

CHAPTER III

RESEARCH METHODOLOGY

3.1 Chemicals

All chemicals will purchase from the Sigma Aldrich Corporation (St. Louis, MO, USA). The cell culture media were purchased from Gibco (Paisley, United Kingdom). Plastic cell culture devices were obtained from SPL Life Sciences (Gyeonggi-do, Republic of Korea), unless otherwise indicated.

3.2 Preparation of feeder cells

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5-day-old OF1 mouse embryos (Charles River, Lyon, France) following the protocol previously described (Afanassieff et al., 2014). MEFs were cultured in MEF medium consisting of Dulbecco's modified eagle medium (DMEM, Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% Non-essential amino acids (NEAA, Gibco), 1% penicillin-streptomycin-glutamine (PSG, Gibco). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were passaged with trypsin-EDTA (Gibco) and frozen at passage 2 (P2) in medium containing 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Frozen-thawed MEFs were cultured to 90% confluence and mitotically inactivated by incubation in fresh MEF medium supplemented with 5 µg/mL mitomycin C (Sigma-Aldrich) at 37 °C for 3 h. Cells were then washed five times with Ca²⁺/Mg²⁺ free PBS (Gibco), trypsinized, incubated for 5 min at 37 °C, centrifuged at 400 × g for 5 min, and resuspended in MEF medium. Mitomycin-treated MEFs (2.5 × 10⁵ cells) were plated in 35 mm culture dishes before seeding rhESCs.

3.3 Preparation of conditioned medium (CM)

MEFs (4 × 10⁶) were plated in 100 mm dishes (SPL Life Sciences, Gyeonggi, Republic of Korea) coated with 0.1% gelatin. One day after plating, MEF medium was replaced with 25 ml N2B27 basal medium containing 48.7% DMEM/F12 (Gibco), 48.7% Neurobasal medium (Gibco), 1% B27 supplement (Gibco), 0.5% N2 supplement (N2, homemade), 0.02% β-mercaptoethanol (Gibco), 1% PSG, and 20 ng/ml FGF-basic human (bFGF, Sigma-Aldrich). After 24 h, the N2B27 conditioned medium (N2B27-CM) was collected and replaced with an equal volume of fresh complete N2B27 medium.

Conditioned medium was collected daily for three consecutive days, pooled, filtered, and stored at -20°C until use.

Table 3.1 Primary antibodies used for immunocytochemistry.

| Marker types | Primary antibodies | Origin | Dilution |
|----------------------------|--|--------|----------|
| Core pluripotency markers | <i>OCT4</i> (Santa Cruz Biotechnologies sc-9081) | Rabbit | 1:100 |
| | <i>NANOG</i> (R&D system AF1997) | Goat | 1:100 |
| | <i>SOX2</i> (R&D system AF2018) | Goat | 1:100 |
| | | | |
| Primed pluripotency marker | <i>OTX2</i> (R&D system AF1979) | Goat | 1:200 |
| Naïve pluripotency markers | <i>TFAP2C</i> (R&D system AF5059) | Goat | 1:100 |
| | <i>KLF17</i> (Atlas Antibodies HPA024629) | Rabbit | 1:100 |
| | <i>ALPPL2</i> (abcam ab96947) | Rabbit | 1:100 |
| | <i>TFCP2L1</i> (abcam ab123354) | Rabbit | 1:100 |

Table 3.2 Secondary antibodies used for immunocytochemistry.

| Secondary antibodies | Origin | Dilution |
|--|--------------------|----------|
| <i>Alexa Fluor 647</i> (Invitrogen A21447) (Far red) | Donkey anti-Goat | 1:500 |
| <i>Alexa Fluor 555</i> (Invitrogen A21432) (Red) | Donkey anti-Goat | 1:500 |
| <i>Alexa Fluor 647</i> (Invitrogen A31573) (Far red) | Donkey anti-Rabbit | 1:500 |
| <i>Alexa Fluor 555</i> (Invitrogen A31572) (Red) | Donkey anti-Rabbit | 1:500 |

3.4 Culture and expansion of rhESCs-FGF2/KOSR

rhESCs-FGF2/KOSR cells were cultured on feeders in 35 mm dishes with 2 ml ESC medium containing 80% KO-DMEM (Gibco), 20% knockout serum replacement (KOSR, Gibco), 1% NEAA, 1% GlutaMAX (Gibco), 0.1mM β -mercaptoethanol (Sigma-Aldrich), and 5 $\mu\text{g}/\text{ml}$ bFGF (Sigma-Aldrich). Frozen-thawed rhESCs were cultured in medium supplemented with 10 μM ROCK inhibitor (Y-27632) (TOCRIS Bioscience, Bristol, UK) for the first 24 h. Cultures were maintained at 37°C , 5% CO_2 and 5% O_2 , with daily medium changes. Cells were passaged every 3–4 days by mechanical dissociation.

3.5 Culture and expansion of rhESCs-ALGöX

rhESCs-ALGöX cells were cultured in 35 mm dishes pre-coated with 5 µg/ml laminin (LN521, STEMCELL Technologies, Lund, Sweden) for 1 h. Cells were maintained in 2 ml ALGöX medium (Pham et al., 2025). consisting of N2B27-CM supplemented with 10 ng/ml Activin A (PeproTech, Cranbury, NJ, USA), 1,000 U/ml LIF (homemade), 1.25 µM Gö6983 (TOCRIS Bioscience), and 2.5 µM XAV939 (Sigma-Aldrich). Cultures were maintained at 37°C, 5% CO₂ and 5% O₂, with daily medium changes until 80% confluence. Cells were passaged every 3–4 days using 1X TrypLE (Gibco) for single-cell dissociation, followed by addition of 10 µM Y-27632 for the first 24 h to promote cell survival.

3.6 Characterization of rhESC by immunocytochemistry

rhESCs-FGF2/KOSR cells and rhESCs-ALGöX cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS for 20 min at room temperature, washed twice with PBS (5 min each), and permeabilized with PBS containing 0.5% Triton X-100. Non-specific binding sites were blocked in PBS with 10% donkey serum for 1 h at room temperature. Cells were incubated overnight at 4°C with primary antibodies (Table 3.1). After three washes, cells were incubated with secondary antibodies (Table 3.2) for 1 h at room temperature. Nuclei were stained with DAPI (0.5 µg/ml), and cells were mounted with M1289 mounting medium (Sigma-Aldrich). Confocal imaging was performed using a DM 6000 CS SP5 microscope (Leica, Wetzlar, Germany) with a 45x/1.25 oil immersion objective (PL APO HCX, Leica) (Aksoy et al., 2021).

3.7 Characterization of rhESC by RNA sequencing

Total RNA was extracted from 4–5 × 10⁶ cells using the RNeasy mini-kit (Qiagen, Hilden, Germany). Libraries were prepared from 200 ng of RNA using the NextFlex Rapid Directional mRNA-Seq kit (Bio-Scientific, Boston, MA, USA), and sequenced on a NextSeq500 platform (Illumina, San Diego, CA, USA) as single-end 75 bp reads. Demultiplexing was performed with bcl2fastq (Illumina), and adapters were trimmed using Cutadapt. Sequencing depth was ~30 million reads per sample. Reads were aligned to the reference genome using HISAT2, and gene counts were generated with HTSeq. RNA-seq data from this study are available under GEO accession number GSE146178 (Amzal et al., 2025).

3.8 Characterization of rhESCs by FPA-FTIR microspectroscopy

Cell pellets were washed three times with 0.9% NaCl, resuspended in 50 μl saline, and 5 μl aliquots were deposited onto IR transparent 2 mm thick barium fluoride (BaF_2) windows. Samples were air-dried and stored in a desiccator until analysis (Figure 3.1). Spectral data was acquired from an FPA-FTIR microscope (Hyperion 3000) with a FPA detector, connected to Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) at the Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand. The acquisition parameters were a 36 \times objective in transmission mode, 64 scans and all data was measured with 8 \times 8 binning at a spectral resolution of 6 cm^{-1} (Heraud et al., 2007). The full area of 64 \times 64 pixels equal 4,096 pixels. The field of view area using a 36 \times objective lens was 70.4 \times 70.4 μm^2 . The biochemical composition distribution was performed by OPUS 7.5 software (Bruker Optics, Ettlingen, Germany).

3.9 Multivariate data analysis of FTIR spectra

Spectral quality control was performed by visual inspection; spectra with weak absorbance (amide I band maximum absorbance <0.2), acquired from regions of the sample where there were no cells, or spectra with very high absorbance (amide I band maximum absorbance >0.8), acquired from regions where cells may have been clumped or overlaid was reject from the analysis. Spectra from sample groups based on differentiation of cell states and passage cells were analyze using PCA. Preprocessing of the data was conduct by first performing a second derivative by the Savitzky Golay method (13 smoothing points) and then normalized using EMSC using the spectral regions from 3000 to 2800 cm^{-1} and from 1800 to 800 cm^{-1} using The Unscrambler X 10.3 software (CAMO, Oslo, Norway). Score plots was use to visualize any clustering of the data, and the loading plot was used to determine which spectral regions contributed most to the variance in the data set, accounting for any clustering of spectra seen in scores plots. Integrate peak areas, was analyze by OPUS 7.5 software. Spectra were analyze using PLS-DA by The Unscrambler X 10.3 software. PLS-DA employed PCA models derived from calibration sets and was used to test the ability to discriminate different cell states using the independent validation set spectra. Calibration and validation of spectral data was employ using data sets that was randomly select comprising two-thirds and one-third of the spectra, respectively. The dataset can utilize to calculate the percentages of specificity and sensitivity. FTIR microspectroscopy combined with multivariate data analysis, in particular PCA, was apply to explain biochemical changes occurring during cellular differentiation.

3.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Data are presented as Standard Error of the Mean (SEM). Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey–Kramer’s Honest Significant Difference (HSD) post hoc test for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant. Different letters (a, b, c, d, e) above the bars indicate a statistically significant difference ($p < 0.05$) between the groups. Identical letters indicate no significant difference. Graphs were generated using Sigma Plot version 15 (Grafiti LLC, Palo Alto, CA, USA).

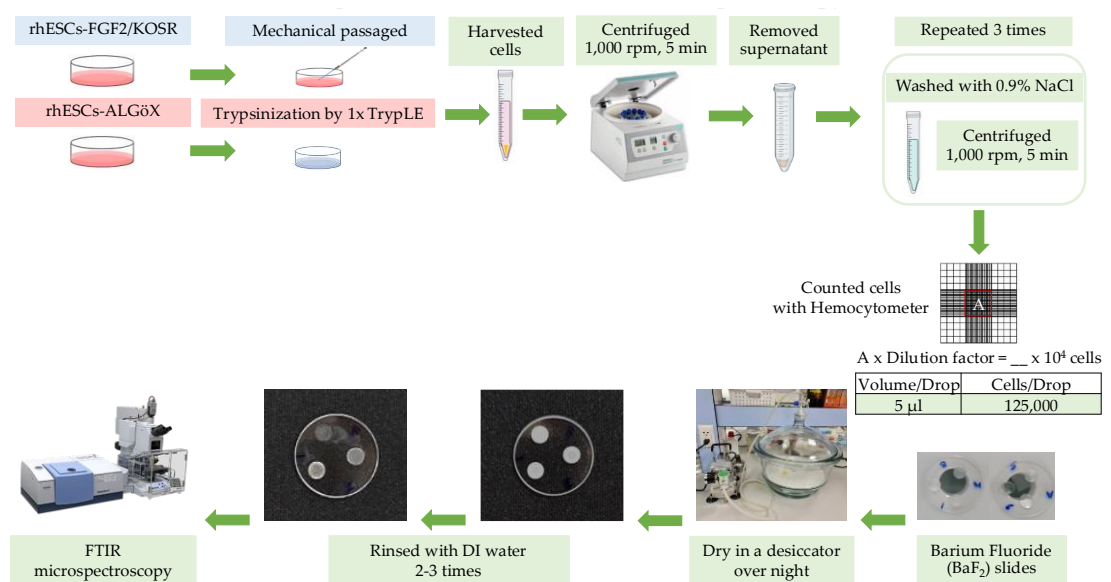


Figure 3.1 Preparation of rhESCs-FGF2/KOSR and rhESCs-ALGöX for characterization by FPA-FTIR Microspectroscopy.

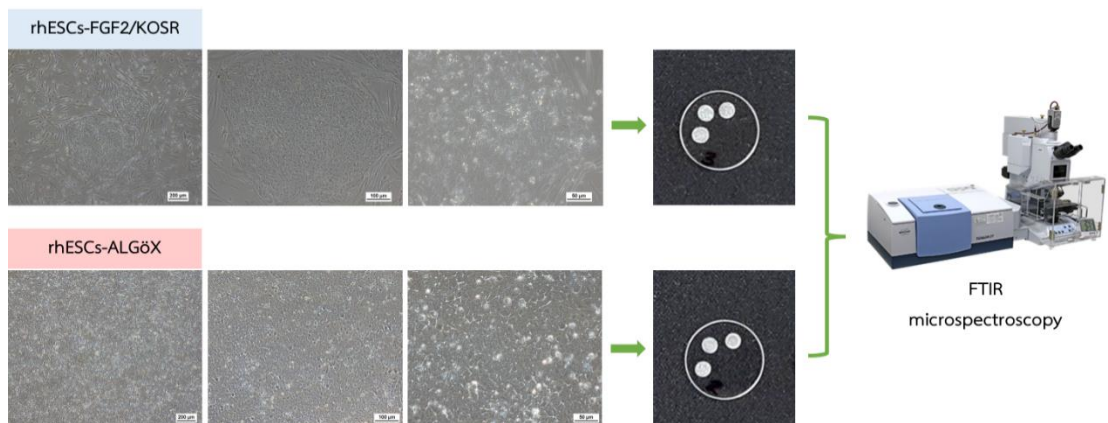


Figure 3.2 Morphology of rhESCs-FGF2/KOSR and rhESCs-ALGöX on culture dishes and morphology of cells on BaF2 slides.