Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

The essential role of osteoclast-derived exosomes in magnetic nanoparticles-infiltrated hydroxyapatite scaffold modulated osteoblast proliferation in osteoporosis model

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Experimental detail:

Preparation of the MHA scaffold

The MHA scaffolds were prepared according to the previous method reported in our previous paper [1-3]. The Fe_3O_4 nanoparticles were first synthesized using thermal decomposition. Briefly, 1.45 g iron (III) acetylacetonate, 5.376g 1, 2- hexadecanediol, 4.8 mL oleylamine and 5.8 mL oleic acid were dissolved in 50 mL benzyl ether. The mixture solution was heated to 200 °C for 2 h, and then refluxed at 280 °C for 1 h with a nitrogen protection atmosphere before being allowed to cool to room temperature. Then the black suspension was washed with ethanol for several times and centrifuged for 5 min at 3500 rpm, the supernatant was removed and the black sediment was dispersed in n-hexane. In order to remove all undispersed residues, the black dispersion liquid was centrifuged for 10 min at 8000 rpm. After discarding the precipitation, the black dispersion of n-hexane was precipitated by ethanol, and then centrifuged for 5 min at 3500 rpm once more to remove the ethanol solvent. Finally, the black MNPs were re-dispersed in n-hexane and stored at 4°C. In order to prepare the MHA scaffolds, HA scaffolds (Φ 14 mm x 2 mm) were immersed in the 10.0 wt% Fe_3O_4 hexane colloid solution for 24 h. The Fe_3O_4 nanoparticles were infiltrated into the pores of the scaffolds by capillary force. Finally, the MHA scaffolds were vacuum-dried overnight to volatilize the hexane completely [1]. The characteristics of MHA scaffolds were analyzed by vibrating sample magnetometry (VSM) and scanning electron microscopy (SEM). The particle size and magnetic performance of Fe_3O_4 nanoparticles were analyzed by dynamic light scattering (DLS) and VSM.

Animal model of osteoporosis

The housing and all procedures involving animal experiments were performed according to the guide for the Care and Use of Laboratory Animals published by the Chinese National Academy of Sciences. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan University and performed at the National Engineering Research Centre for Biomaterials (Sichuan University, China). Twelve female Sprague-Dawley (SD) rats at the age of 3 months (240-300 g weight, Chengdu Dossy experimental Animals Co., LTD.) were subjected to bilateral ovariectomy (OVX) [4]. Micro-computed tomography (Micro-CT) analysis was used to assess bone micro-architectural changes after OVX for three months. Afterward, the OVX rats were used for the isolation of primary osteoblastic and osteoclastic cells.

Primary osteoblastic cell harvesting and culture

The isolation of primary osteoblastic cells was performed according to the protocol from Zhang's group [5]. In detail, the OVX rats were sacrificed and the tibia bones were obtained after scraped thoroughly to remove the soft tissues and then were cut into small explants (1-2 mm in length). After discarded the bone marrow, the explants were washed repeatedly by antibiotic-antimycotic solution to remove the attached bone marrow. The clean bone explants were placed in Dulbecco's modified culture medium containing 10% fetal bovine serum, 1x antibiotic solution and 100 μM L-ascorbate at 37 °C under a humidified incubator of 5% CO_2. The medium was changed every 3 days. During the culture time, osteoblastic cells were continuously exuded from the surface of bone explants and then were attached to the culture plate. Cells within five passage were used in this study.

Primary osteoclastic cell harvesting and culture

Bone marrow monocytes were isolated freshly according to a standard protocol described in previous researches [6, 7]. Simply, the femur and tibia of OVX rats were dissected and the bone marrow was completely flushed by cell culture medium. After centrifuging for 5 minutes at 1200 rpm/min, the cells were suspended in 5 mL red blood cell lysate to remove red blood cells. Then, the cells were washed with PBS and seeded in cell culture dish in DMEM complete medium containing 10 ng/mL M-CSF. After the cells were cultured for 24 h, the non-adherent dependent monocytes were collected. 50 ng/mL M-CSF and 50 ng/mL RANKL were used to

stimulate monocytes differentiation towards osteoclasts. After 7 days, the differentiated cells were fixed and stained by tartrate-resistant acid phosphatase (TRAP activity) following the kit protocol.

The proliferation of osteoblast in cell co-culture system

Osteoblasts with the number of 5×10^3 were cultured on the HA or MHA scaffolds embedded on the bottom of the Transwell plates, and osteoclasts with the number of 5×10^5 were cultured on the Transwell inserts. At 1, 4, and 7 days, osteoblast proliferation was detected by CCK-8 count kit, respectively. Cell morphology was observed at 7 days by laser scanning confocal microscope.

Secretome identification by liquid chromatography mass spectrometry (LC/MS/MS)

After co-culturing for 7 days, the secretion proteins from cells stimulated with HA and MHA scaffolds were collected, respectively. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and LC-MS/MS were selected for protein identification.

Isolation and purification of exosomes by ultracentrifugation

After co-culturing for 7 days, the cell medium was discarded. The cells were washed by PBS for three times and cultured in serum-free medium for another 24 h. Then, the medium was collected and respectively centrifuged at 300g (10 min), 3000g (20 min) to remove the cells and cellular debris. Afterwards, the supernatant was centrifuged at 10000g in a 29.9 mL tube to discard large extracellular vesicles and filtered using a 0.22 um syringe filter. Following a further ultracentrifugation at 100000g for 2 h to discard the supernatant, the exosomes were present in the bottom of the tube. Finally, the exosomes were dispersed in PBS and centrifuged at 100000g for 2 h once more to concentrate. All centrifugation steps were performed at 4 °C, and the obtained exosomes were stored at -80 °C for further use.

Nanoparticle tracking analysis

The obtained exosomes were diluted at a ratio of 1:250,000 in deionized water. Then, the 3-5 mL exosomal suspension were injected into the Zeta View sample cell to achieve the size distribution and the concentration of the exosomes were measured.

Transmission electron microscope analysis

10 μL of the obtained exosome sample was adsorbed onto a copper grid to maintain at room temperature for several minutes, and the sample was stained by 2% uranyl acetate for 3 min under dark conditions. The morphology of exosomes was detected by transmission electron microscope.

Western-blot analysis

The exosome sample was mixed with 5 x loading buffer and boiled for 10 min at 99 °C. The denatured proteins sample was loaded onto 12% SDS-PAGE gel for separation. After electrophoresis, the proteins were transferred to a poly (vinylidene fluoride) (PVDF) membrane for 2 h. After the PVDF membrane were blocked for 1 h at room temperature in 5% non-fat milk solution, the membrane was incubated with the primary antibodies overnight at 4 °C. After washing with PBST for three times, the membrane was incubated in secondary antibodies (1:2000) for 1 h. Finally, the blot was detected through an enhanced chemi-luminescence. The all primary antibodies (β-actin, CD9, CD63, HSP70 and ALIX purchased from Beyotime Biotechnology, System Biosciences and Abcam) were diluted at the ratio of 1:1000.

Osteoblast cellular uptake of exosomes

Osteoclast-like exosomes were collected according to the above methods. 10 μL of the obtained exosomes were added to Diluent C solution to make up the volume to 250 μL. Then, 1 μL PKH26 was added to 249 μL Diluent C solution to make up the volume to 250 μL and mixed gently. The two dilutions were mixed in a new 2 mL tube and incubated for 5 min at 25 °C in a dark place. 500 μL of stopping solution was added to stop the reaction. Then, the mixture was purified by ultracentrifugation at 100000g for 2 h. The PKH26-labeled exosomes

were added to the osteoblasts cultured on HA and MHA scaffolds, respectively. After being co-cultured 6 h, the cells were fixed with 4% paraformaldehyde for 30 min and stained with DAPI solution for 10 min at room-temperature in dark place. Finally, the cellular uptake of exosomes was detected by CLSM.

Statistical analysis

All experiments were conducted three times to ensure reproducibility. All dates were presented as means values ± standard deviation for n = 3. Differences between experimental groups were assessed by one-way analysis of variance (ANOVA) with Bonferoni's method. Probability value (p) <0.05 was taken as showing statistically significant.

Supporting Figures:

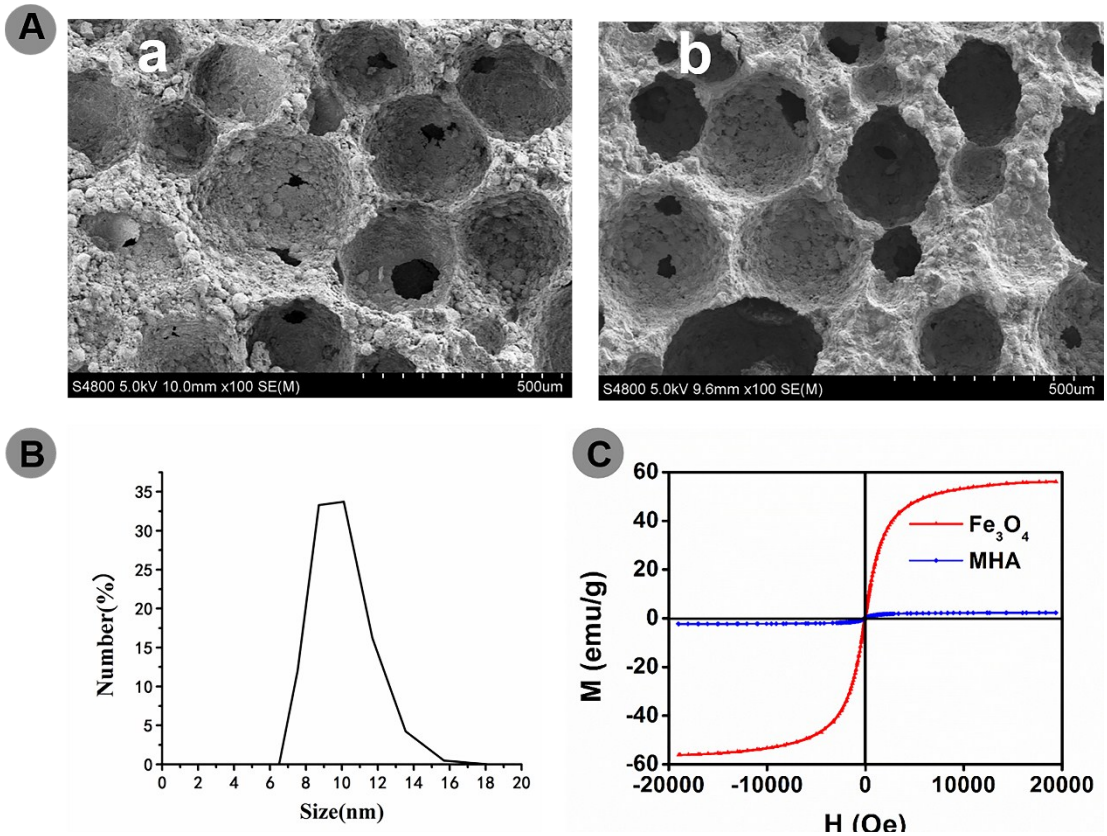

Fig. S1. (A) The morphology of HA and MHA scaffolds, SEM micrographs showed that MHA scaffolds (b) exhibited macro- and microporosity and good interconnectivity, which was similar with HA scaffold (a). (B) The Particle size of Fe_3O_4 nanoparticles measured by DLS; (C) Magnetization curve of MHA scaffold and Fe_3O_4 nanoparticles, MHA scaffold exhibited surperparamagnetism at room temperature and the saturation magnetization of MHA scaffold were 2.31 emu/g.

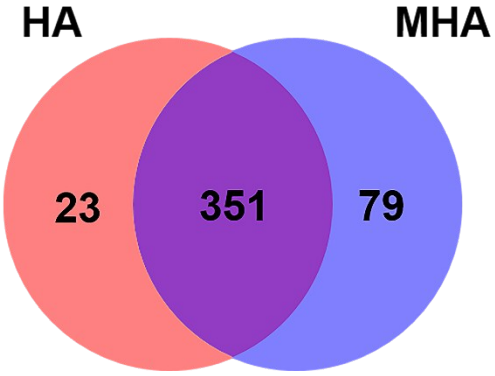

Fig. S2. Venn diagrams reports the number of unique proteins identified in the HA and MHA scaffolds and their respective overlap.

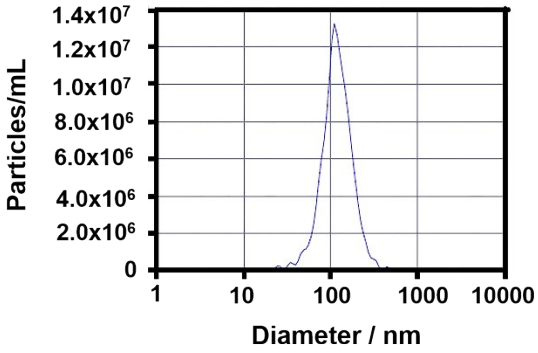

Fig. S3. Nanoparticle tracking analysis for the size and concentration of the exosomes from osteoclast-like cells.

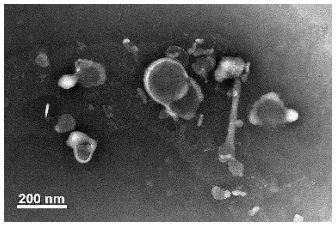

Fig. S4. Transmission electron microscope images of the exosomes from osteoclast-like cells.

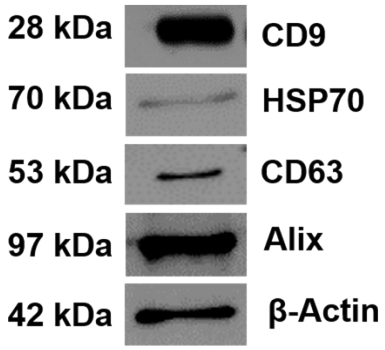

Fig. S5. Western-blotting analysis of specific proteins obtained from osteoclast-like cells.

Table S1. List of the proteins related to Ca^{2+}.

Protein IDs	Names	Up-regulation or Down-regulation
Q71SA3	Thbs1	Down
A0A096P6L8	Fn1	Down
A0A0G2JWK0	Itgb3	Up
F1M7X3	Cdh13	Down
P02680	Fgg	Down
P0DP31	Calm3	Up
P14480	Fgb	Up
P18418	Calr	Up
P20961	Serpine1	Up
P31044	Pebp1	Up
P54311	Gnb1	Down
Q07936	Anxa2	Up
Q3T1J1	Eif5a	Down
Q62658	Fkbp1a	Up
Q6AYC4	Capg	Up
Q91ZN1	Coro1a	Up

Table S2. List of the proteins related to Rho kinase.

Protein IDs	Names	Up-regulation or Down-regulation
P61589	Rhoa	Down
P82995	Hsp90aa1	Up
D3ZMS5	Tiam2	Down
Q5U1Y2	Rac2	Up
Q5XI73	Arhgdia	Up
Q8CFN2	Cdc42	Up

Table S3. List of the proteins related to reactive oxygen species.

Protein IDs	Names	Up-regulation or Down-regulation
A0A0G2JSH9	Prdx2	Down
A0A0G2JSS8	Prdx5	Up
A0A0G2K531	Gpx3	Up
A0A0G2K737	Txnl1	Down
A0A0H2UHM5	Pdia3	Up
B2GUV5	Atp6v1g1	Down
B2RZ27	Sh3bgrl3	Down
F1M983	Cfh	Down
O35244	Prdx6	Down
P04785	P4hb	Up
P05197	Eef2	Up
P05982	Nqo1	Down
P11232	Txn	Down
P14841	Cst3	Down

P07895	Sod2	Down
Q91YB6	Cfh	Down
Q99J86	Atrn	Down
Q9Z0V5	Prdx4	Up
Q3KR76	Plau	Up
A0A0A0MY09	Hsp90b1	Down
P05065	Aldoa	Up
P11980	Pkm	Down

Table S4. List of the proteins related to endopeptidase.

Protein IDs	Names	Up-regulation or Down-regulation
A0A0H2UHM3	Hp	Down
B0BNA5	Cotl1	Up
B2RYM3	Itih1	Up
G3V8U9	Psmb4	Down
M0RBJ7	C3	Down
P01041	Cstb	Up
P06238	A2m	Down
P19804	Nme2	Up
P25113	Pgam1	Up
P47942	Dpysl2	Down
P61983	Ywhag	Down
Q01177	Plg	Down
Q641Z7	Smpdl3a	Down
Q6MG90	C4b	Down
Q6P6Q5	App	Down
Q6P6T6	Ctsd	Down
Q8CIP8	C2	Up
Q8CJH4	rGM2AP	Up
A0A0U1RRP9	Cfb	Up
B2RYC9	Gba	Down
D3ZYK8	Mmp9	Up
O88767	Park7	Up
Q6IN22	Ctsb	Down
I2FHN3	CatL	Down
P30121	Timp2	Down
Q5M7T5	Serpinc1	Down
Q6P734	Serping1	Up
Q80ZA3	Serpinf1	Down
Q9EPB1	Dpp7	Down

Table S5. List of the proteins related to ubiquitination.

Protein IDs	Names	Up-regulation or Down-regulation
P07335	Ckb	Up

P62260	Ywhae	Down
P63259	Actg1	Down
P69897	Tubb5	Up
Q5XIM9	Cct2	Down
Q6P9V9	Tuba1b	Down
P18420	Psma1	Up
P34058	Hsp90ab1	Up
P46462	Vcp	Up
P60901	Psma6	Down
Q6P9V6	Psma5	Down
Q9JI92	Sdcbp	Down

Table S6. List of the proteins related to ATP.

Protein IDs	**Names**	**Up-regulation or Down-regulation**
A0A0G2K1C0	Actr3	Down
D3ZRN3	Actbl2	Down
D4ACB8	Cct8	Up
F1LRV4	Hspa4	Down
G3V6P7	Myh9	Up
P07335	Ckb	Up
P16617	Pgk1	Up
P34058	Hsp90ab1	Up
P50503	St13	Up
P60711	Actb	Down
P63259	Actg1	Down
P68136	Acta1	Down
P82995	Hsp90aa1	Up
Q5BJY2	Cct6a	Down
Q5M7U6	Actr2	Down
Q5XIM9	Cct2	Down
Q6AXQ3	Yes1	Down
Q6P3V8	Eif4a1	Up
Q7TPB1	Cct4	Up
D4A133	Atp6v1a	Down
D4A8F2	Rsu1	Up
F1LP05	Atp5f1a	Down
G3V6D3	Atp5f1b	Down
P04797	Gapdh	Down
P06761	Hspa5	Down
P19804	Nme2	Up
P19945	Rplp0	Up
P50399	Gdi2	Up
Q04679	Fxyd2	Down
Q62636	Rap1b	Down

Q6NYB7	Rab1A	Down

Table S7. List of the proteins related to endocytosis.

Protein IDs	Names	Up-regulation or Down-regulation
F8V328	Rab8a	Down
P02680	Fgg	Down
P0DP31	Calm3	Up
P14480	Fgb	Up
P47942	Dpysl2	Down
P63259	Actg1	Down
Q07936	Anxa2	Up
Q08163	Cap1	Up
P07151	B2m	Up

Table S8. List of the proteins related to phagocytosis.

Protein IDs	Names	Up-regulation or Down-regulation
Q8CFN2	Cdc42	Up
P18418	Calr	Up
Q68FP1	Gsn	Down

Table S9. List of the proteins related to membrane fusion.

Protein IDs	Names	Up-regulation or Down-regulation
P50399	Gdi2	Up
Q9JI92	Sdcbp	Down
A0A0G2JWK0	Itgb3	Up
D3ZRN3	Actbl2	Down
D3ZQ45	Dsg1	Down
D4A8G5	Tgfbi	Up
F1LMV6	Dsp	Up
F1M7X3	Cdh13	Down
G3V6D3	Atp5f1b	Down
P14841	Cst3	Down
P60711	Actb	Down
P45592	Cfl1	Up
P38983	Rpsa	Down
P61589	Rhoa	Down
Q3KR76	Plau	Up
Q71SA3	Thbs1	Down
R9PXU6	Vcl	Up
A1L1J8	Rab5b	Down
B0BMW0	Rab14	Down
Q6NYB7	Rab1A	Down
Q5RKJ9	Rab10	Down
F1LP82	Rab2a	Down

A0A0G2K930	Rab7a	Up
A0A0G2JZR4	Rab11b	Down
Q91ZN1	Coro1a	Up
P68370	Tuba1a	Down
P69897	Tubb5	Up
Q04679	Fxyd2	Down
Q62636	Rap1b	Down
Q5M7U6	Actr2	Down
Q68FP1	Gsn	Down
Q6P6Q5	App	Down

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2 **Table S10.** List of the proteins related to cytoskeleton.

Protein IDs	Names	Up-regulation or Down-regulation
G3V8C3	Vim	Down
A0A0G2K013	Actn4	Down
A0A1W2Q6E9	Msn	Up
G3V6P7	Myh9	Up
P05197	Eef2	Up
P05942	S100a4	Up
P61983	Ywhag	Down
Q07936	Anxa2	Up
A0A0G2K1C0	Actr3	Down
A0A0G2K2J1	Arpc2	Down
B0BNA5	Cotl1	Up
B2GUZ5	Capza1	Up
B2GV73	Arpc3	Down
C0JPT7	Flna	Down
D3ZRX9	Cnn2	Down
F1LMV6	Dsp	Up
G3V7C6	Tubb4b	Up
G3V908	Kb15	Down
O88656	Arpc1b	Down
P04797	Gapdh	Down
P09495	Tpm4	Down
P18418	Calr	Up
P45592	Cfl1	Up
P47942	Dpysl2	Down
P61589	Rhoa	Down
P63259	Actg1	Down
P68136	Acta1	Down
P68370	Tuba1a	Down
P69897	Tubb5	Up
P85108	Tubb2a	Up
Q08163	Cap1	Up

Q3T1K5	Capza2	Up
Q5M7U6	Actr2	Down
Q5RKI0	Wdr1	Down
Q5U1Y2	Rac2	Up
Q5XI32	Capzb	Down
Q5XI38	Lcp1	Up
Q5XIF6	Tuba4a	Up
Q63610	Tpm3	Up
Q68FP1	Gsn	Down
Q6AYC4	Capg	Up
Q6GMN8	Actn1	Down
Q6P9V9	Tuba1b	Down
Q7M0E3	Dstn	Up
Q8CFN2	Cdc42	Up
Q91ZN1	Coro1a	Up
Q9JI92	Sdcbp	Down
R9PXU6	Vcl	Up

Table S11. List of the proteins related to signal.

Protein IDs	Names	Up-regulation or Down-regulation
A0A0G2JSS8	Prdx5	Up
A1L114	Fga	Down
P45592	Cfl1	Up
Q99J86	Atrn	Down
A0A0G2JSH9	Prdx2	Down
A0A0G2JWK0	Itgb3	Up
A0A0H2UHM3	Hp	Down
A0A0H2UHM5	Pdia3	Up
B0BMW0	Rab14	Down
A1L1J8	Rab5b	Down
B2LYI9	Tnc	Down
B2RYC9	Gba	Down
C0JPT7	Flna	Down
D3ZMS5	Tiam2	Down
D3ZYK8	Mmp9	Up
G3V8C3	Vim	Down
G3V8U9	Psmb4	Down
O88767	Park7	Up
P02680	Fgg	Down
P04785	P4hb	Up
P05942	S100a4	Up
P06761	Hspa5	Down
P07895	Sod2	Down
P07943	Akr1b1	Down

P0DP31	Calm3	Up
P14480	Fgb	Up
P18418	Calr	Up
P19804	Nme2	Up
P20059	Hpx	Down
P30121	Timp2	Down
P31044	Pebp1	Up
P34058	Hsp90ab1	Up
P35213	Ywhab	Up
P46462	Vcp	Up
P50399	Gdi2	Up
P54311	Gnb1	Down
P55053	Fabp5	Up
P61589	Rhoa	Down
P61983	Ywhag	Down
P62260	Ywhae	Down
P82995	Hsp90aa1	Up
Q3KR76	Plau	Up
Q5U1Y2	Rac2	Up
Q5XI73	Arhgdia	Up
Q62636	Rap1b	Down
Q62658	Fkbp1a	Up
Q68FP1	Gsn	Down
Q8CFN2	Cdc42	Up
Q91ZN1	Coro1a	Up
Q9JI92	Sdcbp	Down

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2 **Table S12.** List of the proteins related to antigen processing and present.

Protein IDs	**Names**	**Up-regulation or Down-regulation**
Q9JI92	Sdcbp	Down
P46462	Vcp	Up
P69897	Tubb5	Up
A0A0G2K392	Masp2	Down
A0A0U1RRP9	Cfb	Up
A1L1J8	Rab5b	Down
D4A1T6	C1r	Down
F1M983	Cfh	Down
G3V6D3	Atp5f1b	Down
G3V7N9	C1qb	Down
M0RBJ7	C3	Down
O88767	Park7	Up
P02680	Fgg	Down
P04785	P4hb	Up
P06238	A2m	Down

P06761	Hspa5	Down
P07151	B2m	Up
P14480	Fgb	Up
P18418	Calr	Up
P18420	Psma1	Up
P20059	Hpx	Down
P20961	Serpine1	Up
P45592	Cfl1	Up
Q01177	Plg	Down
Q5BKC4	C9	Down
Q5M7T5	Serpinc1	Down
Q5RKI0	Wdr1	Down
Q5RKJ9	Rab10	Down
Q5XFV4	Fabp4	Down
Q6GMN4	Csf1	Down
Q6MG90	C4b	Down
Q6P734	Serping1	Up
Q6P9V9	Tuba1b	Down
Q71SA3	Thbs1	Down
Q80ZA3	Serpinf1	Down
Q8CIP8		Up
Q91YB6	Cfh	Down
Q91ZN1	Coro1a	Up
Q99J86	Atrn	Down
Q9QZK8	Dnase2	Down

Supporting References

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