Single Cell Transcriptome Sequencing Reveals the Potential Mechanism of Heterogeneity in Immunoregulatory Function Between Mesenchymal Stromal Cells

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Abstract

Background: Mesenchymal stromal cells (MSCs) could be applied for the treatment of immune-related diseases. However, some studies have found there is enormous heterogeneity in immunomodulatory function of MSCs isolated from different tissue. At present, the underlying mechanism of heterogeneity in immunoregulatory function is still unclear.

Methods: In this study, the foreskin mesenchymal stromal cells (FSMSCs) and human umbilical cord mesenchymal stromal cells (HuMSCs) were isolated and cultured to the 3rd passage. Cell types were confirmed according to the standard of International Association for Cell Therapy. Then, FSMSCs and HuMSCs were co-cultured with human peripheral blood mononuclear cells (PBMC) stimulated by lipopolysaccharides (LPS) *in vitro* respectively. And the supernatant was sampled for enzyme-linked immunosorbent assay to investigate the secretion of IL-1 β , IL-6, IL-10, TNF- α and TGF- β . Finally, single cell transcriptome sequencing was performed in order to elucidate the mechanism for the difference of immunomodulatory function.

Results: FSMSCs and HuMSCs are successfully identified as MSCs. When co-cultured with LPS pre-treated PBMC, FSMSCs and HuMSCs could effectively reduce the secretion of IL-1 β and TNF- α . But FSMSCs were able to stimulate the PBMC to secrete more IL-10, TGF- β and IL-6. Furthermore, 4 MSCs subsets in integrated data were identified, including Proliferative MSCs, Pericyte, Immune MSCs and Progenitor Proliferative MSCs. Among them, the proportions of Immune MSCs in FSMSCs and HuMSCs were 56% and 10% respectively. Varieties of immune-related genes, gene sets and regulons were enriched in Immune MSCs. And Immune MSCs with powerful transcriptional activity were found to be near to Pericyte at the degree of differentiation and closed to other cell subsets. Finally, the foreskin tissue might be an ideal source of isolating Immune MSCs when comparing the subset composition of MSCs derived from adipose tissue and bone marrow from public database.

Conclusions: Immune MSCs may play a key role in the heterogeneity of immunoregulatory function. It is a new insight that Immune MSCs could be isolated and better applied for the treatment of immune-related diseases without being limited by the heterogeneity of immunomodulatory function derived from different tissues.

Introduction

MSCs, which exist in varieties of tissues, are a kind of stromal cells with multi-directional differentiation potential. MSCs express surface markers CD73, CD90 and CD105, but do not express surface markers HLA-DR, CD11b, CD19, CD34 and CD45[1]. MSCs can differentiate into different types of cells including osteoblasts, adipocytes, chondrocytes, nerve cells, germ cells and so forth. In addition to the huge potential for tissue differentiation and regeneration, MSCs with the powerful immunoregulatory function are also able to applied for the treatment of multiple immune-related diseases, such as graft-versus-host disease (GVHD)[2], multiple sclerosis[3], sepsis[4], systemic lupus erythematosus[5], crohn disease[6],

osteoarthritis[7] and acute lung injury with COVID-19 infection[8]. At present, MSCs are already demonstrated to exert immunomodulatory function by direct cell-cell contact and the secretion of immune regulatory molecules[9]. MSCs were first isolated from bone marrow. But the clinical application of MSCs derived from bone marrow is greatly limited due to ethical disputes, invasive operation and low yield cultured *in vitro*. Therefore, it is urgent to find a new source of MSCs. In recent years, MSCs have been successfully obtained from other tissues such as adipose tissue[10], foreskin tissue[11], umbilical cord[12] and synovial fluid[13]. Among them, foreskin tissue and umbilical cord are considered as biological wastes without ethical issues. Meanwhile, foreskin tissue and umbilical cord can be donated from circumcision or natural birth in which the operations are less invasive and do not cause other dangers to the donors. In addition, FSMSCs and HuMSCs have not only high clone proliferation potential *in vitro* but also huge immunomodulatory ability[11, 14]. Therefore, foreskin tissue and umbilical cord are considered to replace bone marrow and become the main source of MSCs. However, the difference between FSMSCs and HuMSCs remains elusive.

Previous studies have shown MSCs with powerful immunomodulatory function are able to be applied in the therapy of lots of immune-associated diseases[2–8]. However, the clinical application of MSCs still encounters different problems, in which the heterogeneity of MSCs is a major concern[9]. The heterogeneity of immunomodulatory function plays a key role in the heterogeneity of MSCs. It has been shown the difference of immunoregulatory capacity is significant between MSCs derived from different tissues[14]. For example, Ivanova-Todorova *et al*/ found that the immunomodulatory capacity of adipose MSCs was stronger than bone marrow MSCs[15], whereas Xishan *et al*/ found the opposite[16], while Yoo *et al*/ showed that the adipose MSCs and bone marrow MSCs had equal immunomodulatory capacity[17]. Even MSCs isolated from the same tissue may exhibit mixed results in immunotherapy. For example, bone marrow MSCs can both inhibit arthritis by upregulating IL-10[18] and promote arthritis by upregulating IL-6[19]; some studies have shown that bone marrow MSCs can significantly alleviate the clinical symptoms of GVHD[20] while some studies have reported bone marrow MSCs are not effective in improving the outcome of GVHD[21, 22]. These controversies indicate that there is great heterogeneity in the immunomodulatory function of MSCs. And the potential mechanism of the difference in immune regulation function of MSCs from different sources is still unclear.

The main intervention for the heterogeneity of immunomodulatory function of MSCs is to treat MSCs with IFN- γ and/or TNF- α *in vitro* before application, this intervention can differentiate MSCs into anti-inflammatory MSCs[23]. This is based on the theory of immunomodulatory plasticity that MSCs can be induced to differentiate into pro-inflammatory MSCs or anti-inflammatory MSCs under different immune microenvironment[24]. Some researches have shown that the heterogeneity is significantly reduced and immunomodulatory capacity is significantly enhanced when MSCs are pretreated with IFN- γ and/or TNF- α [25]. However, the pretreatment will also promote the expression of immunogenicity-related genes in MSCs that are unfavorable for clinical application[25]. Therefore, we need to explore new ways to solve the problem of immunomodulatory heterogeneity. Recent evidence suggests that NES⁺ MSCs can significantly reduce macrophage infiltration as well as induce macrophage conversion to an anti-

inflammatory M2 phenotype[26]; CD271⁺ MSCs can significantly inhibit T lymphocyte proliferation[27] and have stronger chondrogenic potential[28]; CD106⁺ MSCs can more effectively regulate helper T cells and secrete more immune-related cytokines[29]; CD146⁺ MSCs can inhibit the activation of Th17 cells[30] while they also promote the conversion of M1-type macrophages to M2-type and improve the inflammation or fibrosis of the knee[31]. These findings indicate that MSCs are mixed cell population, and gene markers can help us to distinguish cell subsets with different biological functions in MSCs. A single gene marker may not fully define the biological functions of MSCs subsets. Thus, we need to integrate or combine multiple gene markers to distinguish cell subsets with different biological functions in MSCs. Single-cell transcriptome sequencing, an emerging technology, can detect the difference of gene expression between cells and help us to explore MSCs subsets with different biological functions[32]. In this study, we compared the difference between FSMSCs and HuMSCs in immunomodulatory functions *in vitro*. MSCs subsets with different biological functions in FSMSCs and HuMSCs were identified by single cell transcriptome sequencing. Finally, the potential mechanism of the difference in immunomodulatory function between FSMSCs and HuMSCs was investigated.

Methods

Isolation, culture and expansion of mesenchymal stromal cell

The foreskin tissue and umbilical cord were collected in the sterile tube containing sterile phosphate buffer solution (PBS) respectively. Under sterile conditions, the foreskin tissue and umbilical cord were cleaned with iodophor and PBS in turn, and then transferred to a petri dish. Next, we separated dermis tissue from foreskin tissue and cut up the dermis tissue, and then transfer in a 10-cm petri dish. In umbilical cord, we separated the amniotic membrane, artery and vein. And then, we collected the white connective tissue between the amniotic membrane and blood vessel in a 10-cm petri dish too. Afterwards, the minced dermis tissue and white connective tissue were cultured in incubator (37°C, 5% CO₂) with 5 mL of DMEM/F12 (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) respectively until primary cells migrated from tissue fragments and attached to the plate. When cells became 80% confluent, they were trypsinized and passaged. Finally, cells from the 3rd passage were used for further study and cell morphology was observed under an inverted microscope (Leica, Germany).

The surface markers of mesenchymal stromal cell

According to the identification standard about MSCs of the International Association for Cell Therapy (ISCT)[1], we detected the expression of CD73, CD90, CD105, CD45, CD34, CD14, CD19 and HLA-DR in the third passage of HuMSCs and FSMSCs by flow cytometry. First, we discarded medium and washed cells twice with PBS, and then used trypsin to digest the cells. Next, we stopped the digestion and adjusted the cell concentration to 1x10⁶ cells/mL. We prepared 8 tubes and added 1 mL of suspended cells in each tube. Afterwards, we added 5 uL different antibodies, such as mouse anti-human FITC-CD90 (BD, Germany), mouse anti-human PerCP-CyTM5.5-CD105 (BD, Germany), mouse anti-human APC-CD73 (BD, Germany), mouse anti-human PE-HLA-DR (BD, Germany), mouse anti-human PE-CD11b (BD, Germany),

mouse anti-human PE-CD19 (BD, Germany), mouse anti-human PE-CD45 (BD, Germany) and mouse anti-human PE-CD34 (BD, Germany), in different tubes and incubated them at 37°C for 30 minutes in dark. After incubation, we added 1 mL of PBS to wash out the excess antibodies and then centrifuged cells at 250 g for 10 minutes. Finally, the cells were resuspended in 0.5 mL of PBS and analyzed in Canto II cytometer (BD Bioscience).

The detection of multi-directional differentiation

According to the identification standard about MSCs of ISCT[1], we tested the osteogenic, adipogenic and chondrogenic differentiation ability of FSMSCs and HuMSCs. For osteogenesis: 6×10^4 cells with 0.5 ml MSC NutriStem® XF Medium (BI, 05-200-1) were seeded in one well of 24-well plates pre-coated with MSC Attachment Solution (BI, 05-752-1). The cells were cultured in incubator (37°C, 5% CO₂) for 24 hours to achieve >80% confluence, before induction in 0.5 ml MSCgo™ osteogenic differentiation media (BI, 05-440-1). Afterwards, we incubated the cells for 10-21 days and changed the medium every 2-3 days. Finally, we used 2% Alizarin Red S solution to evaluate the effect of osteogenesis. For adipogenesis: cells were finally grown and induced in 0.5ml MSCgo™ Adipogenic complete medium which consists of supplement mix I (BI, 05-331-1-01), supplement mix II (BI, 05-332-1-15) and MSCgo™ Adipogenic basal medium (BI, 05-330-1B). Next, we incubated the cells for 6-8 days and changed the medium every 2-3 days. After the cells became circular and started floating, we replaced the MSCgo™ Adipogenic complete medium with MSC NutriStem® XF (BI, 05-200-1) for period of 3-6 days. Once lipid droplets formed, Oil Red-O staining was used to evaluate the effect of adipogenesis. For chondrogenesis: 1×10^5 cells with 0.1 ml of MSC NutriStem® XF Medium (BI, 05-200-1) were seeded in 96-well plat. Then, the cells were cultured in incubator (37°C, 5% CO₂) for 24 hours to achieve spheroids. Culture medium was replaced with MSCgo™ Chondrogenic Differentiation Medium (BI, 05-220-1B). Cells were cultured for 14-21 days in incubator (37°C, 5% CO₂) and changed the medium every 3-4 days. Finally, Alcian Blue was used to evaluate the effect of chondrogenesis.

Comparsion of immunomodulatory function

HuMSCs and FSMSCs were cultured to 3rd passage *in vitro*. The cells in the logarithmic growth phase were digested with trypsin and then seeded in 12-well plates (1×10^6 cells per well). The cells were cultured at 37°C until the confluence reaches 50%. Then, we discarded the supernatant and replaced with 1 mL fresh serum-free medium. PBMC were isolated by Ficoll-Hypaque gradient centrifugation from whole blood the health donor providing with informed consent. At the same time, we resuspend the pbmc to 1×10^6 cells/mL in serum-free medium and then PBMC were mixed with MSCs at a 10:1 ratio during the co-culture. The experiment was divided into 4 groups: a) PBMC/HuMSCs/FSMSCs cultured alone, b) PBMC/HuMSCs/FSMSCs stimulated by LPS, c) HuMSCs/FSMSCs co-cultured with PBMC without stimulation, d) HuMSCs/FSMSCs co-cultured with PBMC stimulated by LPS. And the final concentration of LPS (Sigma) was 10 ng/mL. Cells were incubated for 24 hours (37°C, 5% CO₂). Then, the supernatant was centrifuged (1000 g, 5 min) and collected at 2h, 4h, 12h and 24h respectively. Finally, we detected the secretion levels of IL-1β, IL-6, IL-10, TNF-α and TGF-β in the supernatant according to the manual of

enzyme-linked immunosorbent assay (ELISA) kit (NeoBioscience, China). Three replicates are detected for each time point of experimental group. The results were showed as means $\pm$ standard error and visualized by line graph. Kruskal-Wallis test was used for comparison among multiple independent samples, and Dunn's test was used for pairwise comparison. Difference was considered statistically significant when the p value of Kruskal-Wallis test or Dunn's test was less than or equal to 0.05.

Single cell transcriptome sequencing

First, we digested the 3rd passage of FSMSCs and HuMSCs with the cell survival rate higher than 80%. Second, the concentration of cell suspension were adjusted to 1×10^6 cells/ml for 10X Genome Single Cell Platform (Single Cell 3' library and Gel Bead Kit V3). Then, the samples were processed as follows: GEMs creation, thermal cycling, post cycling cleanup, cDNA amplification, library preparation, library quantification and sequencing on Illumina Hiseq3000. Finally, the result of sequencing was mapped to GRCh38 human genome, and reads were counted by cellranger software (version 3.1.0)[33].

Data processing

We performed cell quality control and cluster analysis for single cell transcriptome data by R package Seurat (version 4.0.0)[34]. First, we used the Read10X function of R package Seurat (version 4.0.0)[34] to load the UMI count matrix into R and create the Seurat objec. Secondly, we filtered the low-quality cells with less than 200 genes, more than 20000 genes, more than 10% percentage of mitochondrion genes and more than 10% percentage of erythrocyte genes. Then, in order to correct the sequencing depth of every cell, we ran normalization for single cell transcriptome data and removed the effect of mitochondrial gene during the process of scaling. Afterwards, we executed principal component analysis (PCA) through first 2000 genes with high heterogeneity and evaluated the first 50 PCA dimensions by JackStraw and ElbowPlot function of R package Seurat (version 4.0.0)[34]. And the first 20 PCA dimensions with the most significant p values were used for t-distributed stochastic neighbor embedding (TSNE) and uniform manifold approximation and projection (UMAP). We also used the first 20 principal components (PCs) to cluster cells, and the resolution of the clustering was 0.5. Finally, we annotated the clusters based on the expression results of the surface markers of MSCs[1].

Integration analysis

First, we integrated the subsets annotated as MSCs in FSMSCs and HuMSCs by the FindIntegrationAnchors and IntegrateData function of R package Seurat (version 4.0.0)[34]. Then, we assessed the correction of batch effects based on PCA plots before and after integration. The integrated data was re-normalized, PCA reduced, TSNE reduced, UMAP reduced and cell clustered. In addition, we used the CellCycleScoring function of R package Seurat (version 4.0.0)[34] to infer the cell cycle of each MSCs subset. And we also constructed a clustering tree based on the similarity of MSCs subsets by the BuildClusterTree function of R package Seurat (version 4.0.0)[34] as well as visualized it on dendrogram through the R package ggtree (version 2.4.1)[35]. Finally, we annotated the clusters according to the literature and the marker genes of each subset.

Trajectory inference

Trajectory inference, also known as pseudotime analysis, can sort individual cells in pseudotime and simulate the dynamic changes during the development base on the expression patterns of key genes. Here, we used the R package slingshot (version 1.8.0)[36] to infer the possible differentiation trajectories base on different MSCsubsets. Meanwhile, in order to determine the possible starting points of trajectory, we also used the R package CytoTRACE (version 0.3.3)[37] to infer the degree of differentiation of MSCs subsets.

RNA velocity analysis

RNA velocity can identify the transcriptional status of different MSCs subsets based on the change of mRNA abundance which is inferred by distinguishing unspliced mRNA from spliced mRNA. First, we used the python package velocity.py (version 0.17.17)[38] to generate a loom file containing spliced mRNA, unspliced mRNA and ambiguous mRNA. Secondly, we evaluated the transcriptional status of different MSCsubsets base on the loom files with the help of the R package velocity.R (version 0.6.0)[38].

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) can infer the distribution trend of a predefined gene set between two biological states and thus determine whether a predefined gene set is enriched in specific biological state. First, we used the R package msigdb (version 7.2.1)[39] to download the Hallmark gene set. Next, we performed GSEA to calculate the enrichment scores and p values of Hallmark gene set in different subpopulations of MSCs by R package singleseqset (version 0.1.2.9000)[40], for which we were able to assess the potential biological function for every subset.

Differential gene expression analysis

We compared the gene expression between FSMSCs and HuMSCs by the Findallmarker function of R package Seurat (version 4.0.0)[34] and filter the differential genes with the corrected p value ≤ 0.05 and the absolute value of $\log_2$ fold change (Log_2FC) ≥ 2 . Meanwhile, we also calculated the marker genes in different MSCs subsets by the Findallmarker function of R package Seurat (version 4.0.0)[34] with the corrected P value ≤ 0.05 and $\text{Log}_2\text{FC} \geq 0.8$. In order to analyze the potential immune function of these highly expressed genes, we extracted 2073 immune-related genes from ImmPort database[41] and MSigDB database (Gene Ontology database)[42]. Then, we intersected the immune-related genes with the differential genes and annotated these intersected genes in immune function according to the related literature, ImmPort database and GeneCards database[43]. Finally, we visualized the expression of these intersected genes by R packages ComplexHeatmap (version 2.6.2)[44] and circlize (version 0.4. 12)[45].

Gene regulatory networks analysis

pySCENIC (version 0.10.3) is a python tool which enables us to infer single cell gene regulatory networks from single cell RNA-seq data[46]. The work flow was as follows: a). We inferred the co-expression

modules containing different transcription factors and their target genes from single-cell RNA-seq matrix; b). We retained the significantly enriched motifs in the co-expression module by motif enrichment analysis, and removed the target genes which were less correlated with the significant motif in the co-expression module. Then, the transcription factor and remaining target genes of co-expression module were called regulon; c). We assessed the regulon activity score (RAS) of different regulons in each cell, as well as calculated the RAS threshold for each regulon. This regulon was considered to be activated in cell when RAS was greater than threshold and vice versa. We performed the "0/1" conversion for RAS matrix based on threshold and created binary matrix in order to show the difference of regulon in various cell types best and eliminate technical bias; d). We calculated the regulon specific score (RSS) in different MSCs subsets by using R package *philentropy* (version 0.4.0)[47]. The regulons with RSS > 0.6 were considered as cell-type-specific regulons (CTSRs).

Cell-Cell communication analysis

CellChat (version 1.0.0) is an R package that can infer and visualize intercellular communication network from single-cell RNA-seq data[48]. The work flow was as follows: a). We created a CellChat object and set to human ligand-receptor interactions database; b). We identified overexpressed ligands or receptors and projected gene expression data onto the protein-protein interaction network; c). We assigned a probability value to each ligand-receptor interaction and then performed permutations to infer biologically meaningful interactions; d). We summarized the probabilities of all interactions associated with each signaling pathway and calculated the probability of communication for each signaling pathway; e). We identified the roles of each cell subsets at signaling pathways level by calculating network centrality; f). We finally identified several signaling groups through quantifying the functional similarity of all significant signaling pathways.

Comparsion of MSCs isolated from different tissue sources

In order to evaluate the ideal tissue source for isolating Immune MSCs, we downloaded single-cell transcriptome data from the NCBI public database for adipose mesenchymal stromal cells (ADMSCs) (SRP148833)[10] and bone marrow mesenchymal stromal cells (BMSCs) (GSE115149, GSE162692)[49, 50]. Among them, SRP148833 database was processed by 10x Genomics *cellranger* software (version 3.1.0)[33] for which we were able to acquire the gene expression matrix of ADMSCs. GSE115149 and GSE162692 database only kept the gene expression matrix of untreated BMSCs. After obtaining the gene expression matrix, they were processed according to the same quality control process as above. Finally, we evaluated the proportions of Immune MSCs in ADMSCs and BMSCs based on the integrated data above by R package *SingleR* (version 1.4.0)[51].

Results

Identification of FSMSCs and HuMSCs

According to the minimum identification standard about MSCs of the ISCT, we confirmed the type of cells which were isolated from foreskin tissue and umbilical cord respectively. First, FSMSCs and HuMSCs were able to attach plastic surfaces, and they were observed to be typical fibroblast-like and long spindle shaped under the microscope. Secondly, the result of flow cytometry showed positive surface markers, such as CD73, CD90 and CD105, were both expressed more than 95%, while negative surface markers, such as CD45, CD34, CD14, CD19 and HLA-DR, were expressed less than 2% in FSMSCs and HuMSCs (Figs. 1a-1b). Then, the red spherical vacuoles could be observed by Oil Red-O staining after the induction of adipogenic differentiation in FSMSCs or HuMSCs (Fig. 1c). It meant the lipid droplets were formed in these cells. In addition, after the induction of osteogenic differentiation, we observed scattered, crimson and round nodules in FSMSCs or HuMSCs by 2% ARS staining (Fig. 1c). It revealed that massive calcium was deposited in the cytoplasm. Finally, the blue acid mucopolysaccharides were visible in FSMSCs or HuMSCs by Alcian Blue staining after the induction of chondrocytic differentiation (Fig. 1c), suggesting that the FSMSCs and HuMSCs both had chondrogenic ability. Thus, these assays altogether indicated that the cells isolated from foreskin tissue or umbilical cord were mesenchymal stromal cells according to the identification standard of ISCT[1].

Immunomodulatory function of FSMSCs and HuMSCs

In order to compare the immunomodulatory function between FSMSCs and HuMSCs, the secretion levels of IL-1 β , IL-6, IL-10, TNF- α and TGF- β were measured through ELISA.

First, we compared the differences in autocrine inflammation-related cytokines among FSMSCs, HuMSCs and peripheral blood mononuclear cells (PBMC) (Fig. 2a, Supplementary Table 1). The differences in basal secretion levels of IL-1 β , IL-10 and TGF- β in these three cells were not statistically significant at any time point; The basal secretion level of IL-6 was significantly higher in HuMSCs than that in FSMSCs and PBMC while it showed no significant differences between FSMSCs and PBMC; The basal secretion level of TNF- α was significantly higher in PBMC than that in FSMSCs and HuMSCs, while it showed no significant differences between FSMSCs and HuMSCs. In addition, we also compared whether there were differences in basal secretion levels of inflammation-related cytokines among these three cell types at different time points (Fig. 2a, Supplementary Table 1). The basal secretion level of IL-6 increased with time while the basal secretion level of TGF- β showed fluctuating pattern; The differences in the secretion of IL-1 β , IL-10 and TNF- α were not statistically significant at different time points. In summary, FSMSCs and HuMSCs, like PBMC, were able to secrete inflammation-associated cytokines.

Next, we compared the differences in the secretion of inflammation-associated cytokines between FSMSCs, HuMSCs and PBMS under lipopolysaccharides (LPS) stimulation. The differences in secretion levels of IL-10 and TGF- β were not statistically significant; The secretion levels of IL-1 β and TNF- α were higher in PBMCs while the secretion level of IL-6 was higher in FSMSCs. In addition, we compared whether there were differences in the secretion level of inflammation-associated cytokines among MSCs at different time points after stimulating with LPS (Fig. 2b, Supplementary Table 2). Among them, the secretion level of IL-6 all increased with time while the differences in secretion level of IL-10 was not

statistically significant; The secretion levels of IL-1 β and TNF- α in PBMC increased with time; The secretion level of TGF- β in FSMSCs increased with time. In summary, the secretion levels of pro-inflammatory cytokines IL-1 β and TNF- α were significantly increased in PBMCs after stimulation by LPS. However, FSMSCs and HuMSCs stimulated by LPS showed no significant changes in the secretion levels of pro-inflammatory cytokines IL-1 β and TNF- α .

Moreover, we compared the differences in the secretion of inflammation-related cytokines between FSMSCs and HuMSCs co-cultured with PBMC respectively and PBMC cultured alone (see Fig. 2c, Supplementary Table3). The secretion levels of IL-6, TNF- α , IL-10, and TGF- β were overall higher in FSMSCs co-cultured with PBMC than in HuMSCs co-cultured with PBMC or in PBMC cultured alone; There was no significant difference in the secretion level of IL-1 β among these three conditions. In addition, we compared whether there were differences in the secretion of inflammation-related cytokines at different time points among these three conditions (Fig. 2c, Supplementary Table 3). Among them, the secretion level of IL-6 increased with time; The secretion levels of IL-10 and TNF- α in FSMSCs co-cultured with PBMC increased with time. In summary, FSMSCs were able to stimulate PBMC to secrete TNF- α , IL-6, IL-10 and TGF- β than the HuMSCs.

Finally, we mimicked the inflammatory response by stimulating PBMC with LPS *in vitro*. FSMSCs and HuMSCs were co-cultured with PBMC pre-stimulation respectively. And then, we compared the immunoregulatory ability through detecting the secretion of inflammation-related cytokines (Fig. 2d, Supplementary Table 4). The secretion levels of the pro-inflammatory cytokines IL-1 β and TNF- α were lower in the FSMSCs or HuMSCs than that in the control; The secretion levels of the bidirectional immunomodulatory cytokines IL-6, anti-inflammatory factor IL-10 and TGF- β were higher in FSMSCs than that in HuMSCs. In addition, we investigated whether there were significant differences in the secretion levels of inflammation-associated cytokines in the FSMSCs and HuMSCs at different time points (Fig. 2d, Supplementary Table 4). Among them, the secretion levels of IL-6, IL-10 and TGF- β increased with time in FSMSCs co-cultured with PBMC pre-stimulation; The secretion level of IL-10 increased with time in HuMSCs co-cultured with PBMC pre-stimulation. In summary, FSMSCs and HuMSCs both inhibited the secretion of pro-inflammatory cytokines IL-1 β and TNF- α from PBMC. It implied that FSMSCs and HuMSCs were both able to regulate immune function. Meanwhile, FSMSCs were more significantly able to upregulate the secretion of the bidirectional regulator inflammation IL-6 and the anti-inflammatory cytokines IL-10 and TGF- β than HuMSCs. It indicated that FSMSCs showed stronger immunomodulatory ability than HuMSCs, which was more suitable for the treatment of inflammation-related diseases.

Quality control, cluster and biological annotation

The quality control, cluster and biological annotation were used for FSMSCs and HuMSCs through bioinformatics analysis. First, we created the Seurat object for FSMSCs and HuMSCs separately. Secondly, we performed quality control and filtered the low-quality cells with less than 200 genes, more than 20000 genes, more than 10% percentage of mitochondrion genes and more than 10% percentage of erythrocyte genes. Then, we gained 7335 cells in FSMSCs in which the median of UMI was 33202 and the

median of gene was 5440. We also gained 12542 cells in HuMSCs in which the median of UMI was 15191 and the median of gene was 3831. The result showed that the quality of the transcriptome sequencing data was high. Afterwards, we ran normalization and removed the effect of mitochondrial gene in the process of scale. Next, we executed dimensionality reduction by PCA through first 2000 genes with high heterogeneity and evaluated the first 50 PCs. The first 20 PCs with the most significant p values were used for TSNE and UMAP. We also used the first 20 PCs to cluster cells with a resolution of 0.5, and we finally obtained 7 clusters in FSMSCs and 10 clusters in HuMSCs respectively (Fig. 3a).

Then, we annotated the clusters according to the surface markers of MSCs[1]. In FSMSCs, C0, C1, C2, C3 and C5 highly expressed the positive marker genes CD73, CD90 and CD105, and lacked the expression of the negative marker genes HLA-DRA, CD11b, CD19, CD34 and CD45 (Fig. 3a). It meant that C0, C1, C2, C3 and C5 were possible MSCs. But C4 highly expressed HLA-DRA and C6 was lack of the expression of CD73 and CD105. It indicated that C4 and C6 did not satisfy the identification criteria for MSCs. Similarly, in HuMSCs, C0, C1, C2, C3, C5 and C6 highly expressed the positive marker genes CD73, CD90 and CD105, and lacked the expression of the negative marker genes HLA-DRA, CD11b, CD19, CD34 and CD45, suggesting that C0, C1, C2, C3, C5 and C6 might be MSCs. But C4, C7, C8 and C9 were lack of the expression of CD73 and CD105, therefore C4, C7, C8 and C9 did not satisfy the identification criteria for MSCs (Fig. 3a). In summary, these results suggested that only C0, C1, C2, C3 and C5 were MSCs in FSMSCs and accounted for 93.05% of FSMSCs. The C0, C1, C2, C3, C5 and C6 were MSCs in HuMSCs and accounted for 95.85% of HuMSCs.

Integration analysis

In order to reveals the potential heterogeneity of MSCs, we integrated the subsets which were annotated as MSCs in FSMSCs and HuMSCs. Then, we evaluated the batch effect of integration data by PCA plot in which the cells of FSMSCs and HuMSCs were able to overlap well after integration (Fig. 3d), indicating that the batch effect was corrected well. Afterwards, the integrated data were re-normalized, downscaled and clustered (resolution=0.5) for which we gained 7 major clusters in integration data (Fig. 3e). At the same time, we calculated the proportion of these clusters between FSMSCs and HuMSCs and showed them on bar plot (Fig. 3f).

Moreover, we inferred the cell cycle of each cluster by the CellCycleScoring function of R package Seurat (version 4.0.0)[34]. There was a higher proportion of G1 phase cells in C0, C2 and C6, suggesting a resting state. But G2M or S phase cells occupied a large proportion in C2, C3, C4 and C5 (Fig. 3f). In addition, MKI67, a gene encodes a nuclear protein associated with cell proliferation[52], was highly expressed in C2, C3, C4 and C5 (Fig. 3b). It suggested that the proliferative activity of C2, C3, C4 and C5 was stronger than other subsets. Intriguingly, the result of cellcycle inference were consistent with the result of MSCs cultured *in vitro*. It had been reported that there are three morphologies of MSCs *in vitro*, which are elongated spindle-shaped, large and flattened, and small and prototypical. The first two kinds of cells are larger and proliferated more slowly. The latter cells are smaller and proliferated more rapidly, which are

known as rapid self-renewing cells[53, 54]. It meant there were some cells with more active proliferation in MSCs. Therefore, we named C2, C3, C4 and C5 as Proliferative MSCs.

Secondly, C6 highly expressed the positive marker genes CD146, PDGFRB and NG2, and lacked the expression of the negative marker genes CD31, CD45 and CD34 (Fig. 3b). These genes were proved to be the markers of pericyte which could expressed the markers of MSCs too in prior study[55]. Therefore, C6 were possible pericyte. In addition, CXCL12 and PTGIS were highly expressed in C0 while MKI67 and CD146 were lack of the expression in C0 (Fig. 3b). Among them, The CXCL12 was known to play an important role in immunoregulatory function of MSCs[56]. PTGIS, prostacyclin I2 synthase, is able to catalyze the conversion of prostaglandin H2 to prostaglandin I2, which has been demonstrated to play an important role in immunoregulatory function according to previous research[57]. Meanwhile, we also observed that C1 and C6 could express CXCL12 and PTGIS, but their expression level was lower than C0. Its suggested that C0, C1 and C6 both possessed immunomodulatory ability. But the immunomodulatory function of C0 was most powerful among them. Therefore, we named C0 as Immune MSCs.

Finally, C1 lowly expressed CXCL12 and PTGIS, and lacked expression of MKI67 and CD146 (Fig. 3b). In addition, a few cells in C1 could express MKI67 (Fig. 3b). And we constructed a clustering tree based on the similarity of MSCs subsets as well as visualized it on dendrogram plot. Then, the expression patterns of C1 and C5 were closer (Fig. 3c). In addition, C5 belonged to Proliferative MSCs. It revealed that C1 were possible the precursor cells of C5 while C1 might be a transitional stage from C0 to Proliferative MSCs. Therefore, we named C1 as Progenitor Proliferative MSCs.

In summary, there were 4 subsets in the integrated data (Fig. 3e), such as Proliferative MSCs (MKI67⁺, CD146^{low+}, NG2⁺, PDGFRB⁻), Pericyte (CD146^{high+}, PDGFRB⁺, MKI67⁻, CD31⁻, CD45⁻, CD34⁻), Immune MSCs (CXCL12^{high+}, PTGIS^{high+}, PDGFRB⁺, CD146⁻, MKI67⁻) and Progenitor Proliferative MSCs (CXCL12^{low+}, PTGIS^{low+}, PDGFRB⁺, CD146⁻, MKI67⁻). In addition, we calculated the proportion of different MSCs subsets between FSMSCs and HuMSCs (Fig. 3f). Among them, Proliferative MSCs made up 16% of FSMSCs and 68% of HuMSCs; Pericyte made up 6% of FSMSCs and 4% of HuMSCs; Immune MSCs made up 56% of FSMSCs and 10% of HuMSCs; Progenitor Proliferative MSCs made up 22% of FSMSCs and 18% of HuMSCs. In previous analysis, Pericyte, Progenitor Proliferative MSCs and Immune MSCs could express CXCL12 and PTGIS. It indicated that Pericyte, Progenitor Proliferative MSCs and Immune MSCs all possessed immunoregulatory capacity. However, CXCL12 and PTGIS in Immune MSCs had the highest expression, suggesting Immune MSCs might have stronger immunoregulatory ability. Then, we also observed that the difference of the proportion of Immune MSCs between FSMSCs and HuMSCs was significant, and most of Immune MSCs were derived from FSMSCs. These results demonstrated that Immune MSCs might played a key role in immunomodulatory function between FSMSCs and HuMSCs.

Trajectory inference

First, we inferred the differentiation degree of different MSCs subsets. The cytotrace score which was near to 1.0 indicated a lower degree of differentiation while the cytotrace score was near to 0.0 indicated

a higher degree of differentiation. And the degree of differentiation of Proliferative MSCs and Progenitor Proliferative MSCs were higher than Immune MSCs and Pericyte (Fig. 4b). Some studies had reported that MSCs might originate from Pericyte[58]. Therefore, we defined Pericyte as the starting point of the differentiation trajectory of MSCs. Next, we constructed and finally gained two differentiation trajectories (Fig. 4a). The first trajectory started from Pericyte and differentiated into Proliferative MSCs; the second trajectory started from Pericyte, passed through Immune MSCs and Progenitor Proliferative MSCs, and finally differentiated into Proliferative MSCs. Proliferative MSCs were located at the end of differentiation in both 2 trajectories. And the immunomodulatory capacity of Proliferative MSCs was lower than that of Immune MSCs and Pericyte. These analyses indicated that the immunomodulatory function of MSCs might be influenced by the degree of differentiation.

RNA velocity analysis

The expression of MKI67 in Immune MSCs was lower than that in Proliferative MSCs from above analysis, which meant the proliferative activity of Immune MSCs was much lower. But we find most of arrows in Immune MSCs were longer than that in Proliferative MSCs (Fig. 4c). And the longer the arrow, the stronger the transcriptional activity. It meant that the overall transcriptional activity of Immune MSCs was stronger than Proliferative MSCs. Although Immune MSCs were less differentiated and showed weaker proliferative activity, it still express a large number of genes to maintain its biological function.

Gene set enrichment analysis

In order to assess the potential biological functions, all cell subsets were subjected to gene set enrichment analysis. First, we calculated the enrichment score and adjusted P value of Hallmark gene set in different MSCs subsets (Supplementary Tables 5 and 6) and demonstrated them on heatmap plot (Fig. 4d). Then, we filtered 15 statistically significant gene sets in Immune MSCs with the adjusted P value ≤ 0.05 . Among them, the immune-related gene sets "IL6 JAK STAT3 SIGNALING", "INTERFERON GAMMA RESPONSE" and "INTERFERON ALPHA RESPONSE" were up-regulated in Immune MSCs, indicating a strong immune regulatory ability. In contrast, the mitosis-related gene sets "MITOTIC SPINDLE", "G2M CHECKPOINT", "E2F TARGETS" were down-regulated in Immune MSCs, suggesting weak proliferative activity. Secondly, we filtered 13 and 19 statistically significant gene sets by corrected P value ≤ 0.05 in Progenitor Proliferative MSCs and Pericyte respectively. Among them, the immune-related gene set "TNFA SIGNALING VIA NFKB" was upregulated in both Progenitor Proliferative MSCs and Pericyte, suggesting that these MSCs also had immunoregulatory capacity. In addition, Progenitor Proliferative MSCs tended to enrich gene set related to adipose differentiation, such as "ADIPOGENESIS" and "FATTY ACID METABOLISM", thereby Progenitor Proliferative MSCs might possess a stronger lipogenic potential. However, in Pericyte, it preferred to enrich gene set related to mesenchymal histogenesis, such as "MYOGENESIS", "ANGIOGENESIS" and "EPITHELIAL MESENCHYMAL TRANSITION". It indicated that Pericyte had a strong potential for mesenchymal tissue transformation and regeneration. Next, we filter 20 statistically significant gene sets in Proliferative MSCs with corrected P value ≤ 0.05 . It lacked the immune-related gene sets "IL6 JAK STAT3 SIGNALING", "INTERFERON GAMMA RESPONSE" and

"INTERFERON ALPHA RESPONSE". However, the mitosis-related gene set "MITOTIC SPINDLE", "G2M CHECKPOINT", and "E2F TARGETS" MSCs were significantly up-regulated in Proliferative. It meant that Proliferative MSCs might not possess immune regulatory ability but their activity of proliferation was high. We also found that the hypoxia-related gene set "HYPOXIA" was significantly down-regulated in Proliferative MSCs while it was significantly up-regulated in Immune MSCs and Pericyte. In previous studies, hypoxia was proved to promote the immunoregulatory capacity of MSCs[59]. Thus, it further indicated Immune MSCs and Pericyte may possess strong immunoregulatory capacity.

In summary, "IL6 JAK STAT3 SIGNALING", "INTERFERON GAMMA RESPONSE" and "INTERFERON ALPHA RESPONSE" gene sets were significantly enriched in Immune MSCs (Fig. 4d). "IL6 JAK STAT3 SIGNALING", "INTERFERON GAMMA RESPONSE" and "INTERFERON ALPHA RESPONSE" gene sets were associated with immunomodulatory functions. We concluded that Immune MSCs played an important role in immunomodulatory functions of MSCs.

Differential gene expression analysis

We compared the gene expression between FSMSCs and HuMSCs and filtered 205 differential genes with the adjusted p value ≤ 0.05 and the absolute value of log2 fold change (Log2FC) ≥ 2 (Supplementary Table 7). Then, we intersected the immune-related genes with 205 differential genes, and obtained a total of 37 intersected genes. We annotated the intersected genes for immune functions based on the ImmPort database, Genecard database and relevant literature reports. These intersecting genes could be divided into 7 major categories, which were "Antigen processing and presentation", "Antimicrobials", "Cytokine receptors", "Cytokines", "Cytoskeleton", "Negative regulation of inflammatory response" and "Positive regulation of inflammatory response". Among them, RARRES2, B2M, LGALS3 and ADM which were highly expressed in FSMSCs were in "Antimicrobials" category and reported to possess immunomodulatory and antimicrobial ability [60-63]. In addition, RARRES2 has been found to have anti-inflammatory ability[64]. Then, we showed the expression of these 37 intersected genes between FSMSCs and HuMSCs (Fig. 4e). Next, we calculated the marker genes in different MSCs subsets and filtered 84 differential genes in Immune MSCs with the adjusted P value ≤ 0.05 and the absolute value of Log2FC ≥ 0.8 (Supplementary Table 8). Then, we intersected the immune-related genes with 84 differential genes, and obtained a total of 14 intersected genes. Among them, BIRC5 and PBK were down-regulated while CXCL12, TNFRSF11B, ADM, LGALS3, NUPR1, PTGIS, IGFBP4, FGF7, B2M, CRLF1, ACKR4 and SERPINF1 were highly expressed in Immune MSCs. B2M, LGALS3 and ADM were proved to possess immunomodulatory and antimicrobial ability in the previous studies[61-63]. It indicated that Immune MSCs might have strong immunomodulatory functions. Finally, we visualized the expression of these 14 intersected genes between different MSCs subsets (Fig. 3g).

Gene regulatory networks analysis

Next, we constructed gene regulatory network and identified twenty-two cell type specific regulons (CTSRs). These CTSRs might play an important role in the function of Immune MSCs. Among them, regulons IRF2 and IRF4, were highly expressed in Immune MSCs (Fig. 5a). IRF2 and IRF4 belong to the

interferon regulatory factor family which is associated with interferon regulation in response to viral infection and the regulation of interferon-inducible genes. Usually, members of this family are lymphocyte-specific and can negatively regulate Toll-like receptor signaling. The negative regulation is essential for the activation of innate and adaptive immunity. It had been reported that IRF4 was an inhibitor of TLR-induced inflammatory pathways[65]. Interestingly, IRF2 and IRF4 might regulate the expression of target gene CXCL12 based on single-cell RNA-seq data (Fig. 5b). It further indicates that IRF2 and IRF4 might play a key role in the immunomodulatory function of Immune MSCs. Finally, we showed the expression of all CTSRs among various MSCs subsets on heatmap plot (Fig. 5c).

Cell-Cell communication analysis

Afterwards, we performed Cell-Cell communication analysis to explore the interaction among different MSCs subsets. We identified a total of 2309 ligand-receptor pairs based on single cell transcriptome data (Fig. 6a). And these ligand-receptor pairs were mapped to 47 signaling pathways (Fig. 6e). Among them, there was a close interaction between Immune MSCs and other cell subsets (Fig. 6a). We observed the interaction of CXCL signaling pathways in all MSCs subsets and determined the role of each cell subset at the signaling pathway level by calculating the network centrality index (Figs. 6b-6c). And the Mediator score of Immune MSCs was high but the Influence score was low, suggesting that Immune MSCs were not a network node in the CXCL signal pathway. However, both the Sender score and Receiver score of Immune MSCs were high, indicating that Immune MSCs played an autocrine role in CXCL signaling pathway. Meanwhile, we showed the ligand-receptor pairs which contributed to the CXCL signal pathway on the histogram plot. Among them, CXCL12-ACKR3 made the greatest contribution to the CXCL signal pathway (Fig. 6d). It suggested that CXCL12-ACKR3 might play an autocrine role in Immune MSCs. ACKR3 is the scavenger receptor of CXCL12, which can regulate the concentration gradient of CXCL12[66]. The expression of ACKR3 in Immune MSCs can prevent excessive CXCL12 from causing damage to the cells themselves. In addition, some studies have found that blocking ACKR3 with antagonist AMD3100 can improve the immunosuppressive microenvironment of tumor[67]. It suggests that ACKR3 not only plays an important role in regulating the concentration gradient of CXCL12, but also maintains the immunosuppressive function. Therefore, CXCL12-ACKR3 might plays an important role in the Immune MSCs. Finally, we quantified the functional similarity of all 47 signal pathways and identified 4 signal pathway groups (Fig. 6e). Among them, CXCL, IGF, MIF, IL10, PARs, HGF, NT, GAS, EDN, ACTIVIN and GRN belonged to the same signal pathway, which implied that these signaling pathways might help CXCL pathway maintain the biological functions of Immune MSCs.

Public database analysis

We assessed the proportion of different MSCs subsets between ADMSCs, BMSCs, FSMSCs and HuMSCs which came from public database and private data. We found that Immune MSCs account for 24.36%, 43.46%, 55.65%, and 9.99% of cells in ADMSCs, BMSCs, FSMSCs, and HuMSCs respectively (Fig. 6f). It suggested that foreskin tissue might be an ideal tissue source for Immune MSCs.

Discussion

MSCs are a kind of stromal cells in varieties of tissues, which they are proved to possess the immunomodulatory function and can be used to treat lots of immune diseases. However, MSCs derived from different tissue are significantly heterogeneous in immunomodulatory function. At present, the major way to reduce the heterogeneity is pre-treating MSCs with IFN- γ and/or TNF- α . But it also increases the immunogenicity of MSCs, accelerate the clearance of MSCs *in vivo*, as well as enhances the potential risk of immune rejection. This is detrimental to the clinical application of MSCs. In this study, we compared the immunomodulatory function between FSMSCs and HuMSCs. We found FSMSCs, which had better immunomodulatory capacity, are more suitable for the treatment of inflammation than HuMSCs. Then, single cell transcriptome analysis was applied and we identified four MSCs subsets, such as Proliferative MSCs (MKI67⁺, CD146^{low+}, NG2⁺, PDGFRB⁻), Pericyte (CD146^{high+}, PDGFRB⁺, MKI67⁻, CD31⁻, CD45⁻, CD34⁻), Immune MSCs (CXCL12^{high+}, PTGIS^{high+}, PDGFRB⁺, CD146⁻, MKI67⁻) and Progenitor Proliferative MSCs (CXCL12^{low+}, PTGIS^{low+}, PDGFRB⁺, CD146⁻, MKI67⁻). Among them, the proportions of Immune MSCs in FSMSCs and HUMSCs were 56% and 10% respectively. The proliferative activity of Immune MSCs was low whereas their transcription activity was high. In addition, Immune MSCs, which stayed in close contact with other subsets, were similar to Pericyte at the degree of differentiation. Meanwhile, a variety of immune-related genes, gene sets and regulons were enriched in Immune MSCs. It meant Immune MSCs might play a key role in the heterogeneity of immunomodulatory function among MSCs derived from different tissue. And Immune MSCs are able to be isolate and better applied for the treatment of inflammation-related diseases without being limited by the heterogeneity of immunomodulatory function.

In addition, we assessed the proportion of Immune MSCs between ADMSCs, BMSCs, FSMSCs and HuMSCs, for which the foreskin tissue might be an ideal source of isolating Immune MSCs. We believe MSCs are immunoplastic and susceptible because of external environmental influences. And the foreskin tissue is usually exposed to bacterial and viral attack due to its surrounding dirty environment. Moreover, foreskin tissue is prone to undergo ischemia or hypoxia because of insufficient blood supply. In previous studies, hypoxia is proved to promote the immunoregulatory capacity of MSCs[59]. Thus, MSCs derived from foreskin tissue are more likely to differentiate toward anti-inflammatory MSCs which may be comprised mainly of immune MSCs in our study. In summary, there is a great heterogeneity in the MSCs derived from different tissues. An the Immune MSCs may be the potential mechanism of heterogeneity in immunoregulatory function through single cell transcriptome sequencing. Therefore, our study provides a new insight to explain and address the immunomodulatory heterogeneity of MSCs.

Conclusions

In summary, in the study, FSMSCs and HuMSCs are successfully isolated and identified. We compare the immunoregulatory function between FSMSCs and HuMSCs *in vitro*. Then, the single cell transcriptome sequencing is performed and we find Immune MSCs may play a key role in the heterogeneity of immunoregulatory function through bioinformatics analysis. It is a new insight that Immune MSCs could

be isolated and better applied for the treatment of immune-related diseases without being limited by the heterogeneity of immunomodulatory function derived from different tissues.

Abbreviations

MSCs: mesenchymal stromal cells;

FSMSCs: foreskin mesenchymal stromal cells;

HuMSCs: human umbilical cord mesenchymal stromal cells;

ISCT: International Association for Cell Therapy;

PBMC: peripheral Blood mononuclear cells;

LPS: lipopolysaccharides;

ELISA: enzyme-linked immunosorbent assay;

PBS: phosphate buffer solution;

PCA: principal component analysis;

PCs: principal components;

TSNE: t-distributed stochastic neighbor embedding;

UMAP: uniform manifold approximation and projection;

Log2FC: log2 fold change;

RAS: regulon activity score;

RSS: regulon specific score;

CTSRs: cell-type-specific regulons;

ADMSCs: adipose mesenchymal stromal cells;

BMSCs: bone marrow mesenchymal stromal cells.

Declarations

Ethical approval

After cesarean section or natural birth, umbilical cords were collected with the informed consent of donors' parents. Foreskin tissues were collected after circumcision surgery with informed consent of donors or donors' parents.

Consent for publication

Not applicable

Availability of data and materials

The addition tables and high resolution plots are included in the supplementary materials. And the single cell transcriptome datas are share in Mendeley Data (<https://data.mendeley.com/datasets/f4b2ykfv56/1>).

Competing interests

The authors state that there are no conflicts of interest regarding the publication of this article and there are no financial ties to disclose.

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Authors' contributions

S.C., H.Z., C.F., L.X., A.L. and S.L. finished the exam. S.C., and C.F. performed the bioinformatics analysis. M.L., X.Y., X.S., Y.L., Y.W. administrate project and polished the language. C.F. , H.W., M.W. P.H. and Y.W. wrote the original draft. Y.L., T.W., Y.Z. and L.M. guided, reviewed and edited the manuscript.

Statement

All authors have read and approved the manuscript.

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Figures

Figure 1

The identification of FSMSCs and HuMSCs. (a) Flow cytometric analysis of surface markers in HuMSCs. The control cells without staining are shown in blue while the positive cells with staining are shown in red. (b) Flow cytometric analysis of surface markers in FSMSCs. The control cells without staining are shown in blue while the positive cells with staining are shown in red. (c) The result of adipogenic, osteogenic and chondrogenic differentiation in HuMSCs and FSMSCs.

Figure 2

The results of ELISA assays. (a) The secretion levels of inflammation-related cytokines between different groups. The blue line represents FSMSCs, the yellow line represents HuMSCs, and the grey line represents PBMC. (b) The secretion levels of inflammation-related cytokines between different groups. The blue line represents FSMSCs stimulated by LPS, the yellow line represents HuMSCs stimulated by LPS, and the grey line represents PBMC stimulated by LPS. (c) The secretion levels of inflammation-related cytokines between different groups. The blue line represents FSMSCs cultured with PBMC, the yellow line represents HuMSCs cultured with PBMC, and the grey line represents PBMC cultured alone. (d) The secretion levels of inflammation-related cytokines between different groups. The blue line represents FSMSCs cultured with PBMC stimulated by LPS, the yellow line represents HuMSCs cultured with PBMC stimulated by LPS, and the grey line represents PBMC stimulated by LPS.

Figure 3

The visualization of bioinformatics analysis. (a) The heatmap plots show the expression of MSCs surface markers between FSMSCs and HuMSCs. The bar chart above the heatmap represents different cell subsets with different colors. And the UMAP plots show the distribution of different cell subsets on low dimensional space. Among them, red dots represent MSCs, and green dots represent unknown cells. (b) Violin plots show the distribution of the expression of marker genes among different MSCs subsets with different colors. (c) The dendrogram plot shows the similarity of MSCs subsets. Dots with different colors correspond to different MSCs subsets. The expression patterns of MSCs subsets are closer when the dots are near on the dendrogram plot. (d) The PCA plots show the distribution of FSMSCs and HuMSCs on low dimensional space before and after integration. Among them, red dots represent FSMSCs, and green dots represent HuMSCs. The left plot shows the influence of batch effect before integration. And the right plot shows different colored dots are able to overlap well. (e) The UMAP plots show the distribution of different MSCs subsets on low dimensional space before and after integration. (f) The left bar plots show the proportion of different MSCs subsets between FSMSCs and HuMSCs. Different colors represent different MSCs subsets. The right bar plots show the proportion of different cell cycle phases among different MSCs subsets. Different colors represent different cell cycle

phases. (g) The circle plot shows the expression of immune-related differential gene of Immune MSCs. The color intensity of grid represents the degree of gene expression. The bluer the color, the lower the gene expression and the redder the color, the higher the gene expression.

Figure 4

The results of trajectory inference, RNA velocity analysis, gene set enrichment analysis and differential gene expression analysis. (a) The UMAP plots shows the two differentiation trajectories. The larger the value of the pseudotime, the greener the color of dots. (b) The box plot shows the distribution of cytotrace scores in different MSCs subsets. The cytotrace score which is near to 1.0 indicates a lower degree of differentiation and vice versa. The four asterisks indicate p value of Wilcoxon Test between two groups is less than 0.0001. And the p value of Kruskal-Wallis Test for overall test is less than $2.2e-16$. (c) The UMAP

plot shows the distribution of transcriptional activity. And the longer the arrow, the stronger the transcriptional activity. (d) The heatmap shows the result of hallmark gene set enrichment analysis. The rows are gene sets ,and the columns are cell subsets. The bluer the color of grid, the lower the enrichment and vice versa. The left dendrogram of heatmap represents the similarity of express pattern in different gene set. The asterisks indicate p value is less than 0.05. (e) The heatmap shows the expression of 37 immune-related differential genes between FSMSCs and HuMSCs. The rows are genes, and the columns are differenet groups or clusters. The bluer the color of grid, the lower the gene expression and vice versa. The left dendrogram of heatmap represents the similarity of express pattern in gene. And the left bar with different colors represent different categories.

Figure 5

The results of gene regulatory networks analysis. (a) The scatter plot shows the regulon specific score (RSS) of all regulons which are arranged by RSS and the threshold value of RSS with red line. Only the regulons with RSS > 0.6 are considered as cell type specific regulons (CTSRs). Meanwhile, the IRF2 and IRF4 regulon are marked on the scatter plot. The UMAP plots shows the the distribution of MSCs subsets and the expression of IRF2 and IRF4 regulon. (b) The circle plot shows the target gene of IRF2 and IRF4 regulon. The higher the number of target genes, the larger the circle in which the regulon is located. Each line represents a target gene, and the red line is CXCL12. (c) The heatmap plot shows the expression of

22 CTSRs among different cell subsets. And the darker the color of grid, the higher the expression of regulon. The left dendrogram of heatmap represents the similarity of express pattern in regulon. And the top bar of heatmap represents different cell subsets.

Figure 6

The results of Cell-Cell communication analysis and public database analysis. (a) The chord plot shows a total of 2309 ligand-receptor pairs between different MSCs subsets. The higher the number of ligand receptor pairs, the thicker the line. (b) The chord plot shows the expression of CXCL signaling pathway between different MSCs subsets. (c) The heatmap shows the scores of Sender, Receiver, Mediator and Influence between different MSCs subsets. (d) The histogram plot shows the ligand-receptor pairs which contribute most to the CXCL signal pathway. (e) The left plot shows the 4 signal pathway groups with different colors. And the right plots show all signaling pathways of specific pathway groups respectively. And the smaller the dots, the higher the probability of communication. (f) The circle plot shows the the proportion of different MSCs subsets between ADMSCs, BMSCs, FSMSCs and HuMSCs.

Supplementary Files

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