2.5 Characterization of MSCs – Colony Forming Unit Assay and Differentiation

Two of the main hallmarks of MSCs are their ability to form colonies from a single cell [35] and to differentiate into multiple cell lineages, such as osteocytes [37–39], adipocytes [37, 40, 41], and chondrocytes [37, 42]. When cultured under defined conditions, the cells can be induced to differentiate into numerous other cell types. However, if hMSCs cultures are allowed to become over-confluent in CCM, some of the cells will progress down their default pathway and exhibit characteristics of osteocytes or adipocytes, as evidenced by production of mineral or presence of lipid containing vacuoles, respectively. The protocols given here are for the colony forming unit (CFU) assay and the basic differentiation procedures for inducing osteogenesis, adipogenesis and chondrogenesis in cultures of hMSCs.

2.5.1 Colony Forming Unit (CFU) Assay Procedure

Harvest low density culture plates when cells are between 60 and 80% confluent. Determine cell count and viability using a hemacytometer and trypan blue or using a flow cytometer with Annexin V and PI staining of the cell suspension.

Serially dilute the cell suspension to obtain 100 viable cells in about 500 μl. Add 12 ml of sterile CCM to each of three 10 cm diameter dishes (about 60 cm^2 culture area). Add the 100 viable cells to each dish by dripping the cell suspension in a spiral over the surface of the dish to evenly distribute the cells. Place the cells in a 37°C incubator with humidified 5% CO_2 for 14 days without feeding. After 14 days, remove the media, wash the cells with 10 ml 1X PBS and discard the PBS. Add 5 ml 3.0% Crystal Violet in 100% methanol to each dish. Swirl the solution around to cover bottom of dish. Incubate for 5–10 min at room temperature. Gently flush the dish with tap water until background is clear. Examine dish under an inverted microscope to verify cell staining and, using the naked eye, count the number of colonies that are 2 mm or larger in diameter in each dish. Calculate % CFU for each plate:

$$\frac{\textit{Total Number of Colonies } (> 2\,mm) \textit{ Counted}}{\text{Total cells plated (100 in this case)}} \times 100 \text{ for each dish} = \%\ \text{CFU}$$

To determine "average % CFU" for the preparation, add up the % CFU for each of the three dishes and divide by 3.

The % CFU is an important characteristic of these cells and should be recorded at each cell passage. The % CFUs for early passage hMSCs expanded at low density should be greater than 40%. However, the number of CFUs decreases as the cultures expand from low density cultures enriched for RS-MSCs to high density cultures enriched for SR-MSCs [43]. Plating only 100 cells per dish in this assay provides

enough cells for analysis and increases the probability that the colonies formed are derived from single cells.

2.5.2 Adipogenesis and Osteogenesis Differentiation Procedure

We perform osteogenic and adipogenic differentiation assays in 6 well tissue culture plates. The 6 well plates allow for 2 wells each for control, osteogenic and adipogenic cultures. See Fig. 2.3 for layout of plate.

Label the plates with sample number, passage, date and any other pertinent information. Add 2 ml CCM to each well. Add 100,000 cells in a volume of approximately 100–200 μl (~10,000 cells/cm^2). If you have an insufficient number of cells, a lower density can be substituted as long as each well receives the same number of cells. Incubate cells in humidified incubator at 37°C with 5% CO_2. Every 3–4 days before the cells reach 100% confluency, aspirate media from each well, rinse with 2 ml of PBS, and add 2 ml of fresh CCM. Return to incubator. After the cells have reached between 70 and 80% confluency in 2–8 days, aspirate media from each well and rinse each well with 2 ml of PBS. Do not change to differentiation media before cells have reached 70% confluence or they will not differentiate and do not let the cultures attain greater than 85% confluency.

Add 2 ml CCM to the 2 control wells for no differentiation. To the 2 osteogenic differentiation wells, add 2 ml Osteogenic Differentiation Media (ODM), which

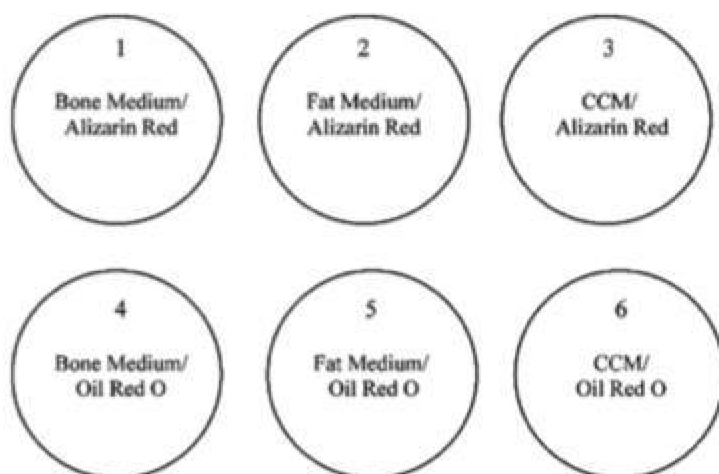

Fig. 2.3 Suggested Layout of 6 well plate for differentiation of MSCs. After MSCs become 60 and 80% confluent in CCM, induce differentiation with specific media. The first column of wells (1 and 4) gets MSCs + ODM (bone medium), second column of wells (2 and 5) get MSCs plus ADM (fat medium) and the third column of wells (3 and 6) gets MSCs plus CCM (control). The first row of wells (1, 2 and 3) gets rinsed with DI water, and then stained with Alizarin Red S. The second row of wells (4, 5 and 6) gets rinsed with PBS and then stained with Oil Red O. Alizarin Red staining for osteogenesis should be strong in Well 1 and absent to light in wells 2 and 3. Oil Red O staining for adipogenesis should be strong in Well 5 and absent to light in the wells 4 and 6. This arrangement allows for controls of media and staining specificity [36]