Physical manipulation of calcium oscillations facilitates osteodifferentiation of human mesenchymal stem cells

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ABSTRACT The role of cytosolic calcium oscillation has long been recognized in the regulation of cellular and molecular interactions. Information embedded in calcium oscillation can provide molecular cues for cell behavior such as cell differentiation. Although calcium dynamics are versatile and likely to depend on the cell type, the calcium dynamics in human mesenchymal stem cells (hMSCs) and its role in differentiation are yet to be fully elucidated. In the present study we characterized the calcium oscillation profiles in hMSCs before and after subjecting the cells to the osteoinductive factors. Our findings indicate that the calcium spikes decreased rapidly with osteodifferentiation to a level observed in terminally differentiated human osteoblasts. In addition, the calcium oscillations appear to serve as a bidirectional signal during hMSC differentiation. While an altered calcium oscillation pattern may be an indicator for hMSC differentiation, it is also likely to be involved in directing hMSC differentiation. Treatment of hMSCs with a noninvasive electrical stimulation, for example, not only altered the calcium oscillations but also facilitated osteodifferentiation. Regulation of calcium oscillation by external physical stimulation could amplify hMSC differentiation into a tissue-specific lineage and may offer an alternate biotechnology to harness the unique properties of stem cells.—Sun, S., Liu, Y., Lipsky, S., Cho, M. Physical manipulation of calcium oscillations facilitates osteodifferentiation of human mesenchymal stem cells.


Key Words: Ca2+ oscillation · hMSCs · electrical stimulation

Over the past several decades, the oscillation of cytosolic Ca2+ concentration ([Ca2+]i) is perhaps one of the important discoveries in the field of intracellular signaling. Understanding the molecular information embedded in the [Ca2+]i oscillations is expected to lead to elucidation of the intracellular signaling mechanisms involved in and regulating the bioactivities of cells (1–3). The [Ca2+]i oscillation is a complex dynamical process and reflects Ca2+ transportation to and from the exterior cell, cytosol, and intracellular stores, exchange between cells, or diffusion and buffering due to the binding of Ca2+ to proteins. The [Ca2+]i oscillations can vary in amplitude, temporal profile, and spatial properties and are likely mediated by at least several influx and efflux pathways depending on the cell type and different cellular processes. It is generally thought that the endoplasmic reticulum (ER) represents the main Ca2+ store in the cell, and Ca2+ release from the ER plays an predominant role in generating sustained [Ca2+]i oscillations by IP3-gated channels and the ryanoindine receptors (4, 5). Cells recognize the [Ca2+]i oscillations through sophisticated mechanisms to decode the information embedded in the Ca2+ dynamics. For example, whereas rapid and localized changes of Ca2+ (i.e., Ca2+ spikes) regulate fast cellular and molecular responses, intercellular or intracellular Ca2+ propagations (i.e., Ca2+ waves) control slower responses (5). In addition, the frequency of the Ca2+ oscillations is known to reflect the strength of the extracellular stimulus. As a well known example, the Ca2+ binding proteins such as troponin C in skeletal muscle cells and calmodulin (CaM) in all eukaryotic cells may serve as transducers of the Ca2+ signals by changing their activity as a function of the [Ca2+]i oscillation frequency (6). Such a frequency-modulated signaling determines the qualitative and quantitative nature of the genomic response, and can be translated into a frequency-dependent cell response such as differentiation (7).

[Ca2+]i oscillation is found to play a key role in cell differentiation (8, 9). Among many cell types, stem cells appear to demonstrate a stronger dependence on the IP3-gated channels (10, 11). Because an increasing amount of research effort has recently focused on stem cell manipulation, studies of the [Ca2+]i oscillations in human mesenchymal stem cells (hMSCs) and a development of potential techniques to regulate the multipotent stem cells would be timely. Extracted from adult mesenchymal tissues such as bone marrow, muscle, synovium, and adipose tissue, hMSCs exhibit a great therapeutic potential due to their multiple differentiation capability (12, 13). These hMSCs can be induced to differentiate into different phenotypic lineages in

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the presence of inductive biochemicals or by extracellular matrix proteins such as collagen or vitronectin (14, 15). Physical forces, including electrical stimulation, have also been shown to alter Ca$^{2+}$ dynamics (16–19) and promote stem cell differentiation (20, 21), but the role of the Ca$^{2+}$ dynamics for hMSC differentiation has not been clearly established. In this study, the [Ca$^{2+}$]i oscillation in the hMSC osteodifferentiation was measured and analyzed. We also tested the hypothesis that externally applied electrical stimulation induces changes in the [Ca$^{2+}$]i oscillation and may influence the hMSC osteodifferentiation. The cell proliferation and bone-specific cellular, extracellular, and molecular markers were measured to assess osteodifferentiation. The [Ca$^{2+}$]i oscillation seems to play a bidirectional role to serve as an indicator of osteodifferentiation and to mediate differentiation.

MATERIALS AND METHODS

Cell culture and osteogenic assay

Bone marrow-derived human MSCs were obtained from the Tulane Center for Gene Therapy (New Orleans, LA, USA). Based on the flow cytometry results, these cells showed negative staining for CD34, CD36, CD45, and CD117 markers (all >2%), and positive staining for CD44, CD90, CD166, CD29, CD49c, CD105, and CD147 markers (all >95%), indicating a minimal heterogeneity in the cell population. hMSCs were cultured in a complete medium that contained α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 2% glutamine, 15% FBS (Atlantic Biologicals, Lawrenceville, GA, USA), 1% antibiotics/antimycotics (Invitrogen; final concentrations: penicillin 100 U/ml, streptomycin 100 μg/ml, and amphotericin B 0.25 μg/ml). Normal human fetal osteoblasts (hFOB 1.19) were purchased from American Tissue Culture Collection (Manassas, VA, USA) and cultured using the same complete medium. Cells were incubated in a culture flask in a humidified atmosphere containing 95% air and 5% CO$_2$ at 37°C; media was changed twice a week.

For osteodifferentiation experiments, hMSCs were seeded in monolayer at a density of 3000 cells/cm$^2$. The osteogenic medium was prepared by adding the soluble factors, including 20 nM dexamethasone, 10 mM β-glycerolphosphate, and 50 μg/ml ascorbic acid. To quantify the initial osteodifferentiation, alkaline phosphatase activity (ALP) and calcium mineralization were determined and evaluated according to methods described elsewhere (22, 23). To image calcium mineralization, cells were first fixed in 3.7% formaldehyde for 20 min at room temperature; the extra dye was removed and differential interference contrast images were obtained using a Nikon Eclipse E800 microscope.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from the cell pellets using TRIzol (Invitrogen) according to the manufacturer’s instruction. The concentration and integrity of RNA were estimated spectrophotometrically. The RNA samples were reverse transcribed into cDNA using SuperScript III First Strand Synthesis SuperMix Kit (Invitrogen Life Technologies). Gene expression was quantitatively measured by real-time PCR using SYBR Green PCR SuperMix-UDG with ROX (Invitrogen) and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Reactions were carried out at 50°C for 2 min, 95°C for 2 min, 50 cycles of 95°C for 15 s, and 60°C for 30 s, followed by melting curve analysis. Samples were loaded in triplicate in 96-well plate with a final volume of 25 μl, containing cDNA generated from 50 ng RNA and mixed with SYBR Green Mix and bone-specific primers. These gene primer sets were purchased from Operon Biotechnologies, Inc. (Huntsville, AL, USA). As listed in Table 1, they were specific for ALP, collagen type Iα2 (Col Iα2), osteocalcin (OC), and osteopontin (OP). Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference for RNA loading of samples. Fold induction and expression levels for each target gene were calculated using the comparative Ct method, which could be expressed as $2^{-\Delta \Delta Ct}$, where $C_t$ is threshold cycle, and $\Delta C_t$ test and $\Delta C_t$ control presented the difference between $C_t$ target and $C_t$ reference of test samples and control samples (24).

Calcium dye loading and fluorescence imaging

hMSCs were cultured and maintained as described. On days 1, 7, 14, 21, and 28, cells were trypsinized and neutralized, centrifuged, and replated at a low density onto 22 × 22 mm coverslips and incubated for 24 h. Cells were then loaded with 5 μM Fluo-4 (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature, then rinsed three times in PBS. The coverslip was mounted on to a Nikon Eclipse E800 microscope. Illuminating light was focused on the sample through a 20×/0.75 numerical aperture (NA) objective and images were recorded using a CCD camera (CoolSnap fx, Roper Scientific, Tucson, AZ, USA). Fluorescence images of [Ca$^{2+}$]i were acquired in real time at 10 s intervals for at least 30 min. Average fluorescence intensities of each cell were determined using the MetaMorph image processor (Universal Imaging, West Chester, PA, USA).

Electrical stimulation

Electrical stimulation has been described in detail elsewhere (20, 25). Briefly, an electrical current from an amplifier

<table>
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<th>TABLE 1. RT-PCR primers</th>
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<td>Gene$^a$</td>
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<td>ALP</td>
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<td>OC</td>
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<td>Col Iα2</td>
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$^a$ALP, alkaline phosphatase; OC, osteocalcin; Col Iα2, collagen type I α2 chain.

EXTERNAL PHYSICAL STIMULATION OF CALCIUM OSCillation

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[Ca^{2+}]_i oscillations

The baseline Ca^{2+} dynamics in hMSCs were first determined using the α-MEM medium without the osteoinductive factors or external physical stimulation. Figure 1 shows a panel of fluorescence images acquired from hMSCs at 10 s intervals, which represent typical changes in [Ca^{2+}]_i. The image acquisition at 10 s intervals has been shown to be sufficient to capture [Ca^{2+}]_i oscillation characteristics in hMSCs (10, 11). The cells show the typical spread morphology, and virtually all hMSCs in the undifferentiated state demonstrated nonsynchronized Ca^{2+} spikes. For example, >95% of hMSCs showed Ca^{2+} spikes (see Table 2) after 1 day subculture onto a coverslip. Determination of the propagation of Ca^{2+} waves (µs to ms dynamics) inside the cell or sometimes between cells is not trivial (26). This rather complex problem was bypassed by integrating the fluorescence signals from the entire cell; therefore, the intracellular Ca^{2+} waves were not monitored.

To demonstrate the different [Ca^{2+}]_i oscillation characteristics in the undifferentiated hMSCs and in terminally differentiated human osteoblasts, the Ca^{2+} dynamics were recorded in these two different cell types for 30 min and the averaged Fluo-4 fluorescence intensities were plotted as a function of time. As shown in Fig. 2, regular and multiple Ca^{2+} spikes were observed in the undifferentiated hMSCs cultured in the α-MEM media without osteoinductive factors (Fig. 2A). In contrast, the terminally differentiated human osteoblasts exhibited an irregular Ca^{2+} spike pattern and a reduction in the number of the Ca^{2+} spikes (Fig. 2B), suggesting that hMSCs require more robust Ca^{2+} activities. The apparent differential Ca^{2+} dynamics were analyzed by determining the number and amplitude (i.e., fluorescence intensity peak) of the Ca^{2+} spikes during the 30 min observation period. More than 50% hMSCs showed Ca^{2+} spikes greater than 8, whereas >50% human osteoblasts demonstrated <2 Ca^{2+} spikes (Fig. 3A). Of the Ca^{2+} spikes monitored, the amplitude of Ca^{2+} spikes was found to be ~2- to 3-fold higher in both hMSCs and osteoblasts (Fig. 3B). These results confirmed that a major difference in the characteristics of the Ca^{2+} dynamics in these two cell types appears to be the Ca^{2+} spiking frequency.

### Table 2. Comparison of Ca^{2+} spiking profiles in hMSCs and human osteoblasts at day 1

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<th>Osteoblast</th>
<th>hMSC</th>
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<tr>
<td></td>
<td>In α-MEM</td>
<td>In α-MEM</td>
</tr>
<tr>
<td>Number of cells</td>
<td>30</td>
<td>97</td>
</tr>
<tr>
<td>Cells showing Ca^{2+} spikes</td>
<td>77%</td>
<td>98%</td>
</tr>
<tr>
<td>Average Ca^{2+} spikes/cell</td>
<td>2.53 ± 2.46</td>
<td>8.06 ± 2.64</td>
</tr>
<tr>
<td>Normalized average Ca^{2+} spike amplitude</td>
<td>2.08 ± 0.68</td>
<td>2.63 ± 0.96</td>
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Effects of osteoinductive factors on Ca$^{2+}$/H$_{11545}$ oscillations and hMSC differentiation

The osteoinductive factors are expected to influence the [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillations in hMSCs. Note that addition of the osteoinductive factors had an immediate effect on the Ca$^{2+}$/H$_{11001}$ dynamics (Table 2). For example, the average Ca$^{2+}$/H$_{11001}$ spikes per cell decreased from 8.06 ± 2.64 to 3.66 ± 2.42 on incubation of hMSCs with the osteoinductive factors for 30 min. This immediate reduction in the Ca$^{2+}$/H$_{11001}$ spiking frequency was reversed by exchanging the buffer that contains no inductive factors (e.g., α-MEM; data not shown). We note that the Ca$^{2+}$/H$_{11001}$ spiking pattern in hMSCs after addition of the osteoinductive factors resembled that found in the terminally differentiated human osteoblasts. In addition, a substantial fraction of hMSCs (~20%) no longer showed [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillations, whereas nearly all hMSCs exhibited [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillations in the α-MEM medium. The average Ca$^{2+}$/H$_{11001}$ spike amplitude did not change significantly, however.

We next monitored changes in the Ca$^{2+}$/H$_{11001}$ dynamics in hMSCs as a function of the osteodifferentiation stage. hMSCs were cultured in the presence of the osteoinductive factors and Ca$^{2+}$/H$_{11001}$ dynamics were determined on days 1 (i.e., start of osteodifferentiation), 7, 14, 21, and 28. The Ca$^{2+}$/H$_{11001}$ spikes were noticeably decreased by day 7 and appeared to have reached the minimum value between days 7 and 14 (Fig. 4A). A longer culture of hMSCs in the osteogenic medium did not abolish the Ca$^{2+}$/H$_{11001}$ spikes, however; rather, by day 14, the Ca$^{2+}$/H$_{11001}$ spikes were statistically indistinguishable from those found in human osteoblasts. The average amplitude of the Ca$^{2+}$/H$_{11001}$ spikes remained essentially unchanged during the 28 day observation period (Fig. 4B).

The osteodifferentiation was verified using real-time RT-PCR. The gene expression levels for the definitive osteogenic markers such as ALP, OC, and Col I were determined and normalized by a sample cultured in regular α-MEM without the osteoinductive factors. As shown in Fig. 5, a large increase (~15-fold) in the ALP gene level was found in response to incubation of hMSCs in the osteogenic medium. Less but nonetheless significant increases were also found in the OC (~2-fold) and Col I (~3-fold) gene levels. When hMSCs were incubated in the osteogenic medium without extracellular Ca$^{2+}$/H$_{11001}$ (denoted as OST-no Ca), gene ex-

Figure 2. Representative time course of typical [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillations in hMSC (A) and human osteoblast (B) in α-MEM medium. Two [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillation profiles in each cell type were obtained on day 1 by continuously recording fluorescence images for 30 min. The fluorescence intensities were normalized by the fluorescence intensity (F$_{0}$) recorded at t = 0. The [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillation profiles were arbitrarily offset for clarity.

Figure 3. Analysis of the [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillations. Histograms of cells exhibiting the Ca$^{2+}$/H$_{11001}$ spikes (A) and amplitude (B) were constructed from 97 hMSCs (open bars) and 30 osteoblasts (hatched bars) on day 1. Because the baseline fluorescence fluctuated, the fluorescence intensities were averaged immediately before and after each Ca$^{2+}$/H$_{11001}$ spike was recorded. The Ca$^{2+}$/H$_{11001}$ spike is then defined as having the fluorescent peak-to-base ratio of greater than 1.5. The average frequency of [Ca$^{2+}$/H$_{11001}$]$_{i}$ oscillations can be estimated by the ratio of the Ca$^{2+}$/H$_{11001}$ spikes per 30 min observation time.
pression for OC and Col I was significantly downregulated, although the ALP gene level was reduced less significantly. These results indicate that depletion of external Ca\textsuperscript{2+} interferes with the proper hMSC osteodifferentiation and thus suggests a critical role for Ca\textsuperscript{2+} influx.

Manipulation of Ca\textsuperscript{2+} oscillations by electrical stimulation

Our earlier reports showed that application of a noninvasive electrical stimulation induces changes in [Ca\textsuperscript{2+}]	extsubscript{i}. (27–30). One potential electrocoupling mechanism involves activation of the cell surface channels to induce Ca\textsuperscript{2+} influx. We therefore formulated and tested the hypothesis that a noninvasive electrical stimulation can be applied to alter the Ca\textsuperscript{2+} dynamics in hMSCs and perhaps affect hMSC osteodifferentiation. Because the strength of the physiologically relevant electrical activities does not exceed \~1 V/cm (31, 32), we tested the effect of 0.1 and 1 V/cm electrical stimulation using either direct current (dc) or 1 Hz sinusoidally oscillating mode. In addition, using a large electrical stimulation (10 V/cm), the effect of an invasive external stimulus was determined for comparison purposes. As shown in Fig. 6A, application of a noninvasive electrical stimulation for 30 min caused a reduction in the Ca\textsuperscript{2+} spikes in hMSCs cultured in the regular α-MEM medium without the osteoinductive factors. Application of a 0.1 or 1 V/cm stimulus in the dc mode or a 1 V/cm stimulus in the oscillatory mode induced the similar effect by reducing the Ca\textsuperscript{2+} spikes by approximately a half. In contrast, application of an invasive 10 V/cm stimulus abolished the [Ca\textsuperscript{2+}] oscillations, probably due to irreversible cell membrane damage. A more detailed analysis of the changes in the

Figure 4. Effects of the osteoinductive factors on [Ca\textsuperscript{2+}] oscillations at the different stages of hMSC osteodifferentiation. The number of Ca\textsuperscript{2+} spikes (A) and the Ca\textsuperscript{2+} spike amplitude (B) were monitored and determined on day 1, 7, 14, 21, and 28. For comparison, the [Ca\textsuperscript{2+}] oscillation characteristics of human osteoblasts were determined on day 28. Each data point represents mean ± sd of >30 cells from 3 experiments.

Figure 5. Real-time PCR analysis of gene expressions in hMSCs cultured in α-MEM medium (white bar), osteogenic medium with (black bar) and without (hatched bar) extra-cellular Ca\textsuperscript{2+}. The relative expression levels for ALP (A), osteocalcin (B), and collagen type I (C) were normalized using α-MEM medium alone. PCR experiments were performed at the end of a 2 wk incubation time.
Ca\textsuperscript{2+} dynamics in response to the smallest electrical stimulation is shown in Fig. 6B. Exposure of hMSCs to a 0.1 V/cm electrical stimulation in the absence of the osteoinductive factors caused a decrease in the Ca\textsuperscript{2+} spikes (filled bars), which now resemble those observed in the terminally differentiated osteoblasts (hatched bars). For example, nearly 70% of hMSCs generated only 2 Ca\textsuperscript{2+} spikes. This is significantly different from those in hMSCs (8%) or osteoblasts (40%) when cells were cultured in the regular α-MEM medium. These findings suggest that electrical stimulation may be applied to an undifferentiated hMSC to alter the

\textbf{Osteodifferentiation by electrical stimulation}

Finally, the role of electrical stimulation is tested in the hMSC osteodifferentiation. After exposing hMSCs to a 0.1 V/cm stimulus 30 min/day for 10 days in the osteogenic medium, cell proliferation was determined and osteodifferentiation was assessed by the ALP level and calcium mineralization. Application of this small electrical stimulation promoted cell growth by ~3-fold (Fig. 7A). We next determined the ALP level and found significantly higher ALP activity in response to a combination of the osteoinductive factors and electrical stimulation (Fig. 7B). Further, the calcium nodules were found to be stained in response to the osteoinductive factors (Fig. 7C). When hMSCs were treated with the osteoinductive factors and exposed to the electrical stimulation concomitantly, much more pronounced calcium mineralization was clearly noticeable (Fig. 7). Taken together, these results suggest that the electrical stimulation used in our experiments facilitates a synergistic hMSC differentiation into the osteogenic lineage.

\textbf{DISCUSSION}

Intracellular Ca\textsuperscript{2+} dynamics in hMSCs were altered in response to the osteoinductive factors and electrical stimulation. In response to osteoinductive factors, Ca\textsuperscript{2+} spikes decreased to a level similar to that found in the terminally differentiated human osteoblasts. The amplitude of the Ca\textsuperscript{2+} spikes remained essentially the same, however. When exposed to a noninvasive electrical stimulation in the presence of the osteoinductive factors, the Ca\textsuperscript{2+} spikes were further reduced; after a 10 day culture, levels of the specific osteogenic cellular and molecular markers were significantly higher than when using the osteoinductive factors alone. In addition to serving as an indicator of hMSC differentiation, the Ca\textsuperscript{2+} dynamics are also likely to be involved in mediating differentiation, and application of a noninvasive electrical stimulation may provide a novel technique for synergistic hMSC differentiation into tissue-specific lineages.

The Ca\textsuperscript{2+} dynamics in hMSCs appear to be rather complex. Moreover, the role of Ca\textsuperscript{2+} dynamics involved in the differentiation of hMSC is yet to be determined. The recently published results indicate that the Ca\textsuperscript{2+} spikes in hMSCs are regulated by the release of Ca\textsuperscript{2+} through the inositol triphosphate receptors (IP\textsubscript{3}R), and voltage-gated Ca\textsuperscript{2+} channels play a minor role (10). We can confirm such results by our observations that the treatment of cells with a potent inhibitor of phospholipase C (U73122, 25 μM) or thapsigargin (25 μM) decreased the number of Ca\textsuperscript{2+} spikes from 8 (e.g., observed in untreated hMSC) to 4 or 2, respectively. Furthermore, Ca\textsuperscript{2+} influx across the plasma membrane
is thought to sustain \([\text{Ca}^{2+}]_i\) oscillations (33), suggesting that \(\text{Ca}^{2+}\) entry is likely required. This is consistent with our findings that \([\text{Ca}^{2+}]_i\) oscillations are significantly reduced depletion of extracellular \(\text{Ca}^{2+}\) (e.g., the \(\text{Ca}^{2+}\) spikes reduced to 4). Together with the recent findings that \(\text{Ca}^{2+}\) influx in the hMSC may be mediated by the membrane \(\text{Ca}^{2+}\) pump and \(\text{Na}^+\)-\(\text{Ca}^{2+}\) exchanger (34), it appears that multiple entry pathways are likely involved in the regulation of \([\text{Ca}^{2+}]_i\) oscillations. It remains to be determined, however, how an electrical stimulation can influence the \([\text{Ca}^{2+}]_i\) oscillations in hMSCs. A simple schematic would be helpful to illustrate the potential coupling mechanism that could mediate the altered \(\text{Ca}^{2+}\) dynamics in hMSCs. As shown in Fig. 8, the inductive soluble factors can diffuse across the cell membrane and produce immediate effects in the \([\text{Ca}^{2+}]_i\) oscillation, as we have demonstrated. We postulate that the electrical stimulation can couple to the cell via at least three mechanisms and alter the \(\text{Ca}^{2+}\) dynamics: 1) cluster and activate important transducers such as integrins; 2) activate ion channels; and 3) redistribute G-protein receptors. We have demonstrated that application of an electrical stimulation is able to couple to phospholipase C (PLC) near the cell surface in human osteoblasts (28) and in hMSCs (present study), and that the stretch-activated cation channels (SACCs) mediate \(\text{Ca}^{2+}\) influx across the cell membrane of human osteoblasts (28). Note that treatment of hMSCs with \(\text{Li}^+\) blocked the \([\text{Ca}^{2+}]_i\) oscillations (34). Since \(\text{Li}^+\) is known to inhibit the SACCs (35), it appears plausible that SACCs mediate the \(\text{Ca}^{2+}\) influx required to sustain the \([\text{Ca}^{2+}]_i\) oscillations in the undifferentiated hMSCs. The electrocoupling mechanisms mediating changes in the \([\text{Ca}^{2+}]_i\) could depend on the strength of simulation and the cell type, however. In response to an electrical stimulation, it is plausible to assume that the membrane potential is altered and the \(\text{Ca}^{2+}\) fluxes are induced (29, 36, 37). But it is not clear how cells can sense and respond to an electrical stimulation as small as 0.1 V/cm (38), which cannot activate the voltage-gated \(\text{Ca}^{2+}\) channels or directly regulate \(\text{Ca}^{2+}\)-dependent subcellular processes. Using a membrane potential sensitive dye (e.g., DiSBAC4, Molecular Probes), we verified that applica-

Figure 7. Effects of electrical stimulation on hMSC osteodifferentiation. hMSCs were cultured in the osteogenic medium with (open bars) or without (black bars) a 0.1 V/cm electrical stimulation applied 1 h per day for 10 days. The cell density (A) and ALP level (B) were measured as described. Calcium deposit (dark regions) was imaged using hMSCs cultured in the osteogenic medium alone (C) and with exposure to a 0.1 V/cm (D).
tion of a 2 V/cm does not alter the membrane potential but that a large invasive stimulus (10 V/cm) depolarizes the membrane potential, which is not restored upon removal of the electrical stimulation (39). Although specific molecular mechanisms mediating the altered Ca\(^{2+}\) spiking pattern in response to noninvasive electrical stimulation remain to be identified, we propose a PLC-coupling mechanism that may lead to activation of G-protein coupled receptors. The PLC-mediated signaling through the release of internal Ca\(^{2+}\) and protein kinase C activation can potentially couple to the mitogen kinase protein (MAP) kinase cascades, which are known to be involved in cell differentiation (40–42).

Finally, we note that, in response to a 0.1 V/cm electrical stimulation, the maximal change in the [Ca\(^{2+}\)]\(_i\) is expected to require a ~80 min exposure (28), which may be long enough to induce cellular responses other than direct changes in [Ca\(^{2+}\)]\(_i\), including cell surface receptor redistribution (e.g., electrosmosis). For example, an electrical stimulation has been shown to induce integrin redistribution, clustering, activation, and assembly of some focal adhesion proteins such as focal adhesion kinase (FAK), paxillin, vinculin, and src (32, 43). Enzymatic activity of assembled proteins (e.g., FAK or src) could then induce the MAP kinase activation (44). Such assembly of adhesion proteins may mimic the integrin-matrix interactions, which are known to regulate the MAP kinase activities. Concomitant activation of the MAP kinases with altered Ca\(^{2+}\) dynamics may facilitate a synergistic hMSC osteodifferentiation. Additional studies are under way to elucidate the mechanism of the MAP kinases.

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