentiation. We further investigated the role of Notch1-Dll4 signaling in regulating hMSCs during osteogenic differentiation. Pharmacological perturbation is applied to disrupt Notch1-Dll4 signaling to investigate the molecular mechanisms that govern osteogenic differentiation. Our results provide convincing evidence supporting that Notch1-Dll4 signaling is involved in regulating hMSCs osteogenic differentiation. Specifically, Notch1-Dll4 signaling is active during osteogenic differentiation. For the first time, we identified that Dll4 is a molecular signature of hMSCs osteogenic differentiation.

II. Material and Methods

A. Cell Culture and Reagents

hMSCs were acquired from Lonza and maintained in mesenchymal stem cell basal medium (MSCBMTM) with GA-1000, L-glutamine, and mesenchymal cell growth supplements. The cells were cultured in a humidified incubator at 37 °C with 5% CO_2 and passaged using 0.25 Trypsin-EDTA (Invitrogen). The cell culture medium was replaced every three days. hMSCs from passages 3-7 were used in the experiments. DAPT was acquired from Sigma Aldrich (DAPT ≥98% (HPLC), solid, D5942). To induce osteogenic differentiation, hMSCs were seeded at a density of 1x 10^6 cells/mL with a volume of 500 µL in 24 well-plates. hMSCs basal medium was replaced with osteogenic induction medium after two days of cell seeding. Osteogenic induction medium were changed every two days. To study the effects of Notch1-Dll4 signaling, hMSCs were treated with 20 µM γ-secretase inhibitor DAPT after osteogenic induction. Images were taken after 3 days, 5 days, 7 days, and 10 days induction, respectively.

B. LNA Probe Design

An LNA probe is a 20-base pair nucleotide sequence with alternating LNA/DNA monomers. A fluorophore (6-FAM) was labeled at the 5' end for fluorescence detection. The design process of LNA probes was reported previously (3). Briefly, the LNA probe was designed to be complementary to the target mRNA sequence with a 100% match. The binding affinity and specificity were optimized using mRold server and NCBI Basic Local Alignment Search Tool (BLAST) database. A quencher is a 10-base pair nucleotide sequence with only DNA monomers that is

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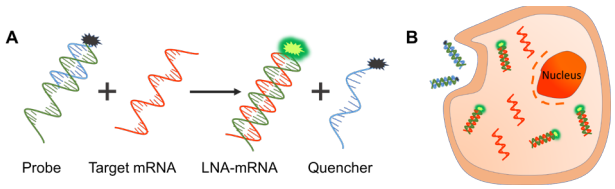

Figure 1. Single cell gene expression analysis of hMSCs. **(A)** Schematic illustration of double-stranded probe's binding scheme for mRNA detection. **(B)** Schematic of mRNA detection in hMSC using dsLNA/DNA probe.

complementary to the 5' end of the LNA probe. An Iowa Black RQ was labeled at the 3' end of the quencher probe. The LNA probe was synthesized by Exiqon Inc. The quencher sequence and corresponding target DNA sequences were synthesized by Integrated DNA Technologies Inc. (IDT).

C. Double-stranded LNA probe preparation

To prepare a double-stranded LNA probe, the LNA probe and quencher were initially prepared in 1x Tris EDTA buffer at a concentration of 100 nM. The LNA probe and quencher were mixed and incubated at 95 ℃ for 5 minutes and cooled down to room temperature over a course of 2 hours. To optimize quenching efficiency, the fluorescent probe and quencher probe were prepared in a number of different ratios to obtain high signal-to-noise ratio. The quenching efficiency was evaluated by measuring fluorescence intensity using a fluorescence microplate reader (BioTek, Synergy 2). The optimized LNA probe and quencher mixer can be stored in a refridgerator for up to 7 days. The prepared double-stranded LNA/DNA probe was transfected into hMSCs using Lipofectamine 2000 following manufacturing instructions.

D. Alkaline Phosphatase Activity (ALP) Staining

To quantify hMSCs osteogenic differentiation, cells were stained for alkaline phosphatase (ALP) using the alkaline phosphatase kit (Sigma-Aldrich) using a modified protocol. First, the staining solution were prepared by mixing fast red violet solution, naphthol AS-BI phosphate solution, and water at a ratio of 2:1:1. Next, hMSCs were fixed using 4 % cold Paraformaldehyde (PFA) for 2 minutes which enable the maintenance of the ALP activation. The staining solution was then added to the fixed cells for 15 minutes under room temperature and protect from light. For nucleus staining, Hoechst 33342 staining solution was prepared in 1x PBS at 1:1000 dilution and added to cells for 15 minutes. The cells were then washed three times with 1x PBS, 15 minutes each time, before fluorescence imaging and analysis.

E. Imaging and Statistical Analysis

Images were captured using ZOE inverted fluorescence microscope or Nikon TE 2000. All fluorescence images were taken with the same setting for comparison. Data collection and imaging analysis were performed using NIH ImageJ software. Experiments were repeated at least three times, and over 100 cells were quantified for each group. Student's t-tests were used to compare two groups. For comparisons of multiple groups, one-way ANOVA with Tukey's post hoc test was used.

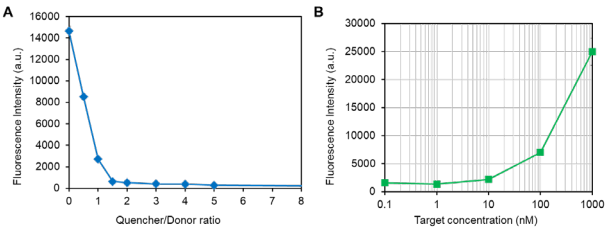

Figure 2. Characterization of dsLNA/DNA probe. (A) Calibration of the quencher-to-probe ratio. (B) Detection dynamic range of the double-stranded LNA probe was determined using 100nM probe and varying concentrations of target strand. N=4.

III. Results

A. Characterization of double-stranded LNA/DNA probe

We first established a double-stranded LNA/DNA (locked nucleic acid) probe for single-cell gene expression analysis during osteogenic differentiation, **Figure 1A**. This probe includes two stands of oligonucleotides, a detecting strand labeled with a fluorophore and a quenching stand labeled with Iowa Black to quench the fluorescence signal in the absence of a target. The LNA probe binds to a quencher probe to form the dsLNA/DNA probe. In close proximity, the fluorophore at the 5' end of LNA probe is quenched simultaneously by a quencher. The dsLNA/DNA probe was transfected into hMSCs to detect endogenous mRNA expression. In the presence of a target mRNA, the LNA probe is thermodynamically displaced from the quencher to bind to the specific target sequences. The displacement reaction permits the fluorophore to fluoresce, detecting the gene expression at the single-cell level, **Figure 1B**.

Before transfecting the LNA/DNA probe into hMSCs, we first characterized quencher-to-probe ratio to minimize the background caused by free fluorophore in the reactions. By adjusting the quencher-to-probe ratios ranged from 0.5 to 10, the binding efficiency was evaluated by measuring the fluorescence intensity. The fluorophore concentration was set to 100 nM. **Figure 2A** shows the fluorescence intensity decreased as the quencher concentration increased. The quenching efficiency was reached to 96% with the

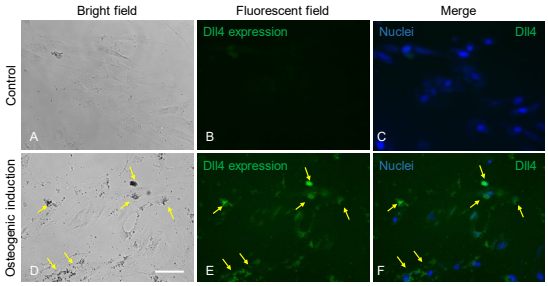

Figure 3. Representative images of hMSCs during oteogenesis in control and osteogenic induction groups. Fluorescent filed images indicate Dll4 mRNA expression at day 7. Green: Dll4 mRNA. Blue: nucleus. Scale bar: 100 μm.

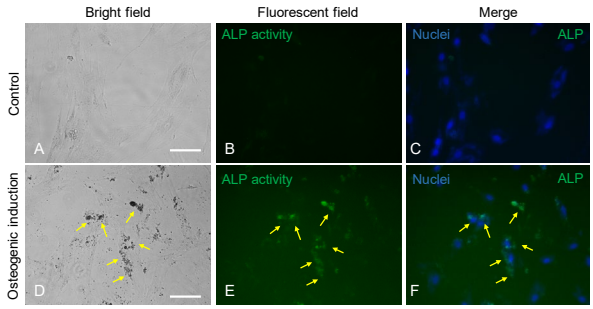

Figure 4. Representative images of hMSCs during oteogenesis at day 7. Fluorescent filed images indicate ALP activity. Green: ALP. Blue: nucleus. Scale bar: 100 μm.

quencher-to-probe ratio of 2, indicating the fluorophore intensity was effectively quenched to a low level (about 4%). Thus, the quencher-to-probe ratio was set at 2:1 for all the subsequent studies. The detectable target concentration range was further estimated by varying the concentration of a synthetic DNA target strand while holding the probe concentration at 100 nM. The titration curve (**Figure 2B**) exhibits a large dynamic range for quantifying the target concentration (from 2 to 1000 nM), providing that a probe of 100 nM is sufficient for large dynamic detection.

B. Dll4 mRNA detection during hMSC osteogenic induciton

To investigate Dll4 mRNA expression during osteogenic differentiation, we compared Dll4 mRNA expression levels in hMSCs cultured in basal growth medium and osteogenic induction medium. After 7 days, the Dll4 mRNA expression was evaluated and compared by measuring the fluorescent intensity. Interestingly, we found the expression of Dll4 mRNA was evaluated in differentiated hMSCs, compared to the control group, **Figure 3**. These results showed the control group (hMSCs in basal medium) has a low level of Dll4 mRNA expression, while the osteogenic induction group has increased Dll4 mRNA expression. Yellow arrows indicate differentiated hMSCs. This finding is consistent with previous results reported by other groups (4). It was reported by Wagley et al. that Notch1-Dll4 signaling is required for bone morphogenetic protein-mediated human osteoblast differentiation (5). Bagheri et al. reported that Notch pathway is avtive during osteogenic differentiation of hMSCs induced by pulsed electromagnetic fields (4). To further confirm Dll4 mRNA expression is evaluated in osteogenic differentiated hMSCs, alkaline phosphatase (ALP, a biochemical marker for bone formation) enzyme activity was further evaluated using ALP living staining assay (ThermoFisher), **Figure 4**. The green fluorescent signal indicates ALP enzyme activity in hMSCs cultured in growth medium, and osteogenic medium. **Figure 4** showed that hMSCs in osteogenic induction medium has higher ALP enzyme activity, indicating higher osteogenic differentaiton efficiency.

C. Dll4 is a molecular signature of differentiated hMSCs

The above results showed Dll4 mRNA expression might relate to osteogenic differentiation of hMSCs. Thus, we hypothesize Dll4 mRNA is a molecular signature of osteogenic differentiated hMSCs. To test our hypothesis,

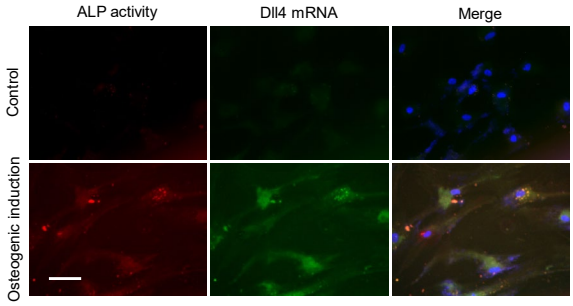

Figure 5. ALP activity and Dll4 mRNA expression after 5 days hMSCs osteogenic induction. Upper panel indicate hMSCs cultured in control growth medium. Bottom panel indicate hMSCs cultured in osteogenic induction medium. Green: Dll4 mRNA. Red: ALP. Blue: nucleus. Scale bar: 100 μm.

we simultaneously detected Dll4 mRNA expression and ALP enzyme activity during hMSCs osteogenic differentiation using LNA/DNA probe and ALP staining assay, **Figure 5**.

Briefly, hMSCs were transfected with LNA/DNA probe one day before ALP enzyme activity analysis. The Dll4 mRNA and ALP activities were evaluated after 5 and 10 days osteogenic induction, respectively. **Figure 5** showed increased ALP activity and Dll4 levels in osteogenic induction group. The fluorescent intensity of ALP activity and Dll4 mRNA expression were further measured and compared. As shown in **Figure 6A**, compared to the control group, ALP activities (measured as red fluorescent signal)

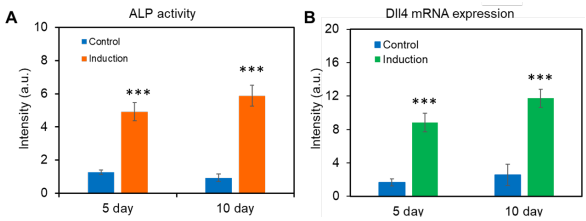

Figure 6. Fluorescent intensity of ALP activity and Dll4 mRNA expression after 5 days and 10 days of hMSCs osteogenic induction. ALP activity **(A)** and Dll4 expression **(B)** comparaion in control and osteogenic group, respectively. Data represent over 100 cells in each group and are expressed as mean± s.e.m. (n=4, ***, P<0.001, **, P<0.01)

in osteogenic induced hMSCs increased 3.2 folds and 6.5 folds after 5 and 10 days of induction, respectively. Accordingly, compared to hMSCs cultured in the basal growth medium, hMSCs cultured in osteogenic induction medium showed an increased Dll4 mRNA expression, with 5.2 folds and 4.56 folds increase at day 5 and day 10, respectively. Thus, our results showed Dll4 mRNA is related to hMSCs osteogenic differentiation. Moreover, Dll4 mRNA expression showed a dynamic profile during the period of osteogenic differentiation. This finding indicates that Dll4 mRNA could potentially be marked as a molecular signature of osteogenic differentiation of hMSCs.

D. Notch1-Dll4 signaling in regulating osteogenic differentiation

In order to investigate the potential involvement of Notch-Dll4 signaling during osteogenic differentiation in hMSCs, we perturbed Notch1-Dll4 signaling by pharmaculeticual inhibition. hMSCs were treated with a γ-secretase inhibitor

DAPT at a concentration of 20 μM (an inhibitor of the Notch signaling pathway) during hMSCs differentiation to observe potential related effects on osteogenesis. A control group was designed without osteogenic induction. First, we evaluated osteogenic differentiation efficiency by quantifying ALP activity after Notch1-Dll4 signaling inhibition. As shown in **Figure 7** and **Figure 8A**, after five days of osteogenic induction, ALP activity was mediated compared to the induction group. Specifically, the ALP enzyme activity decreased by 29.9% ((ALP fluorescence intensity in induction group – ALP fluorescence intensity in the presence of DAPT) / ALP fluorescence intensity in induction group) compared to the osteogenic induction group. These results indicate that inhibition of Notch1-Dll4 signaling using γ-secretase inhibitor mediates osteogenic differentiation of hMSCs. To further investigate the involvement of Notch1-Dll4 signaling during hMSCs induced osteogenic differentiation, we evaluated Notch 1 ligand, Dll4 mRNA expression in osteogenic induced hMSCs. As shown in **Figure 8B**, a significant increase (~ 4 folds) in the expression of the ligand Dll4 mRNA was identifies in cells maintained in osteogenic induction medium compared to control. Further, with DAPT treatment, a significant decrease of Dll4 mRNA (32.7%) was observed compared to osteogenic induction medium only.

As the Notch pathway is triggered by binding specific ligands to receptors, our results indicate that the expression of Notch ligand Dll4 was modulated during osteogenic differentiation. Dll4 mRNA was significantly increased at the end of the differentiation period (Figure 6B). Notch inhibitors induced a significant decrease of ALP enzyme activity and Dll4 mRNA expression at day 7 compared to osteogenic induction.

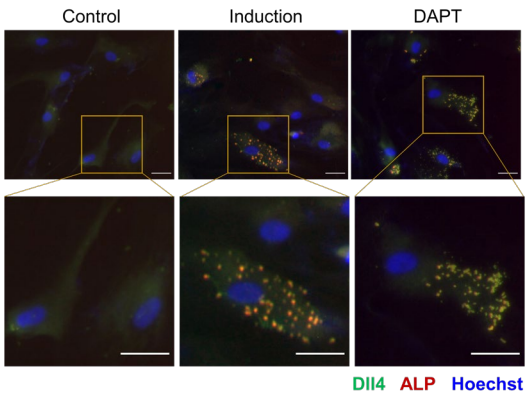

Figure 7. Notch1-Dll4 signaling in the regulation of hMSCs osteogenic differentiation. Representative fluorescence images of hMSCs in control, induction, and DAPT treatment groups. Green: Dll4 mRNA expression. Red and blue indicate ALP activity and nucleus, respectively. Bottom images are enlarged areas of a single hMSC. Scale bar (upper panel): 100 μm.

IV. DISCUSSIONS

In this study, the LNA/DNA biosensor is exploited to monitor the mRNA expression of mesenchymal stem cells during osteogenic differentiation. Unlike conventional techniques, such as RT-PCR, that requires a larege number of cells, this LNA/DNA biosensor detects gene expression at the single cell level. Another advantage of this LNA/DNA biosensor doesn't need cell lysis for fixation, permitting the

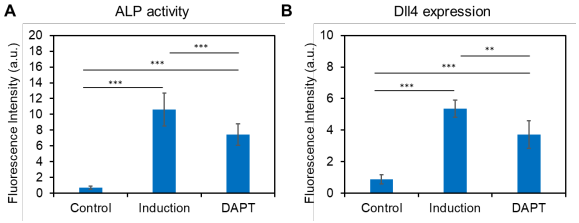

Figure 8. Osteogenic differentiation was analyzed by ALP activity and Dll4 mRNA expression. **(A)** Comparison of ALP activity of differentiated hMSCs in control, induction, and DAPT groups. **(B)** Mean fluorescence intensity of the Dll4 mRNA expression of hMSCs in control, induction, DAPT treatment group.

investigation of dynamic gene expression during osteogenesis. Here, we investigated Notch ligand Dll4 expression dynamics during osteogenic differentiation. This study revealed that Notch1-Dll4 signaling is involved and active during osteogenic differentiation. Dll4 expression levels correlate with the phenotypic and differentiation markers of mesenchymal stem cells during osteogenesis. The ability to monitor Dll4 mRNA in living cells enalbes us to identify the regulatory role of Notch sinaling. Our results suggest the involvement of Notch1-Dll4 signaling in regulating hMSCs osteogenic differentiation. Inhibition of Notch signaling by a gamma secretase enzymatic inhibitor attenuates the alkaline phosphatase enzymatic activity. Our results also demonstrate Dll4 mRNA levels correlated with early osteogenic markers (ALP activity). In the absence of Notch inhibitor, hMSCs, cultured in osteogenic medium, differentiated towards the osteoblast phenotype, has high Dll4 expression.

In conclusion, the results of this study add new information concerning osteogenic differentiation of hMSCs and the involvement of Notch1-Dll4 signaling. Further studies may elucidate the mechanisms underlying Notch1-Dll4 signaling in regulating osteogenesis.

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