Effect of Medium Supplements on Mesenchymal Stromal Cell Development and Permissiveness to Transformation by EWSR1-FLI1 Fusion Protein

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Abstract

Ewing sarcoma is the second most frequent childhood bone tumor and is characterized by FET-ETS translocations. In ~ 85% of cases, the translocation fuses EWSR1 (a member of the FET gene family) with FLI1 (a member of the ETS family). Accurate models are required to study the mechanisms underlying tumorigenesis driven by EWSR1-FLI1, however no such models exist for Ewing sarcoma at present. The main obstacle to establishing a model for Ewing sarcoma is that the cell of origin remains undefined. A growing amount of evidence suggests that Ewing sarcoma originates from a small subset of mesenchymal stromal cells (MSC) permissive to the normally toxic EWSR1-FLI1 fusion protein. Characterizing this permissive subset is hindered by the substantial heterogeneity of MSCs. We sought to address this problem using induced pluripotent stem cells as a model system. It has been shown that some cytokines are capable of influencing the differentiation from iPSCs to MSCs, and the portion of different subpopulations of MSCs can be altered by changing the combination of cytokines that are involved in the differentiation. Herein, we characterized MSC derived from iPSC treated with different combinations of cytokines during differentiation with the ultimate goal of characterizing specific subsets of MSC that are permissive to EWSR1-FLI1.

Key words

Ewing Sarcoma, induced pluripotent stem cells, Cell of origin, mesenchymal stromal cells
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Introduction

Ewing Sarcoma (ES) is the second most frequent childhood bone tumor, accounting for one percent of all pediatric cancer diagnoses with approximately 200 annual cases in the United States. The tumor usually occurs in bone or soft tissue around the bone and patients with metastatic disease are typically resistant to intensive therapies. Five-year survival rates have plateaued at 30% and 60% for patients with and without metastatic disease, respectively\(^1\). Thus, there is a critical need for new strategies to address the basic questions surrounding ES etiology, which may illuminate therapeutic vulnerabilities. ES is characterized by a chromosomal translocation where a member of the FET gene family is fused with an ETS transcription factor\(^2\). In more than 85% of cases\(^3\), the translocation fuses \(EWSR1\) (a member of the FET gene family) with \(FLI1\) (a member of the ETS family).\(^4\) The function of EWSR1 protein is not completely understood, but it has a strong transcription activation domain. The FLI1 protein normally functions as a transcription factor and its DNA binding domain can attach to GGAA microsatellites sites and activate the transcription of nearby genes. The EWSR1-FLI1 fusion protein has the transcription activation domain of EWSR1 protein and the DNA binding domain of FLI1 protein, so it is also able to attach to GGAA microsatellites\(^5\). Since GGAA microsatellites are ubiquitous in human genome, the fusion protein is able to disrupt genetic and epigenetic regulation globally. Reported target genes activated by EWSR1-FLI1 include IGF1, Myc, TOPK, NKX2.2, ID2,
DAX1, GLI1, EZH2, MK-STYX, and PLD2, which are involved in regulating cell survival and proliferation. TGFβ2 and p21 are involved in apoptosis and can be repressed by EWSR1-FLI1. This global alteration of gene regulation leads to transformation in a subset of permissive cells, but more commonly results in senescence or cell death. Even though several targets of EWSR1-FLI1 have been uncovered, the comprehensive pathways and mechanisms of tumorigenesis driven by the EWSR1-FLI1 fusion protein remain unclear, hindering the development of effective treatments. Further complicating the study of transformation in ES is the fact that the cellular origin of ES remains unclear. To overcome these issues, an effective model for ES must be established.

The induced pluripotent stem cell (iPSC) technology provides an ideal platform for cancer modeling. In 2007, Shinya Yamanaka demonstrated a method of reprogramming somatic cells to a pluripotency state via the overexpression of four genes: OCT4, SOX2, KLF4, and c-Myc. Now known as iPSC, Yamanaka’s discovery allows for the generation of pluripotent cells from a variety of genetic backgrounds and for the re-differentiation of these cells back into nearly all somatic cell types by simply changing the environmental cues. The advent of iPSCs in combination with modern genetic engineering strategies, such as the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system, allows for new bottom-up cancer modeling strategies. The pluripotency of iPSCs allows researchers to observe cancer development, which is not possible using tumor-
derived cell lines. Furthermore, iPSC cancer models are also able to better recapitulate the developmental heterogeneity of cancer during its early stages. The genetic backgrounds of cells can be different from patient to patient, but the number of available cell line for a specific type of cancer, such as ES, is limited. Currently, there are only 14 cell lines for ES in public cell banks\(^{12}\), which limits experimental evaluation of the impact of genetic background on ES formation and progression. Conversely, large numbers of iPSC lines exist across a plethora of genetic backgrounds, opening the door to better understanding of the role of genetic background on ES.

A growing amount of evidence suggests that ES originates from a small subset of mesenchymal stromal cells (MSC)\(^{13}\). Most ES tumors arise in bone or soft tissue surrounding bone; thus, it is plausible that the cell of origin is a type of multipotent stem or progenitor cell that can give rise to bone as well as other lineages such as cartilage\(^{14}\). Studies have also shown that inhibition of EWSR1-FLI1 expression by siRNA\(^{15}\) in cancer cell lines induces mRNA expression profiles that converge to that of MSCs. Due to the toxicity of EWSR1-FLI1, most cells do not persist with over expression of the fusion gene, however a small subset of iPSC-derived MSCs shift from spindle-like cells into small, round cells that resemble pathological observations in ES tumors\(^{16}\). This suggests that iPSC-derived MSCs may contain a population permissive to transformation by EWSR1-FLI1, which could represent a candidate cell of origin for ES. However, what exactly constitutes
an MSC is not well defined as MSCs are a heterogeneous population. The primary MSCs isolated from human tissue contain multiple subpopulations that have various differentiation capacity. The iPSC-derived MSCs are only characterized by the expression of standard MSC surface proteins and their ability to give rise to osteocytes, chondrocytes and adipocytes, but the composition of subsets within iPSC-derived MSCs is still not clear. Hence, even though there is evidence that the cell of origin of ES is a subset of MSC, precise characterization of the permissive cell is complicated by the heterogeneity of MSC cultures\textsuperscript{17}.

One way to address this question is to identify differentiation conditions that enrich the frequency of the permissive subset of MSCs that can be transformed by EWSR1-FLI1. In this way, phenotypic profiling of the population could allow correlation to transformation permissiveness. It has been shown that some cytokines are capable of influencing the differentiation from iPSCs to MSCs, thus the proportion of different subpopulations of MSCs can be altered by changing the combination of cytokines involved in the differentiation protocol\textsuperscript{18}. The overarching goal for this study is to establish a defined differentiation system to study the effect of altering cytokine combinations on the heterogeneity of resultant MSC populations, and link observed changes in permissiveness to EWSR1-FLI1 overexpression. The ultimate goal is that this system would allow for identification and characterization of the cell population permissive to EWSR1-FLI1, i.e. the putative cell of origin for ES.
For this reason, we sought to determine the effect of different cytokines on the differentiation of iPSC to MSC. We focused on basic-fibroblast growth factor (bFGF) and activin A, because it has been shown that these two cytokines can alter the osteogenesis and chondrogenesis potential of MSCs\textsuperscript{19}. bFGF can increase the proliferation and osteogenic differentiation potential of MSCs\textsuperscript{20}, while Activin A can promote proliferation, osteogenic, and chondrogenic differentiation potential of MSCs\textsuperscript{21}. Both cytokines influence the differentiation of MSCs by increasing the proportion of progenitors that give rise to osteocytes and chondrocytes versus adipocytes. Since ES tumors are typically formed within and around bone and bone/cartilage growth zones, we hypothesized that the addition of bFGF or activin A could enrich the cell of origin of ES by amplifying a bi-potent (bone and cartilage) subset of MSCs. To test this hypothesis, we initiated MSC differentiation from iPSC and studied the effects of different combinations of cytokines on the differentiation and characteristics of resultant cells.

**Methods**

**Mesoderm Induction**

To set up the iPSC to MSC differentiation, we first seeded the IPS1210 iPSC line in a 12-well plate containing mTeSR-Plus media from STEMCELL technologies. The plates were coated with Geltrex. This culture system is serum-free and feeder-free and maintains the pluripotency of iPSCs. The iPSCs were not
immediately exposed to differentiation inducing media after the thawing process as
the thawing process is harsh to iPSCs. Hence, the cells were kept in this
pluripotency-maintaining culture condition for 24 hours, so that the cells could
restore the viability for downstream treatments.

For our previous study based on the iPSC derived MSCs, we used
Mesodermal Induction Medium (MIM) from STEMCELL technologies for
mesodermal induction. However, the components of MIM are not defined,
precluding the opportunity to systematically test the effect of alternate cytokine
combinations. Further, batch-to-batch variation might lead to the inconsistent
results. To eliminate those unpredictable variables, we sought to develop a defined
culture system for the induction of mesoderm-like cells. For this purpose, we first
started with a small-scale experiment testing a defined culture system against MIM.

TeSR™-E8 (STEMCELL technology) is a minimal iPSC maintaining media
that is widely used. TeSR™-E6 is a formulation that lacks bFGF and TGFβ, which
are key cytokines that maintain the pluripotency of iPSCs. Therefore, TeSR™-E6
can be used as a basal media for the induction of differentiation from iPSCs. Several
groups have shown that BMP4 is a key factor of mesodermal induction\(^2\), it is able
to induce the endogenous FGF and Tgfβ signaling pathway to turn on the mesoderm
induction program\(^2\). Therefore, to establish a defined culture system for
mesodermal induction, we first tested a minimal media of TeSR™-E6 containing
BMP4. The concentration of BMP4 was titrated at the following concentrations: 10ng/ml, 50ng/ml and 100ng/ml.

To induce the differentiation from iPSC to MSC, iPSCs were first differentiated into an intermediate mesodermal cell type\textsuperscript{24} before they were converted to MSCs\textsuperscript{25}. Even though other groups have developed the “one-step” iPSC to MSC induction protocol, it is preferred to direct iPSCs to differentiate into mesoderm-like cells before the MSC stage in cancer modeling, because the purpose is to control and understand the natural development of the cancer and cell of origin of tumor cells.

On day 0, iPSCs were thawed and seeded at ~2750 cells/cm\textsuperscript{2} on pre-warmed Geltrex plate and culture in mTesr1 + 10um ROCK inhibitor from STEMCELL technology. Although 2750 cells/cm\textsuperscript{2} is a relatively low density, it’s important to plate the cells at this density to accommodate the large amount of cell expansion during the 4-day induction period. If too many cells are plated on day 0, cell death may occur by day 4 due to media exhaustion. The addition of ROCK inhibitor facilitated the survival of iPSCs plated on day -2\textsuperscript{26}. On day -1, cells were fed with mTeSR-Plus media, this time the ROCK inhibitor was not added. On day 0, mTesr-Plus media was switched to mesoderm inducing media. mTeSR-Plus was removed, cells were washed with Phosphate-buffered saline (PBS) once to get rid of the dead cells, followed by adding mesoderm induction media. According to the protocol
previously developed in our lab, the mesoderm induction takes four days. So, from day 1 to day 4, cells were fed with mesoderm induction media every 24h.

**MSC Induction**

According to the protocol previously developed in our lab, full MSC induction takes a total of ~21 days. On day 4, the mesodermal induction media was switched to MSC induction media, which is composed of mTeSR-E6 and different combinations of cytokines. The first combination composed EGF and PDGF; the second combination composed EGF, PDGF and Activin A; the third combination composed EGF, PDGF and bFGF; the fourth combination composed EGF, PDGF, bFGF and Activin A. In recent years, a range of iPSC to MSC differentiation protocols have been described. TeSR™-E6 is widely used as basal media in MSC differentiation protocols, and EGF, PDGF and bFGF are standard cytokines for the differentiation. Kenichi Tamama et al. (2010) have shown that the soluble EGF treatment on MSCs can facilitate the proliferation while maintaining the multipotency of MSCs. Mihaylova Z et al. (2017) have demonstrated that proper concentration of PDGF is capable of enhancing the proliferation and increasing the expression of stem cell markers of MSCs. Activin A has been shown to promote formation of biopotent precursors with osteo- and chondro-genic potential within MSCs. To evaluate the functions of FGF and Activin A on resultant MSC, TeSR™-E6 containing combinations of EGF, PDGF, bFGF, and Activin A were evaluated.
for their ability to modify the proportion of different subsets in MSC derived from iPSC.

**Flow cytometry**

To ensure that the iPSCs are differentiating along the pre-designed pathway, the identity of cells needs to be checked at multiple time points. Immunophenotype, which can be measured by flow cytometry, is a good representation of a cell’s identity. Furthermore, for the reproducibility of the differentiation protocol, a detailed profile of immunophenotype needs to be established.

To evaluate the progress of mesoderm to MSC differentiation, standard surface markers for MSC were detected together, they are CD73, CD105 and CD90. All the cells should express all these three markers at the end of differentiation. For the bi-potent MSCs, have shown that CD146⁻, CD164⁺, PDPN⁺ CD73⁺ cells in growth plate of human bone tissue are human skeletal stem cells. These cells can self-renew and give rise to bone and cartilage. So, these four markers for skeletal stem cells were also measured by flow cytometry.

Treated iPSC-MSC cultures from each group were collected at multiple time points for flow cytometry analysis throughout differentiations. To optimize our flow panel, antibodies, compensation, and voltage were tested and optimized using human bone marrow derived MSCs as a reference. The voltages and gates were adjusted to maintain a majority of cells within the acquiring plot. Unstained human
bone marrow derived MSCs and stained bone marrow derived MSCs were recorded for the auto-setting of the compensation. The reason to do the compensation is more than two fluorochrome were used for both MSC and Skeletal stem cells (SSCs) flow cytometry panels. The first time point is day 4, where the cells are supposed to become mesoderm-like cells. The identity of those cells needs to be confirmed by expression of mesodermal cell surface markers KDR and PDGFRα. Then, during the differentiation from day 4 to day 21, a portion of differentiating cells are collected for flow cytometry at every passage to obtain the profile of immunophenotype of differentiating cells. For each flow cytometry analysis, 200,000 cells were collected from control groups and experimental groups. To prepare the sample, the cells were stained for 20 minutes, and washed with fluorescence-activated cell sorting buffer two times. MSC surface marker expression was assessed with LSR II H4760 flow cytometer.
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Table 1. Information about antibodies and fluorochromes used for flow cytometry analysis.
Transduction of EWSR1-FLI1 fusion protein

The EWSR1-FLI1 fusion is toxic to most cells and can activate apoptosis and senescence. The subset of MSC that is able to be transformed into ES needs to tolerate expression of the EWSR1-FLI1 fusion protein. If the cell of origin of ES was enriched after the 21 days differentiation, introduction of EWSR1-FLI1 fusion protein would lead to a higher transformation rate compared to controls.

A previously published\textsuperscript{36} retroviral system was used to insert the \textit{EWSR1-FLI1} cDNA into the cells (kind gift from Dr. Stephen Lessnick). The construct contains \textit{EWSR1-FLI1} fusion cDNA and a hygromycin-resistance cDNA. Cells were plated into 24-well plates in triplicates and were left overnight in MSC maintaining media. On the second day, retrovirus containing \textit{EWSR1-FLI1} fusion cDNA and a hygromycin-resistance cDNA sequence was added to the media. Non-treatment group and an empty vector group were used as control to normalize the effects from media and vector transduction. The cells were incubated overnight after the addition of retrovirus for the transduction. On the third day, the virus containing media was switched back to MSC maintaining media. The cells were incubated in normal condition for 2 days. Hygromycin is added afterwards to select the successfully transduced cells. The number of colonies of EWSR1-FLI1 group are normalized to the number of colonies of vector only group to account for transduction efficiency.
Experimental Design

The purpose of this study is to characterize the effect of different cytokines on the composition of MSC cultures derived from iPSC, and to determine how changes in MSC phenotype impact permissiveness to EWSR1-FLI1 transformation. To do this, iPSCs were differentiated into mesoderm-like cells over the course of 4 to 5 days. After the IPSC-mesoderm induction, the immunophenotype of mesoderm-like cells were analyzed by flow cytometry. Then, the mesoderm-like cells were induced into MSCs in four different conditions (Fig. 1). Each condition has a unique combination of cytokines. The mesoderm to MSC induction was carried out over 17 days with passaging when cells reached confluency. Upon completion of the differentiation process, the immunophenotypes of iPSC-derived MSCs were analyzed by flow cytometry. In order to determine if the ES cell of origin was enriched, retrovirus containing EWSR1-FLI1 fusion cDNA and a hygromycin-resistance cDNA was added to the media to transduce the iPSC-derived MSCs. A non-treatment group and an empty vector group were used as control to normalize the effects from media and vector transduction.
Fig 1. Experimental design. A diagram showing experimental strategy for testing the effects of different combinations of cytokines on MSC development and permissiveness to transformation by EWSR1-FLI1 fusion protein.
Results

Mesodermal induction by MIM

We used STEMdiff™ Mesoderm Induction Medium (MIM) for the generation of mesoderm-like cells, and iPSC maintaining media (mTeSR plus) was used as control. Cells were collected for flow cytometry analysis on day 4 and day 5. Images of cells were taken on both days (Fig. 2A). Compared to cells in iPSC maintaining media, cells treated with MIM showed a slight upregulation of vascular endothelial growth factor receptor 2 (KDR) expression, but the expression level of platelet-derived growth factor receptor alpha (PDGFRa) was low in both MIM treated and control group (Fig. 2B), indicating that at these time points there are few cells that express mesoderm markers. KDR and PDGFRa expression are expected to be detected after day 4, because, according to the protocol, MIM is supposed to induce the mesodermal differentiation program and turn ~80% of iPSCs into mesoderm-like cells. Since MIM is a well-established reagent for mesoderm induction, it is unclear why low expression of KDR and PDGFRa was observed in ours studies. As the recipe of MIM is proprietary it is not feasible to adjust the components to promote the expression of KDR and PDGFRa. Hence, a defined media for mesoderm induction is needed for future study.
Fig 2. **Generation of mesoderm-like cells from human iPSCs.** (A) Representative images of cells in MIM on days 4 and 5, revealing the morphological differences among different conditions. iPSCs in iPSC maintenance media were used as control. Magnification, x10. (B) Mesoderm-like cells were induced by MIM, iPSCs were cultured in iPSC maintenance media as control. Cells were collected for flow cytometry analysis on days 4 and 5.
Validation of skeletal stem cell flow cytometry panel

To identify subpopulations within MSC cultures, we used a surface marker panel originally developed to identify skeletal stem cells (SSC), but which also can identify bone-cartilage-stroma progenitors (BCSP): CD73, PDPN, CD146 and CD164 (Fig. 3A). With this panel, the markers identified SSCs and multipotent progenitors downstream of the SSC, namely BCSP. Both SSC and BCSP are potential subsets of MSCs. We reasoned that this SSC panel could be used to profile subsets of iPSC-derived MSC cultures to determine the proportion of SSC or BCSP present in these cultures. To verify this flow panel, human bone marrow-derived MSC cultures were analyzed by flow cytometry for these four markers (Fig. 3B). We observed MSCs were separated into multiple subpopulations. Cells expressing SSC and BCSP phenotype were found in bone marrow derived MSC cultures. This demonstrates that the markers used to identify SSC can also be used for identification of additional subsets, such as BCSP, within MSC cultures.
Fig 3. Identification of subsets of primary MSCs using SSC markers. (A) Gating strategy for the separating of subsets of iPSC-derived MSCs. (B) Validation of SSC flow cytometry panel using human bone marrow derived MSCs. CD73$^+$ PDPN$^+$ CD164$^+$ CD146$^-$ cells are potentially the bipotent cells that are capable of giving rise to osteocytes and chondrocytes. Bone, cartilage and stromal progenitor cells are immediate downstream cells of SSCs.
**Mesoderm to MSC differentiation**

We next sought to apply the SSC flow cytometry panel to iPSC-derived MSC cultures. Day 4 mesodermal cells were switched from MIM to TeSR™-E6 containing bFGF, EGF and PDGF and cells were collected for flow analysis when they reached confluency. Over the course of 28 days, cells were passaged 5 times. During the early stages (Day 6) MSCs were immature, cells were still differentiating from mesoderm to MSC, and most cells were PDPN positive, which is different from primary human bone marrow derived MSCs and mature iPSC-derived MSCs. When iPSC-derived MSCs were mature (Day 20-day 22), both SSC and BCSP were identified within the MSC population using our optimized flow panel (Fig. 4A). Thus, our SSC panel was able to subfractionate iPSC-MSC cultures, setting the stage for us to observe the effects of different cytokines on the composition of these subsets within intermediate iPSC-MSC populations.
Fig 4. Immunophenotype of intermediate cell populations during iPSC-MSC differentiation.

(A) The expression of SSC markers: CD73, PDPN, CD164 and CD146 on differentiating iPSC derived MSCs, cells were collected for analysis on day 6, day 12, day 20, day 22 and day 28.
Generating MSCs in the presence of different combinations of cytokines

After the SSC flow cytometry panel was verified with human bone marrow derived MSCs, we sought to profile iPSC-MSC using this panel. The iPSC derived MSCs induced by EGF and PDGF; EGF and PDGF plus bFGF; EGF and PDGF plus activin A; and EGF, PDGF, bFGF and activin A were collected on Day 6, Day 12, Day 20, Day 22 and Day 28 for flow cytometry analysis. Images of cells were taken before detaching cells from the dishes (Fig. 5A). All the iPSC-derived MSCs were first analyzed by standard MSC panel, which is composed of CD73, CD90 and CD105. All the iPSC-derived MSCs in four conditions manifested expression of CD73 and CD90 but minimal expression level of CD105 (Fig. 5B). The distribution of all iPSC derived MSCs are different from the distribution pattern of human bone marrow derived MSCs (Fig. 5C), indicating that the sub-populations in iPSC derived MSCs are different from bone-marrow derived MSCs. This may be beneficial in that the iPSC-MSC could contain earlier progenitor populations more permissive to EWSR1-FLI1 transformation. Furthermore, the differences among the proportion of bipotent subsets of iPSC-derived MSCs generated by different conditions suggests that the addition or removal of cytokines may influence the iPSC-derived MSC development and permissiveness to transformation by EWSR1-FLI1 fusion protein (Fig. 5D). Collectively, establishing this phenotyping profile improves or ability to understand and interrogate MSC heterogeneity and potentially link these changes to EWSR1-FLI1 permissiveness.
Fig 5. Generation of MSCs in the presence of different combinations of cytokines. (A): Bright-field images of MIM induced cells in four different conditions on day 20. Magnification, x4 for upper row, x10 for lower row. (B): Verification of iPSC derived MSCs by standard MSC markers: CD73, CD90 and CD 105. (C): The expression of SSC markers: CD73, PDPN, CD164 and CD146 on survived cells after 21 days differentiation. (D): The quantification of bi-potent MSCs (SSC and BCSP) within IPSC-derived MSCs generated by different combinations of cytokines
Generating mesoderm-like cells from human iPSCs in defined conditions

We previously induced mesoderm-like cells using STEMdiff™ Mesoderm Induction Medium (MIM). However, the components of MIM are not clear and resultant mesoderm-like cells have not been well characterized. We sought to derive mesoderm-like cells from iPSCs using a defined condition composed of TeSR™-E6 containing Bone morphogenetic protein 4 (BMP4). The iPSCs were cultured in TeSR™-E6 containing different concentrations of BMP4 while MIM was used as a control (Fig. 6A). Cells were collected for flow cytometry analysis on day 4 and day 5. Images of cells were taken on both days (Fig. 6B, C). Compared to cells in MIM, cells treated with BMP4 were expressing both Vascular endothelial growth factor receptor 2 (KDR) and platelet-derived growth factor receptor alpha (PDGFRα), whereas cells in MIM only express KDR (Fig. 6D,E), indicating that TeSR™-E6 containing BMP4 better recapitulated the heterogeneity of cells within mesoderm. Since PDGFRα is a marker for paraxial mesoderm cells, and paraxial mesoderm cells are believed to be the progenitor cells of MSC, cells treated with 10ng/ml BMP4+ TeSR™-E6 for 4 days may be superior for subsequent MSC induction in terms of the expression of cell surface proteins. However, MSCs generated from the 10ng/ml BMP4 treated mesoderm cells require further characterization by SSC phenotyping, gene expression, and functional assessment of their differentiation capacity.
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Fig 6. Evaluation of defined mesodermal induction media by flow cytometry (A): Diagram of mesodermal induction in escalating concentrations of BMP4, cells were collected for flow cytometry analyzation on days 4 and 5. (B, C): Representative images of cells on days 4 and 5, revealing the morphological differences among different conditions. Magnification ×10 (D, E): Identification of different subsets of mesoderm-like cells on days 4 and 5. KDR is the cell surface marker for lateral plate mesoderm cells, PDGFRα is the cell surface marker for paraxial mesoderm cells.
Discussion

For the induction of iPSC derived MSCs, the commercial media MIM was used for the first step of differentiation because the defined conditions require further characterization. We instead sought to test the effect of different cytokines during MSC differentiation on the frequency of the specific subsets within iPSC-derived MSC cultures that can give rise to bone and cartilage. Expression of SSC markers show that there are distinct cellular subsets within iPSC derived MSC with similar surface markers profiles as skeletal stem cells and multipotent bone and cartilage progenitors. Studies to determine how these different subsets correlate with permissiveness to transformation are ongoing. These studies will evaluate permissiveness as measured by survival and proliferation following transduction with an EWSR1-FLI1 overexpressing vector.

For cancer modeling, the defined nature of conditions at each step of differentiation is important for reproducibility. To develop a defined mesodermal induction condition to replace MIM, we combined TeSR™-E6 and escalating concentrations of BMP4 and applied this mixture to iPSC cultures. Our results suggest that the mesoderm-like cells induced by TeSR™-E6 containing BMP4 show a similar immunophenotype and differentiation potential as what has been described for lateral plate and paraxial mesoderm; the latter being a known developmental origin of MSC. Specifically, our data shows that mesoderm-like cells induced by 10ng/ml BMP4 have higher PDGFRα expression, whereas MIM
induction seems to be less effective in terms of the acquiring this specific mesoderm marker. However, the differentiation of iPSC to mesoderm is very transient, and different conditions for mesoderm induction might alter the kinetics of induction. If iPSCs have a different differentiation rate in MIM compared to cells treated by 10ng/ml BMP4 and TeSR™-E6, there could be a more optimal timepoint to initiate the MSC induction phase.

Although the combination of markers of SSC are informative and divide bulk MSC cultures into observable subsets, it is to be determined if the iPSC-derived MSC subsets expressing SSC or BCSP markers are more permissive to EWSR1-FLI1 transformation. In contrast to bulk MSC, CD73^+CD146^−PDPN^+CD164^+ cells from human growth plate can give rise to bone and cartilage but not adipose tissue. Studies in mice have shown that specific progenitors located within bone growth plates are permissive to transformation by EWSR1-FLI1\textsuperscript{37}. Thus, the SSC or BCSP subsets of iPSC-MSC represent intriguing candidates for the ES cell of origin.

In sum, our studies highlight the utility of iPSC as a model system to better understand cancer development, particularly for ES whose developmental origins remain poorly understood. Our data confirm that iPSC-derived MSC represent a heterogeneous mixture of cells, and that the composition of these cultures can be altered by manipulating cytokine combinations during differentiation. The use of the SSC flow cytometry panel offers a new method to sub fractionate these MSC
subpopulations and should facilitate future studies to define the ES cell of origin with greater resolution.
Reference


