Supporting Information

A Microelectrode Array Chip for Osteogenic Differentiation of Mesenchymal Stem Cells under Electrical Stimulation

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Note 1. Characterization of osteogenic differentiation of MSCs

According to the morphology assay, the MSCs cultured with normal medium were elongated as spindle-shaped or rhomboid after 14 days and as the fluorescent images shown in **Figure S1** (a1), the MSCs grew colonially and distributed in a homogeneous confluent population. By contrast, the MSCs cultured with OM showed a change in morphology from spindle-shape to cuboidal or polygonal shape after 2-3 days. **Figure S1** (a2) exhibited the MSCs cultured with OM for 14 days, and almost all cells changed the shapes associating with aggregation and multilayered formation (as the white arrows indicated in **Figure S1** (a2)) instead of a homogeneous population. This phenomenon became more apparent for the MSCs cultured with OM after 21 days, as shown in **Figure S1** (a4). After 14 days with OM, nodular structures started to appear and cell aggregation was observed (**Figure S1** (a2)), and bare regions were exposed among the nodular structures on Day 21 (**Figure S1** (a4)). For the MSCs with control, the nodular structures and bare region were rarely found after 14 days or 21 days. Although the multilayered growth was observed and the confluent population became unclear, in the control medium after 21 days shown in **Figure S1** (a3), the cell morphology remained spindle-shape and did not show the cuboidal or polygonal growth with OM culture. As **Figure S1** (b1-b4) shown, alizarin red staining on Day 14 and Day 21 exhibited positive results for the MSCs cultured with OM compared to the MSCs cultured in normal medium in which the few of MSCs were stained.

In order to analyze the staining results, image processing was applied and the images shown in Figure S1 (b1-b4) were firstly transferred to greyscale plots with the

luminance values from 0 to 255. Afterwards, a threshold (79 of the luminance 0-255) was applied to extract the stained nodular structures, in which a low luminance represented the dark area with a high intensity of staining referring to more mineral deposition. **Figure S1** (c1-c4) showed the quantified histograms of the pixels with different luminance indicating the change of alizarin red stained intensity under different conditions. Accordingly, the OM groups on both Day 14 and Day 21 exhibited much larger areas of dark values than the relevant control groups, which corresponded to more intensive nodular aggregation of MSCs due to their osteogenic differentiation. Moreover, the OM group on Day 21 presented a larger stained area (over 7%) of low luminance than Day 14 (0.66%) corresponding to more blank areas exposing on the substrate surface due to the intensive aggregation and nodular structures generation. In the case of control culture, stained nodular structures were slightly increased from Day 14 (0.01%) to Day 21 (0.15%), which was also indicated by the histograms of pixels with low luminance values.

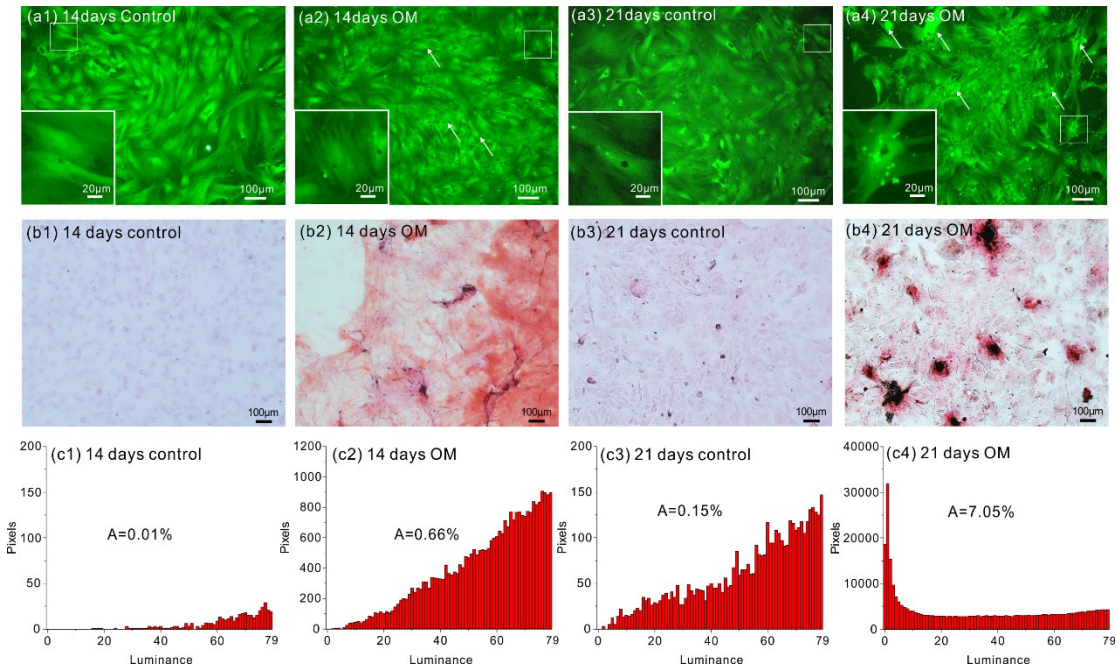

Figure S1. (a1-a4) Cellular morphology cultured with normal medium (control) and osteogenic medium (OM) after 14 and 21 days. The insets show the magnified images for single-cells indicated by the white frames. The white arrows indicate the nodular structures formed during the MSCs differentiation. (b1-b4) Alizarin red staining results on Day 14 and Day 21 in the cases of control culture and OM culture. Qutitive analysis was performed to the luminace lower than the threshold of 79 indicating stained nodual structures and transfer the pixels with different luminace to histograms (c1-c4). The variable *A* represents the stained area proportion.

Quantitative analysis for the sizes of nodulor structures

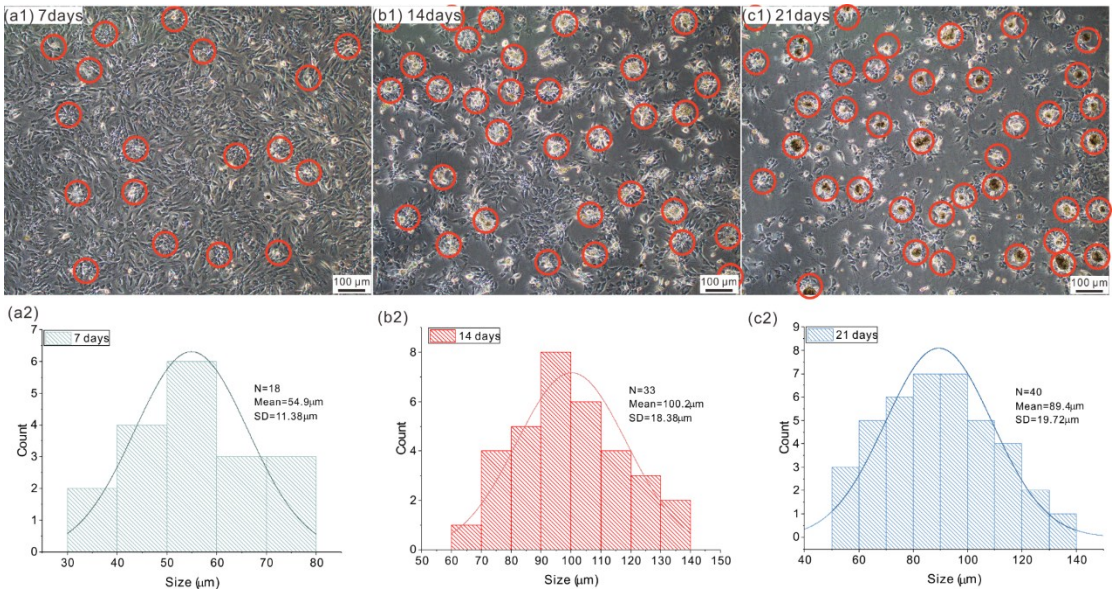

Figure S2. (a1-c1) Extended microscopic images of Figure 2 (a-c). The red circulars mark the cell aggregation areas (on Day 7) and the generated nodular structures (on Day 14 and Day 21). (a2-c2) Histograms of the sizes of the cell aggregation and nodular structures marked in (a1-c1). The average sizes of the generated nudular structures are around 100 μm.

Image processing for extracting areas of alizatin red staining

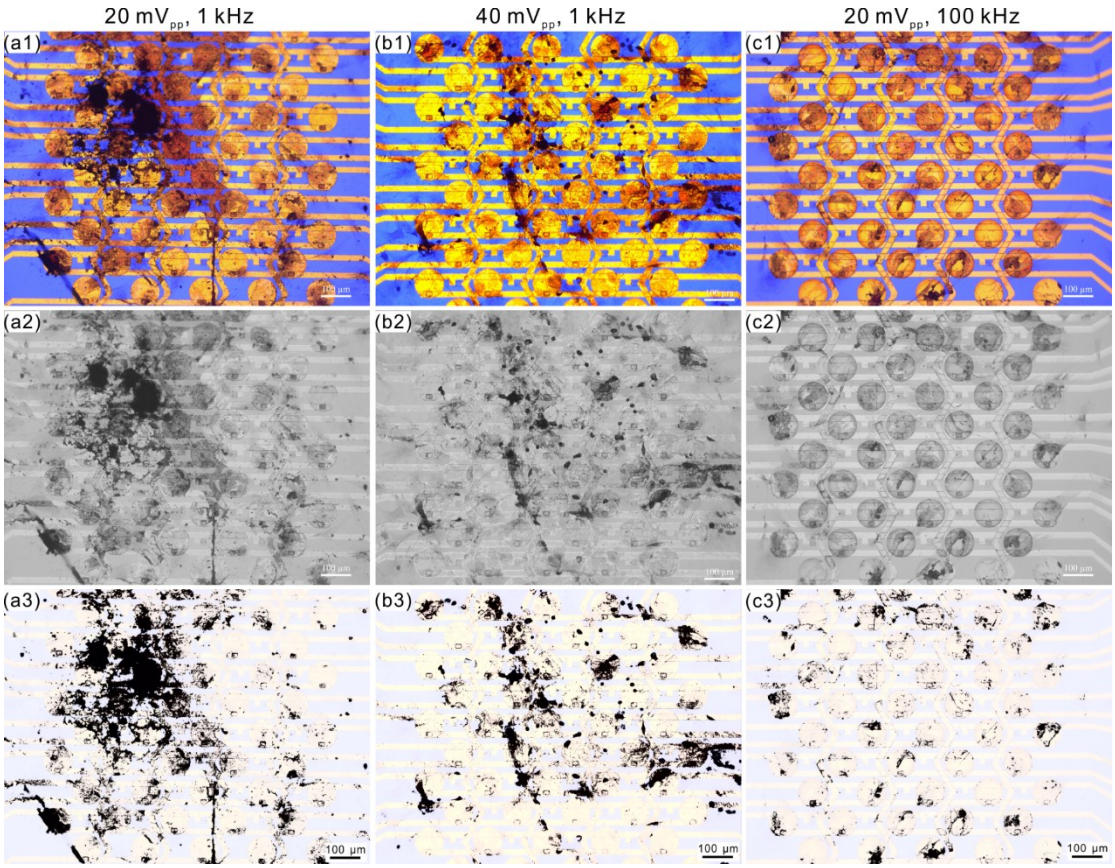

Figure S3. Image processing using an open source software ImageJ to extract alizarin red staining areas from the results of 21 days shown in **Figure 4**. The stained areas were extracted via thresholding method to transfer the microscopic RGB images (a1-c1) to grayscale maps (a2-c2). Setting an appropriate threshold, the red was transferred to black leaving out the background areas as white. (a3-c3) illustrate the binary images overlaid with the original RGB images. With the same approach, the cell areas in the fluorescent images dyed with Calcein-AM were also extracted.

Optimiztion of stimulation duration

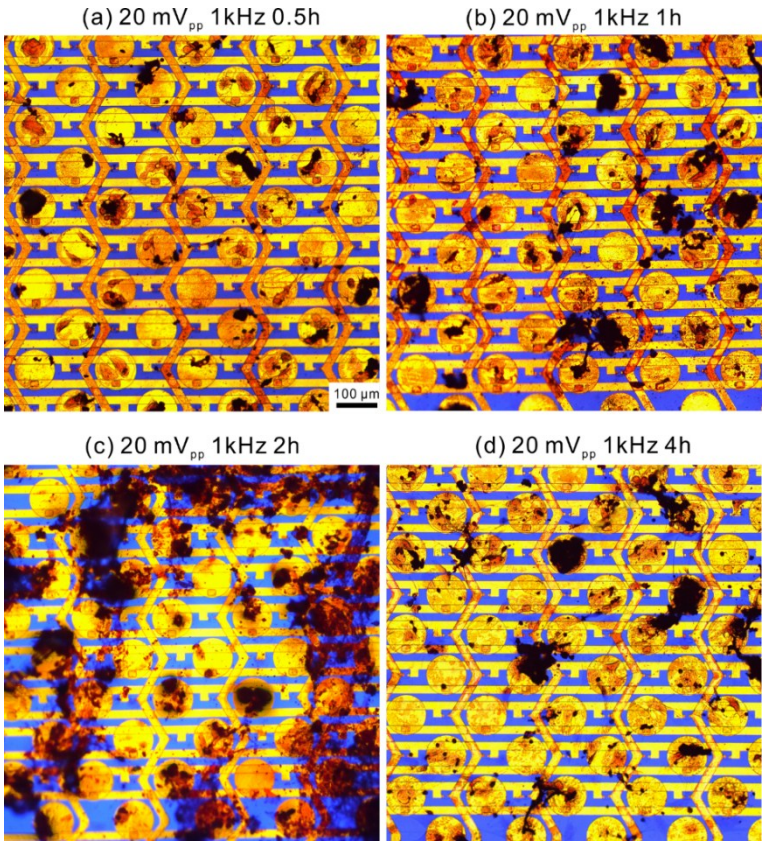

Figure S4. Alizarin red staining results on Day 21 for osteogenic differentiation of MSCs under different stimulation durations including 0.5 hour, 1 hour, 2 hours and 4 hours per day. The largest areas of calcium deposition were generated on the microelectrode array with the stimulation duration of 2 hours per day.

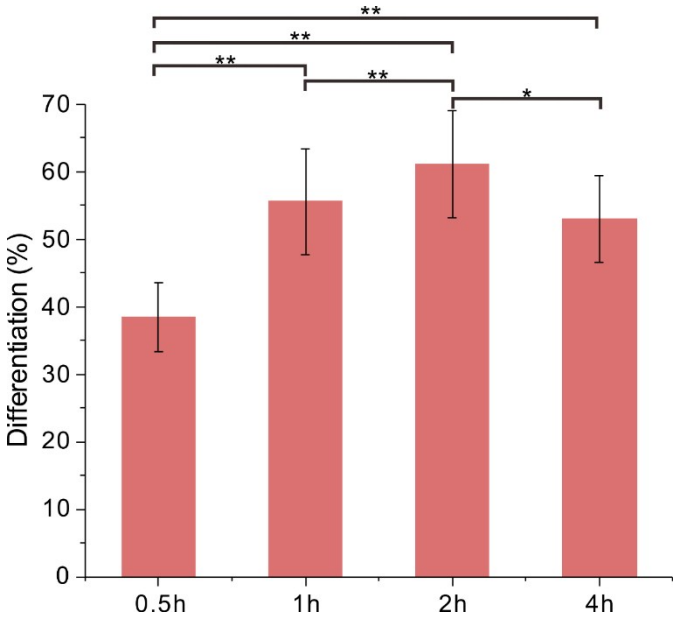

Figure S5. Histogram of calcium deposition ratio under different stimulation durations, including 0.5 hour, 1 hour, 2 hours and 4 hours per day, according to **Figure S2**. The ES with stimulation duration of 2 hours per day accelerated tremendously osteoblast proliferation and differentiation, the highest efficiency of calcium deposition was obtained. The error bars represent the standard deviations with at least three independent experiments. (*$p<0.01$, **$p<0.05$)

Alizarin staining intensity analysis

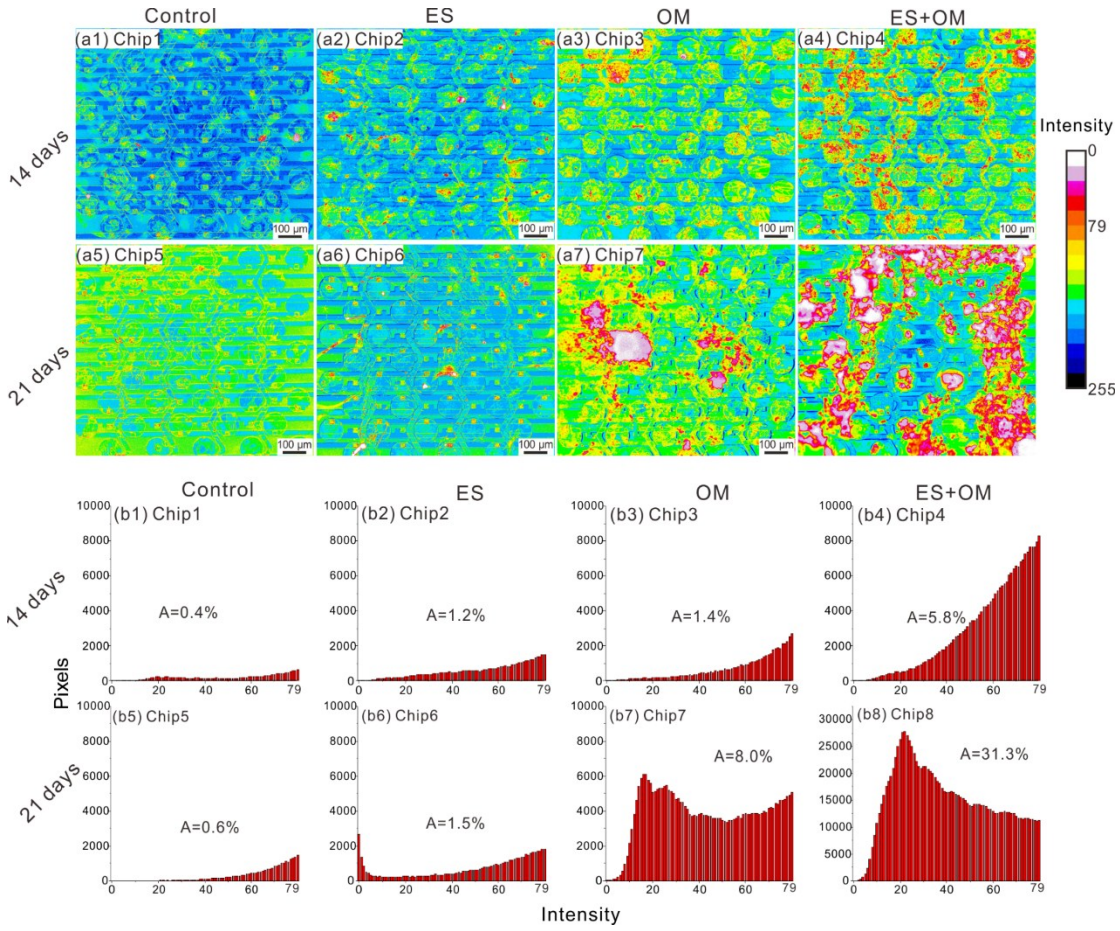

Figure S6. Image processing for staining intensity analysis with thresholding method. The images in **Figure 4** were firstly transfered to greyscale images. Dark areas with low luminance represented the stained areas. In order to exhibit the staining intensity, 16-color plots were transferred from these greyscale images and the results are shown in (a1-a8). The color gradient respresents the different luminaces with diverse staining intensity. Qutitive analysis was performed with these 16-color-plots and the areas with the luminace lower than the threshold of 79 were transferred to histograms (b1-b8). The areas with the luminances below this threshold value represented higher staining intensity.

Cell viability assay

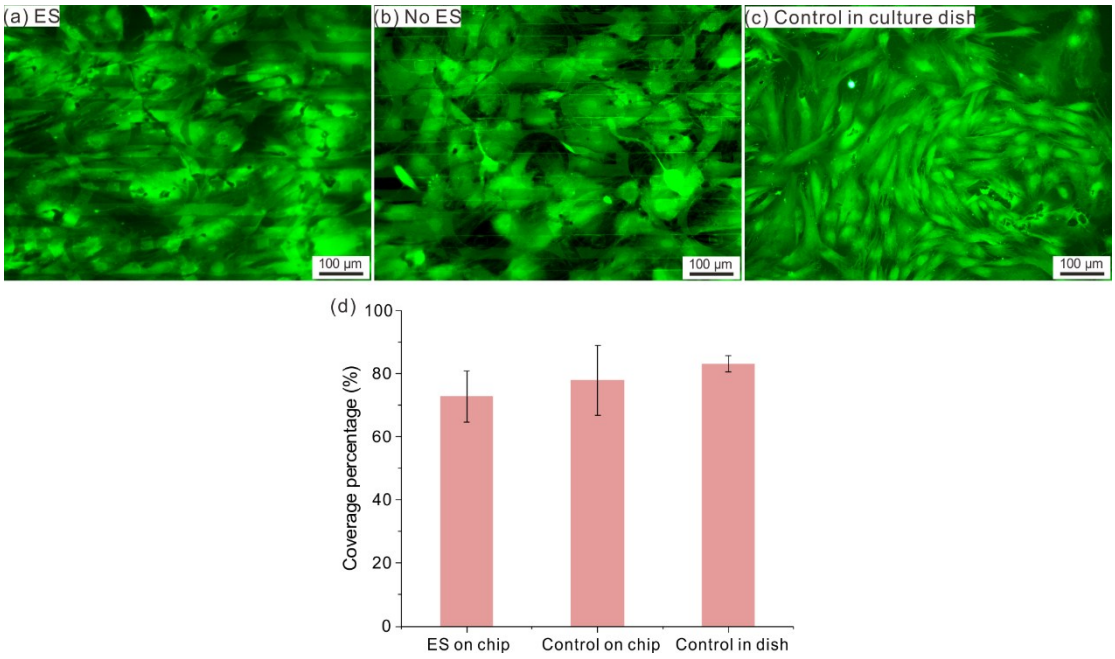

Figure S7. Cell viability assay with Calcein-AM dyeing for the MSCs cultured in normal proliferation medium without induced differentition for 14 days. The green fluorecent areas represent the coverage of live cells. (a-c) The fluorecent images of the dyeing results with different conditions, including ES on chip, control on chip as well as control in culture dish. (d) Histogram of the percentages of the coverage areas of the green fluorence indicating live cells. Although ES or on chip slightly infulenced the cell viability, the coverage percentages of live cells were suffiecient high (>70%) which validated good biocompability of ES with microchip. The error bars represent the standard deviations calculated from at least three independent experiments.