Embryonic Stem Cells Morphology

Cryo-SEM and Light Microscopy Evaluation of STEM Cells

Morphological characterisation of embryonic stem cells is important in many ways. In culture, the morphology indicates the status of the cells, e.g., undifferentiated or differentiated, and also provides clues about the general health and condition of the cells, e.g., apoptotic, necrotic or mycoplasma contaminated. While there are a vast range of studies characterising stem cell markers by light microscopy, there is limited information about the fine structure of hES cells and colonies by electron microscopy. In the present work, undifferentiated human embryonic stem cells were phenotypically characterised by light and electron microscopy. The hES colonies were analysed for the presence of pluripotency markers Oct4, Tra-1-60 and SSEA-4 by confocal and epifluorescence microscopy. We also analysed the morphology of the colonies by brightfield microscopy and cryo-SEM. A better understanding of the structure of hES cells during in vitro growth helps to identify changes related to their differentiation.

Human Embryonic Stem Cell Culture and Imaging of Live Cells

Shef7 cell line was cultured on mitotically inactivated mouse embryonic fibroblasts (iMEF) plated at 16,000 cells/cm² in medium composed by knockout DMEM (Gibco) containing 20% knockout serum replacement (Gibco), 1% non-essential amino acids (Gibco), 1% Glutamax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen) and 4 ng/mL of recombinant human bFGF (Invitrogen). The medium was changed daily and the cells were passaged once a week. Brightfield (Hoffman Interference Contrast) pictures were taken from live stem cells using Olympus IX81 microscope with environmentally control chamber at 37°C and 5% CO₂. Because this technique does not need exogenous contrast agents, the cells can be readily observed in their native state (Figure 2). Undifferentiated hES cells usually form round colonies with clear margins (Figure 2A and C). Based on the location of the cells in the colony, different cell features may be identified. Cells at the centre of the colony exhibited high nucleus/cytoplasm ratio with prominent nucleoli, they are rounded and lie tightly packed with each other suggesting close cell membrane contact.
Compared to the cells at the core, stem cells at the periphery of colonies exhibited epithelial cell morphological features and consequently lower nucleus/cytoplasm ratio (Figure 2B and D).

**Immunofluorescence and Confocal Microscopy**
Cells were fixed at room temperature for 1 hour using 2% paraformaldehyde in PBS. After the fixation step, the cells were washed with PBS, permeabilised and blocked using 0.2% Triton X and 3% BSA in PBS for 1 hour. Primary and secondary antibodies were diluted in permeabilisation/blocking solution. The cells were incubated at room temperature for 2 hours with the following primary antibodies, mouse IgG anti-Oct3/4 (1:100, Santa Cruz Biotechnologies), mouse IgM anti-Tra-1-60 (1:50, Abcam) and mouse IgG anti-SSEA-4 (1:50, Abcam). After the incubation period, the cells were washed (3 times for 5 minutes) in PBS and subsequently incubated for 1 hour at room temperature with secondary antibodies, conjugated goat anti-mouse IgG Alexa Fluor 488 (1:1000, Invitrogen) and conjugated goat anti-mouse IgM Alexa Fluor 594 (1:1000, Invitrogen). Again, the cells were washed (4 times for 5 minutes) in PBS and counterstained with Hoechst 33342. The immunolabelled cells were examined using a confocal laser scanning microscope (Leica SP2). The immunofluorescent labelling of hES colonies showed clear positive labelling against TRA-1-60, Oct4 and SSEA-4 (Figure 3). The identification of the status of hES cells, e.g., differentiated or undifferentiated, relies on ready detection of pluripotency and markers associated with undifferentiated cell status. TRA-1-60 labelling displayed heterogeneous distribution on undifferentiated cells (Fig.3A). The labelling pattern suggested these proteins were present in the membrane as aggregates. This idea is supported by the previous studies on Tra-1-81 antigen distribution [1]. On close examination, Oct-4 (Fig.3B) expression was detected in the nuclei, whereas Tra-1-60 and SSEA-4 (Fig.3C) were mainly found in the cell surface, as expected.

**High-pressure Freezing Followed by Freeze-fracture and Cryo-SEM**

Stem cell colonies were manually cut from the culture dishes and enclosed between two aluminium planchettes with 100 um gap and flat carrier facing each other. The samples were high-pressure frozen with HPM010 (Bal-Tec, now RMC products by Boeckeler), mounted on a Bal-Tec holder and transferred to the BAF060 freeze-etching device (Bal-Tec, now Leica). The temperature of the sample stage was raised from -160ºC to -120ºC under 1 x 10^-7 mbar vacuum. The samples were then fractured and etched (3 minutes at -115ºC). The stage was rotated and multi-angular (20 to 90 degrees) evaporative coating of the sample with carbon (10 nm) performed. The samples were transferred to JEOL JSM-7401F FEG-SEM and imaged at -140ºC. The figure 4A shows an overview of a stem cell colony fragment. The cells are round-shaped, tightly packed and with rounded nucleus confirming previous observations made by light microscopy (Figure 4A). The arrows in figure 4B, C and D point at cells connected by small protrusions. The embryonic stem cells exhibited smooth membrane surface. Their scarce cytoplasm contained a few
organelles but variable amount of fat vacuoles and pools of glycogen particles were consistently present.

**Neural Differentiation of Human Embryonic Stem Cells**

Shef 7 hES cell line was induced to differentiate into neurons using a five steps protocol [2]. The undifferentiated stem cells were grown on mouse feeder layer and expanded by manual passaging (stage 1). The stem cell colonies were enzymatically dissociated with collagenase IV to form embryonic bodies (EBs). The EBs were cultured in suspension for 3 days (stage 2). The embryonic bodies were then plated under adherent conditions and stimulated to differentiate in the presence of 200 ng/mL of noggin and 5 µg/mL of fibronectin during 4 days (stage 3). The risen neural precursors were incubated expanded with bFGF for 7 days (stage 4) and then matured for another 7 days with cAMP (stage 5). Following treatment (21 days in total), the neural differentiation process was assessed and characterised by epifluorescence microscopy. Differentiated cells resemble morphologically primary neuronal cultures exhibiting elaborate network. Immunoflurescence studies confirmed the presence of βIII-tubulin (neuron marker) at the end of the differentiation (Figure 5).

**References:**


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