

Selective vulnerability of human-induced
pluripotent stem cells to dihydroorotate
dehydrogenase inhibition during
mesenchymal stem/stromal cell purification

ジヒドロオロト酸デヒドロゲナーゼ阻害剤による
間葉系幹／間質細胞からの未分化 iPS 細胞の選択的除去

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Selective vulnerability of human-induced pluripotent stem cells to dihydroorotate dehydrogenase inhibition during mesenchymal stem/stromal cell purification

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The use of induced mesenchymal stem/stromal cells (iMSCs) derived from human induced pluripotent stem cells (hiPSCs) in regenerative medicine involves the risk of teratoma formation due to hiPSCs contamination in iMSCs. Therefore, eradicating the remaining undifferentiated hiPSCs is crucial for the effectiveness of the strategy. The present study demonstrates the Brequinar (BRQ)-induced inhibition of dihydroorotate dehydrogenase (DHODH), a key enzyme in *de novo* pyrimidine biosynthesis, selectively induces apoptosis, cell cycle arrest, and differentiation; furthermore, it promotes transcriptional changes and prevents the growth of 3-dimensional hiPSC aggregates. Contrastingly, BRQ-treated iMSCs showed no changes in survival, differentiation potential, or gene expression. The results suggest that BRQ is a potential agent for the effective purification of iMSCs from a mixed population of iMSCs and hiPSCs, which is a crucial step in successful iMSC-based therapy.

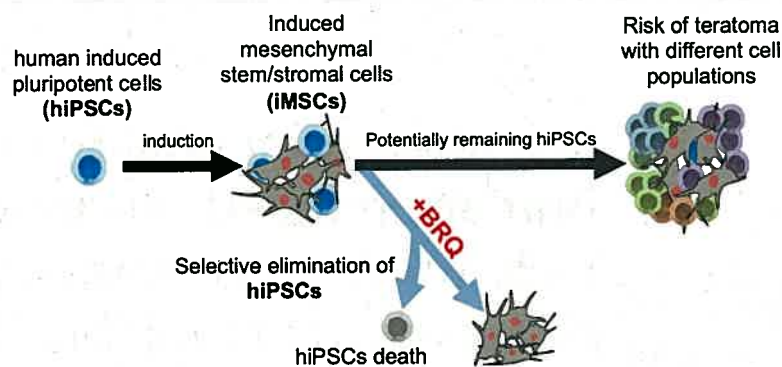
KEYWORDS

mesenchymal stem/stromal cells (MSCs), induced mesenchymal stromal cells (iMSCs), induced pluripotent stem cells (hiPSC), pyrimidine biosynthesis, dihydroorotate dehydrogenase (DHODH), brequinar (BRQ), survival

1 Introduction

Mesenchymal stem/stromal cells (MSCs) are present in almost all types of human tissues and contribute to tissue regeneration either directly or by paracrine-mediated effects (Han et al., 2019; Shammaa et al., 2020). MSC-based therapy is introduced in modern medicines. They are used for the treatment of graft-versus-host disease and are under investigation for their implementation in regulating the immune response in COVID-19 patients for the treatment of acute respiratory distress syndrome (ARDS), alleviating cardiac damage in post-myocardial infarction, and accelerating wound healing with increased vascularization (Wu et al., 2007; Duran et al., 2013; Liang et al., 2020).

Despite their advantages and wide spectrum of applications, there are limitations to MSC acquisition in terms of invasiveness, proliferation, and variation in biological characteristics (Abbas et al., 2019; Kozłowska et al., 2019; Sarkar et al., 2019). Induced MSCs (iMSCs) derived from human induced pluripotent stem cells (hiPSCs) provide rejuvenated, easily scalable, and



GRAPHICAL ABSTRACT

homogeneous populations of MSCs (Eto et al., 2018; Spitzthorn et al., 2019; Zhang et al., 2021). However, hiPSC-derived cell therapy carries the risk of teratoma formation if some pluripotent cells remain undifferentiated (Ben-David and Benvenisty, 2011). Therefore, the complete eradication of unwanted hiPSCs is a crucial step in safe iMSC-based therapies.

Different methods have been proposed for the elimination of pluripotent cells by inhibiting fatty acid synthesis, genetic modulation, changing the culture medium concentration of certain amino acids, or inhibiting the rate-limiting enzyme in *de novo* pyrimidine synthesis (Nagashima et al., 2018; Kimura et al., 2019; Tanosaki et al., 2020; Kondo, 2021). Fast proliferating cells rely solely on the *de novo* pyrimidine biosynthesis pathway to meet the high demand for nitrogenous bases (Walter and Herr, 2022). Dihydroorotate dehydrogenase (DHODH) is a rate-limiting enzyme in the *de novo* pyrimidine biosynthesis pathway; thus, depletion of cellular pyrimidines is achieved by inhibition of DHODH (Desler et al., 2010; Chen et al., 2019). The expression of DHODH is regulated by the oncogene MYC, which is significantly upregulated in cancer cells and pluripotent cells. DHODH inhibitors suppress MYC expression, presumably due to the induction of apoptosis and cell cycle arrest in cancer cells (Dorasamy et al., 2017; Christian et al., 2019).

Although it was previously reported that DHODH inhibitors selectively eliminated murine pluripotent cells, it is still unknown and worth confirming in human cells. We hypothesized that *de novo* pyrimidine biosynthesis is indispensable for the high nitrogenous base demand in hiPSCs and that iMSCs can utilize the pyrimidine salvage pathway. In this study, we treated iMSCs with the potent DHODH inhibitor Brequinar (BRQ) and analyzed the cell survival, differentiation potential, gene expression, and functional characteristics to determine the effectivity and safety of using the DHODH inhibitor for the elimination of unwanted hiPSCs, which will provide a fundamental step for the future of iMSC-based therapy.

2 Materials and methods

2.1 iPSCs culture

For the present study, hiPSCs (1231A3) reprogrammed by episomal vectors (Nakagawa et al., 2014), RPCiPS771-2 reprogrammed by RNA (Stemgent), Ff-XT28s05-Abo-To

reprogrammed by episomal vectors and knockout of HLA-A,B, and CIITA genes (provided by CiRA Foundation) were cultured in plastic dishes coated with iMatrix-511 silk (Nippi, 892021) fed with StemFit AK03N (Ajinomoto) under xeno-free culture conditions. Cells were dissociated into a cell suspension using Accutase® (Sigma Aldrich, A6964) and passaged when they reached more than 70% confluence.

2.2 iPSC cell aggregates assay

For 3-dimensional cell aggregates experiment, hiPSCs were dissociated into cell suspensions and seeded into 96 well U-bottom ultra-low attachment plates (PrimeSurface®, Sumitomo Bakelite, MS-9096U) in StemFit Basic03 (equivalent to AK03N without bFGF, Ajinomoto) in the presence of ROCK inhibitor (Y-27632, Wako, 034-24024). After aggregates with clear edges were formed, the culture medium was replaced with DMEM/F12 supplemented with 0.1% human serum albumin (HSA, Wako, 014-21543) and 10 ng bFGF (R&D, 236-EG). Aggregate cross-sectional area sizes were measured using Fiji ImageJ software (Schindelin et al., 2012). 1231A3 hiPSCs were used in all experiments unless otherwise stated.

2.3 iMSCs culture

iMSCs were generated by inducing hiPSCs into neural crest cells (NCCs), and then into iMSCs (Kamiya et al., 2022), which were then maintained in a plastic dish coated with Fibronectin (Chemicon, FC010-10 MG) and fed with PRIME-XV™ MSC Expansion XSFM (IrvineScientific, 91149). Analyses were performed during passages 3–5.

2.4 iMSCs differentiation

For the adipogenic differentiation of iMSCs (Figure 5D), 7 days BRQ (Selleck, S3565)-or DMSO (Nacalai, 13408-64)-pretreated iMSCs were seeded on fibronectin-coated wells, and cells were maintained until they reached full confluence, then adipogenic induction medium made of DMEM (High Glucose, Nacalai, 08459-64), 10% fetal bovine serum (FBS) (Nichirei, 171012), 0.5% penicillin/streptomycin (GIBCO, 15140122), 10 µg/ml insulin (Wako, 097-06474), 1 µM

dexamethasone (Wako, 047-18863), 200 μ M indomethacin (Wako, 093-02473), and 500 μ M IBMX (Wako, 095-03413) were added to it. After 3 weeks of adipogenic induction, cells were stained with Oil Red O (Nacalai, 25633-92). For osteogenic differentiation (Figure 5E), 7 days BRQ- or DMSO-pretreated iMSCs were seeded on gelatin-coated wells, maintained until they reached full confluence, and then treated with osteogenic induction medium made of MEM-Alpha GlutaMAX (GIBCO, 32571-036), 10% FBS, 0.5% penicillin/streptomycin, β -glycerophosphate disodium salt hydrate (SIGMA, G9422), and 100 nM dexamethasone. After 3 weeks of osteogenic induction, the cells were stained with Alizarin Red S solution (Muto Pure Chemicals, 17971). For chondrogenic differentiation using the micromass method (Figure 5F), a drop of 5 μ l of 7 days BRQ- or DMSO-pretreated iMSCs suspension in the center of a well (24-well plate) coated with fibronectin. After incubation at 37°C for 1 h, the hMSC Chondrogenic Differentiation BulletKit (Lonza, PT-3003), 100 ng/ml TGF β 3 (R&D, 243-B3-200), and 100 ng/ml BMP7 (R&D, 354-BP-010V) were added. After 7 days of differentiation, Alcian blue solution (pH = 1; Muto Pure Chemicals, 40862) was added. We used DMSO as the control treatment at similar volume to BRQ since BRQ was dissolved in DMSO.

2.5 Cell survival

To examine cell survival, cells were cultured with the DHODH inhibitors teriflunomide (Selleck, S4169), ASLAN003 (Selleck, S9721-5 MG), and BRQ. For the rescue experiment, 100 μ M of uridine (Sigma Aldrich, U3003-5G) was added. Then, using a highly water-soluble tetrazolium salt-based cell counting kit (CCK-8 Dojindo, CK04-05), absorption was detected using a 450 nm plate reader (PerkinElmer, 2104 EnVision). The survival percentage was calculated relative to the non-treated control and by subtracting the background absorbance value.

2.6 Flow cytometry analysis

Apoptosis was evaluated using the Annexin V-FITC Apoptosis Detection Kit (Nacalai, 15342-54), which includes an Annexin V-FITC conjugate and propidium iodide (PI) according to the protocol provided by the manufacturer, and was further analyzed using flow cytometry. For the cell cycle analysis, Cell Cycle Assay Solution Deep Red (Dojindo, C548) was used; the solution provided with the kit was added to the cell suspension, followed by incubation according to the manufacturer's protocol, and a flow cytometric analysis. For staining, CD105-APC (eBioscience, 17-1057-42), CD90-PE (eBioscience, 555596), CD73-PE (eBioscience, 550257), CD44-PE (eBioscience, 550989), CD34-PE (eBioscience, 560941), CD31-Alexa Fluor[®] 647 (BioLegend, 303112), and TRA-1-60-PE (Thermo Fisher, 12-8863-82) (1:50) was added to the cell suspension and incubated for 30 min before the flow cytometric analysis. BD FACSAria[™] III was used to detect fluorescence. Results were plotted using FlowJo_v10.8.1 software.

2.7 Immunocytochemistry

Cells were fixed using 4% PFA, stained with anti-POU5F1 (Santa Cruz Biotechnology, sc-5279), and detected using Alexa Fluor

555 goat anti-mouse (Thermo Fisher, A21422) and DAPI counterstaining. They were visualized and imaged with a Keyence microscope system (BZ-X710 and BZ-X810).

2.8 Real-time quantitative PCR analysis

RNA samples were extracted using the RNeasy[®] Mini kit (Qiagen) and reverse-transcribed to cDNA. Real-time quantitative PCR (RT-qPCR) was performed using THUNDERBIRD[™] Next SYBR[®] (TOYOBO, QPX-201), QuantStudio[™] 3 Real-Time PCR System, and QuantStudio[™] 7 Flex Real-Time PCR System (Applied Biosystems) with specifically designed primers (Supplementary Table S1). Data from three biological replicates were analyzed to calculate the relative fold change ($2^{-\Delta\Delta CT}$). GraphPad Prism 9 software was used to plot the graphs.

2.9 RNAseq data analysis

Libraries were prepared using the Ion AmpliSeq Transcriptome Human Gene Expression Panel, Chef-Ready Kit (A31446), and Ion Torrent Ion Chef Instrument (4484177) (Thermo Fisher Scientific, Tokyo, Japan). In brief, 10 ng of RNA was reverse transcribed with SuperScript VILO cDNA Synthesis Kit (11754050), PCR conditions were 13 cycles of 16 min extension reaction using 10 ng of cDNA, qPCR Ion Library TaqMan Quantitation Kit (4468802). 50 p.m. was used for template preparation and sequencing using Ion Chef. Sequencing was performed using the Ion Torrent Ion GeneStudio S5 Prime System. Then, the count data analyses were performed using R studio with the 'DESeq2' package normalization method for the detection of significantly ($p \leq 0.05$) differentially expressed genes (Love et al., 2014).

2.10 Statistical analysis

For statistical analyses, ANOVA with Dunnett's multiple comparisons test or unpaired parametric Welch's *t*-test was performed using GraphPad Prism 9 software ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$).

3 Results

3.1 Vulnerability of hiPSCs to DHODH inhibition

To test the viability of hiPSCs treated with different DHODH inhibitors, we cultured cells in 96-well plates and treated cells in each experiment with one of the different DHODH inhibitors, teriflunomide, ASLAN003, and BRQ, for 7 days at different concentrations. After 7 days of treatment, the CCK-8 kit solution was added and incubated for 4 h. Absorbance was measured using a 450 nm filter plate reader. Approximately 50 nM of BRQ resulted in the complete eradication of hiPSCs, which was more potent than ASLAN (250–500 nM), while the hiPSCs showed higher tolerance to teriflunomide and 50% survival even at the highest concentration tested (Figure 1A). Contrastingly, iMSCs were more resilient to the

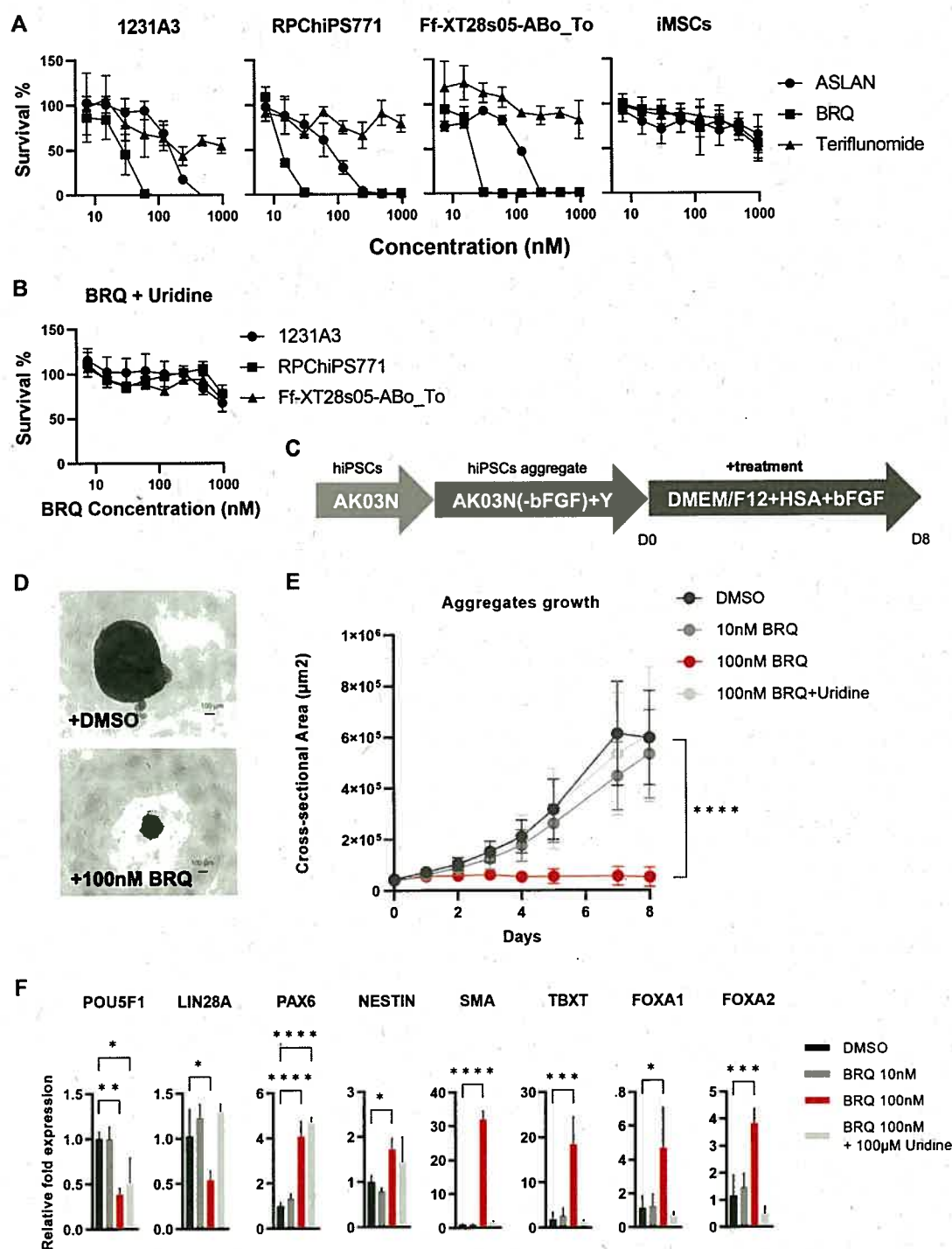


FIGURE 1

Vulnerability of hiPSCs to DHODH inhibition (A) Survival measurement using water-soluble formazan dye after the treatment with DHODH inhibitors, Teriflunomide (Triangle), ASLAN (Circle), and Brequinar (Square) for 7 days on 1231A3, RPChiPS771, Ff-XT28s05-ABO_To hiPSCs lines and iMSCs (From left to right) (B) Rescued survival of 1231A3 (Circle), RPChiPS771 (Square), and Ff-XT28s05-ABO_To (Triangle) BRQ-treated hiPSCs lines by 100 μM Uridine supplementation. Data plotted as mean with error bar S.D., minimum of triplicates per hiPSCs line and triplicates of three biological repeats of the iMSCs. (C) Schematic representation of iPS cell aggregates formation, hiPSCs were collected in single cell suspension then cultured in ultra-low attachment U-bottom 96-well plates in StemFit Basic03 in the presence of ROCK inhibitor (Y) for 2–3 days until aggregates with clear edges are formed. Medium then was replaced with DMEM/F-12 supplemented with 0.1% HSA and 10 ng bFGF and (D) phase contrast images were taken, then the (E) area of each aggregate was measured, dots represent mean and error bar S.D., $n = 6$, biological replicates. (F) Relative mRNA expression of pluripotency markers (POU5F1 and LIN28), ectodermal markers (PAX6 and NESTIN), mesodermal markers (SMA and TBXT), and endodermal markers (FOXA1 and FOXA2). mRNA of minimum 4 aggregates were extracted per sample after 3 days of treatment then expression was detected by RT-qPCR, DMSO (Black), 10 nM BRQ (Dark gray), 100 nM BRQ (Red) and 100 nM BRQ with 100 μM Uridine (Light gray). Bar: mean, Error bar: S.D., $n = 3$, biological replicates.

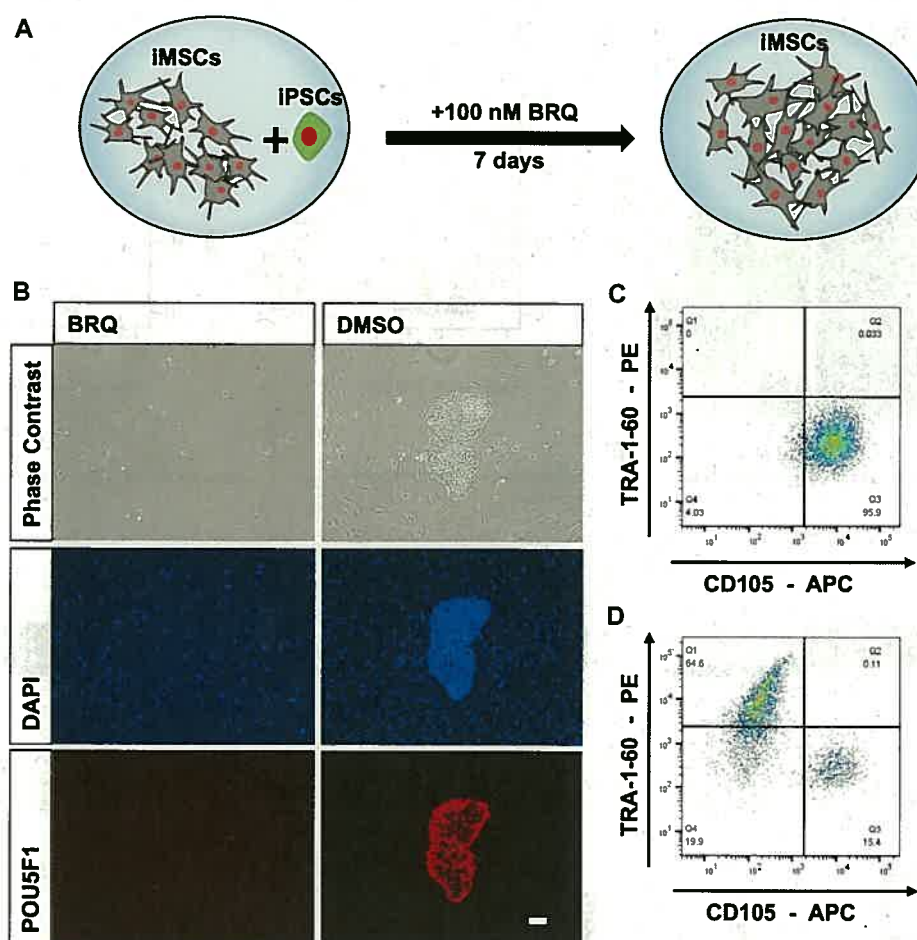


FIGURE 2

Selective elimination of hiPSCs by BRQ in iMSC-hiPSC mixed culture. **(A)** Schematic representation of the mixed culture condition by mixing iMSCs with 10% hiPSCs and culturing both cell types in the presence of BRQ for 7 days. **(B)** iMSCs mixed with 10% hiPSCs cultured for 1 week in the presence of 100 nM BRQ or DMSO control. Cells were fixed using 4% PFA then immunostained using anti-POU5F1 (Red) antibody and nuclear stain DAPI (Blue), scale bar 100 μ m. Mixed culture was repeated. **(C)** BRQ- and **(D)** DMSO-treated cells were stained with surface antibodies CD105 and TRA-1-60. Cells were then passed through the cell sorter for analysis. Experiments were performed in three biological repeats, representative figures.

DHODH inhibitors and showed >50% survival, even at the highest concentration tested. hiPSCs were rescued by adding 100 μ M uridine, which suppressed the inhibitory effects of BRQ (Figure 1B). The effects of DHODH inhibitors and the uridine-induced rescue from the BRQ effect were similar in the three hiPSC lines tested. These results indicate that 100 nM BRQ is sufficient to fully eradicate hiPSCs, but not iMSCs. While the effect of BRQ on hiPSCs can be prevented by adding uridine that saturates the salvage pathways, iMSCs utilize the salvage pathway for pyrimidine biosynthesis, which is sufficient to sustain the viability of iMSCs without uridine supplementation.

To reveal the effects of BRQ on 3-dimensional (3D) cell condition, we cultured 1231A3 hiPSCs in 96-well U-bottom plates as 3D cell aggregates, and then replaced the culture medium with DMEM/F12 medium supplemented with 0.1% HSA and 10 ng bFGF to allow the hiPSCs to spontaneously differentiate. Subsequently, the treatment of cell aggregates with 100 nM BRQ, 10 nM BRQ, 100 nM

BRQ supplemented with 100 μ M uridine, and DMSO control was done for 8 days (Figure 1C). Aggregates treated with 100 nM BRQ failed to grow and were significantly smaller ($p < 0.0001$) than DMSO-treated aggregates (Figures 1D, E). We collected samples at an early stage of treatment (day 3) and extracted mRNA for gene expression analyses. Despite their smaller sizes, BRQ-treated aggregates revealed significantly upregulated ectodermal (PAX6 and NESTIN, $p < 0.0001$ and $p = 0.0499$), mesodermal (SMA and TBXT, $p < 0.0001$ and $p = 0.0007$), and endodermal (FOXA1 and FOXA2, $p = 0.0249$ and $p = 0.0009$) gene markers of the germ layers and significantly downregulated of the pluripotency marker (POU5F1, $p = 0.0044$). The 100 nM BRQ-treated aggregates also revealed significant downregulation of LIN28A ($p = 0.0268$) which is a marker for residual undifferentiated hiPSCs (Kuroda et al., 2012) (Figure 1F). These results suggest that BRQ is effective in eliminating undifferentiated hiPSCs, preventing the growth of hiPSC aggregates and spare differentiated cells.

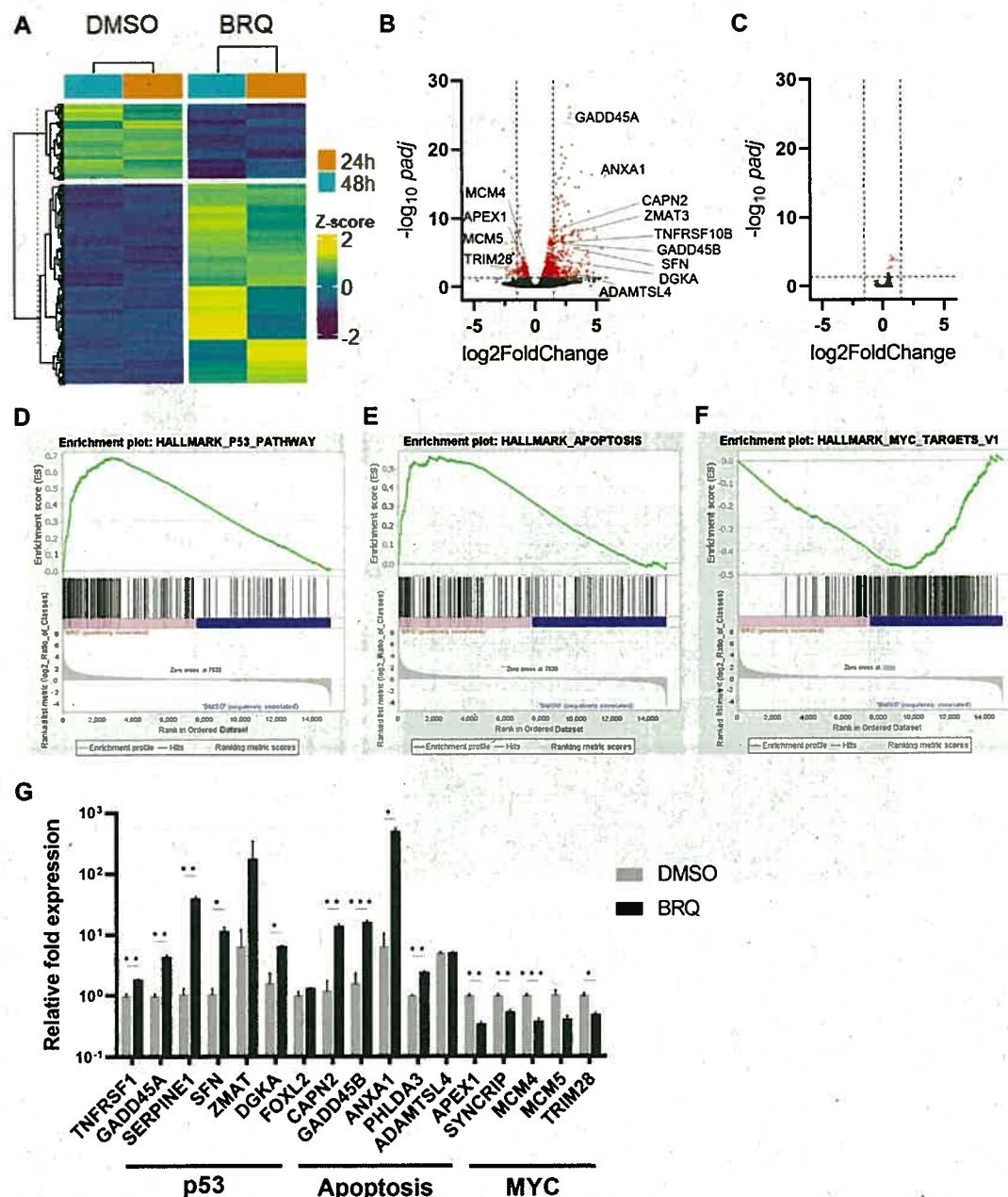


FIGURE 3

Relevant transcriptional changes of BRQ-treated hiPSCs. (A) hiPSCs treated for 24 h and 48 h, hierarchical clustering of samples of the significantly differentially expressed genes (100 nM BRQ vs. DMSO control), bright yellow color indicates higher expression of gene and blue for lower expression. Volcano plots of significance ($-\log_{10} p\text{-adj}$) vs $\log_2$ fold change showing the differentially expressed genes of 100 nM BRQ vs. DMSO control of (B) hiPSCs, and (C) iMSCs. Red color dots indicate genes showing significantly differentially expressed genes ($p \leq 0.05$ horizontal dashed) that are transcriptionally downregulated or upregulated ($\log_2$ fold change ≥ 1.5 or ≤ -1.5 vertical dashed lines). Gene Set Enrichment Analysis (GSEA) of the normalized counts data of BRQ- and DMSO-treated hiPSCs shows a correlation with (D) Apoptosis targets, (E) p53 pathway, and (F) MYC targets. (G) 48 h BRQ-treated hiPSCs relative mRNA expression of Apoptosis, p53, and MYC targets detected by RT-qPCR. Bars indicate the mean, error bars indicate S.D., $n = 3$, biological replicates.

3.2 BRQ selectively eliminates hiPSCs in iMSC-iPSC mixed culture

To further investigate the effectiveness of BRQ in application settings and cell type selectivity, we intentionally contaminated iMSCs derived from the 1231A3 hiPSC line with 10% hiPSCs from

the same cell line, and co-cultured both cell types for a week in one culture well in the presence of BRQ or DMSO (Figure 2A). The mixed cells were cultured in 6-well plates coated with both fibronectin and iMatrix-511, fed with Stemfit Basic03, and then treated with 100 nM BRQ or DMSO. We visually examined cell cultures and found that 100 nM BRQ-treated mixed culture

prevented hiPSCs from proliferating and forming colonies during the 7 days of cell culture, whereas colonies were formed in DMSO-treated mixed culture (Figure 2B). The cells were fixed with 4% PFA, immunostained using anti-POU5F1 antibody and nuclear stain (DAPI), and fluorescence microscopic imaging was done. The results showed that the BRQ-treated mixed culture fully eliminated hiPSCs, as indicated by the absence of POU5F1 positive colonies. Similar results were obtained using cell sorting analyses. The colonies were dissociated to form a single-cell suspension and stained with TRA-1-60 and CD105 conjugated antibodies, which are surface markers for human iPSCs and iMSCs, respectively. The stained cells were then passed through a cell sorter for analysis. The results revealed the absence of TRA-1-60 positive cells in BRQ-treated samples (Figure 2C), and the presence of 64.6% of positive cells in the DMSO control (Figure 2D) was detected. These results show the effectiveness of BRQ in eliminating unwanted hiPSCs, even in the presence of different cells in the same culture well, by selectively eliminating hiPSCs and sparing iMSCs derived from the same 1231A3 hiPSC line.

3.3 BRQ-treated hiPSCs show relevant transcriptional changes

To reveal the effect of BRQ on global gene expression, we performed next-generation RNA sequencing (RNAseq) of hiPSCs treated with 100 nM BRQ for 24 h and 48 h, and the normalized count data of the differentially expressed genes (DEGs) were analyzed. The BRQ and DMSO samples revealed distinct clusters of highly and less expressed genes, and both samples of each condition showed two hierarchical clusters (Figure 3A). Gene expression analysis of BRQ vs. DMSO samples revealed 646 significant ($p \leq 0.05$) DEGs. Approximately 33.7% of the genes were transcriptionally upregulated ($\log_2$ fold-change ≥ 1.5), and approximately 4.33% of genes were downregulated ($\log_2$ fold-change ≤ -1.5) in BRQ-treated samples. Results were plotted to show the significance vs. log fold change (Figure 3B), and we found that the genes relevant to DNA damage and cell cycle regulation, regulation of apoptosis, and MYC targets were significantly differentially expressed (Fornace et al., 1989; Zeller et al., 2003; Yang et al., 2006; Kawase et al., 2009; Weyd et al., 2013; Dubail and Apte, 2015; Yang et al., 2020). Contrastingly, RNAseq data analyses of BRQ-treated iMSCs revealed only 14 DEGs (Supplementary Figure S1A), and expressions of none of these genes were altered at the transcriptional level ($\log_2$ fold-change ≥ 1.5 or ≤ -1.5) (Figure 3C).

Gene Set Enrichment Analysis (GSEA) using the hallmark data set against the normalized count data of BRQ- and DMSO-treated hiPSCs revealed a positive correlation of apoptosis and p53, and a negative correlation of MYC targets with BRQ-treated samples' expression data (Figures 3C–E). Moreover, RT-qPCR analyses revealed upregulation of genes relevant to p53 and apoptosis pathways, and downregulation of MYC targets in 48 h BRQ-treated hiPSCs relative to DMSO control (Figure 3G), however, 7 days BRQ-treated iMSCs showed relatively unchanged expression (Supplementary Figure S1B), which supported the findings in RNAseq data. These results suggest that the BRQ treated hiPSCs lost their oncogenic property and under the instructions of programmed cell death (PCD).

3.4 BRQ induces apoptosis and cell cycle arrest in hiPSCs but not in iMSCs

To further investigate the cause of death in BRQ-treated cells, we tested the 24 h and 48 h BRQ-treated hiPSCs and 7 days BRQ-treated iMSCs by adding Annexin-V and propidium iodide (PI). Damage to membrane lipids permits the Annexin-V binding, whereas fully damaged lipid membranes expose the cells, allowing PI binding to the cell content, indicating that the cells underwent PCD. If only PI was detected, the cells were considered to have died from necrosis. It is important to note that hiPSCs have higher apoptosis than iMSCs in control conditions. hiPSCs in small colonies or when cells are dissociated are prone to be eliminated by apoptosis due to mechanical stress. On the other hand, stress induced proliferation and did not affect apoptosis in the MSCs (Choi et al., 2007; Ohgushi et al., 2010; Frank et al., 2016). The results demonstrated a shift of hiPSCs treated with 100 nM BRQ to the double positive quadrant (51.2% at 24 h and 84.2% at 48 h), in which cells were positive for both Annexin-V and PI, indicating the late-apoptotic stage, while the iMSCs treated for 7 days revealed no marked change in the percentages of living cells or cells undergoing apoptosis (Figure 4A). Cell cycle analysis revealed that BRQ-treated hiPSCs caused cell cycle arrest in the S/G2 phase after 48 h of treatment, with a significant decrease in the percentage of cells in the G1 phase, while iMSCs treatment for 7 days showed no changes in the cell cycle (Figure 4B). These data further verify that BRQ treatment causes cell cycle arrest and commences PCD only in hiPSCs and the resilience of iMSCs to BRQ effects.

3.5 BRQ treated iMSCs maintain their characteristics and differentiation potentials

We investigated whether BRQ-treated iMSCs maintained their gene expression profiles. For 7 days, iMSCs were treated with 100 nM BRQ or DMSO. On the day 7, dissociated cell suspensions were collected and stained with MSC-specific positive surface markers CD105, CD90, CD73, and CD44 in addition to the MSC-specific negative surface markers CD34, and CD31; stained cells passed through a cell sorter were analyzed. iMSCs treated with BRQ expressed a fluorescence pattern similar to that of the DMSO control for the positive and the negative markers (Figure 5A). Although CD31 is a negative marker for iMSCs, we detected a slight percentage (6.33%) of cells that were positive for CD31. CD31 is expressed on the surface of endothelial cells and platelets (Duncan et al., 1999). Therefore, low detection level of CD31⁺ cells in iMSCs suggested partial differentiation of iMSCs into endothelial cells, and may be due to the fact that the iMSCs were derived from NCCs, one of whose derivatives is endothelial cells, or that the parent hiPSCs from which the iMSCs were derived were induced from a sample of peripheral blood. To further confirm these similarities, we evaluated mRNA expression of the same MSCs markers; BRQ-treated iMSCs showed relatively similar gene expression to that of the control (Figure 5B). Previous studies revealed that MSCs treated with interferon- γ (IFN- γ) showed upregulation of immunomodulatory genes IDO1 and PD-L1 granting MSCs their potential application in inflammatory diseases (Duncan et al., 1999; Wobma et al.,

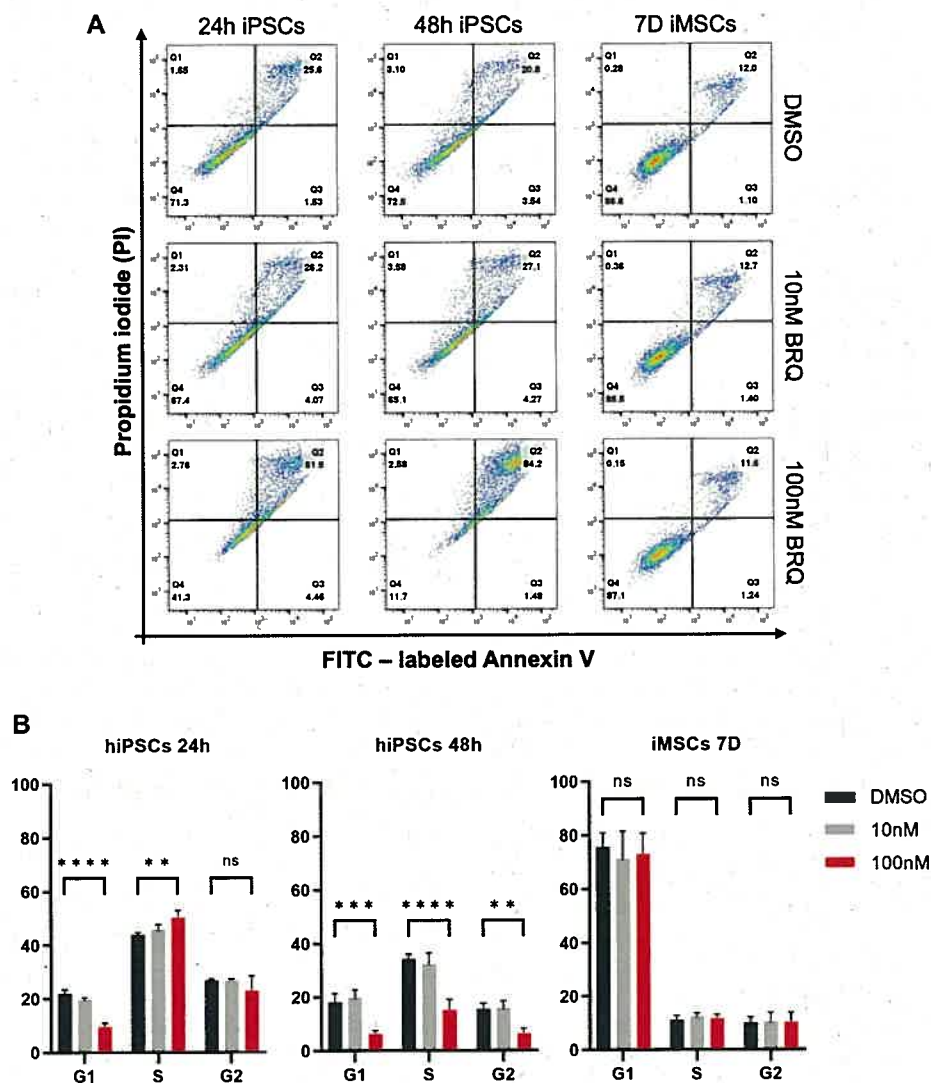


FIGURE 4

Induction of apoptosis and cell cycle arrest in hiPSCs but not iMSCs by BRQ treatment. hiPSCs were cultured until 70% confluence was reached, and iMSCs were treated directly after plating for 7 days. (A) Apoptosis detection in 24 h and 48 h BRQ- and DMSO-treated hiPSCs and 7 days BRQ and DMSO-treated iMSCs. Dissociated cells were collected in cell suspension, stained with FITC labeled Annexin V and propidium iodide (PI) using an Annexin Apoptosis Detection Kit, and analyzed by flow cytometry. $n = 3$, representative figures. (B) Cell cycle analysis of 24 h and 48 h BRQ- and DMSO-treated hiPSCs, and 7 days BRQ- and DMSO-treated iMSCs using a Cell Cycle Assay kit and analyzed using flow cytometry. $n = 3$, biological replicates.

2018). Therefore, we supplemented the 100 nM BRQ pretreated iMSCs with IFN- γ then checked their mRNA expression (Figure 5C). The results showed that BRQ pretreated iMSCs maintained their reactivity to IFN- γ significantly ($p < 0.0001$), upregulating both IDO1 and PD-L1. The induced expression was similar to that observed in the DMSO control. Furthermore, iMSCs are multipotent cells that can differentiate into lineage-specific cells, such as chondrocytes, osteoblasts, and adipocytes (Jones and McTaggart, 2008). We examined the differentiation potential of iMSCs after 7 days of BRQ treatment (Figures 5D–F), and these cells were detected to maintain their differentiation potential and were successfully differentiated into the lineage-specific cells of adipocytes, osteoblasts, and chondrocytes, as

confirmed by Alcian blue, Alizarin red, and Oil red O staining analyses, respectively.

4 Discussion

The stepwise induction of iMSCs from hiPSCs is controlled through selective cell sorting which utilizes specific antibody-mediated selection to eliminate unwanted cell populations (Kamiya et al., 2022). However, hiPSCs potentially remain undifferentiated and are able to evade selective cell sorting, potentially giving rise to teratomas, especially if large numbers of cells are transplanted in clinical settings (Ben-David and Benvenisty, 2011). Our study revealed

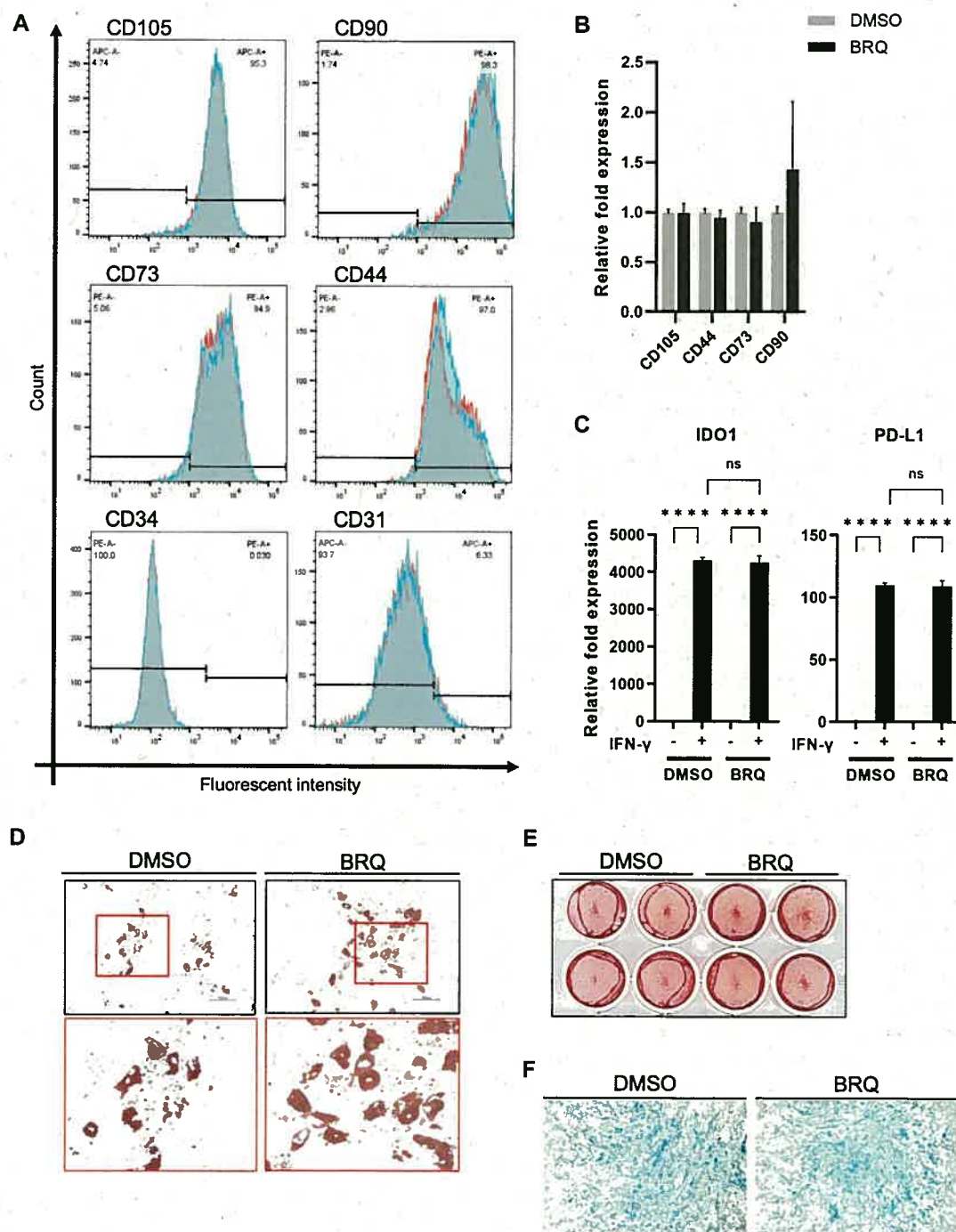


FIGURE 5

BRQ-treated iMSCs maintain their characteristics and differentiation potentials (A) Overlay histogram of the CD105, CD90, CD73, CD44, CD34, and CD31 in DMSO (Blue) and BRQ (Red) treated iMSCs for 7 days, detected by staining with conjugated antibodies then passed through cell sorter for analysis' n = 3, biological replicates, representative figures (B) mRNA expression of CD105, CD90, CD73 and CD44 detected by RT-qPCR, 100 nM BRQ 7 days treated iMSCs (Black) relative fold expression to DMSO treated iMSCs (Gray) (C) mRNA expression of IDO1 (left) and PD-L1 (right) in DMSO pretreated iMSC or BRQ pretreated iMSCs with or without 10 ng/ml IFN- γ for 24 h mRNA expression relative to DMSO without IFN- γ of each gene was analyzed using RT-qPCR. Bars indicate the mean, error bar is SD, n = 3, biological replicates. Differentiation of BRQ and DMSO pretreated iMSCs into (D) adipocytes detected by Oil red staining, (E) osteoblasts detected by Alizarin red staining, and (F) magnified image of the chondrocytes micromass detected by Alcian Blue staining

the successful selective elimination of hiPSCs by BRQ-induced inhibition of the enzyme DHODH during *de novo* pyrimidine synthesis.

Here, we demonstrated that BRQ-induced inhibition of DHODH in hiPSCs prevented cell survival, caused cell cycle arrest, and induced apoptosis. Although *in vivo* teratoma formation assay is still the golden

standard for the assessment of iPSCs pluripotency (Dominici et al., 2006). 100 nM BRQ eliminated all the living cells after few days, and it was not possible to obtain the required number of cells for the assay. Therefore, we allowed the hiPSCs to spontaneously differentiate as 3D aggregates utilizing the features of iPSCs to differentiate into cells of the three germ layers (ectoderm, mesoderm, and endoderm), and exploited suboptimal spontaneous differentiation in which some cells retained pluripotency (Hentze et al., 2009). It was previously reported that iPSCs cultured in mouse metanephron as a scaffold allowed the stem cells to differentiate without immune rejection and formed *in vitro* teratoma assay (Yamamoto et al., 2018). Similarly in our condition we applied ultra-low attachment plastic plates that sustained the cells in suspension forming aggregates of differentiated and undifferentiated iPSCs which revealed that treatment with 100 nM BRQ completely prevented growth of those aggregates. Remarkably, the 100 nM BRQ-treated aggregates, despite their smaller sizes, also showed higher gene expression of germ layer markers than that in control. This could potentially mean that the remaining cells in the 100 nM BRQ-treated aggregates had enhanced differentiation potential which could be the result of BRQ-induced differentiation due to cellular stress with the BRQ-sparing only differentiated cells and allowing those cells to proliferate. Interestingly, the BRQ-treated, Uridine-rescue aggregates revealed distinct upregulation of ectodermal marker. Furthermore, next-generation RNA sequencing indicated positive enrichment of apoptosis and p53 pathways in BRQ-treated hiPSCs, while negative enrichment of the MYC target pathway was detected. These findings demonstrate that the effect of pyrimidine bases depletion caused by BRQ drove the cells into arrest and initiation of programmed cell death with loss of tumorigenicity/pluripotency. Our findings were parallel with previous reports on the effects of BRQ on cancer stem cells and mouse pluripotent stem cells. Contrastingly, iMSCs were resilient to the effects of BRQ. After 1 week of treatment with 100 nM BRQ, iMSCs maintained their gene expression profile, lineage-specific differentiation potential, and IFN- γ -induced expression of IDO1 and PD-L1. RNA sequencing data revealed only 14 significantly differentially expressed genes, none of which showed a fold change greater than 1.5. This novel finding indicates that the pyrimidine requirement of iMSCs for proliferation is sufficed only by the salvage pathway, therefore inhibited key enzyme for the *de novo* pyrimidine synthesis (DHODH) sustained the status of the iMSC; so far, this criterion of iMSCs was not revealed by previous studies.

Cells acquire pyrimidine bases *via* either *de novo* biosynthesis or the salvage pathway. Highly proliferative cells (such as cancer or pluripotent cells) share a similar preference depending on the *de novo* pathway for pyrimidine biosynthesis, which is regulated by the rate-limiting DHODH (Walter and Herr, 2022). DHODH inhibitors are used in the treatment of autoimmune diseases and are currently under investigation for their antiviral and anticancer potencies. In general, DHODH inhibitors prevent cellular proliferation, but some of them, such as BAY 2402234, induce differentiation of acute myeloid leukemia (AML) cells (Christian et al., 2019). BRQ also inhibited tyrosine phosphorylation in lymphocytes which was not corrected by uridine supplementation, suggesting its additional mode of activity (Masuda et al., 2012). Additionally, while the MYC family of oncogenes regulates the expression of DHODH, the key enzymes in *de novo* pyrimidine synthesis, DHODH inhibitors downregulate c-MYC in melanoma, myeloma, and

lymphoma cells. Although it is unclear why DHODH inhibition leads to c-MYC downregulation, it is thought to be a result of cell cycle arrest and apoptosis activation (Dorasamy et al., 2017). Or perhaps cellular instability and halted proliferation lead to the deactivation of the MYC family of oncogenes, whose main purpose is to promote proliferation. Another possible scenario is that the inhibition of the DHODH enzyme led to its accumulation or the accumulation of the substrate, which signaled feedback inhibition on the MYC transcription family genes through an unknown mechanism. This makes DHODH inhibitors a potential drug for their anticancer effects in solid and non-solid tumors (Christian et al., 2019). BRQ is the most potent human DHODH inhibitor ($IC_{50} = 1.8$ nM), with known potential to specifically decrease the proliferation of glioblastoma cells (Xu et al., 1998); and it is in clinical trials for application against COVID-19 and AML (go.drugbank.com/drugs/DB03523).

The tumorigenic potential of iMSCs has been well assessed in previous research (Harada et al., 2021; Yoshimatsu et al., 2021). These cells are typically induced from iNCCs using established protocols and fluorescent cell sorting techniques. While there is currently no evidence to suggest that hiPSCs remain contaminating iMSCs after induction, it is important to demonstrate that hiPSCs can be effectively and selectively eliminated without affecting the characteristics of iMSCs. BRQ has been identified as a chemical that may be able to achieve this goal, and we investigated its potential as a means of purifying iMSCs derived from hiPSCs. We suggest using BRQ as an additional precautionary measure to eliminate unwanted undifferentiated hiPSCs.

In conclusion, it is evident that cell proliferation can be terminated by DHODH inhibition using BRQ. Although we only tested iMSCs reactivity to DHODH inhibition, most terminally differentiated cells could fully utilize the pyrimidine salvage pathway. It has been previously reported that mouse neural stem cells and astrocyte survival are not affected by BRQ (Kondo, 2021). Further investigation of other differentiated cell types is required before this purification method can be fully adopted for hiPSC-derived regenerative cell therapy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee at CiRA, Kyoto University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZA, TK, TK, and MI contributed to the conception and design of the study. ZA created figures and wrote the manuscript. ZA and MI have

reviewed the manuscript. ZA conducted the experiments presented in Figure 1, Figure 2, Figure 3, ZA and DZ conducted the assays presented in Figures 4, 5, except for that of Figure 5C, which was conducted by DK. All authors have read and agreed to the final submitted version.

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Conflict of interest

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Supplementary material

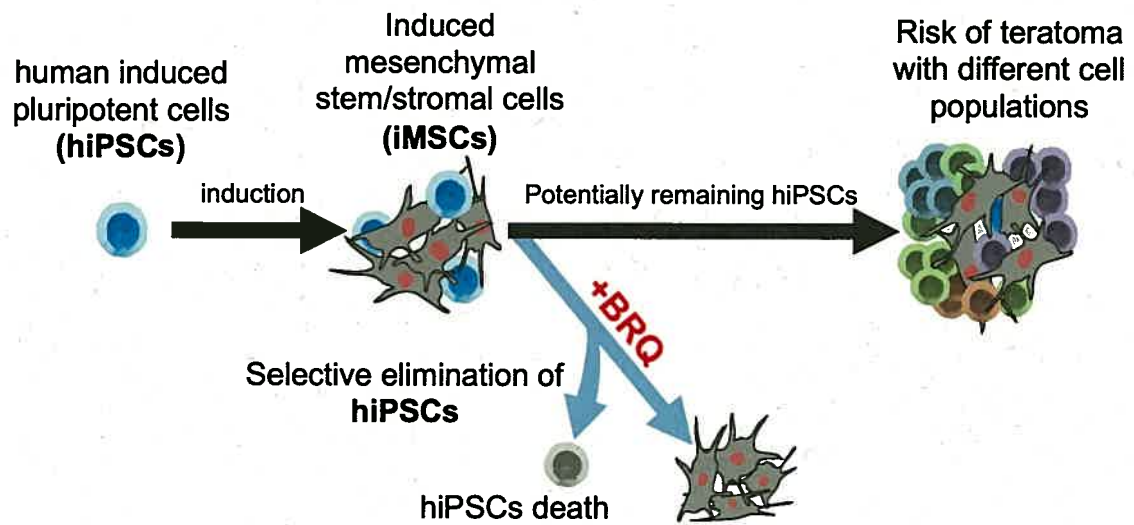
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SUPPLEMENTARY FIGURE S1

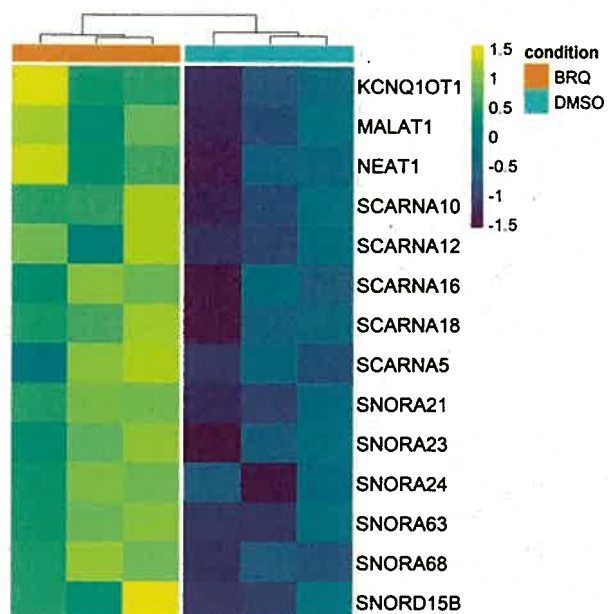
Unaltered gene expressions of BRQ treated iMSCs (A) iMSCs treated for 7 days with BRQ or DMSO. hierarchical clustering of samples of the significantly differentially expressed genes (100 nM BRQ vs. DMSO control), bright yellow color indicates higher expression of gene and dark blue for lower expression. (B) RT-qPCR of pathway markers for the 7 days BRQ-treated iMSCs

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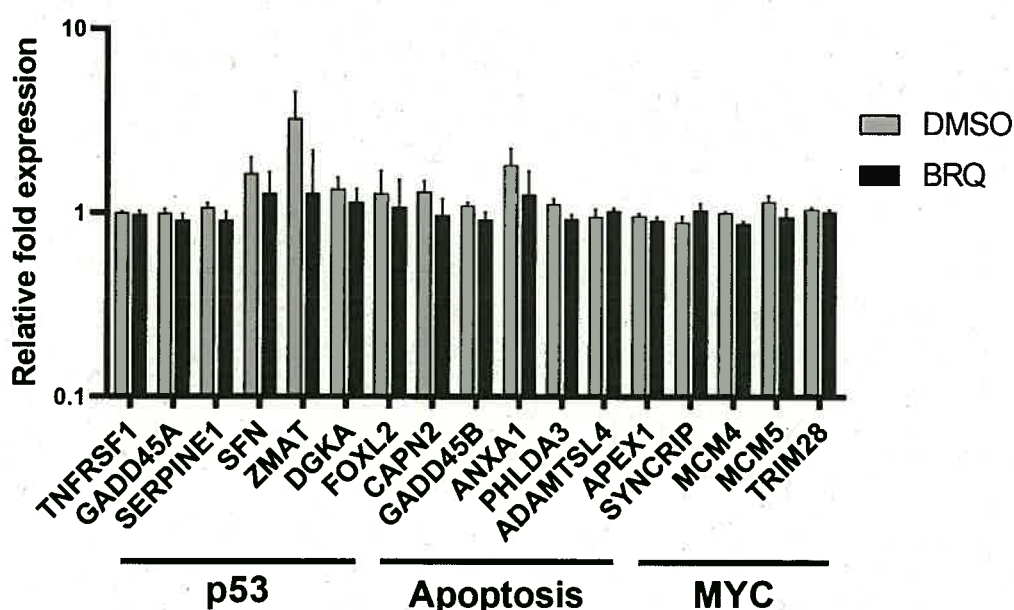
Graphical Abstract



A



B



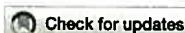
Supplementary Figure 1 |

Unaltered gene expressions of BRQ treated iMSCs (A) iMSCs treated for 7 days with BRQ or DMSO, hierarchical clustering of samples of the significantly differentially expressed genes (100 nM BRQ vs. DMSO control), bright yellow color indicates higher expression of gene and dark blue for lower expression. (B) RT-qPCR of pathway markers for the 7 days BRQ-treated iMSCs.

Supplementary Material

Genes	Forward	Reverse
ACTB	AGGTCTTTGCGGATGTCCACGT	CACCATTGGCAATGAGCGGTTC
ADAMTSL4	CAGAAGGGTTAACGGGCCAC	CGACCCTGTCCTCCAAGTTC
ANXA1	AATCCATCCTCGGATGTGCGC	TCAGTGTTTCATCCAGGGGC
APEX1	GCAGATACGGGGTTGCTCTT	TTCGGCATTCCCGTTACGAA
CAPN2	AGTCGCTCTCAAAGGACACG	ACCATGTTTCCCAGTTGGCT
CD105	CACTAGCCCAGGTCTCGAAGG	CTGAGGACCAGAAGCACCTC
CD44	GCAATGCTTCTCAGACCACA	GAGGGGAGAGGGTAGACAGG
CD73	GCCGCTTTAGAGAATGCAAC	CTCGACACTTGGTGCAAAGA
CD90	TGGATTAAGGATGAGGCCCG	CCTAAGTCACTCGCCATCCC
DGKA	GAAGAGGTCCAAGCAACGGA	TTGCAGCTGGGCAAAATCAC
FOXA2	TGTTTCGAGAACGGCTGCTAC	CCCCGAGTTGAGCCTGTGAG
FOXL2	GCGAAGTTCCCGTTCTACGA	CTCGTTGAGGCTGAGGTTGT
GADD45A	TTTTGCCGGGAAAGTCGCTA	AGATGCCATCACCGTTCAGG
GADD45B	CCTGCAAATCCACTTCACGC	GTGTGAGGGTTCTGTACCAG
IDO1	GCCAGCTTCGAGAAAGAGTTG	ATCCCAGAACTAGACGTGCAA
MCM4	CTCCGAGCACTATGTCGTCC	GTGGAATCCTCGCCTCTACG
MCM5	ATCGAGAAGCAGCTCAAGCG	ACTTGAGGCGGTAGAGAACC
PAX6	CAGGAAGGAGGGGGAGAGAA	CTTTGCAGCTTCCGCTTCAG
PD-L1	TGGCATTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT
PHLDA3	CCCTCGTGTCTTAAACCACC	AGCCAGAGGGAACAACGAAG
POU5F1	GCAAAGCAGAAACCCTCGTG	GATCTGCTGCAGTGTGGGT
SERPINE1	AGAGCGCTGTCAAGAAGACC	AGTTCTCAGAGGTGCCTTGC
SFN	CCACTACGAGATCGCCAACA	CGTCCACAGTGTGAGGTTGT
SMA	TCCGGAGCGCAAATACTCTG	CCCGGCTTCATCGTATTCCT
SYNCRIP	GCGGTAGAGCCGGTTATTCA	TTTGTTGTTGGGCACCTCCT
TNFRSF10B	CTGCGCCACAAAATACACC	GTTCTGTCCCCGTTGTCCA
TRIM28	GAGGAAGAGGGACGGATTGC	CAGTGGGGTATGCGAACCTT
ZMAT3	TATCGAAGGGAGGGGAGCAA	GGAGGGACTGGAACAACCTGG

Supplementary Table 1 | Table of forward and reverse primers used to detect the gene expression in alphabetical order.



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Enhanced chondrogenic differentiation of iPS cell-derived mesenchymal stem/stromal cells via neural crest cell induction for hyaline cartilage repair

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Background: To date, there is no effective long-lasting treatment for cartilage tissue repair. Primary chondrocytes and mesenchymal stem/stromal cells are the most commonly used cell sources in regenerative medicine. However, both cell types have limitations, such as dedifferentiation, donor morbidity, and limited expansion. Here, we report a stepwise differentiation method to generate matrix-rich cartilage spheroids from induced pluripotent stem cell-derived mesenchymal stem/stromal cells (iMSCs) via the induction of neural crest cells under xeno-free conditions.

Methods: The genes and signaling pathways regulating the chondrogenic susceptibility of iMSCs generated under different conditions were studied. Enhanced chondrogenic differentiation was achieved using a combination of growth factors and small-molecule inducers.

Results: We demonstrated that the use of a thienopyridine derivative, TD-198946, synergistically improves chondrogenesis in iMSCs. The proposed strategy produced controlled-size spheroids and increased cartilage extracellular matrix production with no signs of dedifferentiation, fibrotic cartilage formation, or hypertrophy *in vivo*.

Conclusion: These findings provide a novel cell source for stem cell-based cartilage repair. Furthermore, since chondrogenic spheroids have the potential to fuse within a few days, they can be used as building blocks for biofabrication of larger cartilage tissues using technologies such as the Zenan Bioprinting method.

Abbreviations: BMPs, bone morphogenetic proteins (BMPs); BM-MSCs, bone marrow-derived MSCs (BM-MSCs); Dex, dexamethasone (Dex); ECM, extracellular matrix (ECM); DEGs, differentially expressed genes (DEGs); iMSCs, induced pluripotent stem cell-derived mesenchymal stem/stromal cells (iMSCs); iPSCs, induced pluripotent stem cells (iPSCs); MSCs, mesenchymal stromal/stem cells (MSCs); iNCC, iPSC-derived neural crest-like cells (iNCC); TGF- β , Transforming growth factor- β (TGF- β); RNAseq, RNA sequencing (RNAseq); TD, TD-198946 (TD); GAGs, glycosaminoglycans (GAGs).

KEYWORDS

mesenchymal stem/stromal cells, induced mesenchymal stem/stromal cells, induced pluripotent stem cells, chondrogenesis, cartilage, chondrocytes, tissue engineering, cell-based therapy

1 Introduction

Cartilage tissue has limited self-healing capacity owing to the lack of blood vessels and insufficient blood supply necessary to promote cell proliferation and differentiation *in situ* (Ulrich-Vinther et al., 2003; Sophia Fox et al., 2009). Therefore, damaged cartilage is difficult to repair, and sound long-term therapeutic effects have not yet been obtained. Cell-based therapies have been explored as alternative approaches for treating cartilage defects. Primary chondrocytes and mesenchymal stromal/stem cells (MSCs) isolated from adult tissues are the most common cell sources used in regenerative medicine (Mardones et al., 2015). However, both cell types have limitations such as dedifferentiation, donor morbidity, and limited expansion, resulting in heterogeneous and inconsistent cell products and poor clinical outcomes.

Induced pluripotent stem cells (iPSCs) are promising alternatives to generate a large number of chondrogenic precursors for cell therapy (Boreström et al., 2014). The advantages of using iPSC-derived mesenchymal stromal/stem-like cells (iMSCs) over adult tissue-derived MSCs include extensive cell expansion *ex vivo* and the elimination of invasive biopsies. In addition, iMSCs can be genetically modified to increase their differentiation potential, reduce their immunogenicity, and introduce patient-specific mutations for research purposes and disease modelling (Zhao and Ikeya, 2018).

Chondrogenesis is a sequential process that occurs mainly during the embryonic stages. Chondrocytes are derived from mesenchymal precursors originating from the paraxial mesoderm, lateral plate mesoderm, and neural crest. The embryonic nature of iPSCs provides the opportunity to recapitulate the developmental path of chondrocyte differentiation. Several protocols have been established to generate chondrocytes and cartilage-like tissues from iMSCs (Umeda et al., 2012; Koyama et al., 2013; Rodríguez Ruiz et al., 2021; Rodríguez Ruiz et al., 2021).

Our team as well as others have successfully established strategies to generate iPSC-derived neural crest-like cells (iNCC) as an intermediate source of iMSCs (Lee et al., 2010; Menendez et al., 2011; Fukuta et al., 2014; Umeda et al., 2015; Umeda et al., 2015; Kamiya et al., 2022; Kamiya et al., 2022). We have previously shown that the resulting iMSCs are highly expandable, cryogenically preservable, and can differentiate into osteoblasts, chondrocytes, and adipocytes (Fukuta et al., 2014; Zhao and Ikeya, 2018). We have extensively explored the use of iMSCs in drug discovery and disease modeling (Zhao and Ikeya, 2018; Nakajima and Ikeya, 2019). Our efforts to generate cells for regenerative medicine prompted us to translate our protocol into a xeno-free system for generating functional iMSCs (Kamiya et al., 2022). We have demonstrated the *in vivo* therapeutic effect of iMSCs on musculoskeletal tissues, including muscle, bone (Kamiya et al., 2022), and laryngeal cartilage (Yoshimatsu et al., 2021).

Here, we attempted to extend the applications of iMSCs to regenerative medicine and tissue engineering by generating high-quality cartilage spheroids *in vitro*. Because successful neocartilage

formation may depend on both the susceptibility of the precursor cells to chondrogenesis and the proper manipulation of the signaling pathways involved in chondrocyte commitment, our approach included screening the chondrogenic potential of two types of iMSCs and optimizing the chondrocyte differentiation strategy. Particularly, we propose the use of TD-198946 (TD), a small molecule used to enhance chondrogenic differentiation of various human progenitor cells, including BM-MSCs (Yano et al., 2013b), synovium-derived stem cells (Chijimatsu et al., 2019; Kobayashi et al., 2020), and nucleus pulposus cells (Kushioka et al., 2020).

2 Results

2.1 iMSCs display different differentiation potential towards chondrogenesis

To establish an efficient differentiation strategy for generating cartilage-like tissue from iMSCs, we first studied the characteristics and differentiation potential of iMSCs generated from the iNCC lineage under different conditions. Cryopreserved iNCCs were differentiated and expanded using our previously established protocol (Kamiya et al., 2022) and were further differentiated into iMSCs using two types of xeno-free culture media, XSF and T1 media (Figure 1A). After three passages, iMSCs differentiated with T1 (T1-iMSCs) or XSF (XSF-iMSCs) were analyzed. The NCC markers *SOX10* and *NGFR* were downregulated, while the typical MSC markers (*CD44*, *CD73*, *CD90*, and *CD105*) were upregulated in both types of iMSCs compared to the iNCCs, and no significant differences were found between T1-iMSCs and XSF-iMSCs for any of the markers analyzed (Figure 1B). Flow cytometry analysis also confirmed that nearly all of the T1- and XSF-iMSCs expressed MSC marker proteins (*CD44*, *CD73*, *CD90*, and *CD105*) at comparable levels (Figure 1C). Next, we examined the three-lineage differentiation potential of the iMSCs. Human bone marrow-derived MSCs (BM-MSCs) were also differentiated as a reference. T1- and XSF-iMSCs were able to differentiate into chondrocytes, osteoblasts, and adipocytes (Figures 1D–F). However, T1-iMSCs showed superior chondrogenic potential compared to XSF-iMSCs and BM-MSCs, as indicated by increased deposition of sulfated proteoglycans (as assessed by Alcian blue staining) and significant upregulation of the chondrogenic markers *COL2A1*, and *ACAN* with reduced expression of *COL1A1* (Figure 1D). Conversely, T1-iMSCs displayed decreased extracellular matrix (ECM) calcification and expression of the osteoblast master transcription factor *RUNX2* compared with XSF-iMSCs during osteogenic induction (Figure 1E). The osteogenic culture of BM-MSCs showed high expression of the osteoblast marker *SP7* but decreased expression of *BGLAP*, a *bona fide* marker for mature osteoblasts. This observation could potentially account for the extensive alizarin red staining, but fewer dark calcified areas when comparing to iMSC osteogenic cultures. When subjected to adipogenic differentiation, T1-iMSCs synthesized fewer lipid vacuoles and showed decreased mRNA

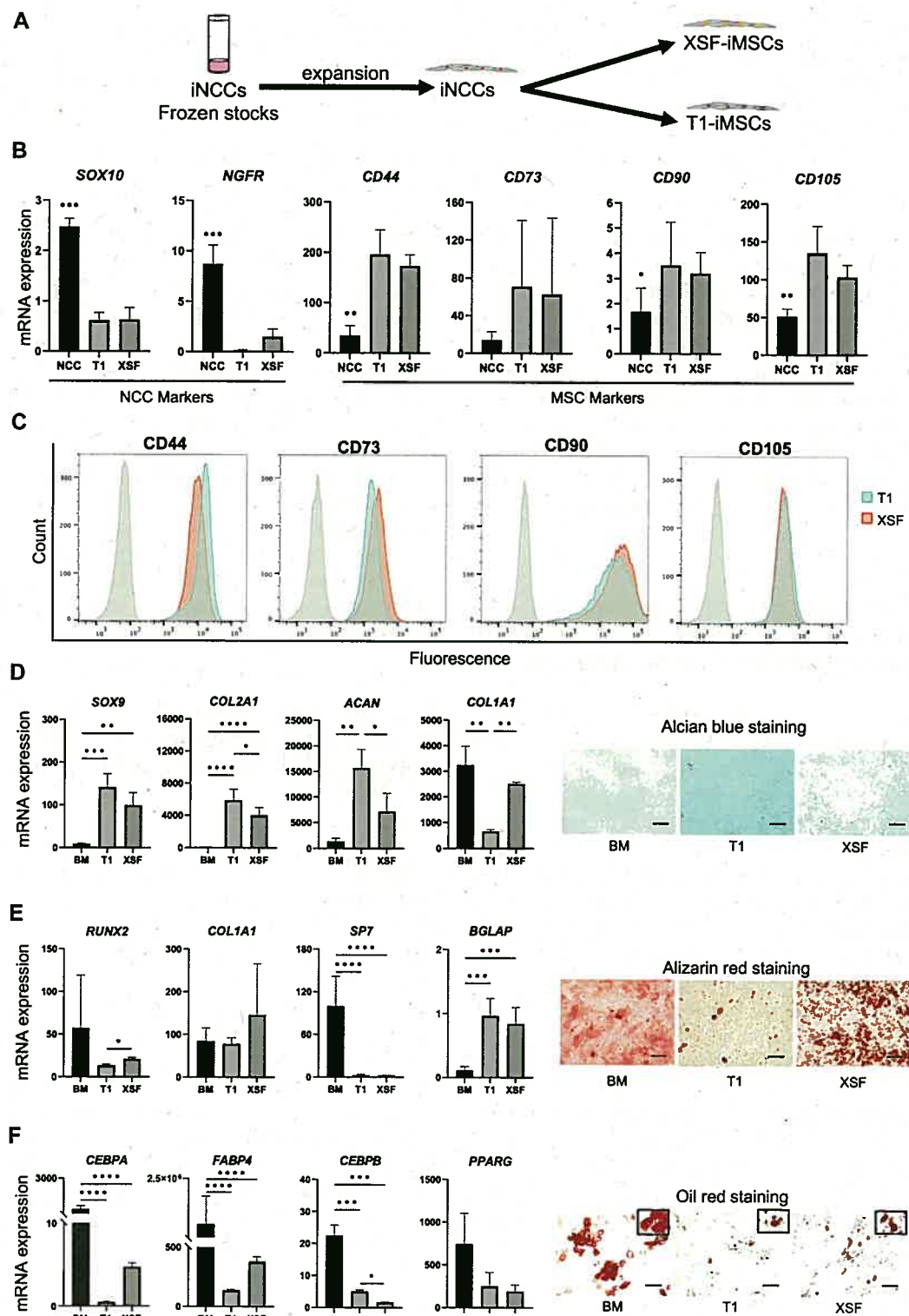


FIGURE 1

Characterization of iMSCs generated from expanded iNCCs with two culture media under xeno-free conditions (A) Schematic diagram of the induction protocol for iMSC differentiation from iNCCs (B) mRNA expression of NCC and MSC markers determined by RT-qPCR in iMSCs generated using T1 and XSF media. Data is presented as folds relative to iPSCs, and are the means $\pm$ SD from four independent experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$ in NCC vs. all others. No significant difference was found in T1 vs. XSF for any marker (C) Flow cytometry analysis of the distinctive MSC markers in the iMSCs generated using T1 and XSF media (D) Characterization of BM-MSCs, T1-iMSCs and XSF-iMSCs differentiated to chondrogenic cells in micromass culture. mRNA expression levels of chondrocyte markers (left) and representative pictures of alcian blue staining (right) (Continued)

FIGURE 1 (Continued)

are shown, scale bar: 100 μ m. (E) Characterization of BM-MSCs, T1-iMSCs and XSF-iMSCs differentiated to osteogenic cells at 3 weeks. mRNA expression levels of osteoblast markers (left) and representative pictures of alizarin red staining (right) are shown, scale bar: 100 μ m. (F) Characterization of BM-MSCs, T1-iMSCs and XSF-iMSCs differentiated to adipogenic cells at 3 weeks. mRNA expression levels of adipocyte markers (left) and representative pictures of Oil Red O staining (right) are shown, scale bar: 100 μ m. RT-qPCR data (D), (E) and (F) is presented as folds relative to iPSCs, and are the means $\pm$ SD of three independent experiments ($n = 3$) for iMSCs and one experiment for BM-MSCs ($n = 6$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$, and **** $p \leq 0.0001$ as indicated.

expression of the adipogenic markers *CEBPA* and *FABP4* compared with XSF-iMSCs (Figure 1F). In contrast, the early adipogenic marker *CEBPA* was significantly upregulated in T1-iMSCs. BM-MSCs showed remarkable adipogenic potential compared to iMSCs as evidenced by greater lipid production and the upregulation of the major adipogenic markers. These data suggest that although the iMSCs were derived from common progenitor cells and showed indistinguishable characteristics by conventional characterization methods, the differentiation potential was differently modulated by the medium composition during iMSCs differentiation. Notably, T1-iMSCs selectively gained a greater chondrogenic potential during expansion.

2.2 Transforming growth factor- β (TGF- β) signaling pathway positively correlates with iMSC susceptibility to chondrogenesis

To elucidate the differences between iMSCs leading to improved chondrogenesis, we performed a comparative global gene expression analysis of parental iPSCs, iNCCs, T1-iMSCs, and XSF-iMSCs using next-generation RNA sequencing (RNAseq). Figure 2A shows a heatmap of the normalized counts, highlighting the transcriptomic differences between the samples. Five clusters were identified by k-means clustering. Cluster 1 included genes that were specifically upregulated in iPSCs, while cluster 2 comprised genes upregulated only in iNCCs. Cluster 3 showed genes that were upregulated in both iMSCs but not in iNCCs or iPSCs. Importantly, cluster 4 & cluster 5 revealed a different set of upregulated genes in XSF-iMSC and T1-iMSC, respectively. To further investigate these differences, we performed a differential gene expression analysis. Figure 2B shows volcano plots of the significantly differentially expressed genes (DEGs) in T1-iMSCs vs. iPSCs (left panel), XSF-iMSCs vs. iPSCs (middle panel), and T1-iMSCs vs. XSF-iMSCs (right panel) with p -value ≤ 0.05 . To further narrow down our analysis, we restricted the threshold to log2 fold-change greater than 1.5 with p -value ≤ 0.05 (red dots in Figure 2B). We then extracted the list of DEGs upregulated in T1-iMSCs vs. iPSCs (log2 fold-change ≥ 1.5) and performed a pathway analysis using EnrichR (Chen et al., 2013; Kulshov et al., 2016; Xie et al., 2021), a gene set search engine. The same procedure was followed for the XSF-iMSC versus iPSCs dataset. Figure 2C shows a plot of the top five enriched pathways by the combined score obtained from two gene set libraries, BioPlanet (Huang et al., 2019) and Panther (Thomas et al., 2022). Results from both libraries showed enrichment of TGF β -related pathways known to play a key role in chondrogenic differentiation of MSCs. Notably, T1-iMSCs showed greater enrichment scores for these terms compared to XSF-iMSCs. Enrichment of integrin-related signaling pathways, recently

proposed to regulate various chondrocyte functions such as differentiation and matrix production (Knudson and Loeser, 2002), was also found to be superior in T1-iMSCs.

Therefore, we performed a pathway enrichment analysis of DEGs upregulated in T1-iMSCs versus XSF-iMSCs. Consistently, the TGF- β and integrin signaling pathways were among the top 10 enriched terms by the combined score (Supplementary Table S1). Given the relevance of TGF- β in chondrogenesis, we decided to confirm the results by extracting the overlapping genes with the TGF- β pathway and evaluating mRNA expression in T1-iMSCs and XSF-iMSCs. Figure 2D shows that out of the ten identified genes, eight were upregulated in T1-iMSCs. Overall, these data suggest that the susceptibility of T1-iMSCs to chondrogenesis may be mediated, at least partially, through the TGF- β signaling pathway. The integrin signaling pathway may also contribute to these findings.

2.3 The small molecule TD-198946 and a stepwise differentiation strategy widely enhances the chondrogenic phenotype in T1-iMSCs

To generate an efficient chondrogenic differentiation method, we first examined whether the thienopyridone derivative TD-198946 (TD) could enhance chondrogenesis and synthesis of cartilage ECM. T1-iMSCs were treated with different concentrations of TD (1, 10, and 100 nM) in micromass cultures. Figure 3A shows that T1-iMSCs treated with TD at 100 nM showed increased expression of *SOX9*, *ACAN*, and *COL2A1* compared to all other concentrations tested, with no significant effect on *COL1A1* expression. Alcian blue staining also showed increased sulfated ECM deposition following TD treatment in a concentration-dependent manner. Given the positive effect of TD on chondrogenesis, we investigated whether the addition of TD (100 nM) during chondrogenic differentiation may alter the observed differences between T1-iMSCs and XSF-iMSCs. The chondrogenic potential of T1-iMSCs remained higher even in the presence of TD (Supplementary Figure S1).

Next, we examined whether TD added to chondrogenic medium could enhance differentiation of T1-iMSCs in 3D spheroid cultures in the presence BMP4 known for its potent chondrogenic effect (Miljkovic et al., 2008). Spheroids treated with 100 nM showed enhanced production of sulfated proteoglycans and glycosaminoglycans (GAGs) compared to untreated ones, as indicated by a quantitative dye-binding assay and Alcian blue staining (Figure 3B).

Chondrogenic differentiation of MSCs is traditionally performed by sustained stimulation with factors such as TGF- β and bone morphogenetic proteins (BMPs). Recently, stepwise

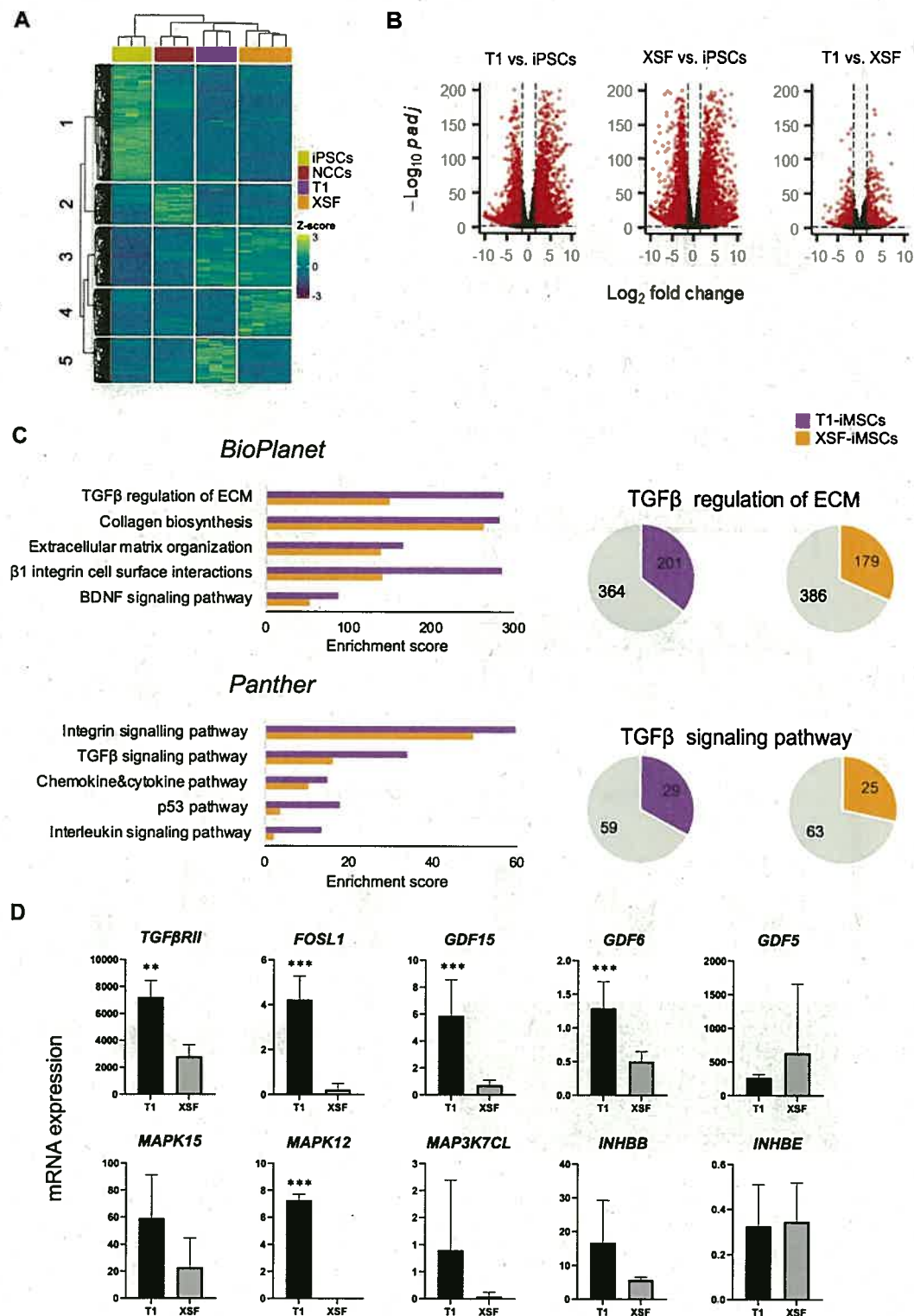
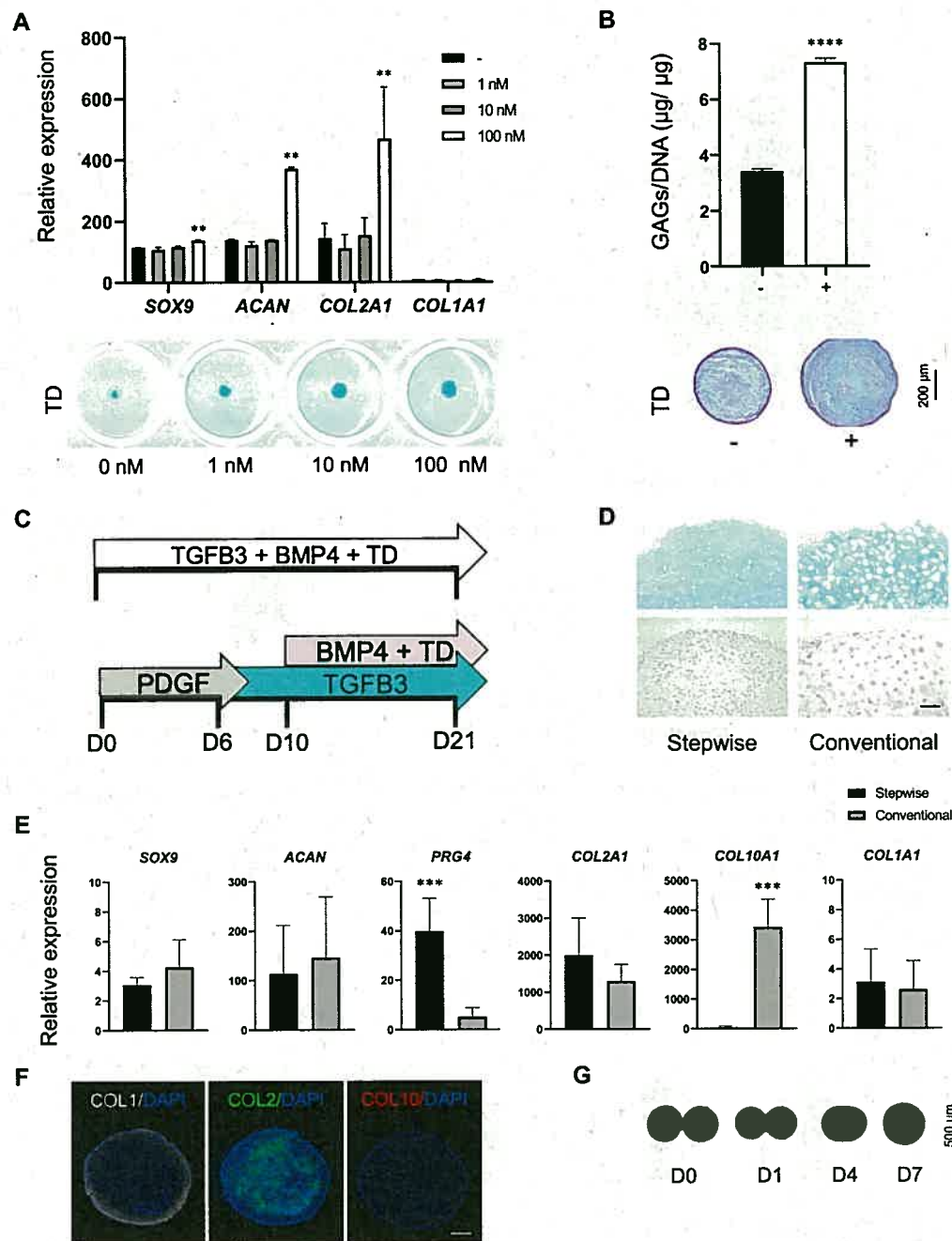
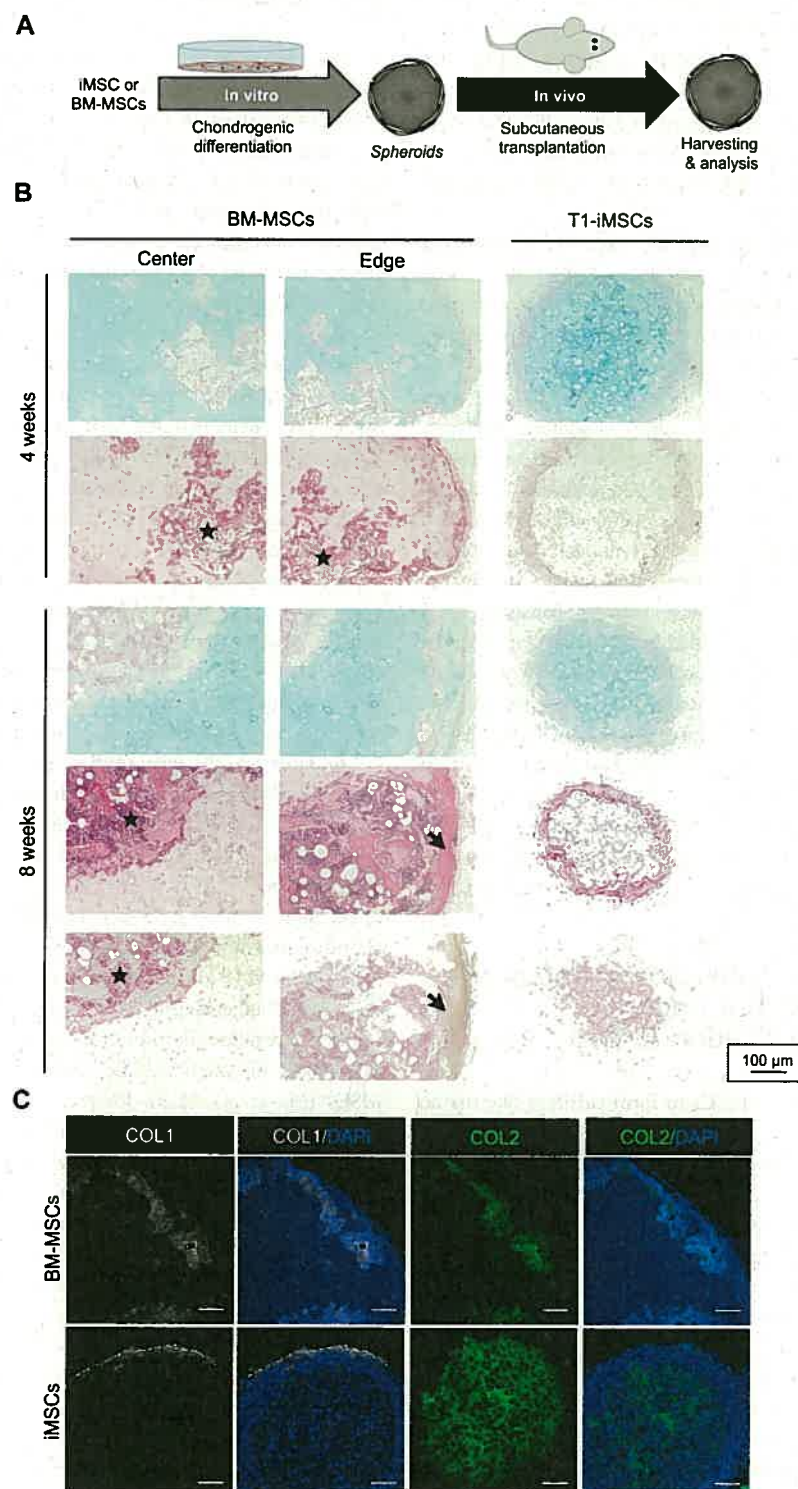


FIGURE 2

Global gene expression profile of iMSCs and parental iPSCs (A) Heatmap illustrating the count data of the RNAseq results and k-means clustering of the parental iPSCs ($n = 3$), iNCCs ($n = 3$), T1-iMSCs ($n = 3$) and XSF-iMSCs ($n = 4$). (B) Volcano plots of the differentially expressed genes as indicated. Red color dots show DEGs ($p \leq 0.05$) that are transcriptionally up- or down-regulated ($\log_2$ fold change ≥ 1.5 or ≤ -1.5 respectively). (C) Top five enriched pathways in T1-iMSCs versus iPSCs (T1), and XSF-iMSCs versus iPSCs (XSF) (left) as well as graphic representation of the overlapped genes in selected pathways. (D) The mRNA expression of identified genes from the TGF- β pathway in T1-iMSCs (T1) and XSF-iMSCs (XSF) determined by RT-qPCR. Data represent the means $\pm$ SD of three independent experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.005$ as indicated.

**FIGURE 3**

Optimization of the chondrogenic differentiation strategy (A) mRNA expression levels of chondrogenic markers in T1-iMSCs subjected to chondrogenic differentiation using different concentrations of TD determined by RT-qPCR and representative images of Alcian blue staining. (B) Quantification of GAG content ($n = 4$) and representative images of T1-iMSC chondrogenic spheroids stained with Alcian blue on day 21, differentiated in the presence or absence of TD (100 nM). (C) Schematic diagram of the differentiation strategies for the induction of chondrogenesis. (D) Histological analysis of chondrogenic spheroids on day 21 generated from T1-iMSCs using different strategies. H&E staining (upper panel) and Alcian blue staining (lower panel) is shown. Scale bar: 50 μm . (E) mRNA expression levels of chondrogenic markers determined by RT-qPCR in T1-iMSCs subjected to chondrogenic differentiation using different strategies. (F) Immunostaining of chondrogenic spheroids for COL1, COL2, and COL10 on day 21 differentiated using the stepwise strategy from T1-iMSCs. Scale bar: 100 μm . (G) Representative images of the fusion process of two chondrogenic spheroids. mRNA data represent the means $\pm$ SD of three independent experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ as indicated.

**FIGURE 4**

Subcutaneous transplantation of chondrogenic spheroids (A) Schematic diagram of the experimental procedure (B) Histological analysis of the retrieved spheroids after four (upper panel) and eight (lower panel) weeks. Representative pictures of Alcian blue staining (top), H&E staining (middle), and von Kossa staining (bottom). Stars represent potential cell infiltration of the host cells and arrows indicate calcified areas (C) Immunostaining of COL1 and COL2 in the retrieved BM-MSC and T1-iMSC chondrogenic spheroids at 8 weeks. Scale bar: 100 μ m.

differentiation strategies have emerged (Nakayama et al., 2003; Umeda et al., 2012). Therefore, we aimed to compare the chondrogenic phenotype of T1-iMSCs cultured using the conventional protocol *versus* a stepwise differentiation strategy in the presence of 100 nM TD, as depicted in Figure 3C. The small molecule TD was added during the BMP4 stimulation period, as previous reports have shown that TD acts in a BMP-dependent manner (Supplementary Figure S2).

Histological analysis revealed that the stepwise differentiated spheroids showed richer sulfated proteoglycans ECM than those differentiated using the conventional method (Figure 3D). Increased cell size, a characteristic commonly observed in hypertrophic chondrocytes, was detected in spheroids produced via the conventional method. SOX9, ACAN, COL2A1, and COL1A1 were expressed at similar levels under both culture conditions. However, PRG4, a marker expressed exclusively in the superficial zone of articular cartilage, was upregulated in the differentiated spheroids using the stepwise strategy, whereas COL10A1, a marker for hypertrophic chondrocytes, was upregulated by the conventional differentiation method (Figure 3E). Furthermore, immunohistochemistry in the spheroids differentiated in a stepwise manner consisted of a COL2A1-rich inner matrix surrounded by a COL1A1-rich layer, while COL10A1 was not detected (Figure 3F). In contrast, spheroids from iMSCs or BM-MSCs differentiated with the conventional method showed limited COL2A1. The expression of COL1A1 was restricted to the outer layer and COL10A1 was detected only in spheroids derived from BM-MSCs (Supplementary Figure S3). Finally, the iMSC chondrogenic spheroids generated with the optimized stepwise strategy also had the potential to fuse completely within 7 days (Figure 3G, Supplementary Video S1).

2.4 Chondrogenic spheroids maintain the phenotype and do not undergo endochondral ossification *in vivo*

Given the potential of T1-iMSCs to form cartilage-like tissues *in vitro*, we determined their ability to maintain chondrogenic phenotype *in vivo*. Following *in vitro* differentiation, the cell spheroids were subcutaneously transplanted into immunodeficient mice (Figure 4A). Chondrogenic spheroids derived from BM-MSCs were used as positive controls to study endochondral ossification. The spheroids were harvested after four and 8 weeks for further analysis. Figure 4B shows that vascular penetration was detected within the cartilage matrix in the retrieved BM-MSCs spheroids after 4 weeks. In contrast, chondrogenic spheroids from T1-iMSCs did not show cell infiltration in the host, and Alcian blue staining revealed a homogeneous cartilage-like matrix. After 8 weeks, developing bone-like tissue was clearly identified in BM-MSCs spheroids by a well-defined edge between the remaining cartilage-like tissue and the calcified tissue that was positive for von Kossa staining. The iMSC spheroids remained as cartilage, although the size decreased, and the lacunae appeared enlarged compared to those maintained *in vitro*. Unlike BM-MSC derived spheroids retrieved at 8 weeks, the spheroids from iMSCs still showed a COL2A1-rich inner matrix surrounded by a COL1A1 layer, suggesting the maintenance of stable cartilage-like tissue *in vivo* (Figure 4C).

3 Discussion

The repair of articular cartilage defects using regenerative medicine requires suitable cell sources and the establishment of effective expansion and differentiation methods. In this study, we established a stepwise differentiation strategy to generate stable matrix-rich chondrogenic spheroids from iMSCs via induction of neural crest cells under defined conditions. Our approach included the generation and selection of iMSCs with a greater potential for chondrogenesis and optimization of the differentiation strategy.

The generation and cryopreservation of cells that cannot be isolated from adult tissues constitute the unique advantage of using iPSCs for cell-based therapy and *in vitro* studies of various skeletal tissues. We demonstrated that cryopreserved iNCCs could be differentiated into functional iMSCs and expanded with selectively enhanced chondrogenic potential using a commercially available xeno-free culture medium. It is known that cell culture media and expansion conditions influence the properties of tissue-derived MSCs and can prime the cells to a specific cell type by promoting or suppressing important factors regulating lineage-specific gene expression (Baer et al., 2010; Hudson et al., 2011; Gharibi and Hughes, 2012; Liu et al., 2017; Noronha et al., 2019). Under our culture conditions, enrichment of TGF- β and integrin-related signaling are hallmarks of pro-chondrogenic T1-iMSCs.

In vitro and *in vivo* studies have shown that TGF- β plays essential roles at all stages of chondrogenesis (Li et al., 2005; Wang et al., 2014). Initially, MSC condensation, which is required for chondrogenesis, was promoted by TGF β -induced upregulation of N-cadherin and fibronectin expression (Tuli et al., 2003; Song et al., 2007). TGF- β has been shown to support chondrogenic differentiation of MSCs, partially through stimulatory activities on MAP kinases and modulation of Wnt signaling (Fischer et al., 2002; Tuli et al., 2003). In particular, p38 MAPK signaling was found to regulate chondrocyte-specific genes by mediating the interaction between TGF- β 1 and Smad1/4 molecules in BM-MSCs (Ma et al., 2019). RNAseq analysis of the DEGs in T1-iMSCs vs. XSF-iMSCs identified the p38 MAPK pathway (P05918) and the cadherin signaling pathway (P00012) within the top ten enriched terms (Supplementary Table S1). Following chondrogenesis, TGF- β signaling is also positively correlated with chondrocyte proliferation and ECM deposition, while it inhibits terminal differentiation into hypertrophic chondrocytes (Yang et al., 2001; Li et al., 2005; Wang et al., 2014). TGF- β is the only known effective inducer of chondrogenic activity in cultured MSCs (Somoza et al., 2018). For instance, a brief pretreatment with TGF- β increased the precartilaginous condensing capacity of ectomesenchymal cells generated from iNCCs expanded under conditions similar to ours (Umeda et al., 2015). Integrins are transmembrane cell surface receptors that interact with the ECM and regulate cell functions including adhesion, migration, and differentiation. Integrin-mediated mechanisms can stimulate intracellular signal transduction in MSCs. The transition from collagen type I to type II is correlated with a switch from α 1 integrin to α 3 integrin during MSC chondrogenic differentiation (Shakibaei et al., 1995). Likewise, deregulation of integrin signaling is associated with osteoarthritis (Knudson and Loeser, 2002). Transcriptome-wide analyses of high-quality articular-like cartilage

pellets derived from human neonatal articular cartilage cells showed strong enrichment of integrin pathways compared to chondrogenic MSC-derived pellets with limited chondrogenic potential (Somoza et al., 2018). Furthermore, growing evidence demonstrates crosstalk between the integrin and TGF- β pathways in cartilage (Munger and Sheppard, 2011).

According to our analysis, it is likely that soluble factors present in T1 medium can stimulate both TGF- β and integrin signaling. It is possible that TGF- β signaling is activated indirectly by an integrin-mediated pathway. It is known that TGF- β must be released from its latent complex to interact with its cell surface receptors, and integrins are central to this process in at least two of the three TGF- β isoforms. We also observed significant upregulation of TGF β RII in T1-iMSCs compared to that in XSF-iMSCs, suggesting that TGF- β induces rapid translocation of its own receptors to the cell surface, thus amplifying its own response as shown in previous report (Duan and Derynck, 2019). Expression of GDF15 and GDF6 but not GDF5 was higher in T1-iMSCs compared to XSF-iMSCs. The effect of GDFs, and their functions on chondrogenesis are still largely unknown compared with other members of TGF-beta super family. Functional differences among GDFs should be also further studied. For instance, GDF6 stimulation of MSCs resulted in a significant increase in a higher aggrecan and collagen type II gene expression, and higher GAG production compared with TGF- β or GDF5 stimulation (Clarke et al., 2014). Therefore, several aspects of the nature of T1 medium stimulation remain unclear and will be the subject of future investigation in our laboratory.

One limitation of this study is that the composition of T1 and XSF media is largely unknown; it was disclosed that T1 medium contains dexamethasone (Dex) at 90 nM concentration along with 0.2 μ g/ml recombinant human Laminin-511 E8 fragment (iMatrix). Although Dex is a common chemical factor used for the differentiation of MSCs, lineage specification is highly dependent on the concentration used and other specific culture conditions. For example, Dex can promote adipocyte differentiation by upregulating *C/EBP α* expression but inhibits adipogenesis via *RUNX2* (Zhou et al., 2019). Dex used at concentrations below 10 nM promotes chondrogenesis in human synovial MSCs but attenuates chondrogenesis at concentrations higher than 100 nM (Chijimatsu et al., 2018). Moreover, low-dose Dex treatment (10^{-8} M) preserves the stemness of expanded human MSCs (Xiao et al., 2010). In contrast, laminins promote adhesion and expansion of various cells, including embryonic stem cells, iPSCs, and MSCs (Miyazaki et al., 2012). Further studies have shown that laminins participate in chondrogenic differentiation by upregulating *COL2A1* expression in human chondrogenic progenitor cells and GAG content in human MSCs (Schminke et al., 2016; Sun et al., 2017). Although the exact mechanisms by which laminins influence stem cells are complex, they involve interactions with integrins, which can bind with broad specificity and high affinities. In particular, laminin-511 exhibited the highest affinity for $\alpha 6$ integrins (ITGA6). Notably, *ITGA6* was upregulated among the DEGs in T1-iMSCs versus XSF-iMSCs.

Protocols to induce chondrogenesis in MSCs *in vitro* coincide in two aspects: high cell density (pellets or micromasses) and stimulation with TGF- β 1 or TGF- β 3. We hypothesized that T1-iMSCs with greater susceptibility to integrin and TGF- β signaling

account for their increased chondrogenic potential when subjected to further differentiation. Moreover, our results suggest that the expansion and generation of iMSCs in the presence of Dex, and iMatrix may be a powerful approach for generating an MSC population with enhanced chondrogenic potential.

TD has been shown to be a chondrogenic factor as potent as insulin, BMP2, and TGF- β 1 (Saito et al., 2013). Administration of TD also induces cartilage regeneration in murine models of osteoarthritis (Yano et al., 2013a) and intervertebral disc degeneration (Chijimatsu et al., 2019) *in vivo*. Here, we show for the first time the chondrogenic potential of TD on iPSC-derived MSCs. Although the exact mechanism by which TD supports chondrogenesis is unclear, TD has been reported to upregulate *Runx1*, *Sox9*, and *Col2a1* (Yano et al., 2013a; Hamamoto et al., 2020). T1-iMSCs treated with 100 nM TD showed upregulation of *SOX9*, *ACAN*, and *COL2A1* as well as increased GAG production without a noticeable effect on hypertrophy. Consistent with previous reports, TD and BMP stimulation had a positive synergistic effect on chondrogenesis. These findings could potentially contribute to the use of TD in regenerative medicine using iPSC-derived chondrogenic progenitors. Finally, we demonstrated that more relevant protocols are required to efficiently induce chondrogenesis in 3D aggregates. In particular, the traditional method of continuously stimulating cells with chondrogenic factors such as TGF- β s and BMPs often produces hypertrophic phenotypes and a less rich cartilage matrix. In contrast, the stepwise differentiation approach with a sequential transition from platelet-derived growth factor to TGF- β 3 and BMP4 (Umeda et al., 2012) in combination with TD molecules, formed spheroids with stable cartilage-like tissue that was resistant to vascular invasion and calcification *in vivo*. Given the potential of the generated spheroids to fuse within a few days, our method is compatible with modern tools for tissue engineering such as bioprinting using the Kenzan method (Nakayama, 2013; Nakamura et al., 2021).

Overall, this study presents a novel method for producing and expanding clinically relevant pro-chondrogenic iMSCs as well as an improved chondrogenic differentiation strategy for the stable generation of cartilage-like tissue. The insights provided here will help identify and select stem cell progenitors with superior chondrogenic potential for regenerative medicine and cartilage tissue engineering.

4 Methods

4.1 Generation, expansion, and cryopreservation of iNCCs from iPSCs under xeno-free conditions

Human iPSCs 1231A3 reprogrammed with episomal vectors (kindly provided by Yamanaka Laboratory) were maintained as described previously (Nakagawa et al., 2014). For NCC induction, 3×10^4 cells/well were seeded in 6-well plates coated with iMatrix-511 (Nippi, Tokyo, Japan) in StemFit AK03N medium (Ajinomoto, Tokyo, Japan). After 4 days, the medium was replaced with NCC induction medium containing 10 μ M SB431542 (FUJIFILM Wako Pure Chemical Corporation, Japan) and 1 μ M CHIR99021 (Axon Medchem, Reston, VA, United States), as described previously

(Kamiya et al., 2022). On day 10, CD271high positive cells were sorted and replated on fibronectin-coated plates at a density of 1×10^4 cells/cm² in NCC expansion medium: Basic03 medium supplemented with 10 μ M SB431542, 20 ng/ml EGF (FUJIFILM Wako Pure Chemical Corp.), and 20 ng/ml FGF2 (FUJIFILM Wako Pure Chemical Corp.). The medium was changed every 2–3 days. For cell passage, the cells were dissociated with Accutase (Innovative Cell Technologies, San Diego, CA, United States). iNCC stocks were prepared using STEM-CELLBANKER GMP grade (Takara Bio Inc., Kusatsu, Japan).

4.2 Differentiation of cryopreserved iNCCs into iMSCs under xeno-free conditions

iNCCs from frozen stocks at passage number (PN2) were cultured and expanded to PN5 in fibronectin-coated plates. Then, the medium was replaced with either PRIME-XV MSC Expansion XSFM (FUJIFILM Irvine Scientific, Tokyo, Japan) to generate XSF-iMSCs or T1 medium composed of StemFit For MSC (Ajinomoto, Tokyo, Japan) supplemented with 90 nM Dex and 0.2 μ g/ml iMatrix-511 to generate T1-iMSCs. Cells were transferred to fibronectin-coated plates at a density of 1×10^4 cells/cm² up to seven times before further differentiation. iMSCs stocks in PN2 were prepared using the STEM-CELLBANKER GMP grade.

4.3 Real-time quantitative PCR analysis

Total RNA was purified using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and reverse-transcribed to cDNA. Real-time quantitative PCR (RT-qPCR) was performed using THUNDERBIRD™ Next SYBR® qPCR mix (QPX-201; Toyobo Co., Ltd.), QuantStudio™ 3 Real-Time PCR System, and QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). Primer sequences are summarized in [Supplementary Table S2](#). Data from at least three biological replicates were analyzed to calculate the relative fold-change ($2^{-\Delta\Delta C_T}$). All data were plotted as fold change relative to iPSCs using GraphPad Prism 9 software.

4.4 Flow cytometry analysis

T1-iMSCs and XSF-iMSCs (PN3–PN4) were stained on ice for 30 min with the following antibodies (1:50 dilution in FACS buffer): CD105-APC (eBioscience, 17-1057-42), CD90-PE (eBioscience, 555596), CD73-PE (eBioscience, 550257), CD44-PE (eBioscience, 550989), CD105APC (eBioscience, 17-1057-42), PE-isotype (BD Bioscience, 551438), and APC isotype (BD Bioscience, 565381). After washing, BD FACSAria™ III Cell Sorter was used to detect the fluorescence. The results were plotted using FlowJo_v10.8.1.

4.5 Three lineage differentiation of iMSCs

The same procedure was followed for the differentiation of T1-iMSCs and XSF-iMSCs (collectively called iMSCs), and all

experiments were carried out in parallel with cells at the same passage number (PN4–PN7). As a reference, human BM-MSCs (PT-2501, (Batch 20TL262529; Lonza, Durham, NC, United States) at PN5 were also differentiated under the same conditions. Chondrogenic differentiation was performed using micromass culture onto fibronectin-coated 24-well plates. Briefly, 1.5×10^5 iMSCs were resuspended in 5 μ L of chondrogenic medium consisting of DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, United States), 1% (v/v) ITS + premix (Corning, Corning, NY, United States), 0.17 mM AA2P (Sigma-Aldrich, St. Louis, MO, United States), 0.35 mM Proline (Sigma-Aldrich), 0.1 mM Dex (Sigma-Aldrich), 0.15% (v/v) glucose (Sigma-Aldrich), 1 mM sodium-pyruvate (Thermo Fisher Scientific), and 2 mM GlutaMAX (Thermo Fisher Scientific) supplemented with 10 ng/ml TGF- β 3 (R&D Systems, Minneapolis, MN, United States). BMP7, commonly used for 2D chondrogenic cultures (Caron et al., 2013), was also added to the differentiation medium at 50 ng/ml (R&D Systems). After 1 h incubation, 1 ml of chondrogenic medium was added to each well and the cells were cultured for 7 days. Chondrogenesis was assessed by Alcian blue staining. Briefly, induced cells were fixed for 30 min with 4% paraformaldehyde (PFA) (FUJIFILM Wako Pure Chemicals Corp.) and rinsed with phosphate buffered saline. The cells were then incubated with 1% Alcian Blue solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 1 h at room temperature and washed five times with phosphate buffered saline before imaging. For osteogenic differentiation, 5×10^4 iMSCs were seeded onto gelatin-coated wells, maintained until they reached full confluence, and then cultured in osteogenic induction medium containing MEM-Alpha GlutaMAX (Gibco, 32571-036), 10% fetal bovine serum (Thermo Fisher Scientific), 0.5% penicillin/streptomycin, β -glycerophosphate disodium salt hydrate (Sigma-Aldrich, G9422), and 100 nM Dex. The medium was changed every 2–3 days. After 3 weeks, the PFA-fixed cells were stained with Alizarin Red S solution (Muto Pure Chemicals Co., Ltd., 17971). For adipogenic differentiation, 5×10^4 iMSCs were seeded on fibronectin-coated 12-well plates, and adipogenic induction was initiated when they reached full confluence by replacing the MSC medium with adipogenic induction medium containing DMEM (08459-64, High Glucose; Nacalai Tesque, Japan), 10% fetal bovine serum (Nichirei, 171012), 0.5% penicillin/streptomycin (Gibco, 15140122), 10 μ g/ml insulin (Wako Pure Chemicals Corp., 097-06474), 1 μ M Dex (Wako Pure Chemicals Corp., 047-18863), 200 μ M indomethacin (Wako Pure Chemicals Corp., 093-02473), and 500 μ M IBMX (Wako Pure Chemicals Corp., 095-03413). PFA-fixed cells were washed with water, incubated with 60% isopropanol for 5 min, and stained with Oil Red O (Nacalai Tesque, 25633-92) dissolved in 60% isopropanol. Non-specific staining was removed by washing several times with water.

4.6 RNAseq data analysis

Total RNA was purified using an RNeasy Micro Kit (Qiagen) and treated with a DNase I kit (Qiagen) to remove genomic DNA. We reverse-transcribed 10 ng of total RNA to obtain single-stranded cDNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). We synthesized cDNA libraries for the Ion Ampliseq transcriptome analysis using the Ion AmpliSeq

Transcriptome Human Gene Expression Core Panel (Thermo Fisher Scientific) and Ion AmpliSeq Library Kit Plus (Thermo Fisher Scientific), according to the manufacturer's protocol. Briefly, cDNA was amplified for 12 cycles with an Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel using a thermal cycler. Primer sequences were partially digested with the FuPa reagent by sequentially performing 10 min at 50°C, 10 min at 55°C, and 20 min at 60°C. Barcode ligation was performed using an Ion Xpress Barcode for 30 min at 22°C. Barcode-labeled cDNA libraries were purified using DNA Clean & Concentrator™-5 (Zymo Research, CA, United States) and analyzed using the Ion S5 XL System (Thermo Fisher Scientific) and Ion 540 Chip Kit (Thermo Fisher Scientific). Count data analyses were performed using R studio with the "DESeq2" package normalization method for the detection of significantly ($p \leq 0.05$) differentially expressed genes (Love et al., 2014).

4.7 Optimization of chondrogenic differentiation strategy

The micromass culture described in the previous section was used to evaluate the small-molecule TD-198946 (TD) (MedChemExpress, HY-15642/CS-6860) in T1-iMSCs. Different concentrations were tested, and the results are shown in Figure 3A. Subsequently, 3D culture was used to produce chondrogenic spheroids. Briefly, 2×10^4 T1-iMSCs/well were plated onto ultra-low attachment 96 U-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in hMSC Chondrogenic Basal Medium (PT-3925) and hMSC Chondrogenic SingleQuots™ Kit Supplement (PT-4121). Chondrogenic inducers were added at specific time points, as indicated in Figure 3C, including 40 ng/ml platelet-derived growth factor-BB (PDGF-BB; R&D Systems), 10 ng/ml TGF- β 3 (Peprotech Inc., Rocky Hill, NJ, United States), 50 ng/ml BMP4, and 100 nM TD. Cells were cultured for 21 days and the medium was changed every 3–4 days. At day 21, spheroids were isolated for mRNA expression, quantification of sulfated proteoglycans and GAGs, immunostaining, and histological analysis.

4.8 Production of sulfated proteoglycans & GAGs

Chondrogenic spheroids were collected on day 21 and digested for 6 h in 50 μ g/ml papain solution (Sigma-Aldrich) at 65°C. The obtained extracts were used to quantify the GAG content using a Blyscan™—sulfated glycosaminoglycan (sGAG) assay kit, following the manufacturer's instructions. DNA content was determined using Quant-iT™ PicoGreen™ dsDNA Assay Kits and dsDNA Reagents (Invitrogen, Waltham, MA, United States).

4.9 Fusion experiment

A set of two chondrogenic spheroids (day 21) per well was placed in 96 U-well plates and cultured in chondrogenic medium for an additional week. The medium was replaced on day 4. Images were obtained on days 0, 1, 4, and 7. To enable accurate monitoring of the fusion process while maintaining stable environmental conditions,

we used an automated system for capturing images every 30 min during the first 4 days (BioStation CT, Nikon). The images were combined to produce a video (Supplementary Video S1).

4.10 Immunocytochemistry

Prior to immunostaining, paraffin sections or cryosections of PFA-fixed spheroids were prepared. Staining was performed as previously reported (Zujur et al., 2017) and DAPI (1:1000; Thermo Fisher Scientific) was used to counterstain nuclei. The primary antibodies used in this study are listed in Supplementary Table S3. Observations and assessments of the samples were performed using a BZ-X700 Fluorescence Microscope (Keyence, Osaka, Japan) or Olympus FV3000 confocal laser scanning microscope.

4.11 Subcutaneous transplantation of chondrogenic spheroids

Chondrogenic spheroids (day 21) of approximately 500 μ m generated from iMSCs or human BM-MSCs (PT-2501, (Batch 20TL262529; Lonza, Durham, NC, United States) were subcutaneously transplanted into a small incision on the back of eight-week-old female CB17/IcrJcl-Prkdc^{scid} mice purchased from CLEA Japan, Inc. (Tokyo, Japan) ($n = 6$ per group). For this procedure, the mice were anesthetized with 3% forane inhalant liquid (AbbVie, North Chicago, IL, United States). Ethical approval was obtained from the Animal Care Committee of Kyoto University (16-73-13). The mice were sacrificed after four or 8 weeks, and the spheroids were retrieved for further analysis. The calcium stain kit of modified von Kossa (ScyTek Laboratories, UT, United States) was used following the manufacturer's instructions for the visualization of calcium deposits in paraffin sections.

4.12 Statistical analysis

The means of groups were compared by analysis of variance using GraphPad Prism 9 software. Significance of differences was determined by Dunnett's test in the case of multiple group comparisons to a single control.

Data availability statement

RNA-seq data presented in the study was deposited in the Gene Expression Omnibus (GEO) database. Accession numbers GSE230303 (overall normalized data and the raw data corresponding the iMSCs) and GSE206048 (raw data of the iPSC and iNCC samples).

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for

participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Animal Care Committee of Kyoto University.

Author contributions

DZ, CZ, KN, and MI contributed to the study conception and design. DZ wrote the manuscript. DZ, WT, and DK performed the experiments. DZ and ZA-A analyzed the data and created the figures. KT and SA contributed the reagents and materials. All authors have read and agreed to the final version of the manuscript.

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Conflict of interest

This study was funded by HEALIOS K.K., Ajinomoto Co., Inc., and Takeda Pharmaceutical Company Limited. The funders were not involved in the study design, collection, analysis, interpretation of data, writing of this article, or decision to submit it for publication. KN is a co-founder and share-holder of Cyfuse Biomedical K.K. KT and SA are employees of Ajinomoto Co., Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2023.1140717/full#supplementary-material>

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