Development of a research platform to improve local bone marrow stromal cell transplantation in the treatment of osteonecrosis

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INTRODUCTION: Ischemic osteonecrosis (ON) is characterized by death of osteocytes and bone marrow cells due to disruption of blood supply to the bone. ON commonly affects the hip, knee, shoulder, elbow, foot, and hand joints. It produces a necrotic bone environment with insufficient recruitment of osteoprogenitors, which impairs the bone repair process and leads to irreversible bone deformity. Bone marrow stromal cell (BMSC) therapy is a promising therapy for ON, which can be applied following drilling or core decompression procedures. Yet, the role of BMSC therapy for ON remains controversial, and the optimal method for local delivery of BMSC is unknown. Several key questions related to BMSC transplantation, such as BMSC retention rate, survival, and participation in bone repair, are largely unanswered. The purposes of this study were to develop a rat model of ON for local BMSC transplantation, to optimize the local cell delivery by using gelatin hydrogel, and to determine the early transcriptomic profile of the transplanted BMSCs using a genetic cell labeling method.

METHODS: To prepare for BMSC transplantation, a compound rat of *CAG-Tdtomato* was generated by crossing *Tg (CAG-Ncre) and Rosa26 (CAG-LSL-Tdtomato) rats*. BMSCs were isolated from the femur and tibia of the *CAG-tdtomato* rats (4 weeks old) and cultured in vitro for BMSC selection. Passage 1 (P1) BMSCs were used for transplantation. Flow cytometry was used to determine the purity of the mesenchymal population in the P1 BMSCs. Rat ischemic ON model was created by local vessel disruption of the distal femoral epiphysis using microsurgical technique and characterized by microangiography and histology as previously described [1]. The experiment for quantifying BMSC retention is illustrated in Fig.1f. The animals were sacrificed right after the cell transplantation (T0). The distal femoral epiphysis was harvested, and the specimens were processed with tissue clearance protocol for 3D imaging [2] to determine the cell number and distribution. The experiment to investigate the early fate of the transplanted BMSC is illustrated in Fig. 2a. The animals were sacrificed 24 hours after local BMSC transplantation. The specimens were dissected and digested using Collagenase II (0.2%) to release the cells from the bone marrow. The transplanted BMSCs (tdt^+ cells) were sorted for BulkRNAseq. The non-transplanted cells were used as a control. Gene enrichment analyses of GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used to reveal the early transcriptomic profile of the transplanted cells.

RESULTS: *BMSC preparation:* The P1 BMSCs derived from the bone marrow of tdtomato rats showed a red fluorescence by a green laser (Fig.1a, left upper image). Flow cytometry showed that the P1 BMSCs are CD45-, CD73+, and CD90+ (Fig.1a, right upper and lower panel images). When the BMSCs were cultured in an osteogenic medium, mineralization was present in the extracellular matrix. *Rat model*: Microangiography and H&E images showed a successful induction of ON on Sprague-Dawley rats. Compared to the sham control, ON-induced rats had an absence of vessels (Fig.1b upper panel) and extensive empty lacunae and necrotic marrow cells in the distal femoral epiphysis 1 week following the ON induction. *Local BMSC delivery:* For successful cell delivery, stepwise surgical techniques were developed (Fig.1 c-e), including the surgical position, instrument settings (Fig. 1 c left panel), drillings (Fig. 1 c middle and right panels), necrotic bone wash procedure (Fig. 1 d), and application of cell carrier (gelatin hydrogel, Fig. 1 e). Following the development of these techniques, local cell delivery efficiency using PBS vs. gelatin hydrogel was compared. 10^6 BMSCs were loaded in 25ul PBS or gelatin hydrogel and injected into the distal femoral epiphysis. The 3D reconstructed images showed a lower cell retention in the PBS group compared to the hydrogel group (Fig. 1 g). 3D measurement (Fig.1h, Matlab 3D reconstruction images for 3D distribution measurement) showed only 25% of the necrotic epiphysis retained BMSCs in the PBS group. In contrast, over 70% of the necrotic epiphysis retained BMSCs in the hydrogel group (Fig.2i, $p<0.05$). 3D cell counting showed that only 1.9 x 10^4 cell was detected in the PBS group compared to 1.4 x 10^5 in the hydrogel group (>7 fold increase, Fig.2j, $p<0.05$). *Bulk RNAseq:* Following successful cell delivery, the injected cells were isolated 24 hours after the transplantation via flow cytometry sorting of isolated cells to obtain the RNA. The bulk RNAseq analysis showed a total of 2098 different expression genes (DEGs) between the transplanted BMSC and the non-transplanted BMSC (control) group. GO analysis showed that *cell necrotic death* ranked as #1 and *response to oxygen* ranked as #10, where the related genes were upregulated in the transplanted BMSCs compared to the non-transplanted BMSCs (Fig.2 c). In KEGG analysis, *HIF-1 signaling pathway* ranked #5, where the related genes were upregulated in the transplanted BMSCs compared to the non-transplanted BMSCs (Fig.2 d). Interestingly, the genes related to amino acids/sugar/lipid metabolism processes were upregulated in the transplanted BMSCs compared to the non-transplanted BMSCs (Table).

DISCUSSION: A rat model of ON for investigating cell transplantation was developed by using donor rats with fluorescence reporter, ON induction on wild-type host rats, and application of gelatin hydrogel for BMSC retention following an intraosseous injection. In the rat study, we found significantly improved BMSC retention using the gelatin hydrogel carrier. Transcriptomic analysis of transplanted BMSCs, however, revealed upregulation of cell necrotic death, attributable to the low oxygen environment. Oxygen-releasing materials might be a potential resolution and warrant further investigation to improve BMSC viability in the hypoxic environment.

CLINICAL RELEVANCE:

In the United, over 20,000 people develop ON every year. Currently, there is no effective treatment for ON. BMSC transplantation is a promising therapy, but no standardized protocol based on scientific evidence has been established. The current study created a research platform to optimize cell-based therapy for ON.

[1] Ma Chi, et al., Acta Biomaterialia, 2021, PMID 33588127; [2] D Jing, et al., Cell Research, 2018 PMID: 29844583

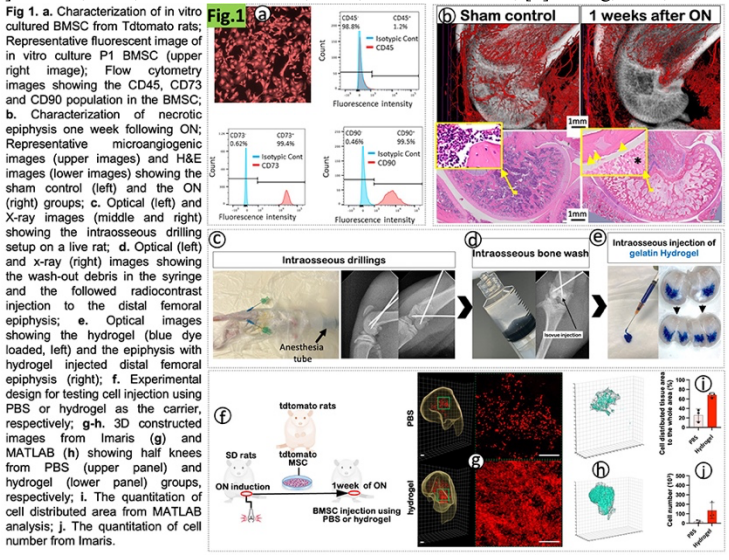

Fig 1. a. Characterization of in vitro cultured BMSC from Tdtomato rats; Representative fluorescent image of in vitro culture P1 BMSC (upper right image); Flow cytometry images showing the CD45, CD73 and CD90 population in the BMSC; **b.** Characterization of necrotic epiphysis one week following ON; Representative microangiogenic images (upper images) and H&E images (lower images) showing the sham control (left) and the ON (right) groups; **c.** Optical (left) and X-ray images (middle and right) showing the intraosseous drilling setup on a live rat; **d.** Optical (left) and x-ray (right) images showing the wash-out debris in the syringe and the followed radiocontrast injection to the distal femoral epiphysis; **e.** Optical images showing the hydrogel (blue dye loaded, left) and the epiphysis with hydrogel injected distal femoral epiphysis (right); **f.** Experimental design for testing cell injection using PBS or hydrogel as the carrier, respectively; **g-h.** 3D constructed images from Imaris (**g**) and MATLAB (**h**) showing half knees from PBS (upper panel) and hydrogel (lower panel) groups, respectively; **i.** The quantitation of cell distributed area from MATLAB analysis; **j.** The quantitation of cell number from Imaris.

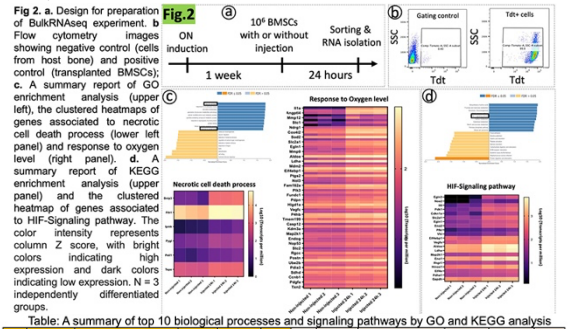

Fig 2. a. Design for preparation of BulkRNAseq experiment. **b** Flow cytometry images showing negative control (cells from host bone) and positive control (transplanted BMSCs); **c.** A summary report of GO enrichment analysis (upper left), the clustered heatmaps of genes associated to necrotic cell death process (lower left panel) and response to oxygen level (right panel). **d.** A summary report of KEGG enrichment analysis (upper panel) and the clustered heatmap of genes associated to HIF-Signaling pathway. The color intensity represents column Z score, with bright colors indicating high expression and dark colors indicating low expression. N = 3 independently differentiated groups.

Table: A summary of top 10 biological processes and signaling pathways by GO and KEGG analysis

	Gene Set ID	Description	Size of the gene set	leading edge subset	FDR
GO	GO:0045454	Cell redox homeostasis	48	18	0.0126
GO	GO:0006575	Cellular modified amino acid metabolic process	118	37	0.0128
GO	GO:0072524	Pyridine-containing compound metabolic process	97	26	0.0133
GO	GO:0006091	Generation of precursor metabolites and energy	248	75	0.015
GO	GO:0035902	Response to immobilization stress	33	9	0.0159
GO	GO:0006457	Protein folding	117	53	0.0169
GO	GO:0035966	Response to topologically incorrect protein	83	26	0.0174
GO	GO:0070265	Necrotic cell death	31	6	0.0224
GO	GO:0070482	Response to oxygen levels	331	75	0.0238
GO	GO:0019693	Ribose phosphate metabolic process	303	91	0.0238

	Gene Set ID	Description	Size of the gene set	leading edge subset	FDR
KEGG	rno03320	PPAR signaling pathway	67	4	0.0008
KEGG	rno01230	Biosynthesis of amino acids	65	23	0.001
KEGG	rno00010	Glycolysis / Gluconeogenesis	52	19	0.001
KEGG	rno00051	Fructose and mannose metabolism	30	13	0.0016
KEGG	rno04066	HIF-1 signaling pathway	89	21	0.0019
KEGG	rno01200	Carbon metabolism	101	43	0.0019
KEGG	rno00052	Galactose metabolism	25	10	0.0055
KEGG	rno00500	Starch and sucrose metabolism	24	7	0.0084
KEGG	rno00030	Pentose phosphate pathway	24	10	0.0103
KEGG	rno05211	Renal cell carcinoma	63	15	0.0112