**IDG-SW3 Protocol – Updated for IU**

### Reagents

- Alpha-MEM (α-MEM, Hyclone, SH30265.01)
- Fetal bovine serum (FBS, Serum A17006, Atlanta Biologicals)
- Penicillin-Streptomycin 100X (P/S, Sigma, P4333)
- Interferon-gamma recombinant mouse protein (IFN-γ, Gibco, PMC4034)
- L-Ascorbic acid (Sigma, A4544)
- B-glycerophosphate disodium salt hydrate (βGP, Sigma, G9422)
- Type I collagen coated plates (prepare before use and store at 4°C)

### Proliferation media

- α-MEM supplemented with 10% FBS, 1% P/S and 50 U/ml IFN-γ

### Differentiation media

- A-MEM supplemented with 10% FBS, 1% P/S, 50 µg/ml L-Ascorbic acid and 4 mm βGP

### Cell culture

1. Quickly thaw a cryovial of IDG-SW3 cells in a water bath at 37°C.
2. Transfer thawed cells to a 15 ml Falcon tube and carefully add 5 ml of proliferation media. Mix gently.
3. Centrifuge at 1200 rpm at room temperature for 5 mins to pellet the cells.
4. Gently aspirate and discard media from cell pellet and re-suspend the cells in 1 ml of proliferation media.
5. Add the cell suspension to a collagen-coated 50 cm² culture dish containing 12 ml of proliferation media. Gently agitate the dish to evenly distribute the cells.
6. Culture at **33°C in 5% CO₂** until 80-90% confluent. Remove the media, wash the cell layer with 5 ml sterile PBS and add 2.5 ml Trypsin/EDTA. Place in culture incubator at 37°C for 5 mins.
7. Check under microscope to ensure cells have detached. Transfer trypsin/cells to 15 ml Falcon tube. Wash the dish with 5 ml proliferation media and add to the Falcon tube containing the trypsin/cells. Mix gently.
8. Centrifuge at 1200 rpm for 5 mins at room temp. Gently aspirate and discard media and re-suspend the cell pellet in 1 ml proliferation media.
9. Add the cell suspension to a collagen-coated 150 cm$^2$ culture dish containing 22 ml proliferation media. Gently agitate the dish to evenly distribute the cells.

10. Culture at **33°C in 5% CO$_2$** until 80-90% confluent. If a large number of cells are required, the cells in this dish can be trypsinized and split between multiple 150 cm$^2$ dishes and then cultured as above until 80-90% confluent. Each 150 cm$^2$ dish at 80% confluence contains approximately 4-5 x 10$^6$ cells.

**Plating cells for experiments**

1. Trypsinize the cells from the culture dish(es), centrifuge, remove trypsin and re-suspend in 10 ml proliferation media.
2. Count the number of cells using a hemocytometer.
3. Plate the cells at a density of 4 x 10$^4$ cells/cm$^2$ in proliferation media in collagen-coated culture plates (e.g. one well of a 12 well plate has an area of 3.8 cm$^2$, so 1.52 x 10$^5$ cells would be needed per well in 1 ml of media, so 1.824 x 10$^6$ cells would be needed in 12 ml of media per 12 well plate).
4. Gently agitate the plate to evenly distribute the cells and culture at **33°C in 5% CO$_2$** until completely confluent (usually takes 48 hrs).

**Differentiation**

1. Prepare differentiation media by adding 50 µg/ml of L-Ascorbic acid and 4mM βGP to α-MEM containing 10% FBS and 1% P/S.
2. Remove proliferation media from the wells and replace with differentiation media (1 ml per well for a 12 well plate).
3. Culture cells at **37°C in 8% CO$_2$** for 21-28 days, replacing the media every 2-3 days.
4. Mineral deposits will normally be observed from day 7 onwards, GFP expression is initially seen at day 7-9 and increases throughout differentiation.
5. The culture time will depend on the experiment. Strong mineralization, Dmp1 and Sost expression are present by day 21 of differentiation. Fgf23 expression takes longer, so it is advised to culture the cells for 28-35 days to see robust changes in Fgf23.