Germ layer differentiation protocol

1. iPSCs were cultured until a stable confluency was achieved.
2. Cells were passaged using 0.5 mM EDTA.
3. iPSCs were plated at approximately 30-50% confluency onto four Matrigel-coated wells (one for undifferentiated iPSCs and one for each germ layer) using mTeSR+ medium supplemented with 5 µM ROCK inhibitor.
4. On the following day, cells were washed with 1x PBS, and the medium was replaced with basal differentiation medium (Day 0).
   Mesoderm differentiation:
   Cells cultured in APEL™2 medium with 1% P/S, 50 ng/mL BMP4, and 3 µM CHIR99021. Differentiation was performed for 2 days with daily medium changes.
   Endoderm differentiation:
   Cells cultured in DMEM high glucose with 10% FBS, 1x P/S, and 50 ng/mL Activin A. Differentiation was performed for 7 days with medium changes every 2 days.
   Ectoderm differentiation:
   Cells cultured in DMEM high glucose with 10% FBS, 1x P/S, and 1x B27 supplement. Differentiation was performed for 14 days with medium changes every 2 days.
5. After differentiation, cells were collected for RNA extraction. cDNA synthesis was performed and gene expression was analyzed by RT-qPCR.

Differentiation markers (qPCR)

TBX6 (Mesoderm)	F: CATCCACGAGAATTGTACCCG
	R: AGCAATCCAGTTTAGGGGTGT
TBXT (Mesoderm)	F: ACCCAGTTCATAGCGGTGAC
	R: CCATTGGGAGTACCCAGGTT
SOX17 (Endoderm)	F: GAGCCAAGGGCGAGTCCCGTA
	R: CCTTCCACGACTTGCCCAGCAT
FOXA2 (Endoderm)	F: GGAGCAGCTACTATGCAGAGC
	R: CGTGTTCATGCCGTTCATCC
PAX6 (Ectoderm)	F: TGGGCAGGTATTACGAGACTG
	R: ACTCCCGCTTATACTGGGCTA
MAP2 (Ectoderm)	F: GGCCCAAGCTAAAGTTGGTTCTC
	R: GCAGTGACATCCTCAGCCAAAG