Modeling Osteocyte Differentiation

Introduction

The ability to differentiate into multiple lineages is a fundamental characteristic of mesenchymal stem cells. iCell® Mesenchymal Stem Cells, human induced pluripotent stem cell (iPSC)-derived mesenchymal stem cells, recapitulate the physiological characteristics of native human mesenchymal stem cells. Due to their human origin, high purity, functional relevance, and ease of use, iCell Mesenchymal Stem Cells represent an optimal in vitro test system for interrogating mesenchymal stem cell multiple lineage differentiation in basic research and many areas of regenerative biology.

The Application Protocol presented here has demonstrated utility in inducing differentiation of iCell Mesenchymal Stem Cells into osteocytes as assessed by alizarin red S or alkaline phosphatase staining.

Required Equipment and Consumables

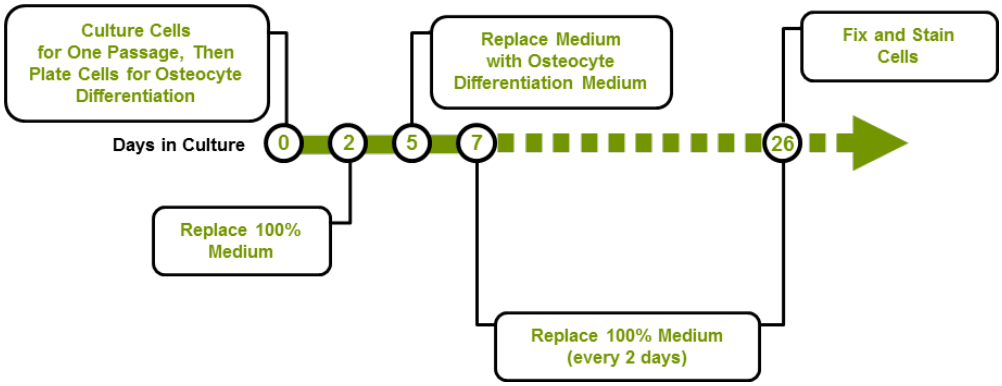
The following equipment and consumables are required in addition to the materials specified in the iCell Mesenchymal Stem Cells Prototype User's Guide.

Item	Vendor	Catalog Number
Equipment		
Bright Field Microscope	Multiple Vendors	
Consumables		
iCell Mesenchymal Stem Cells Prototype	Cellular Dynamics International (CDI)	MSC 301-010-001-PT
1M Tris-HCl Buffer, pH 8.2 - 8.5	Teknova	T1082
6-well Cell Culture Plates	Multiple Vendors	
Alizarin Red S Solution*	Millipore	2003999
Alpha-MEM	Gibco	11900-024
Ascorbic Acid	Wako	013-19641
B-glycerophosphate	Sigma	G9422-10G
Buffered Formaldehyde	Fisher Scientific	SF93-4
Dexamethasone	Sigma	D8893
Distilled Water	Multiple Vendors	
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Life Technologies	14190
Fetal Bovine Serum (FBS)	Hyclone	SH30071.03
GlutaMAX Supplement	Life Technologies	35050
Penicillin/Streptomycin	Multiple Vendors	
Vector Blue Alkaline Phosphatase Substrate Kit*	Vector laboratories	SK-5300

* Optional. The protocol presented here provides instructions for staining with alizarin red S or vector blue alkaline phosphatase.

Workflow

iCell Mesenchymal Stem Cells are thawed and plated into a tissue culture treated plate. When iCell Mesenchymal Stem Cells reach 80% - 90% confluency (approximately day 5), cells are passaged and plated for osteocyte differentiation. When iCell Mesenchymal Stem Cells reach confluency, 100% of spent medium is replaced with Osteocyte Differentiation Medium and every 2 days thereafter. After 14 days of osteocyte differentiation, 100% of spent medium is replaced with Osteocyte Mineralization Medium. Optimal osteocyte differentiation is observed at day 21 post-osteocyte induction.



Methods

Preparing the Osteocyte Differentiation Medium

Using sterile technique, combine the following components at the final concentrations specified to prepare the Osteocyte Differentiation Medium. Scale the reagents as needed.

Component	Amount (ml)	Final Concentration
Alpha-MEM	722.5	80%
Ascorbic Acid, 20 mg/ml	2.5	50 µg/ml
B-glycerophosphate	5	5 mM
FBS	200	20%
GlutaMAX Supplement	10	1%
Penicillin/Streptomycin	10	1%

Preparing the Osteocyte Mineralization Medium

Using sterile technique, combine the following components at the final concentrations specified to make the Osteocyte Mineralization Medium:

Component	Amount (ml)	Final Concentration
Osteocyte Differentiation Medium	1,000	100%
Dexamethasone, 5 mM	0.002	10 nM

Notes

Notes

Thawing iCell Mesenchymal Stem Cells

1. Thaw iCell Mesenchymal Stem Cells according to their User's Guide.
2. Remove a sample of cells to confirm viability using a hemocytometer (using trypan blue exclusion to identify viable cells) or an automated cell counter.
3. Dilute the cell suspension in Maintenance Medium to achieve a cell density of 35,000 cells/cm^2.

Performing the Osteocyte Differentiation

1. When iCell Mesenchymal Stem Cells reach 80% - 90% confluency, passage the cells using TrypLE according to the User's Guide, quenching the enzyme with an equal volume of Plating Medium.
2. Remove a sample of cells to confirm viability using a hemocytometer (using trypan blue exclusion to identify viable cells) or an automated cell counter.
3. Centrifuge the cell suspension at room temperature at 400 x g for 5 minutes.
4. Carefully aspirate the supernatant, taking care not to disturb the cell pellet.
5. Resuspend the cells in the appropriate volume of Maintenance Medium to plate the cells at a density of 25,000 viable cells/cm^2.
6. Culture the cells in a cell culture incubator at 37°C, 5% CO_2.
7. When the cells reach 80 - 90% confluency (approximately day 5 - 7), replace 100% of spent medium with Osteocyte Differentiation Medium.
8. Replace the spent medium every 2 days with Osteocyte Differentiation Medium.
9. 14 days post-osteocyte differentiation, replace spent medium with Osteocyte Mineralization Medium.
10. Replace spent medium every 2 days with Osteocyte Mineralization Medium. At day 21 post-osteocyte differentiation, the cells can be labeled cells for osteocyte-specific markers or used for downstream applications.

Staining Differentiated Osteocytes

1. Fix the cells with buffered formaldehyde:
    a. Aspirate the spent medium.
    b. Carefully wash the cells 2 times with D-PBS.

    Note: *Do not disrupt the cell monolayer.*

    c. Carefully aspirate the D-PBS.
    d. Add a volume of buffered formaldehyde to cover the area of the cell monolayer.
    e. Incubate the plates at room temperature for 20 minutes.
    f. Carefully aspirate the buffered formaldehyde.
    g. Wash the cells 2 times with D-PBS.

2. Stain the cells with alizarin red S solution:
   a. Carefully aspirate the D-PBS.
   b. Add a volume of alizarin red S solution to cover the cell monolayer.
   c. Incubate the plates at room temperature for 5 - 10 minutes.
   d. Aspirate the alizarin red S solution.
   e. Wash the cells 4 times with distilled water.
   f. Visualize the cells using the bright field microscope.
3. Alternatively, stain the cells with alkaline phosphatase:
   a. Prepare 100 mM solution of Tris-HCl buffer, pH 8.2 - 8.5.
   b. Aspirate the spent medium.
   c. Carefully wash the cells with D-PBS.

      ***Note**: Do not disrupt the cell monolayer.*
   d. Prepare the Vector Blue substrate staining solution immediately before use:

      Add 2 drops of Reagent 1 to 5 ml of 100 mM Tris-HCl buffer, pH 8.2 - 8.5, and mix well.

      Add 2 drops of Reagent 2 and mix well.

      Add 2 drops of Reagent 3 and mix well.

      ***Note:** It is important to prepare the Vector Blue substrate working solution in 100 mM Tris-HCl, pH 8.2 - 8.5.*
   e. Add a volume of Vector Blue substrate working solution to cover the cell monolayer.
   f. Incubate the plates at room temperature for 20 - 30 minutes.
   g. Rinse the cells in water. Visualize the cells using the bright field microscope.

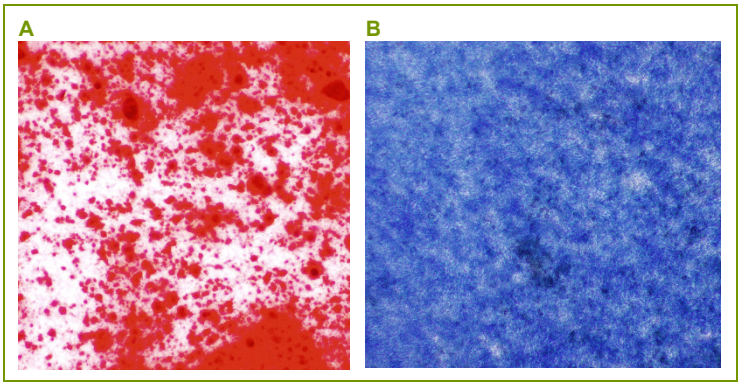


Figure 1: Differentiation into Osteocytes
In this representative experiment, iCell Mesenchymal Stem Cells differentiated into osteocytes as indicated by (A) alizarin red S or (B) alkaline phosphatase staining. The osteocytes display extracellular calcium deposits, in vitro, indicative of bone formation as assessed by the presence of alizarin red S stain.

Notes

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Summary

iCell Mesenchymal Stem Cells are derived from human iPSCs and provide an in vitro cellular system for osteocyte differentiation. The methods and data presented here highlight a reproducible cell culture protocol for inducing osteocyte differentiation as assessed by alizarin red S or alkaline phosphatase staining.

Revision History
Version 1.0: December 2016
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