The precursor osteoblast-like cell, MC3T3-E1 cell line, enhances sodium–calcium exchanger 1 (Ncx1) gene expression by stretch stimuli prior to osteoblast differentiation

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ABSTRACT. This study evaluated the expression of genes involved in the concentration of Ca^{2+} in precursor osteoblast-like cell, MC3T3-E1 subjected to stretching stimuli. Transient receptor potential vanilloid 4 (Trpv4) gene expression, the factor that is activated by stretch stimulation and enables inflow of Ca^{2+} from the extracellular space, was not affected as a result of stretch stimulation; conversely, the expression of sodium-calcium exchanger 1 (Ncx1) gene involved in outflow of intracellular Ca^{2+} increased, depending on stimulation intensity. Localization of Ca^{2+} correlated with the positioning of the endoplasmic reticulum, and intracellular Ca^{2+} decreased in inverse proportion to the intensity of the stretching force. These results suggest that stretch stimulation activates intracellular Ca^{2+} elimination rather than Ca^{2+} uptake before osteoblast differentiation.

KEY WORDS: Ncx1, Ncx3, osteoblast differentiation, stretch stimulus, Trpv4

Osteoblasts differentiate from mesenchymal stem cells, and they secrete bone matrix around themselves to form bone tissue [13]. Both physical and chemical signals have been reported to affect osteoblast differentiation in addition to various physiologically active substances. Examples of physical signals include shear and stretch stresses. These kinds of mechanical stimuli promote osteogenic activity [18, 21]. Examples of chemical signals include Ca^{2+} and Zn^{2+} ions, which have also been reported to promote osteoblast differentiation [2, 19].

Osteoblasts have a mechanism for regulating intracellular Ca^{2+} concentrations by controlling the uptake of Ca^{2+} into the endoplasmic reticulum (ER) and its outflow to the extracellular space [20]. The presence of multiple ion channels has been reported to be a factor regulating Ca^{2+} inflow [2], and the transient receptor potential vanilloid (TRPV) family of proteins is known as a channel responsive to changes in temperature, osmotic pressure, and mechanical stimuli [1]. Among these proteins, TRPV4 selectively infuses cations such as Ca^{2+} and Mg^{2+} into cells via a 6-transmembrane channel activated by cell stretching [9, 12]. Meanwhile, the sodium–calcium exchanger (NCX) protein acts as a Ca^{2+} excretion mechanism by utilizing molecules comprised of 3 Na^{+} ions to eject a single Ca^{2+} ion [17]. NCX can exist as subtype 1, 2, or 3, and osteoblasts have been confirmed to express NCX subtypes 1 and 3 [10, 14, 15]. Although osteoblast differentiation is induced in response to physical and chemical stresses, the extent of exposure to stretch stimulation and changes in the expression of factors related to the mechanism of Ca^{2+} regulation are not fully elucidated in an undifferentiated osteoblast. In this study, the precursor osteoblast-like cell, MC3T3-E1 cell line was used as the target of an analysis of the effect of stretch stimulation provided to an undifferentiated osteoblast with focus on Trpv4 and Ncx genes.

Cells from the MC3T3-E1 mouse osteoblast-like cell line (DS Pharma Biomedical, Osaka, Japan) were cultured in an incubator using a minimum essential medium alpha (MEMα; Wako, Osaka, Japan) containing 5% fetal bovine serum (FBS; Atlas

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Biologically, and the results of our intracellular Ca
t+ localization analysis were supported by this previous study.

In our experiment, we predicted that Ca2+ entered cells as a result of stretch-related stress, and the cells would accumulate more Ca
+ for calcification. In contrast, however, the intracellular Ca
+ concentration (Trpv4, Ncx1, and Ncx3) was measured to be significantly lower in the experimental group cells compared with the control group. TRPV4, a Ca
+ ion-permeable channel, is known to be responsive to mechanical stimuli [4, 16]. At the start of the experiment, we predicted that Ca2+ entered cells as a result of stretch-related stress, and the cells would accumulate more Ca2+ for calcification. In contrast, however, the intracellular Ca2+ concentration was markedly decreased in 8% of the experimental group cells compared with the control group.

Function of transcription factors is important for the initiation of osteoblast differentiation, and Runx2 is a representative factor for confirming the gene expression of osteoblast differentiation markers (Runx2, Runt-related transcription factor 2; Alpl, alkaline phosphatase liver/bone/kidney; Bglap, bone gamma carboxyglutamate protein [osteocalcin]; and Colla1, collagen type I alpha 1) and regulating factors involved in intracellular Ca
+ concentration (Trpv4, Ncx1, and Ncx3). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal standard. Statistical analyses were performed using Student’s t-test or one-way analysis of variance with Bonferroni-Dunn’s post hoc tests. P values of <0.05 were considered statistically significant.

Fluorescence microscopic observation revealed that Ca2+ was distributed throughout the cytoplasm in MC3T3-E1 cells in the control group (0% stretch stimulus), and its localization was found to be consistent with the positioning of the ER (Fig. 2). On examining the localization of intracellular Ca2+ by CaTM-2AM, the presence of Ca2+ was also reported in the smooth ER (Figs. 2 and 3).

Total RNA was extracted from cells using the QIAGEN RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, 30 ng/µl of RNA was reverse transcribed into cDNA using the ReverTra Ace qPCR RT with gDNA Remover Kit (Toyobo, Osaka, Japan). Quantitative PCR for comparing the rates of gene expression in each experimental group was performed using FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) and various primers (Table 1) for confirmation of gene expression of osteoblast differentiation markers (Runx2, Runt-related transcription factor 2; Alpl, alkaline phosphatase liver/bone/kidney; Bglap, bone gamma carboxyglutamate protein [osteocalcin]; and Colla1, collagen type I alpha 1) and regulating factors involved in intracellular Ca
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Table 1. Forward and reverse primer sequences for real time RT-PCR

| Gene   | Primer sequence | Product size (bp) | Gene accession number |
|--------|-----------------|------------------|----------------------|
| Runx2  | 5′- AGC TTC TCC AGC GCA GTG AC-3′ | 59 | NM_001146038 |
|        | 5′- CTG GTG CTC GGA TCC CAA-3′   |   |                     |
| Alpl   | 5′- TCC TGG CTC TGC CTT TAT TCC-3′ | 62 | NM_007431 |
|        | 5′- TGC CCA AGA GAG AAA CCT GCT-3′ |   |                     |
| Colla1 | 5′- CCT GGC CTT GGA GAA AAT TTT-3′ | 73 | NM_007742 |
|        | 5′- GCA CGG AAA CTC CAG CTG ATT-3′ |   |                     |
| Bglap  | 5′- AAG CCT TCA TGT CCA AGC AGG-3′ | 170 | NM_007541 |
|        | 5′- TTT GTA GGC GTT CTT CAA GCC-3′ |   |                     |
| Trpv4  | 5′- TCA CCT TCG TGC TCC TGT TG-3′ | 84 | NM_022017 |
|        | 5′- AGA TGT GCT TGC TCT CTT TG-3′ |   |                     |
| Ncx1   | 5′- CTG GGG AAA TTC ATG AGG ATG-3′ | 122 | NM_011406 |
|        | 5′- TTC TGT AGG TGG GAC GAA G-3′ |   |                     |
| Ncx3   | 5′- AGG AGG GGA TGA GGA TGA AGG-3′ | 161 | NM_01167920 |
|        | 5′- TGA GGA TGG AGA CCA CGA AGG-3′ |   |                     |
| Gapdh  | 5′- AGG TGG TGG AGC GAT TGG TG-3′ | 123 | NM_008084 |
|        | 5′- TGT AGA CCA TGT AGT TGA GGT CA-3′ |   |                     |
To differentiate MC3T3-E1 cells into osteoblasts, osteogenic differentiation medium is usually used. In this study, we did not use such medium for culturing MC3T3-E1 cells, but interestingly, the stretch stimulation significantly increased Runx2 expression (Fig. 3). It was inferred that the stretch stimulation provided a clue to switch on osteogenic differentiation in the osteoblast precursor.

In the differentiation of osteoblasts, the synthesis levels of proteins such as alkaline phosphatase (ALPL), type I collagen (COL1A1), and osteocalcin (BGLAP) change at each stage of differentiation. The expression levels of Alpl and Col1a1 mRNA increase in the early and middle stages of osteoblast differentiation, whereas Bglap expression is associated with terminal differentiation [8]. In this study, the stretch stimulation significantly increased the Alpl mRNA level; however, the expression of Col1a1 and Bglap showed no change (Fig. 3). Although Alpl and Col1a1 are known as markers expressed from the early stages of osteogenic differentiation, Alpl, whose protein serves to hydrolyze extracellular pyrophosphate, an inhibitor of calcium crystal formation in bone [11], is suggested to be more responsive to mechanical stimuli than Col1a1.

A comparison of gene expression of regulating factors involved in intracellular Ca\textsuperscript{2+} concentration, except for Ncx1, revealed no difference (Fig. 3). Although it is believed that TRPV4 is activated by mechanical stimuli [9, 12], no change was observed.

Fig. 2. Fluorescent staining images of osteoblast endoplasmic reticula and intracellular Ca\textsuperscript{2+} in MC3T3-E1 cells following stretch stimulation. Endoplasmic reticulum was stained green, Ca\textsuperscript{2+} was stained red, and cell nuclei were stained blue using Hoechst. A marked decrease in intracellular Ca\textsuperscript{2+} staining was observed in the cells that underwent 8% stretch stimulation compared with the control cells (0% stretch stimulus).

Fig. 3. Gene expression of osteoblast differentiation markers (Runx2, Alpl, Col1a1, and Bglap) and regulating factors involved in intracellular Ca\textsuperscript{2+} concentration (Trpv4, Ncx1, and Ncx3) in the undifferentiated MC3T3-E1 cells exposed to 8% stretch stimulus. Each gene expression level was defined as the value obtained from realtime RT-PCR analysis divided by the Gapdh value. The data were analyzed using Student’s t-test. Mean ± SD, n=5, *P<0.05, **P<0.01.
in mRNA expression after stretch stimulation. This result suggests that, in undifferentiated MC3T3-E1 cells, stretch stimulation does not affect Trpv4 gene expression. In contrast, expression of Ncx1 mRNA increased significantly (P<0.01) after exposure to 8% stretch stimulation (2.7 times compared to the control group; Fig. 3). To confirm whether the Ncx1 gene responds to lower stretch stimuli than 8%, qPCR analysis of Ncx1 expression in cells after 2, 5, and 8% stretch stimuli was performed. As a result, Ncx1 increased in proportion to the intensity of the stretch stimulus (Fig. 4). NCX typically plays a role in pumping Na⁺ into cells and Ca²⁺ out of cells along the Na⁺ concentration gradient via the cell membrane (forward mode). However, in pathological states, a Ca²⁺ flow reversal phenomenon (reverse mode) can occur [5]. The intracellular Ca²⁺ staining performed in this experiment was attenuated as stretch stimulation intensity increased, and the stretch stimuli applied in this experiment caused stretch intensity-dependent forward-mode activation rather than nonphysiological reverse-mode activation, as described in Fig. 2. Sosnoski and Gay [14] have reported that NCX3 is a major contributor to Ca²⁺ translocation out of differentiated MC3T3-E1 osteoblasts into calcifying bone matrix and that NCX1 has little to no involvement. Considering our results of Ncx1 and Ncx3 gene expression patterns after stretch stimulation, the MC3T3-E1 cells may initially use NCX1 as the main NCX and gradually shift from NCX1 to NCX3 with progressive differentiation. In this experiment, whether increased Ncx1 expression was the direct result of the influence of stretch stimulation or whether the increase was due to uptake of Ca²⁺ via TRPV remains unclear. Analysis of restrictions on the flow of Ca²⁺ in and out of cells using TRPV4 antagonists such as HC-067047 [3] or Na⁺/Ca²⁺ exchanger inhibitors such as KB-R7943 [6] is believed to be necessary. Based on the above, a conclusion can be drawn that exposure to stretch stimulation facilitates greater Ca²⁺ excretion than inflow from the extracellular space in undifferentiated MC3T3-E1 cells due to NCX1 activity and promotes osteogenesis following osteoblast differentiation.

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