

Individual Heterogeneity Screened Umbilical Cord Derived Mesenchymal Stem Cells With High Treg Promotion Significantly Recovered the Mouse Liver Fibrosis

Yuanyuan Xie

Nanjing Drum Tower Hospital: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Shuo Liu

Nanjing Drum Tower Hospital: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Liudi Wang

Nanjing Drum Tower Hospital: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Hui Yang

Nanjing Drum Tower Hospital: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Chenxu Tai

Nanjing Drum Tower Hospital: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Li Ling

Huazhong University of Science and Technology

Libo Chen

Huazhong University of Science and Technology

Shanshan Liu

Nanjing Drum Tower Hospital: Nanjing University Medical School Affiliated Nanjing Drum Tower Hospital

Bin Wang (✉ wangbin022800@126.com)

The Affiliated Drum Tower Hospital of Nanjing University Medical School <https://orcid.org/0000-0003-3981-8849>

Research Article

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Abstract

Background

To compare the heterogeneities of human umbilical cord mesenchymal stem cells (HUCMSCs) derived from different donors and test their therapeutic variations in mouse liver fibrosis model.

Methods

The HUCMSCs derived multiple donors were performed comprehensively analysis and potent assays including expressions of surface markers, viability, growth curve, karyotype analysis, tumorigenicity, differentiation potentials, and immune regulation capability. Then the HUCMSCs with distinct immunomodulatory effects were tested for treating liver fibrosis in mice and then therapeutic effects were observed.

Results

The HUCMSCs derived multiple donors kept a high consistency in surface marker expressions, viability, growth curve, tumorigenicity in nude mic, but had robust heterogeneities in differentiation potentials and immune regulations. In addition, three HUCMSC lines applied to mice liver fibrosis model had different therapeutic outcomes, in line with individual immune regulation capability.

Conclusion

The HUCMSCs derived different donors have individual heterogeneity, which potentially lead to distinct therapeutic outcomes in mouse liver fibrosis, indicating we could make use of the donor-variation of MSCs to screen out guaranteed general indicators of MSCs for specific diseases in further stem cell therapy.

Background

Mesenchymal stem cells (MSCs) have been widely tested for treating a variety of refractory medical indications such as type 1 diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis and Crohn's disease due to their multiple differentiation potentials and immunomodulation capability [1–5]. MSCs could be derived from a series of tissues including but not limited to umbilical cord, placenta, adipose tissue, bone marrow, gingiva, and dental pulp [5–12]. These MSCs derived different tissues do not have uniform characteristics, differing in expression profiles of surface markers and biological functions after certain stimulation such as pro-inflammatory mediators [5, 13].

The heterogeneity of MSCs discussed above hampers the comparison of the therapeutic among different MSC products when applied to clinic use. To achieve a general definition of MSCs, the International Society for Cellular Therapy (ISCT) raised a minimal set of standard to specify MSCs as following: (i) MSCs present plastic-adherence in standard culture conditions; (ii) MSCs should positively express surface markers of CD73, CD90, CD105, and negatively express CD14/CD11b, CD34, CD45, CD79a/CD19, and human leukocyte antigen (HLA)-DR; and (iii) MSCs maintains the differentiation potentials of adipocytes, osteoblasts, and chondroblasts *in vitro* [14]. But these criteria of MSCs have been debated ever since and each MSC product is deemed to be unique. In spite of tissue origin, many other elements such as culture method and further modulations also influence the heterogeneity of MSCs, leading to differential gene expression profiles, growth phenotype, and differentiation potentials [15–17].

Over the past few years, MSCs researches have achieved some inspiring results and some of which moved up to clinic period from preclinical phases, resulting in the marketing approval of a few cell-based therapy products (CTPs) by different national regulatory authorities [18]. According to China's new regulatory policies, CTPs will be classified as biological drug and be regulated according to the principles of drug review and monitoring [19]. CTPs are considered to be the most complicated healing drugs in the history of human medical care due to their intricate biological features. MSCs, if considered as a CTP in therapeutic use, have a huge challenge to achieve stable and uniform biological characteristics for ensuring safety and effectiveness in patients received MSCs treatment.

Our hospital is approved to be qualified for implementing stem cell clinic trials by government agencies [19]. We established a Good Manufacturing Practice (GMP) grade cell facility to produce clinic-grade human umbilical cord derived MSCs (HUCMSCs) for treating premature ovarian failure (POF) and recurrent uterine adhesion [18, 20]. In our practice of MSCs-based therapy, we set up a quality evaluation system to guarantee the security of therapeutic HUCMSCs as a CTP in clinic [21]. This quality evaluation system assayed various biological features of MSCs including but not limited to cell viability, proliferation, apoptosis, growth curve, differentiation potentials, karyotype analysis, expression of surface markers, tumorigenicity, and immunoregulation ability. In this study, based on the quality evaluation of HUCMSCs derived from each donor, we compared the heterogeneities of HUCMSCs derived from different donors and tested their therapeutic

effects in mouse liver fibrosis model. Results showed HUCMSCs derived multiple donors had remarkable individual heterogeneities in differentiation potentials and immune regulations. We proposed that MSCs with individual heterogeneity could display functional variations when applied to certain disease treatment, by which we could make use of the donor-variation of MSCs to screen out guaranteed general indicators of MSCs for specific diseases in further stem cell therapy. Thus, based on the individual immunoregulatory heterogeneity, we screened out three HUCMSC strains with different immune phenotype and applied them to mouse tetrachloromethane (CCl4) induced-live fibrosis treatment to examine their therapeutic efficacy. As we expected, though all three test strains of MSCs displayed effective outcomes in treating mouse liver fibrosis. MSCs owing distinct immune phenotypes, had distinct therapeutic efficacy. The MSCs strain with high regulatory T cells (Tregs) promotion phenotype had the best therapeutic outcomes in treating the mouse liver fibrosis by altering the endogenous T subset differentiation. Thus we could take the advantage of individual heterogeneity to screen out seeding cells with the best criteria for specific disease.

Materials And Methods

This research was supported by the Research Ethics Board of Nanjing Drum Tower Hospital. Written consent was obtained from the puerperia who are willing to donate the umbilical cords for isolating MSCs after childbirth.

Donor screening criteria

Cell-based therapies have the probability to spread infectious diseases. The delivery type, age and health condition of the donor can as well influence the quality and function of MSCs. Therefore, a strict donor screening is required to be carried out before sampling, including physical examination, explicit medical history, and infectious disease detection. For the sake of excluding the window phase of viral infections, three months after the sample donation, we will have another serological tests of infectious diseases for the donor three months after umbilical cord donation [22]. The general features of umbilical cord donors are listed in supplementary table 1.

Supplementary table 1. The general features of umbilical cord donors

	Age	Gestation	Delivery mode	Parity	Neonate gender
UC1	32	39 + 5	Vaginal	2	Female
UC2	25	36 + 6	Vaginal	1	Female
UC3	26	38 + 3	Vaginal	1	Female
UC4	31	37 + 3	Vaginal	1	Female
UC5	32	38 + 2	Vaginal	1	Male
UC6	29	38 + 2	Vaginal	3	Male
UC7	29	40	Vaginal	1	Male
UC8	28	39 + 4	Vaginal	1	Male
UC9	27	39 + 4	Vaginal	1	Female
UC10	26	38 + 3	Vaginal	1	Male
UC11	28	39	Vaginal	1	Female
UC12	25	40 + 2	Vaginal	1	Male

HUCMSCs culture

MSCs are manufactured in clean environments in accordance with requirements of current Good Manufacturing Practice (cGMP) [18]. The critical raw materials and reagents applied in MSCs culturing containing fetal bovine serum (FBS, Gibco, USA), tryple and culture medium. First of all, it is necessary to guarantee that the materials and reagents applied in cell therapy are bought from capable manufacturers, which must ensure their GMP requirements, and the credentials should be gained as well. As demanded in the laws and regulations, GMP compliant FBS can be used for preparing therapeutic grade stem cells, but the serum must be free of Bovine Spongiform Encephalopathy/Transmissible Spongiform Encephalopathy (BSE/TSE). Tryple must be certified to be free from animal viruses and porcine mycoplasmas.

The primary HUCMSCs were isolated putting to use tissue explant method [22]. The detailed experimental reagents and methods could be referred to our previous research [21]. The cells of fourth generation were harvested for further studies. To avoid the differences caused by culture method, HUCMSCs from each donor were cultured by two well-trained cell culture operators in strict accordance with standard procedures.

Cell counts and viability

The number of cells was measured by automatic cell counter (Nexcelom, cellometer Mini, USA), and trypan blue exclusion method was used for cell viability detection. Moreover, the forth passage cells were harvested for CCK8 and cell cycle assays as a complementary experiment to describe the viability of cells. The Cell Counting Kit (Beyotime, China) was carried out according to the manufacturer's instruction and then the growth curve was drawn. The BD Cycletest Plus DNA Reagent Kit (BD, USA) was used to determine cell cycle.

Surface marker expressions

The final identification of cells is the first problem that requires to be settled in cell therapy products. The settings of the cell recognition criteria contribute to the data exchange among research workers and make a distinction between blended cell population. According to guidelines from Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular therapy (ISCT), MSCs have three minimal definition criteria including adhesion to plastic, expressions of specific surface markers (CD105, CD73, CD90, positive cells $\geq 95\%$; CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR negative cells $\leq 2\%$), and multi-lineage differentiation potentials of adipogenesis, osteogenesis, and chondrogenesis[14]. The detailed experimental reagents and methods could be referred to our previous research [21].

Multi-lineage differentiation assays

In regard to multi-lineage differentiation, MSCs at the forth passage were harvested and were replated in 24-well tissue culture plate at a density of 1×10^4 cells /well. The detailed experimental reagents and methods could be referred to our previous research [21]. Briefly, HUCMSCs were cultured in adipogenic, osteogenic, or chondrogenic medium (Gibco, USA) to induce adipogenesis, osteogenesis or chondrogenesis for 21 days and stained with Oil red (Sigma-Aldrich, USA), Alizarin Red S (Sigma-Aldrich, USA) or Alcian Blue (Sigma-Aldrich, USA) to assess the adipogenic, osteogenic or chondrogenic differentiations, respectively.

Safety evaluation of tumorigenicity and karyotype

Researches have indicated that the possibility of chromosomal abnormality of MSCs was 4 % during the *in vitro* culturing and the tumorigenicity of stem cells is as well a potential safety hazard in clinic application [21]. Tumorigenicity analysis was performed within severe combined immunodeficient (SCID) mice to determine that the MSCs had no tumorigenicity risk and Giemsa banding technique was adopted for karyotype analysis to verify the genetic stability of MSCs. The detailed experimental reagents and methods could be referred to our previous research [21]. Chromosomal abnormalities include the number abnormalities and morphological distortion. In terms of tumorigenicity, male SCID mice received subcutaneous injection of hUCMSCs, human embryonic stem cells (hESCs) as positive control or PBS as negative control and the tumor formation was recorded once a week for 4 months. The mice were euthanized and main organs were sectioned for hematoxylin-eosin (H&E) staining.

Immunomodulation assay

Increasingly evidence indicated that the immunomodulatory function of MSCs is the basis for the treatment of systemic lupus erythematosus (SLE), osteoarthritis and other diseases. It is recommended as a potency as well a release standard for advanced period clinical trials by the ISCT [23]. The detailed experimental reagents and methods could be referred to our previous research [21]. Briefly, the immunomodulatory effects of HUCMSCs on Th1 (CD3 + CD8 - IFN- γ +), Th17 (CD3 + CD8 - IL17A +), and Tregs (CD4 + CD25 + Foxp3 +) were detected by co-culturing HUCMSCs with human peripheral blood mononuclear cells (PBMCs) in our evaluation system. Flow cytometry (BD facsariatm, USA) was used to analyze the cells, and the data was analyzed by FACS software.

Mouse liver fibrosis model and HUCMSCs treatment via open-flow microperfusion (OFM)

All the animal experiments were performed in accordance with the guidelines and regulations from the Institutional Animal Care and Use Committee of *Nanjing University*. Animal care was provided in compliance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Mouse liver fibrosis was induced by CCl₄. CCl₄ was dissolved in corn oil(V/V,25 %), which was injected into the peritoneum of 10-weeks mice with a dose of 1g/kg CCl₄. The CCl₄ administration was performed twice a week and lasted for 8 weeks. Mice in control group were injected same volume of corn oil into peritoneum. The abdominal cavity was opened to confirm the success of liver fibrosis and the mice were used for subsequent experimental operations. HUCMSCs were re-suspended into PBS (2×10^6 /mL) and were administrated by liver orthotopic injection via OFM as following: Mice are anesthetized, fixed on

their backs and shaved. The abdominal cavity of the mice is cut to expose the liver after epidermal disinfection. The OFM guide cannula is implanted in the left lobe of the liver, and the OFM probe is inserted into the guide cannula. Then, the guide cannula is removed and the porous section of the OFM probe is left in the liver. The OFM probe is connected to a microdialysis pump and a syringe. 50 μ L cell suspension is injected into the liver with a flow rate of 2 μ L/min. After the injection, the mouse abdominal muscle layer and skin layer are sutured separately. Three weeks later, mice are anesthetized and the abdominal cavity is open to observe efficacy.

In this study, the level of hepatic fibrosis was researched by Masson staining (BASO, China), alanine aminotransferase (ALT) (blood samples which taken from mice' eyeballs were sent to the laboratory for testing) and immunohistochemical staining of α -SMA\Col I: slices were put at 65 \sim 80°C for 2 h, set into xylene I, xylene II, xylene III for 3 min in turn and rinsed under tap water for 30 s to 1 min after immersing in anhydrous ethanol, 95 % alcohol, 80 % alcohol, 75 % alcohol in turn for 2 min. Incubated with 3 % hydrogen peroxide at room temperature for 15 min to remove endogenous peroxidase, the slices antigen were repaired by pressure cooker method (1:100 dilution of antigen repair solution into ddH₂O). Then the slices were sealed with 5 % BSA or goat serum 60 min at room temperature. After adding appropriately diluted primary antibody at 4 °C overnight, the slices rinsed with PBST and 50 \sim 100 μ L secondary antibody was added to the tissue and incubated at room temperature for 30 \sim 60 min. The color developed by DAB, the hematlignin redyeing 1 \sim 5 min and ammonia solution returned to blue for 5 \sim 10 S. 75 % alcohol, 80 % alcohol, 95 % alcohol, anhydrous ethanol I and anhydrous ethanol II used for 3 min to dehydrate and xylene I and xylene II used for 5 min to permeabilize the tissues. Slices were sealed by neutral resin after dring the tissues. The staining results were observed under a microscope.

Statistical analysis

Data were showed by mean value \pm standard deviation (SD) from three or more tests. The statistical analysis of data was executed using Graphpad prism 6 software (GraphPad Software, USA). The quantitative data were compared by one-way ANOVA (S-NK). *P*-value < 0.05 was deemed statistically meaning.

Results

HUCMSCs stably expressing surface markers

In present study, we isolated HUCMSCs from 12 donors and analyzed their individual heterogeneity and therapeutic effects in liver fibrosis model. First, we examine the surface markers of HUCMSCs by FCM and the results showed that HUCMSCs from 12 donors stably expressed positive surface markers of CD105, CD90, and CD73 (over 95 % percentage), and negative surface markers of CD14, CD34, CD45, CD19, and HLA-DR (less than 2 % percentage) (Fig. 1). The surface markers of 12 HUCMSCs strains are listed in supplementary table 2.

Supplementary table 2. The surface markers of HUCMSCs

	UC1	UC2	UC3	UC4	UC5	UC6	UC7	UC8	UC9	UC10	UC11	UC12
CD14	0.2	0.0	0.1	0.0	0.2	1.4	0.4	0.1	0.0	0.0	0.0	0.2
CD19	1.8	0.0	0.1	0.2	0.1	0.5	0.2	0.9	1.4	0.1	0.1	0.1
CD34	0.1	0.5	0.3	0.2	0.1	0.7	0.3	0.1	0.6	0.0	0.0	0.1
CD45	0.2	0.0	0.0	0.2	0.2	0.4	0.2	0.0	0.0	0.0	0.0	0.2
CD73	100.0	99.6	100.0	99.8	99.3	97.9	99.6	99.9	99.7	99.7	99.5	99.3
CD90	100.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
CD105	99.8	99.8	99.7	96.2	98.8	99.1	99.7	99.9	99.9	100.0	100.0	98.8
HLA-DR	0.1	1.3	0.1	0.4	0.1	1.9	0.3	0.3	0.6	0.3	0.1	0.1

Viability and growth

The viability and growth are two important characteristics of MSCs. The trypan blue exclusion method was performed to assay the viability at different stages (Master cell bank, Work cell bank, and Releasing) and results showed a similar viability (over 90 %) among 12 HUCMSCs strains (Fig. 2B). We carried out CCK8, cell cycle assays, and Edu staining to examine the growth of HUCMSCs. The viability and cell cycle testing results of 12 HUCMSCs strains are listed in supplementary table 3. Cell cycle and CCK8 assays showed there was certain similarity in growth curve and each stage of cell cycle among 12 HUCMSCs strains. Further Edu staining revealed that there was not much difference overall among 12 HUCMSCs strains, only UC4 HUCMSCs had a markedly higher proliferation rate than UC1 HUCMSCs (*p* < 0.001) (Fig. 2F).

Supplementary table 3. The viability and cell cycle testing results of HUCMSCs

		UC1	UC2	UC3	UC4	UC5	UC6	UC7	UC8	UC9	UC10	UC11	UC12	$\bar{X} \pm SD$
Viability (%)	MCB	96.30	95.50	97.80	93.00	96.10	97.70	96.00	92.90	92.20	99.40	96.60	96.20	95.81 \pm 2.15
	WCB	95.40	97.90	96.80	97.70	97.40	95.80	96.30	98.30	96.30	98.80	94.50	96.50	96.81 \pm 1.26
Cell Cycle (%)	G0/G1	84.78	62.54	66.28	72.79	81.90	83.37	87.54	64.94	66.69	50.80	54.10	49.30	68.75 \pm 13.46
	S	12.04	31.34	29.56	17.21	12.09	11.32	9.23	25.06	13.10	31.10	25.20	29.00	20.52 \pm 8.77
	G2/M	3.18	6.13	4.16	10.00	6.01	5.31	3.23	10.00	20.21	17.50	20.10	19.60	10.45 \pm 6.95

MCB: Master Cell Bank; WCB: Working Cell Bank.

HUCMSCs having vast individual heterogeneity of osteogenic differentiation

It is well known that MSCs have differentiation potentials including osteocytes, adipocytes, and chondrocytes as their crucial characteristics. The osteogenic differentiation assay showed there was a vast individual heterogeneity among 12 HUCMSCs strains, varying from 1 % to 70 % osteogenesis (Fig. 3A and B). We wondered that whether gender could affect the osteogenic differentiation of HUCMSCs. The quantified data displayed that HUCMSCs from male infants had significantly higher potential of osteogenesis *in vitro* (about 10 folds) than HUCMSCs from female infants ($p < 0.01$), although there was also obvious heterogeneity among the 6 HUCMSCs strains from male infants (Fig. 3C). In robust contrast, there was no distinct individual heterogeneity in adipogenesis and chondrogenesis *in vitro* (Fig. 4).

Consistency in tumorigenicity and karyotype analysis among 12 HUCMSCs strains

The tumorigenesis risk is a major concern for MSCs application in clinic. The SCID mice were subcutaneously injected HUCMSCs to monitor the tumor formation during a 4-month observation period. Human embryonic stem cells (HESCs) injection was positive control and tumor was formed within about 1 month after transplantation. Among the transplantation of 12 HUCMSCs strains, there was no tumorigenicity and no observation of tumor cells infiltration by H&E staining at injection sites and main organs such as heart, liver, spleen, lung, muscle, and kidney (Supplementary Fig. 1A, 1B and Supplementary table 4). In karyotype analysis, all test HUCMSCs have normal karyotype of 46 chromosomes (XX/XY) and stable genetic stability including normal morphology, number, length, size, centromere position in karyotypes, without any abnormality in deletion, reduplication, inversion, translocation, insertion, and Ring-chromosome (Supplementary Fig. 1C and Supplementary Table 5). These results displayed that there was consistency in tumorigenicity and karyotype analysis among 12 HUCMSCs strains.

Supplementary table 4. Tumorigenicity of HUCMSCs

	UC1	UC2	UC3	UC4	UC5	UC6	UC7	UC8	UC9	UC10	UC11	UC12	PBS control	HESCs control
Skin	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Muscle	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Heart	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovary	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Uterus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovary	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary table 5. Chromosome karyotype analysis

	UC1	UC2	UC3	UC4	UC5	UC6	UC7	UC8	UC9	UC10	UC11	UC12
Karyotype	XX	XX	XX	XX	XY	XY	XY	XY	XX	XY	XX	XY
Number	46	46	46	46	46	46	46	46	46	46	46	46
Morphology	N	N	N	N	N	N	N	N	N	N	N	N
Length	N	N	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N	N	N
The centromere position	N	N	N	N	N	N	N	N	N	N	N	N
Deletion	N	N	N	N	N	N	N	N	N	N	N	N
Reduplication	N	N	N	N	N	N	N	N	N	N	N	N
Inversion	N	N	N	N	N	N	N	N	N	N	N	N
Translocation	N	N	N	N	N	N	N	N	N	N	N	N
Insertion	N	N	N	N	N	N	N	N	N	N	N	N
Ring-chromosome	N	N	N	N	N	N	N	N	N	N	N	N

Remarkable individual heterogeneity in immunomodulation effects of HUCMSCs

MSCs were capable to secrete immune mediators or directly interact with immune cells in recipients so as to play a therapeutic role in various immune diseases. We detected T cells subpopulation of PBMCs after being co-cultured with each HUCMSCs strain to estimate the immunoregulatory effects *in vitro*. HUCMSCs strains significantly inhibited the activation and differentiations of CD4 + T cells into Th1 and Th17 subpopulations and significantly promoted the maturation of Tregs subpopulation in PBMCs induced by IL-2 (Fig. 5). We noted that there was remarkable individual heterogeneity in immune regulation among all test HUCMSCs, and the same cell strain also had a different capability in every aspect of immune regulation. For example, UC12 HUCMSCs strain had the strongest capability to promote Treg subpopulation differentiation of PBMCs among all test HUCMSCs strains, but common suppression potential of Th1 and Th17 subpopulation differentiation. UC11 HUCMSCs only had very limited capability to promote Treg subpopulation differentiation and suppress Th1 and Th17 subpopulation differentiation. UC5 HUCMSCs strain had the strongest capability to inhibit Th1 subpopulations differentiation of PBMCs. We assumed the remarkable individual heterogeneity of HUCMSCs in immune regulation could lead to differential therapeutic effects in curing diseases. Thus next we applied UC5, UC11, and UC12 HUCMSCs strains to mouse liver fibrosis treatment to examine their therapeutic efficacy.

Individual heterogeneity of HUCMSCs affecting T cells subpopulation differentiation of splenocytes in liver fibrosis model

The mice were treated by CCl₄ to induce liver fibrosis. We isolated splenocytes from control and liver fibrosis (LF) model mice to induce the differentiation of CD4 + T cell subsets by Cell Stimulation Cocktail *ex vivo*. The results showed that liver fibrosis caused a significant increase in the proportion of CD4 + IFN- γ + T-cells (Th1), CD4 + IL-17A + T-cells (Th17), and CD4 + CD25 + Foxp3 + regulatory T-cells (Tregs), compared with the control group ($p < 0.05$) (Fig. 6). All three HUCMSCs strains treatment decreased the proportion of CD4 + Th1 subset, compared with LF group ($p < 0.05$). UC5 HUCMSCs treatment caused a lower CD4 + Th1 subset differentiation of splenocytes, compared with UC11 and UC12 HUCMSCs ($p < 0.05$). In addition, UC12 HUCMSCs treatment significantly promoted the Tregs differentiation of splenocytes, compared with UC5 and UC11 HUCMSCs treatments ($p < 0.05$). These results revealed that the individual heterogeneity of HUCMSCs was capable to alter the immune responses in liver fibrosis model, which probably contributed to different therapeutic efficacy in liver fibrosis treatment.

Individual heterogeneity resulting in differential therapeutic effects on mouse liver fibrosis

UC5 HUCMSCs, not UC11 and UC12 HUCMSCs, markedly decreased the interferon-gamma (IFN- γ) mRNA level in splenocytes, compared with LF group ($p < 0.05$). The elevated ALT level in serum was a general marker of liver damage. Serum ALT level was elevated by CCl₄ induced-liver fibrosis in mice, but was markedly alleviated by all three HUCMSCs strains. Further UC12 HUCMSCs better increased the ALT level than other UC5 and UC11 HUCMSCs ($p < 0.05$). UC12 HUCMSCs, not UC5 and UC11 HUCMSCs, significantly improved about 8 folds mRNA levels

of Foxp3 in splenocytes, compared with LF group ($p < 0.001$). Liver fibrosis could cause a heavy deposit of collagens in liver tissue, which lead to heavy Masson, alpha-smooth muscle actin (α -SMA), and Collagen I staining in liver tissue section. In our study, we could observe obvious nodules of liver fibrosis on the liver surface in CCl4 treated mice and heavily positive staining of Masson, α -SMA, and Collagen I in liver sections (Fig. 7). All three HUCMSCs strains markedly alleviated the amount and size of fibrosis nodules of liver surfaces, as well as the positive staining of Masson, α -SMA, and Collagen I in liver sections. Among three HUCMSCs, UC12 HUCMSCs had the best therapeutic potential in liver fibrosis.

Discussion

If MSCs are considered one type of drug, they beyond doubt are the most complex medicine in human medicine history due to their various biological characteristics. Cell-based therapy products need stable and uniform biological features to achieve consistent therapeutic effects in patients received treatment. However, each MSCs product owns unique characteristics and biological activities, because of different donors (gender and age), origin tissue, isolation and culture method, passage number, and further modulations [15, 19]. Even there is distinct individual heterogeneity among MSCs derived from same tissue origins of different donors. In this study, we compared the characteristics of HUCMSCs from 12 donors using our once established systemic quality evaluation for MSCs as a cell-based therapy product [21] and found distinct individual heterogeneity among donors. Based on individual heterogeneity, we screened HUCMSCs with high Tregs promotion to treat mouse liver fibrosis, and obtained therapeutic benefit by altering the endogenous T subset differentiation.

In our study, all 12 strains of HUCMSCs met the standard criteria of MSCs according to ISCT proposal in 2006. All MSCs present plastic-adherence in our GMP-complaint culture conditions and stably expressed positive surface markers (CD105, CD90, and CD73), and negative surface markers (CD14, CD45, CD19, CD34, and HLA-DR). Meanwhile all strains of MSCs maintained the differentiation potentials of adipocytes, osteoblasts, and chondroblasts *in vitro*. In addition, all 12 strains of MSCs collectively shared similar characteristics in viability, cell cycle, growth, and proliferation.

However, we noted that there was evident individual heterogeneity among 12 strains in several vital biological properties. One hallmark of the MSCs is their multipotency to differentiate into multiple lineages. We found that there was a vast individual heterogeneity among 12 HUCMSCs strains, varying from 1 % to 70 % osteogenesis. Moreover, HUCMSCs from male infants had robust higher potential of osteogenesis *in vitro* (about 10 folds) than HUCMSCs from female infants, indicating the gender remarkably affect the osteogenesis capability of MSCs. In robust contrast, there was no distinct individual heterogeneity in adipogenesis and chondrogenesis.

To our acknowledgement, this is first time to report the gender remarkably promoted the ability to give rise to osteogenic cells *in vitro*. In karyotype analysis, MSCs derived from male infants and female infants had the karyotype of 46 chromosomes (XY) and chromosomes (XX), respectively. The genetic variance between chromosome X and Y probably is the main reason which causes the robust gender-related osteogenesis potential in MSCs and the exact mechanism should be elucidated in further study. Obviously, the MSCs derived male infant with remarkably high osteogenesis could be applied to repair bone defect in clinic to obtain better therapeutic outcomes. Similarity to osteogenesis, we found that there was robust individual heterogeneity in immune phenotype among 12 strains of HUCMSCs.

It was well known that MSCs were capable to secrete immune mediators or directly interact with immune cells to play a role of immune regulation. HUCMSCs strains remarkably suppressed the activation and differentiations of CD4 + T cells into Th1 and Th17 subpopulations and significantly promoted the maturation of Tregs subpopulation in PBMCs induced by IL-2 *in vitro*. We observed that there was remarkable individual heterogeneity in immune regulation among all test HUCMSCs. Interestingly, the same cell strain also had a different capability in every aspect of immune regulation. For example, UC12 HUCMSCs strain had the strongest capability to promote Treg subpopulation differentiation of PBMCs among all test HUCMSCs strains, but mean suppression potential of Th1 and Th17 subpopulation differentiation. Accumulating evidences have showed that human MSCs derived different donors have individual heterogeneity [24–26]. For example, Phinney et al reported that there were dramatic differences in levels of bone-specific gene expressions and alkaline phosphatase enzyme activity among MSCs populations derived from posterior iliac crest marrow of 17 healthy donors [27]. Summarily, the subtle intrinsic variability in MSCs populations derived from different donors is a common phenomenon, apart from the inconformity of cultivation procedure and conditions.

Undoubtedly, the intrinsic individual heterogeneity in MSCs populations is a major obstacle for obtaining consistent cell-based therapeutic products aiming at MSCs. We proposed that MSCs with individual heterogeneity could display functional variations when applied to certain disease treatment. Thus we could make use of the donor-variation of MSCs to screen out guaranteed general indicators of MSCs for specific diseases in further stem cell therapy. Based on the individual immunoregulatory heterogeneity, we screened UC5, UC11, and UC12 strains of MSCs with different immune phenotype and applied them to mouse CCl4 induced-live fibrosis treatment to examine their therapeutic efficacy.

As we expected, though all three test strains of MSCs displayed effective outcomes in treating mouse liver fibrosis, MSCs derived from different donors, owing to distinct immune phenotypes, had distinct therapeutic efficacy. Liver fibrosis could cause a heavy deposit of collagens in liver tissue, which leads to heavy Masson, α -smooth muscle actin (α -SMA), and Collagen I staining in liver tissue sections. All three HUCMSCs strains markedly alleviated the amount and size of fibrosis nodules of liver surfaces, as well as the positive staining of Masson, α -SMA, and Collagen I in liver sections. Among three HUCMSCs, UC12 HUCMSCs had the best therapeutic potential in liver fibrosis. UC12 strain of MSCs had the highest potential of Treg cell differentiation and we also observed UC12 strain of MSCs could promote Treg differentiation in phenotype, proving the heterogeneity of MSCs owned the functional discrepancy in disease treatment.

MSCs have the potential of liver differentiation, immunomodulatory function, and the ability to produce nutritional factors, making them ideal drugs for the treatment of liver fibrosis [28]. A number of animal studies have shown that MSCs can safely reverse liver fibrosis and improve liver function [29–31]. In recent years, the immunomodulatory function of MSCs has gradually become the main research target in the treatment of liver fibrosis. *In vitro* experiments have proved the effects of HUCMSCs on different immune cell subsets while these effects are still need to be confirmed in animal models of liver fibrosis [28]. We selected three strains of HUCMSCs, with significantly different *in vitro* immune regulation capability. Among them, UC12 has the highest immune regulation capability of Tregs subsets *in vitro*, and exerts the strongest anti-fibrosis effect in the mouse liver fibrosis model induced by carbon tetrachloride. It is consistent with the anti-fibrosis effect of Tregs reported in many literatures. Claassen et al found that there were a large number of Treg cells in the liver of hepatitis C-related liver fibrosis model, and the degree of liver fibrosis decreased with the increase of the number of Treg cells, suggesting that Tregs had an inhibitory effect on the formation of liver fibrosis [32]. Treg cells are a subgroup of CD4+ T cells with reverse regulatory function. It plays an irreplaceable role in the immune tolerance and over-effect regulation by inhibiting the inflammatory stimulation response, preventing the excessive effect response and maintaining the immune balance. On the one hand, we speculate that the orthotopic transplanted MSCs secrete many soluble factors (such as prostaglandin E2, IDO, and IL-10) in the liver, which can change the microenvironment, exert anti-inflammatory effect, eliminate effector cells, significantly inhibit hepatocyte apoptosis and promote the proliferation of hepatic cell, so as to achieve the purpose of protecting liver tissue [33]. On the other hand, MSCs exerts the effect of systemic immunity and changes the proportion of immune effector cells in mice. The increased Tregs cell subsets reduce the infiltration of CD8+ lymphocytes into the liver, decrease the level of pro-inflammatory factors such as tumor necrosis factor- α in circulation, inhibit the proliferation of activated HSCs and collagen synthesis, and finally reduces fibrosis [34]. Hence, compared with the other two cell strains, UC12 improved the immune subsets of Tregs in mice to a greater extent, and the degree of liver fibrosis was significantly reversed.

Our study revealed that although MSCs are derived from same tissue origin such as human umbilical cord, they own donor-related heterogeneity, which could contribute to the explanation of experimental and clinical discrepancy. It is a huge challenge for researchers to obtain consistent qualified MSCs in clinic use because of the individual heterogeneity. In other hand, we could take the advantage of individual heterogeneity to screen seeding cells with the best criteria for certain disease treatment. We could establish an optimized criterion, beyond the general standards of MSCs to provide therapeutic benefit for various diseases, according to the disease pathogenesis, mechanism, and process. For example, the MSCs derived from male infant with high potential of osteogenesis could be applied to recover the huge bone defect. MSCs with a strong immunoregulatory effect were screened out for treating immune-related diseases.

Conclusion

Above all, we first proposed that MSCs should be established grading standards as a cell-based therapeutic product. The class standard is the general standard of MSCs as ISCT proposed, including certain surface marker expressions, plastic-adherence, differentiation potentials of adipocytes, osteoblasts, and chondroblasts *in vitro*, as well as immune phenotypes. The class II standard should go beyond the general standard of MSCs, namely the internal standards in each manufacture. The class III standard of MSCs is established for specific disease therapy based on the individual heterogeneity screening according to the disease pathogenesis, mechanism, and process, as well as the strategy of therapy.

Abbreviations

MSCs: mesenchymal stromal cells; **ISCT:** the International Society for Cellular Therapy; **HLA:** human leukocyte antigen; **CTP:** cell-based therapy product; **GMP:** Good Manufacturing Practice; **HUCMSCs:** human umbilical cord mesenchymal stromal cells; **POF:** premature ovarian failure; **CCl4:** tetrachloromethane; **Tregs:** regulatory T cells; **BSE:** Bovine Spongiform Encephalopathy; **TSE:** Transmissible Spongiform Encephalopathy; **SLE:** systemic lupus erythematosus; **ISCT:** International Society for Cellular therapy; **PBMCs:** peripheral blood mononuclear cells (PBMCs); **OFM:** open-flow microperfusion; **ALT:** alanine aminotransferase; **H&E:** hematoxylin-eosin; **SD:** standard deviation; **MCB:** Master Cell Bank; **WCB:** Working Cell Bank; **LF:** liver fibrosis; **FBS:** fetal bovine serum; **PBS:** phosphate buffered saline; **SCID:** severe combined immune deficiency; **HESCs:** human embryonic stem cells; **SLE:** systemic lupus erythematosus.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BW, YyX and SL designed the study. LdW, HY and CxT carried out the isolation and culture of HUCMSCs. YyX, SL, LL, and LbC performed the cell detections and animal experiments and analyzed the data. YyX wrote the first draft of the manuscript and all authors read, edited, and approved the final manuscript.

Ethics approval and consent to participate

All umbilical cord samples were taken after informed and written consent, and the study was approved by the Research Ethics Board of Nanjing Drum Tower Hospital (permit number: 2017–161-01).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

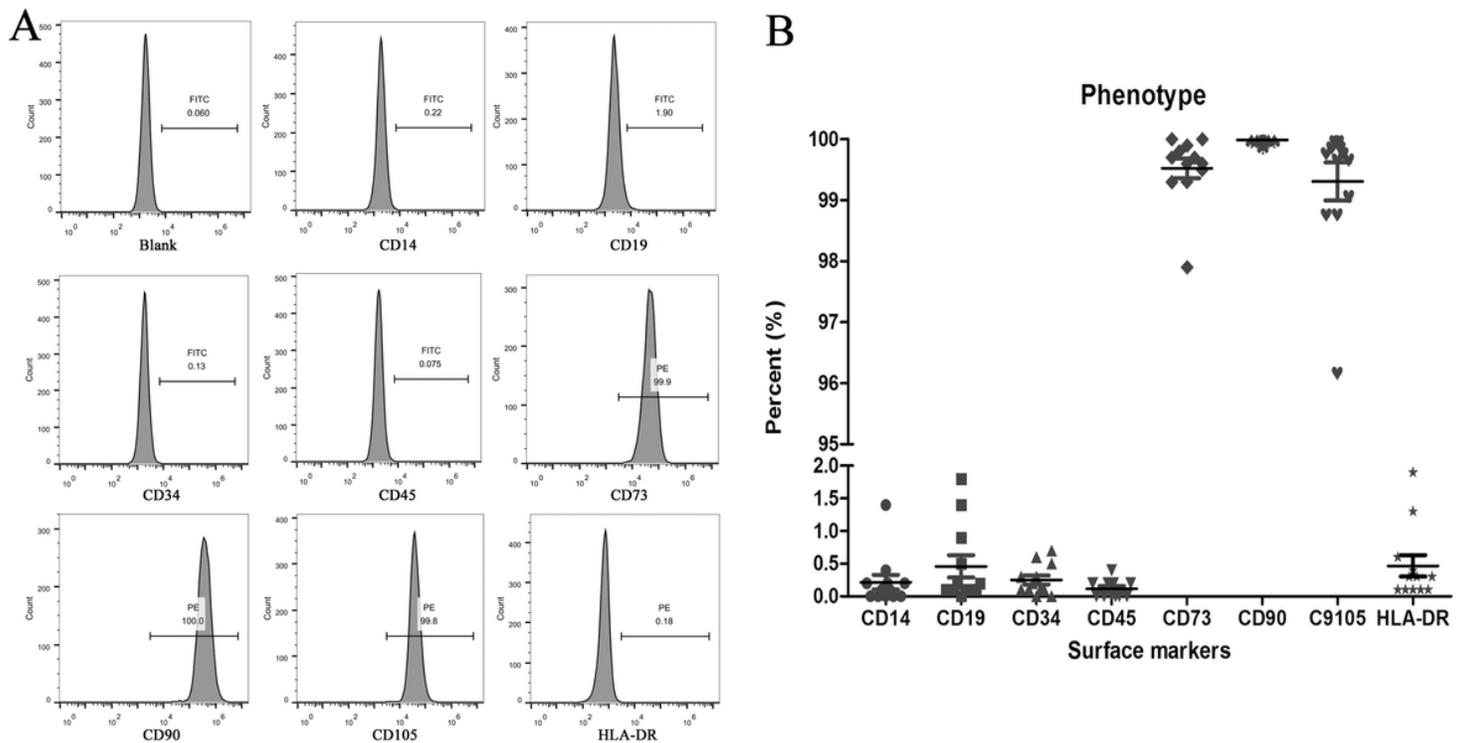


Figure 1

Surface markers of HUCMSCs. A: Flow cytometry was used to detect the surface markers in HUCMSCs, CD14, CD19, CD34, CD45 and HLA-DR were negative, while CD73, CD90 and CD105 were positive. B: Statistical analysis of all the mentioned markers of 12 HUCMSCs strains.

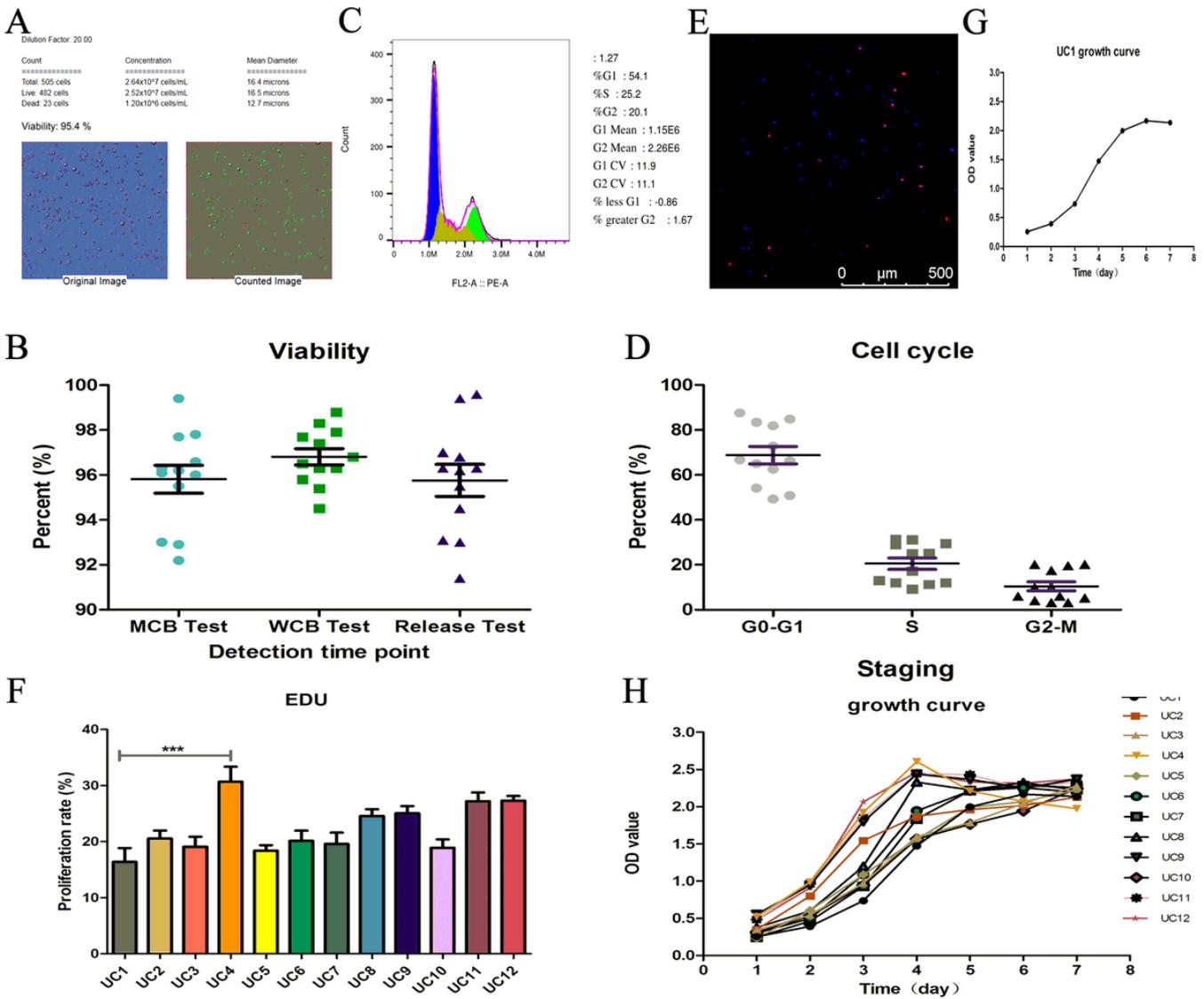


Figure 2

The viability and growth of 12 HUCMSCs. A: The trypan blue exclusion data for viability. B: The viability at different stages (Master cell bank, Work cell bank, and Releasing) with a similar viability. C and D: FCM analysis data for 12 HUCMSCs' cell cycle. E: The red nuclei cells were identified as proliferating cells in EDU immunostaining for proliferation rate. F: there was not much difference overall among 12 HUCMSCs strains. G and H: HUCMSCs had a 'S' growth curve. (***, $p < 0.001$).

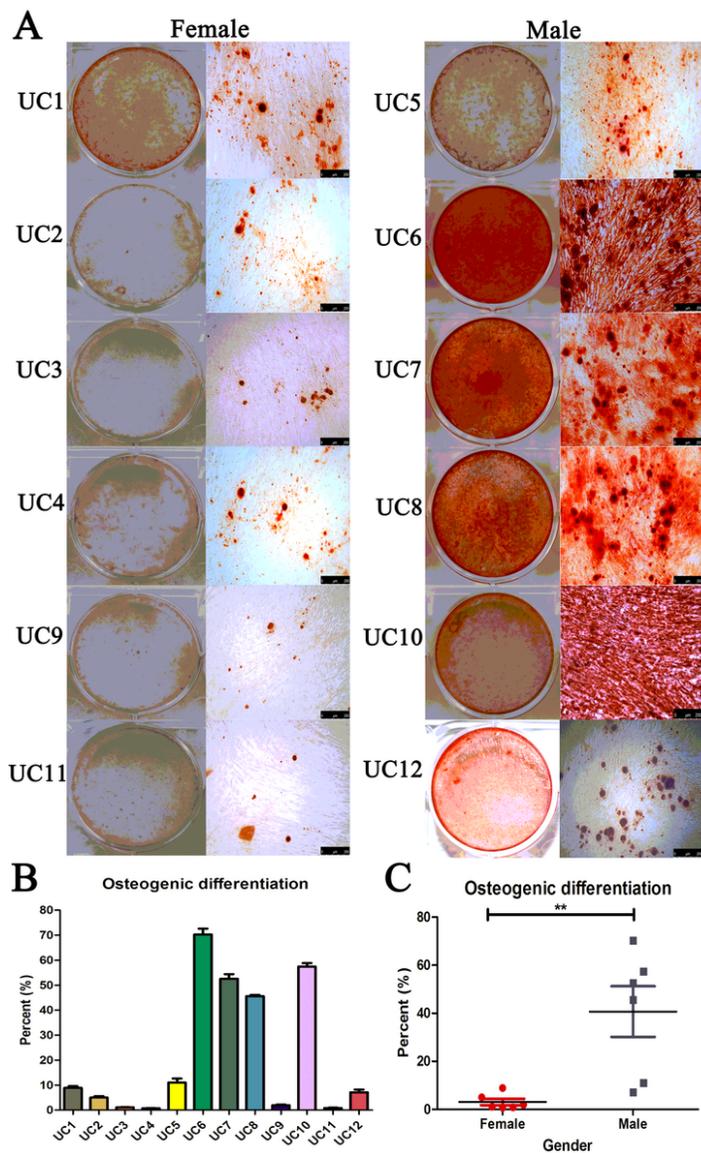


Figure 3

Alizarin red-S staining showed HUCMSCs were induced into osteogenic. There was a vast individual heterogeneity among 12 HUCMSCs strains in osteogenic differentiation assay. HUCMSCs from male infants had significantly higher potential of osteogenesis in vitro than HUCMSCs from female infants (**, $p < 0.01$).

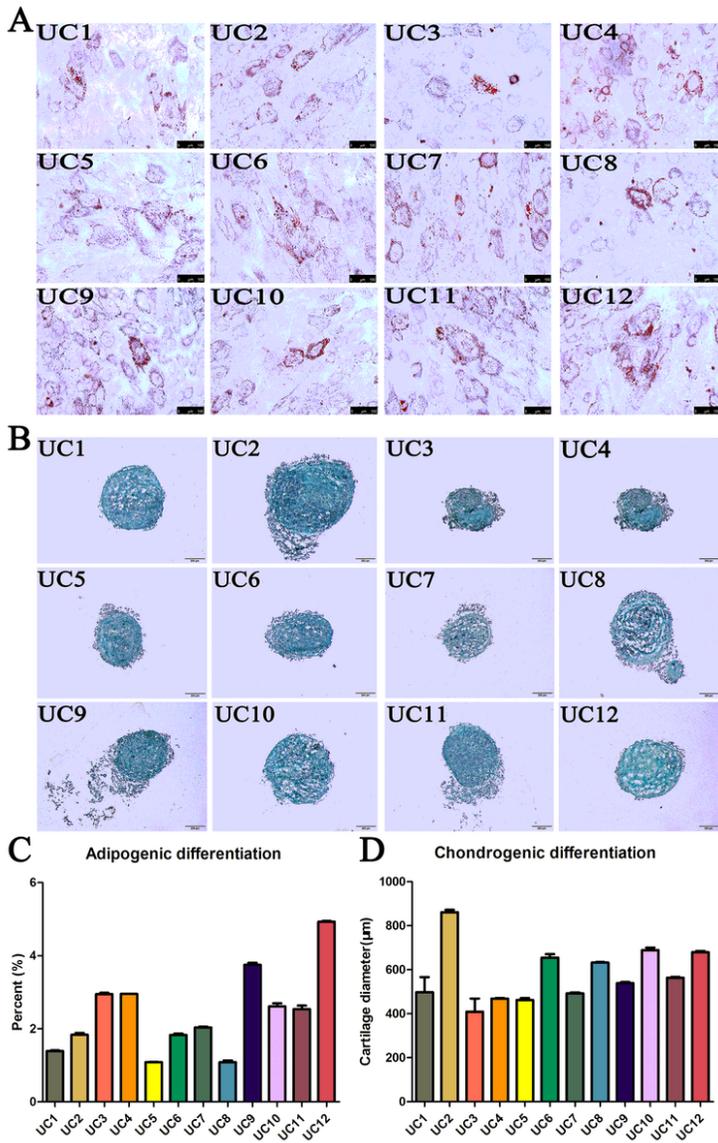


Figure 4

All the 12 strains of HUCMSCs were induced into adipocytes and chondrocytes detected by oil red O staining and alcian blue staining, respectively. There was no distinct individual heterogeneity in adipogenesis and chondrogenesis in vitro.

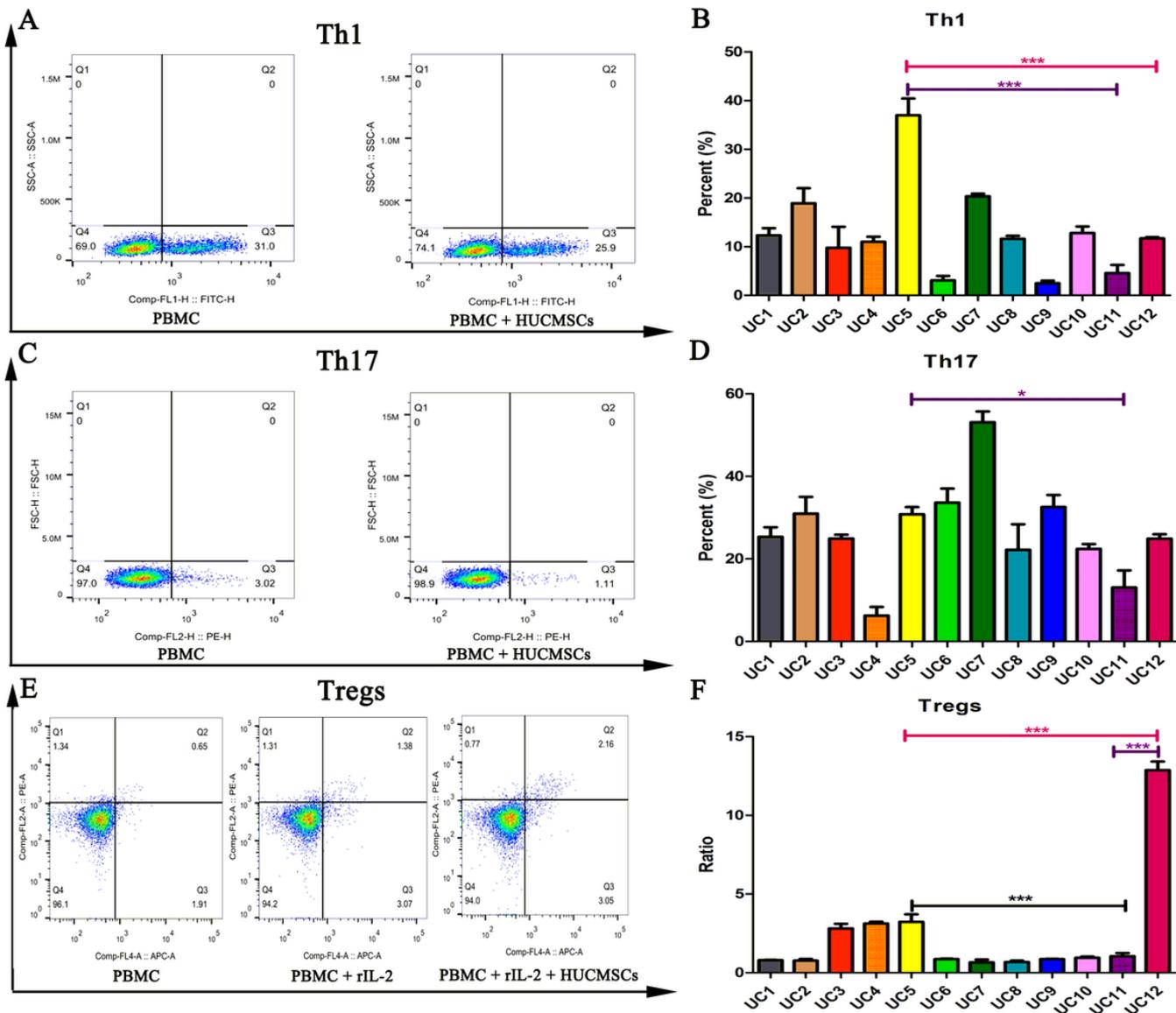


Figure 5

HUCMSCs inhibited the differentiations of CD4 + T cells into Th1 and Th17 substs, promoted the maturation of Tregs subpopulation in PBMCs induced by IL-2. There was remarkable individual heterogeneity in immune regulation among 12 test HUCMSCs, and the same cell strain also had a different capability in every aspect of immune regulation. (*, $p < 0.05$, ***, $p < 0.001$)

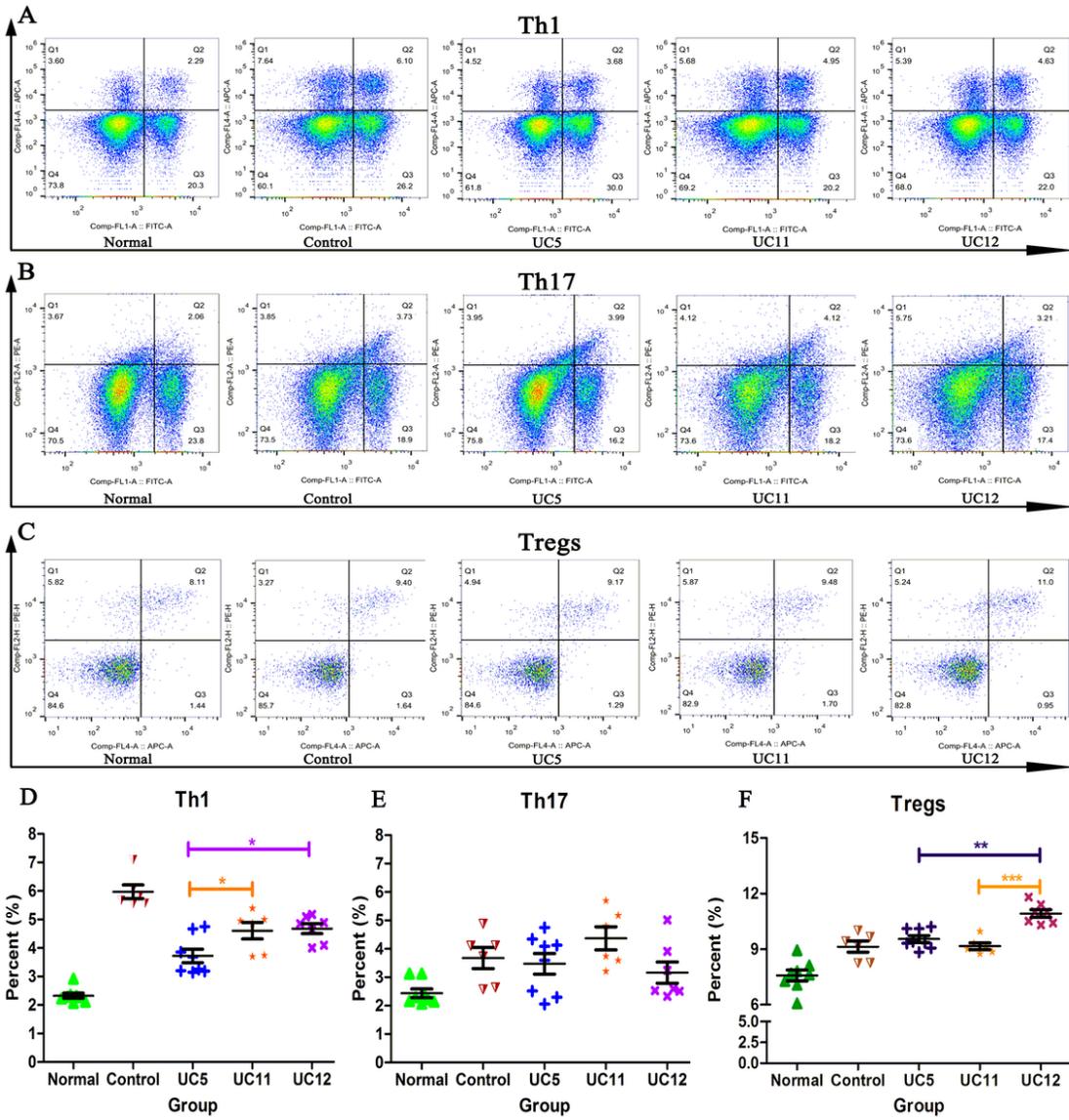


Figure 6

HUCMSCs treatment liver fibrosis mice decreased the proportion of CD4+IFN- γ + T-cells (Th1) subset, compared with liver fibrosis group. UC5 HUCMSCs treatment caused a lower CD4+ Th1 subset differentiation of splenocytes, compared with UC11 and UC12 HUCMSCs. UC12 HUCMSCs treatment significantly promoted the Tregs differentiation of splenocytes, compared with UC5 and UC11 HUCMSCs treatments. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$)

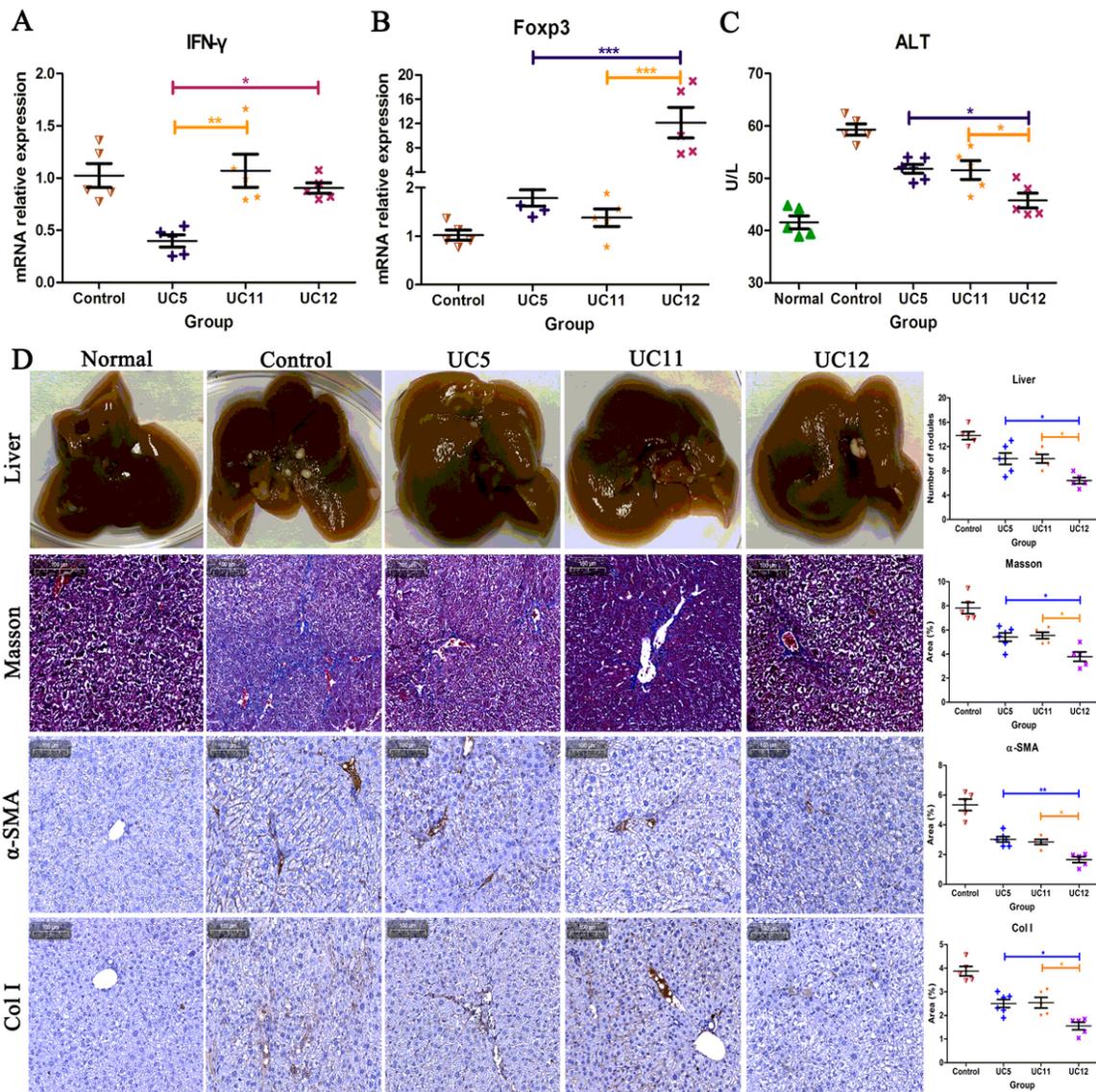


Figure 7

UC5 markedly decreased the IFN- γ mRNA level and UC12 significantly improved the mRNA level of Foxp3 in splenocytes, compared with LF group. UC12 HUCMSCs better increased the ALT level than other UC5 and UC11 HUCMSCs. Three HUCMSCs strains markedly alleviated the amount and size of fibrosis nodules of liver surfaces, as well as the positive staining of Masson, α -SMA, and Col I in liver sections and UC12 HUCMSCs had the best therapeutic potential in liver fibrosis. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$)

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