Osteogenic and Adipogenic Differentiation of Osteosarcoma Cells

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[Abstract] Osteosarcomas are the most common primary, non-hematologic malignant tumors in childhood and adolescence, comprising almost 60% of all bone sarcomas. Although these tumors are osteoblastic in nature, the characteristics of the specific tumor-initiating cells are unclear. Osteosarcomas contain highly proliferative undifferentiated malignant cells with a disrupted bone differentiation program. Cancer stem cells (CSCs) that have tumor-initiating properties and capacity of symmetric and asymmetric division have now been described in many solid tumors. For osteosarcomas, the CSC hypothesis has received support from recently reported findings that both human and murine osteosarcomas contain a sub-population of multipotent cells that that express various mesenchymal stem cell surface markers and are capable of undergoing differentiation in multiple mesenchymal lineages such as osteoblasts and adipocytes. Differentiation into these different lineages can be easily assessed by growing cells in specific medium and assaying for differentiation markers.

Materials and Reagents

1. Osteosarcoma cells (Basu-Roy *et al.*, 2012)
2. DMEM – high Glucose from Invitrogen (Life Technologies, Invitrogen™, catalog number: 11330-032)
3. Fetal bovine serum (FBS)
4. Penicillin-streptomycin

5. Beta-glycerol phosphate (Sigma-Aldrich, catalog number: G9422)
6. Ascorbic acid (Sigma-Aldrich, catalog number: A4034)
7. Earle's balanced salt solution (EBSS) from Invitrogen (Life Technologies, Invitrogen™, catalog number: 14155-063)
8. HCl
9. Ethanol
10. DMSO (Sigma-Aldrich, catalog number: D8418)
11. Phosphate buffered saline (PBS)
12. Paraformaldehyde
13. Isopropanol
14. Acetone
15. Double-distilled H_2O (ddw)
16. Citrate concentrated solution (20 ml) (Sigma-Aldrich, catalog number: 854C)
17. Naphthol AS-MX phosphate alkaline solution, 0.25% (20 ml) (Sigma-Aldrich, catalog number: 855)
18. COMPLETE capsule of Fast Blue RR salt (Sigma-Aldrich, catalog number: FBS25-10 CAP)
19. Oil-Red-O (Sigma-Aldrich, catalog number: O0625)
20. Mayer's HemTox stain (Sigma-Aldrich, catalog number: MHS1)
21. Alizarin Red S certified by the biological stain commission (Sigma-Aldrich, catalog number: A5533)
22. Beta-glycerol phosphate stock solution (see Recipes)
23. 1,000x ascorbic acid solution (see Recipes)
24. Dexamethasone (Sigma-Aldrich, catalog number: D4902) (see Recipes)
25. 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, catalog number: I5879) (see Recipes)
26. Indomethacin (Sigma-Aldrich, catalog number: I8280) (see Recipes)
27. Insulin (recombinant human) (Sigma-Aldrich, catalog number: I2643) (see Recipes)

28. Fixation solution (see Recipes)
29. Staining solution for osteogenesis (see Recipes)
30. Oil-Red-O stock solution (see Recipes)
31. Staining solution for mineralized osteoblasts (see Recipes)

Equipment

1. Tissue culture set up
2. Pasteur pipette
3. Pasteur pipette tip
4. Light microscope
5. Pall Life Sciences acrodisc syringe filter (0.2 μM HT Tuffryn membrane low-protein binding non-pyogenic) (Life Sciences, catalog number: PN 4192)
6. 0.2 μM nylon filter
7. 24-well plates

Procedure

A. Plating cells for differentiation

1. Plate 50,000-60,000 osteosarcoma cells per well in 24-well plates in DMEM/10% FBS/ Penicillin-Streptomycin. This can be your cell line of interest too. Change medium according to your cell line of interest.
2. Next day or when they reach confluence (usually in 36 h), switch medium to differentiation medium. Usually cells will not differentiate unless they are at least 80-90% confluent. All differentiation medium is regular DMEM/10% FBS/PS supplemented with the differentiation cocktail indicated in steps 3 and 4.

3. For osteogenesis, use 10 mM beta-glycerol phosphate and 100 µg/ml ascorbic acid. These are the final concentrations.
4. For adipogenesis, use 10 µg/ml Insulin, 100 nM dexamethasone, 250 µM IBMX and 200 µM Indomethacim. These are the final concentrations. How to make stocks is described later.
5. Feed cells every 2-3 days with fresh differentiation medium made each time during feeding. While feeding make sure Pasteur pipette tip used for aspirating medium does not touch cell layer as cells attach loosely during differentiation, especially during adipogenesis.
6. Stain cells for osteogenesis and adipogenesis at 2, 5, 8, 10, 12 and 15 days.

B. Alkaline phosphatase assay to measure osteoblast differentiation.

This is a colorimetric assay where the yellow substrate. 4-Benzoylamino-2, 5-dimethoxybenzenediazonium chloride hemi (zinc chloride) salt, Azoic Diazo No. 24 is hydrolyzed by alkaline phosphatase to yield a purple dye precipitate.

Prepare fixation solution and staining solution before starting assay. Protect solutions from light fiter preparation.

1. Plate required cells in differentiation medium (usually 50,000 cells/well in 24-well plates).
2. Aspirate off medium from cells plated in differentiation medium at designated time.
3. Wash once with 1x PBS. Each washing step involved adding 1x PBS followed by aspiration.
4. Add 1 ml of fixation solution per well and incubate at RT for 1 min.
5. Wash wells with 1x PBS once.
6. Add I ml of staining solution per well.
7. Incubate in the dark for 30 min at RT and check color development. Development of purple-violet stained areas in well indicates positive reaction. If necessary, continue reaction for another 30 min at RT. Record increased incubation time. Do not exceed 1 h.

8. Stop reaction by washing quickly with dd water or tap water.
9. Do not forget to photograph plates before proceeding to Alizarin red S staining for mineralization.

C. Alizarin red S staining to detect mineralization

Alizarin red S is a calcium-sensing dye. Differentiated osteoblasts deposit large amounts of extracelluar calcium (a hallmark of mineralization) that can be detected by complexing with Alizarin red S. Calcium deposits appear as bright orange-red stained areas.

1. After photographing the plates stained for alkaline phosphatase, proceed to Alizarin red S staining by washing the stained plated twice with 1x PBS whose pH has been adjusted to 4.2 with 0.1% NH_4OH.
2. Aspirate the PBS and add enough Alizarin red S staining solution to cover the cellular monolayer. Incubate the plate at RT in the dark for 45 min.
3. Carefully aspirate the Alizarin red S solution and wash the plate fours time wits distilled water.
4. Extracellular calcium deposits appear as brightly stained orange-red areas.

D. Oil red O staining assay to measure adipogenic differentiation

This staining assay is based on the principle that the lipophilic dye Oil Red O is retained in fat globules of adipocytes that can be seen under a microscope.

5. Aspirate medium and wash cells once in PBS.
6. Fix cells in 4% PFA at RT for 15 min.
7. Wash twice with ddH_2O for 2 x 5 min.
8. Stain with Oil-Red-O working solution for 15 min at RT.
9. Wash with 1x PBS or ddH_2O for 3 x 5 min.
10. Rinse cells with 50% isopropanol once at RT.
11. Rinse cells with 1x PBS or ddH_2O.

12. The red stained lipid droplets can be visualized by light microscopy. The adipocytes are also readily visualized in normal microscopy through their rounded morphology and markedly enhanced light reflection of larger lipid droplets.
13. To count total cells, stain with Mayer's HemTox stain for 15 min at RT and wash 2x with dd water.

Recipes

1. Beta-glycerol phosphate can be prepared by making a 100x stock of 1 M solution. Pre-heat water for ease of dissolution. The formula weight of the compound depends on the degree of hydration and can be determined by using the Lot Number provided on the bottle. Filter using a Pall Life Sciences Acrodisc syringe filter. Make 1 ml aliquots and freeze in -20 °C.
2. 1,000x ascorbic acid solution can be prepared by dissolving 1 gram of Ascorbic acid in 10 ml of ddw (double-distilled water). Filter using a Pall Life Sciences Acrodisc Syringe filter. Make 1 ml aliquots and freeze in -20 °C.
3. Dexamethasone (FW 392.5)

   Dissolve 0.0196 g in 10 ml absolute ethanol to yield 5 mM stock; store at -80 °C. Prepare fresh working stock by diluting in 100% ethanol to 100 µM.
4. 3-Isobutyl-1-methylxanthine (IBMX) (FW 222.2)

   Dissolve 100 mg IBMX in 1.8 ml DMSO and sterile filter through 0.2 µM nylon filter to make 250 mM stock (1,000x). Aliquot and store at -20 °C or below.
5. Indomethacin (FW 357.8)

   Dissolve 0.5367 g indomethacin in 7.5 ml DMSO, and filter through 0.2 µm nylon filter to sterilize for 200 mM (1,000x) stock. Dispense 1-2 ml aliquots in sterile cryovials, and store in opaque box to protect from light. Store at -70 °C or below.
6. Insulin, recombinant human

Dissolve 50 mg insulin in 10 ml EBSS acidified with 50 µl of 0.005 N HCl to yield 500x stock, and sterile filter. Do not forget to use low protein binding filters, as described in equipment. Dispense 1 ml aliquots in sterile cryovials and store at -70 °C or below for up to 2 years. Thawed, working stock can be stored at 4 °C for up to 1 month. Alternatively, sterile-filter acidified EBSS and use the sterile solution to make insulin under sterile conditions.

7. Fixation solution for osteogenesis

   20 ml dd water + 30 ml acetone + 400 µl citrate concentrated solution.

8. Staining solution for osteogenesis

   48 ml dd water + 2 ml Naphthol AS-MX phosphate alkaline solution, 0.25% + 1 complete capsule of fast blue RR salt

9. Oil-Red-O stock solution for staining adipocytes

   Dissolve 500 mg of Oil-Red-O in 100 ml of isopropanol. For working stock, dilute stock as 2 parts dd water + 3 parts Oil Red O stock. Shake, Incubate at RT for 15 min and filter using a Pall Life Sciences Acrodisc Syringe filter. Use within 1 h of preparation.

10. Staining solution for mineralized osteoblasts

    Dissolve 2 g Alizarin Red S in 100 ml distilled water, mix, and adjust pH to 4.2 with 0.1% NH_4OH to prepare the Alizarin Red S staining solution. Filter the dark-brown solution using a using a Pall Life Sciences Acrodisc Syringe filter. And store it in the dark in a foil-covered or amber colored bottle.

    Note: The correct pH of the solution is critical. The solution is stable at RT for 2-3 weeks. If you plan on using it after a month, check pH to ensure pH is still 4.2.

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