Stem cell conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms

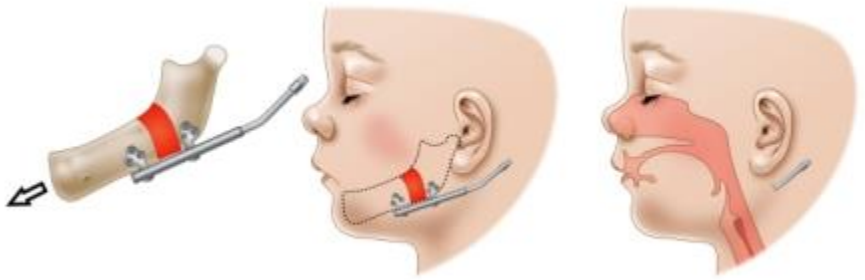
By Yuji Ando et al; Akihito Yamamoto

Bone Volume 61 (2014) pages 82-90

Background

Background

Distraction Osteogenesis



- Used in Orthopedic and craniofacial surgeries
- Lengthens the skeleton
- Osteotomy followed by gradual distraction
- Neocallus formation, neoangiogenesis and cell recruitment required
- Long treatment periods (~12 months)
- External fixator – risk of infections

McCarthy JG et al Plast Reconstr Surg 2001;107:1812-27.
Royal childrens hospital Melbourne (picture)

Background

Stem Cells

- Undifferentiated cells, able to differentiate into specialized cells, can renew themselves.
  - embryonic stem cells: pluripotent
    - isolated from blastocysts of an early-stage pre-implantation embryo
  - adult stem cells = ”repair system of the body”
    - Can be acquired from: periosteum, bone marrow, adipose tissue, umbilical cord, etc.

Background

Mesenchymal stem cells

- Mesenchymal stem cells are multipotent stromal cells
- Can differentiate into:
    - Osteoblasts
    - Chrondrocytes
    - Myocytes
    - Adipocytes
- Initially isolated from bone marrow, today adipose tissue is preferred (less invasive).
- MSC paracrine signaling is increasingly believed to be more important than the use of MSC cells.

Background

Bone healing

- 3 phases:
  - 1. Reactive (inflammatory) phase:
    - 5-7 days
    - Clot and granulation tissue forms
    - Blood cells and macrophages release TNFα, IL-1, IL-6, IL-11, IL-18
  - 2. Reparative phase
    - 7 days – 4 weeks
    - Cartilage callus formation (periostal cells -> chondroblasts, osteoblasts, etc.
    - Lamellar bone deposition
  - 3. Remodeling phase
    - 3 weeks – 5 years
    - Remodeling to original bone contour

Background

IL-3/-6, MCP-1/-3

- IL-3/-6
  - IL-1 induces inflammatory responses, promotes prostaglandin production, the growth of leukocytes and induces acute phase reaction.
  - IL-6 accelerates inflammation by promoting the differentiation of cytotoxic T cells and Th17 cells.
- MCP-1/MCP-3 (Monocyte chemoattractant protein 1) (= **Chemokine (C-C motif) ligand 2/7** (CCL2/7)
  - MCP-1 accelerates inflammation by recruiting macrophages, monocytes, leucocytes, memory T lymphocytes and natural killer cells.
  - MCP-3 attracts monocytes and regulates macrophage function. It also interacts with MMP2 (= matrix metalloproteinase-2)

Bachmann MF et al. Nat Rev Immunol 2006;6:159-64.
Nishimoto N. et al. Clin Pharmacol Ther 2010;87:483-7.

Background

Complications

- Infections (superficial, deep, osteomyelitis)
- Premature union (requiring repeat corticotomy)
- Delayed union/non-union (requiring bone grafting or internal fixation)
- Fracture
- Axis deviation
- Pin loosening or breaking
- Joint subluxation
- Joint contractures

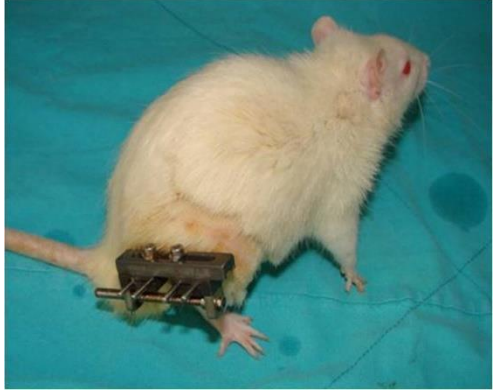
Material & Methods

Methods

Cell culture and Conditioned Medium

- Human bone marrow MSCs & human skin fibroblasts
  - Suppliers = Lonza Walkersville and Health Science Research Resources Bank Japan
- Cultured in Dulbecco´s modified Eagle´s medium (DMEM) containing 10% fetal bovine serum (FBS)
  - 37° C, 5% CO2 atmosphere
- 70-80% confluence -> serum free DMEM + incubated for 48h
- Afterwards Conditioned Medium collected and centrifuged for 4-5 min at 4°C.

Methods



Mouse distraction osteogenesis model = control model (C-DO)

- 8- to 10-week-old female ICR (Institute of cancer research) mice (Swiss albino mice commonly used)
- Fixator out of 2 acrylic resin rings and an expansion screw
  - Total weight 2,7g
- Anterior longitudinal incision to the right leg followed by fibulotomy with scissors.
- Osteotomy in the middle of the diaphysis
- Begin of distraction after 3 day latency
- Distraction for 8 days, 0,2 mm/12 h -> 3,2mm in total

Methods

High speed distraction osteogenesis (H-DO) model

- Same mouse model + procedure
- n = 10 per group
- Distraction rate 0,4mm/12h -> 3,2 mm in 4 days
  - Compared to 3,2 mm in 8 days
- 6 High speed DO groups:
  - No additional treatment
  - Mesenchymal stem cells (MSCs) transplanted into distraction zone
  - Fibroblasts (FBs)
  - MSC Conditioned Medium (MSC-CM) inserted on days 3, 5, 7
  - Dulbecco´s modiefied Eagle´s medium (DMEM) inserted on days 3, 5, 7
  - Fibroblast Conditioned Medium (FB-CM) inserted on days 3, 5, 7

Methods

histology

- Perfusing mice with a 4% paraformaldehyde solution
- Tibial segments harvested
  - embedded in SCEM gel
  - frozen in cooled isopentane
- Non-decalcified tibial bone sections generated using Kawamoto´s film method
- Cryostat sections (5 µm) stained with hematoxylin eosin
- Histomorphometric analysis from digital images obtained (percentage of callus formation activity)

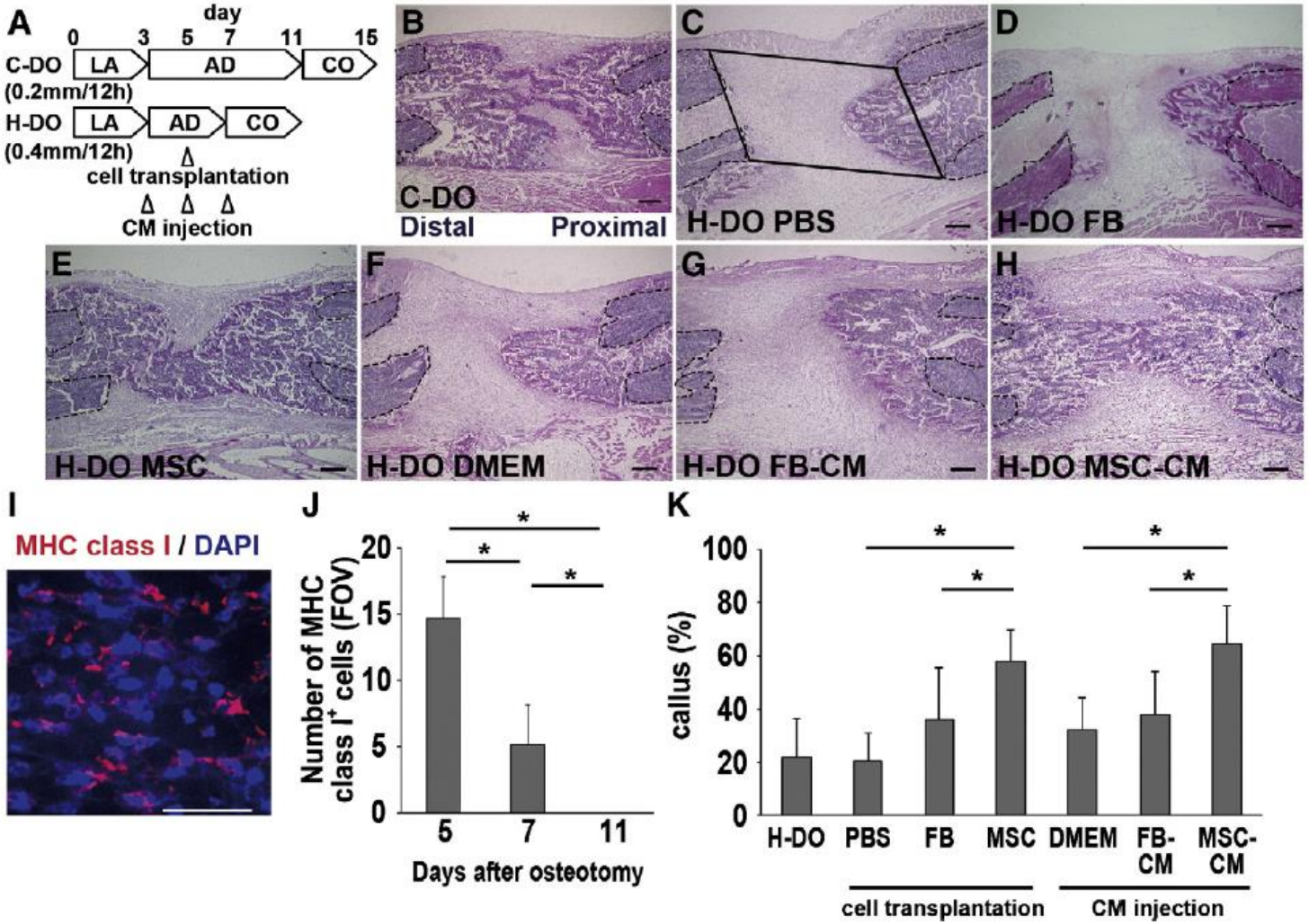


Fig. 1. MSCs accelerate callus formation through paracrine mechanisms. (A) Distraction protocols and experimental design. After a 3-day latency period, distraction was conducted over a period of 8 days (C-DO) at 0.2 mm/12 h, or a period of 4 (H-DO) days at a rate of 4 mm/12 h (H-DO), for a total length increase of 3.2 mm. Time points: white arrowheads, MSC transplantation or CM injections. LA: latency period; AD: active distraction period; CO: consolidation period. (B–H) Representative micrographs of hematoxylin–eosin (HE)-stained DO-gap sections, shown distal (left) to proximal (right); bar = 300 μm. (C) Schematic of the distraction zone. At the end of the consolidation period, neocallus had formed in the C-DO (B) but not in H-DO (C) gaps. Transplanted MSCs (E) but not FBs (D) promoted callus formation in the H-DO gap. Locally injected MSC-CM (H) but not DMEM (F) or FB-CM (G) promoted callus formation in the H-DO gap. (I) Immunohistochemical analysis of H-DO gaps: sections were prepared immediately after engrafting MSCs (post-surgery day 5) and stained with specific antibodies against human MHC class I (red) and DAPI (blue). Bar = 50 μm. (J) Quantification of transplanted human MHC class I^+ MSCs in the H-DO gap over time: MSCs had completely disappeared from the gap by the end of the consolidation period. FOV: field of view. Data represent mean ± SD; $^*p < 0.05$; n = 5 per group. (K) Histomorphometric analysis of callus formation in the distraction zone at the end of the consolidation period. Note that the callus area was significantly larger in the MSC-CM groups than in the DMEM or FB-CM groups. Data represent mean ± SD; $^*p < 0.05$; n = 10 per group.

Methods

Immunohistochemical analysis of H-DO gaps

- n =5 per group
- Samples sectioned at 5 µm
- Fixed in 99,5% ethanol – 10 min
- Blocked with 5% bovine serum – albumin/PBS (phosphate buffered saline)
- Stained with primary antibodies (in blocking buffer for 1 h)
- Several antibodies were used for immunostaining
- Secondary antibodies conjugated with Fluor 488/ Fluor 555

Methods

Immunohistochemical analysis of H-DO gaps

- Cell nuclei were counted in randomly selected fields
  - Positively stained MHC Class I
  - CD11b cells (neutrophils, monocytes, nk cells, lymphocytes)
  - Microvessel supply measured by measuring density and number of CD31 positive cells around muscle fibers

J Allergy Clin Immunol. 2005 Jul;116(1):192-7. Kawai K. Et al.

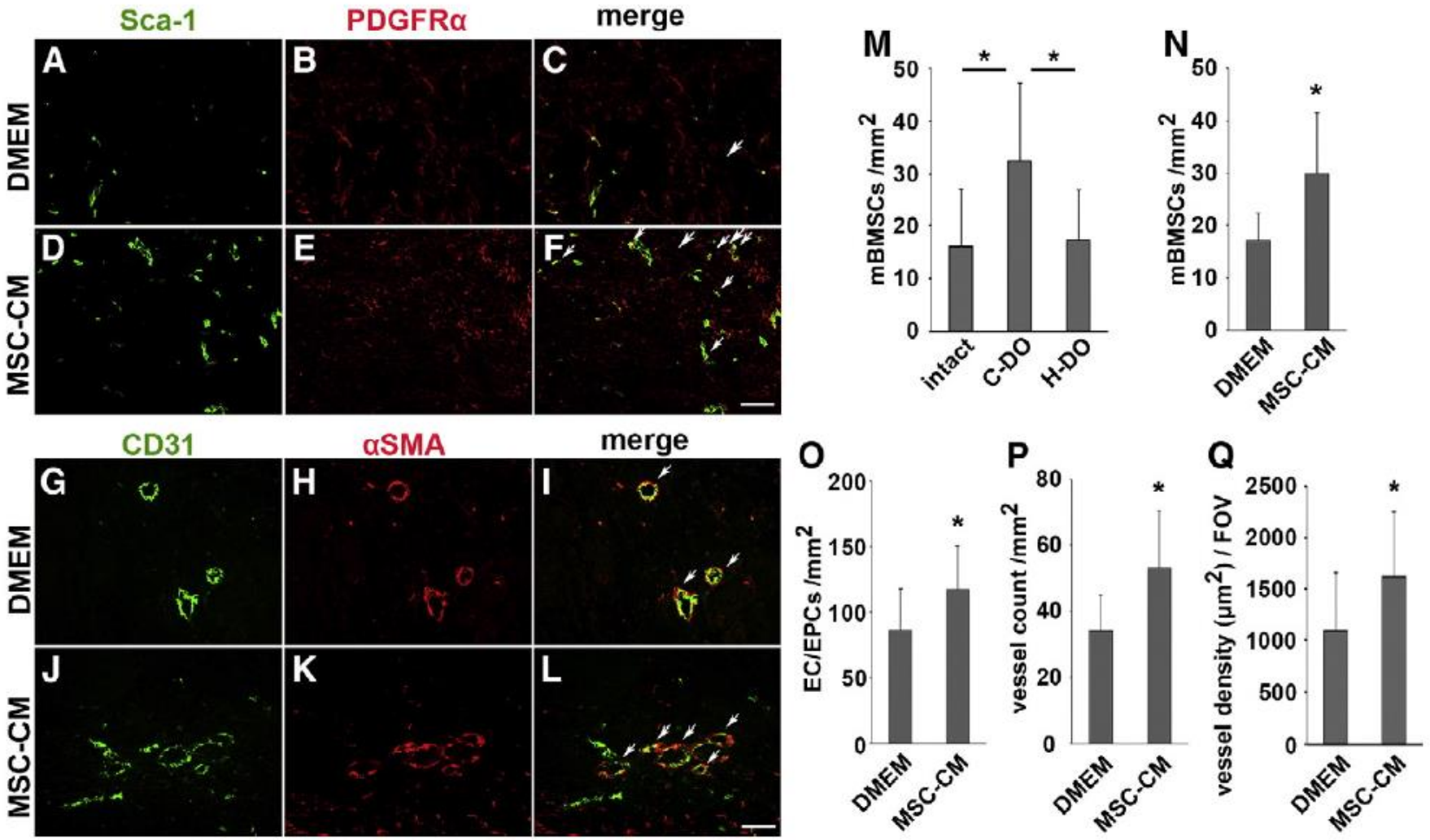


Fig. 2. MSC-CM accelerates the recruitment of endogenous mBMSCs and formation of blood vessels. (A–L) Representative images showing the immunohistochemical staining of H-DO gaps treated with DMEM (A–C, G–I) or MSC-CM (D–F, J–L); samples were obtained at the end of active distraction. White arrowheads (C, F) indicate mBMSCs detected with Sca-1 (green) and PDGFRα (red) staining (A–F). EC/EPCs and vascular smooth muscle cells were detected with CD31 (green) and αSMA (red) (G–L) staining, respectively. White arrowheads (I, L) indicate mature blood vessels consisting of CD31-positive endothelial cells and αSMA-positive vascular smooth muscle cells; these vessels were increased in the MSC-CM group. Bar = 50 μm. (M–O) Quantification of Sca-1$^+$/PDGFRα$^+$ mBMSCs (M and N, n = 10), CD31$^+$ EC/EPCs (O, n = 10), vessel counts (P, n = 10), and vessel density (Q, n = 10). Data represent mean ± SD; *$p < 0.05$.

Methods

Cytokine antibody assay

- n = 10
- Fibroblast Conditoned medium (FB-CM) and Mesenchymal stem cell conditioned medium (MSC-CM) used
- CCL2/MCP-1, CCL7/MCP-3 levels measured
  - (CCL2/MCP-1) = C-C motif chemokine 2/monocyte chemoattractant protein-1
  - (CCL7/MCP-3) = C-C motif chemokine 7/monocyte chemoattractant protein-7
- IL-3, IL-6 levels measured
- Stromal-cell-derived factor 1 (SDF-1)

Methods

Cytokine antibody assay

- ELISA kit used for measurement
- MCP1 & MCP3 neutralizing antibodies to deplete MSC -CM of MCP1/3 proteins
  - Antibodies pre attached to Protein G Sepharose
  - Antibodies removed by centrifugation
- Control of depletion via ELISA
- Control Conditioned Medium (CM) same treatment, except for anti-SDF-1 antibody

A

MSC recruitment	intensity	references
MCP-1	11765.7	51
MCP-3	201.0	52
RANTES	41.6	53

EC/EPC recruitment	intensity	references
IL-6	1136.7	54
IL-3	68.2	55
RANTES	41.6	56

osteogenesis	intensity	references
IL-6	1136.7	57, 58
IL-3	68.2	59

angiogenesis	intensity	references
MCP-1	11765.7	43, 60
osteoprotegerin	2236.2	61
IL-6	1136.7	62
VEGF-C	451.8	63
angiogenin	335.6	64
IL-3	68.2	55
osteopontin	63.5	65

cell proliferation	intensity	references
osteoprotegerin	2236.2	61
IL-6	1136.7	54, 66
VEGF-C	451.8	63
angiogenin	335.6	64
MCP-3	201.0	67
IL-3	68.2	55, 66
osteopontin	63.5	65

anti-inflamatation	intensity	references
IL-22	126.0	68

B

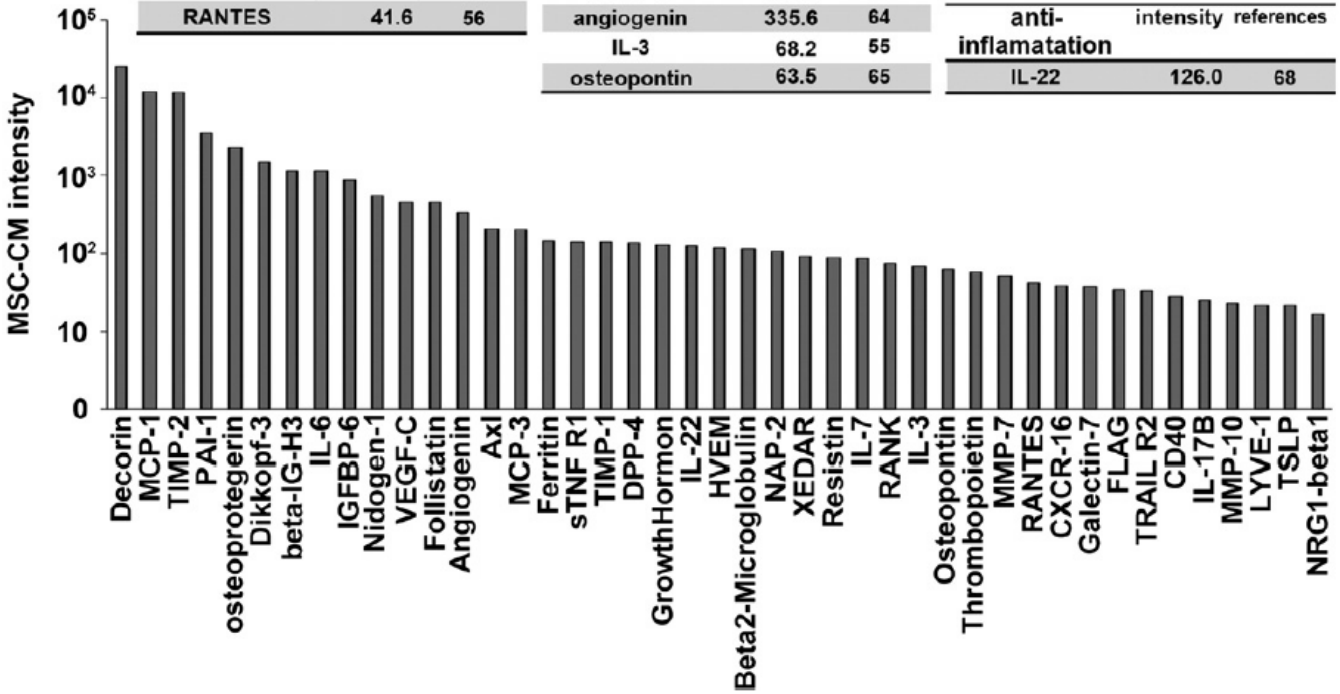


Fig. 3. Soluble factors contained in MSC-CM. (A) Classification of MSC-CM factors: MSC-CM contained previously annotated proteins that recruit MSCs and EC/EPCs, promote osteogenesis, angiogenesis, and cell proliferation, and inhibit inflammation. (B) Factors expressed in BMSC-CM at levels 1.5-fold or greater than those in fresh DMEM medium without cell culture. X-axis: the intensity levels of factors in MSC-CM, with the intensity of the medium control set arbitrarily as 1.0. Fig. 3 cites the following references: [43,51–68]. Abbreviations: MCP-1: monocyte chemoattractant protein-1; TIMP-2: tissue inhibitor of metalloproteinase-2; PAI-1: plasminogen activator inhibitor-1; beta-IG-H3: transforming growth factor beta-induced (TGFBI); IL-6: interleukin-6; IGFBP-6: insulin-like growth factor-binding protein-6; VEGF-C: vascular endothelial growth factor-C; MCP-3: monocyte chemoattractant protein-3; sTNF R1: soluble tumor necrosis factor receptor 1; TIMP-1: tissue inhibitor of metalloproteinase-1; DPP-4: dipeptidyl peptidase-4; IL-22: interleukin-22; HVEM: herpesvirus entry mediator; NAP-2: neutrophil-activating protein-2; XEDAR: X-linked ectodysplasin-A2 receptor; IL-7: interleukin-7; RANK: receptor activator of nuclear factor kappa-B; IL-3: interleukin-3; MMP-7: matrix metalloproteinase-7; RANTES: regulated upon activation normal T-cell expressed and secreted (CCL5, chemokine C-C motif ligand 5); CXCR16: chemokine C-X-C motif receptor 16; TRAIL R3: TNF related apoptosis-inducing ligand receptor 3; IL-17B: interleukin-17B; MMP-10: matrix metalloproteinase-10; LYVE-1: lymphatic vessel endothelial receptor 1; TSLP: thymic stromal lymphopoietin; NRG1-beta1, neuregulin-1-beta1.

Methods

Concentration of cytokines in CM

Table 1
Concentration of cytokines in CM (pg/ml).

Trophic factors	MSC-CM	MSC-CM control	MSC-CM-MCP-1/-3	MSC-CM-SDF-1	FB-CM
MCP-1	205 ± 2.3	222 ± 9.9	ND	177 ± 9.3	70 ± 9.7
MCP-3	102 ± 10.9	79 ± 9.9	ND	76 ± 5.7	ND
IL-3	181 ± 21.9	190 ± 8.7	113 ± 10.9	194 ± 7.6	ND
IL-6	106 ± 3.3	110 ± 4.2	111 ± 0.3	165 ± 14.0	76 ± 3.0
SDF-1	ND	ND	ND	ND	ND

Abbreviations: MCP-1: monocyte chemoattractant protein-1; MCP-3: monocyte chemoattractant protein-3; IL-3: interleukin-3; IL-6: interleukin-6; SDF-1: stromal- cell-derived factor-1; ND: not detected.

Methods

Isolation of mouse bone marrow mononuclear cells (mBMMNCs)

- mBMMNCs collected from femurs of 8-10 week old female ICR mice
- Mice euthanized with CO2
- Bone marrow cavity of proximal femur exposed
- Marrow flushed out with saline
- Aspirations centrifuged and cell layer washed in PBS (phosphate buffered saline)
- Re-suspended in DMEM with 10% FBS

Methods

Migration studies

- Human umbilical vein endothelial cells (HUVECs) bought
- Migration abilities through transmembrane chambers assessed;
    - Pore size 8 µm (as previously described)
- mBMMNCs & HUVECs seeded into upper membrane chambers
- DMEM containing MSC-CM or MSC-CM containing neutralizing antibodies added to lower chambers (MCP-1,MCP-3;,IL-3,IL-6)
- Incubated for 8 h (mBMMNCs)/16 h (HUVECs)
- Membranes fixed in 4% paraformaldehyde and stained with hematoxylin + counted

Methods

Migration studies

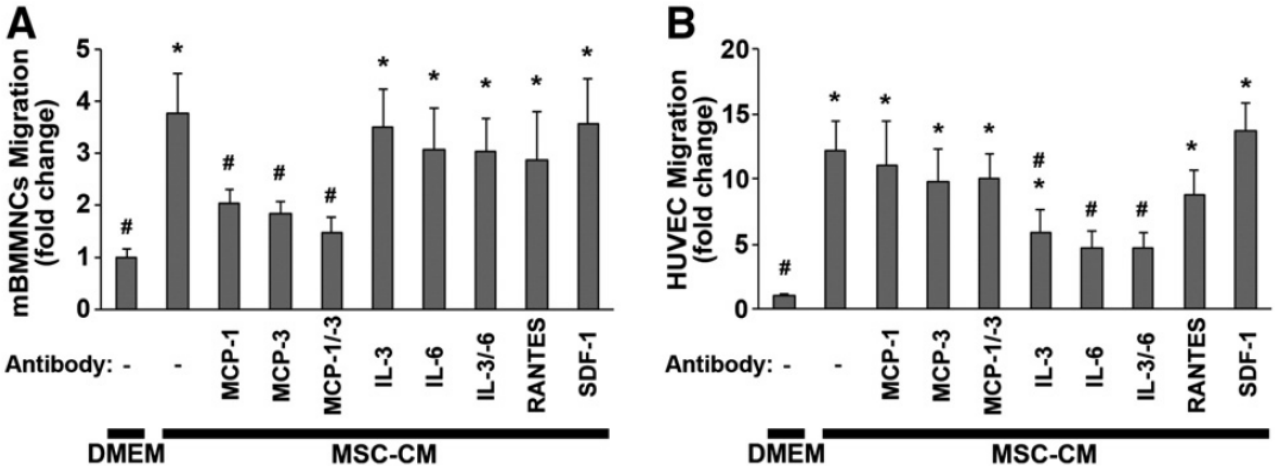


Fig. 4. Effect of MSC-CM on cell migration. The migratory response of freshly isolated mouse bone marrow mononuclear cells (mBMMNCs) (A) and HUVECs (B) to MSC-CM was measured by a modified Boyden chamber migration assay (see Materials and methods). X-axis: migratory response relative to that for fresh DMEM medium. Neutralizing antibodies for MCP-1/-3 and IL-3/-6, respectively, suppressed the MSC-CM-induced migration of mBMMNCs (A) and HUVECs (B). In contrast, antibodies against RANTES (CCL5) or SDF-1 (CXCL12) had little or no effect on cell migration. Triple wells were used for each treatment. Data shown represent mean ± SD of three independent experiments; $^{*}p < 0.05$ vs DMEM, $\#p < 0.05$ vs MSC-CM.

Methods

Osteoblast differentiation

- Multipotential progenitor cells derived from mBMSCs plated into well-plates for the alkaline phosphatase activity assay
    - DMEM (control)
    - MSC-CM
    - MSC-CM containing neutralizing antibodies against IL-3, IL-6, Rantes, SDF-1 used as test media
- mBMSCs incubated with osteogenic supplements (10%FBS, 10 mMβ-glycerophosphate, 50 µg/ml ascorbic acid, and 100 nM dexamethasone) in DMEM or MSC-CM
- medium exchanged three times per week
- After 7 days -> the protein level was determined (BCA Protein Assay Reagent Kit)
- ALP activity determined by measuring absorbance at 415nm (n-nitro-phenyl-phosphate)
- After 14 days of induction culture mBMSCs fixed and stained (Alizarin red-S) to detect mineralized nodules
    - 4% paraformaldehyde, incubated with Alizarin red-S for 10 min -> washing with destilled water -> pictures taken
    - Alazarin dye extracted with 10% formic acid -> absorbande at 415nm determined and normalized to protein concentration.

Results

Results

Callus formation

- C-DO filled with Callus
- H-DO filled with fibrotic tissue
- Distal end of the gap failed to generate new callus
- MSCs but not FBs promoted callus formation in H-DO
- MSCs disappeared from gap within 11 days
    - -> paracrine mechanisms?
- MSC-CM promoted callus formation
- DMEM & FB-CM no noticeable effect
- Callus formation occurred outside of the DO gap
    - (amount not significant)

Results

BMSC recruitment & neoangiogenesis

- mBMSCs that express Sca-1 and PDGFR-α have a robust potential to differentiate into osteoblasts
- Their recruitment was increased in C-DO and H-DO by MSC-CM
    - Not in the H-DO gap alone
- MSC-CM - H-DO group showed a large number of mature blood vessels composed of CD-31, Ecs and αSMA+ pericytes
    - 1,5 x those of the DMEM group
- MSC-CM treatment increases recruitment of mBMSCs and EC/EPCs and the formation of blood vessels -> restores callus formation in the H-DO gap.

Morikawa S et al. J Exp Med 2009;206:2483-96.

Results

Recruitment of progenitor cells

- Transwell migration assays:
- For mBMMNCs and HUVECs, MSC-CM was more chemoattractive than DMEM
- mBMMNC migration inhibited by neutralizing MCP-1 & MCP-3
- HUVEC migration was inhibited by neutralizing IL-3 or IL-6

Results

Mineralization

- Mineralization of KUM9 cells (osteoblblast progenitor cells) increased by MSC-CM; More precisely by IL-3 and IL-6 but not RANTES or SDF-1

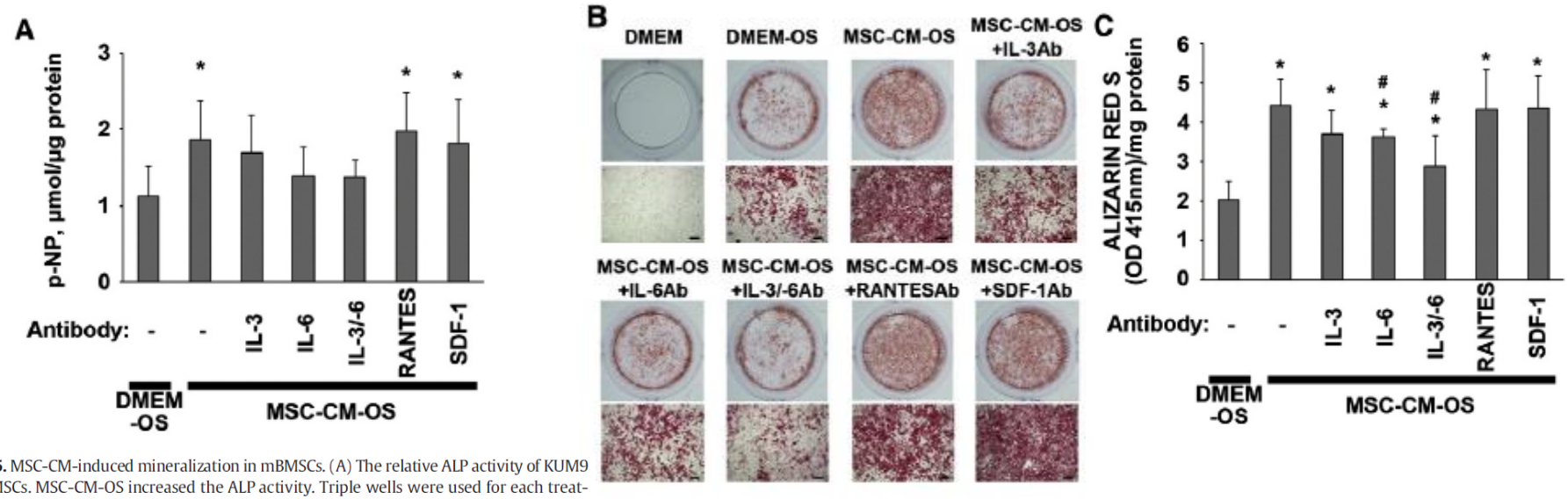


Fig. 5. MSC-CM-induced mineralization in mBMSCs. (A) The relative ALP activity of KUM9 mBMSCs. MSC-CM-OS increased the ALP activity. Triple wells were used for each treatment. Data shown represent mean ± SD of three independent experiments; $^{*}p < 0.05$ vs DMEM-OS. (B) Representative images of KUM9 mBMSCs: those cultured in MSC-CM with osteogenic supplements (OS; see Materials and methods) were stained more strongly by Alizarin red-S than those cultured in OS medium alone (B, C). Bar = 300 µm. MSC-CM-OS increased the Alizarin staining; this increase was suppressed by neutralizing antibodies for IL-3 and/or IL-6 (C). Triple wells were used for each treatment. Data shown represent mean ± SD of three independent experiments; $^{*}p < 0.05$ vs DMEM-OS; $\#p < 0.05$ vs MSC-CM-OS.

Results

MSC-CM depleted of MCP-1/-3 failed to recruit mBMSCs or restore bone callus formation.

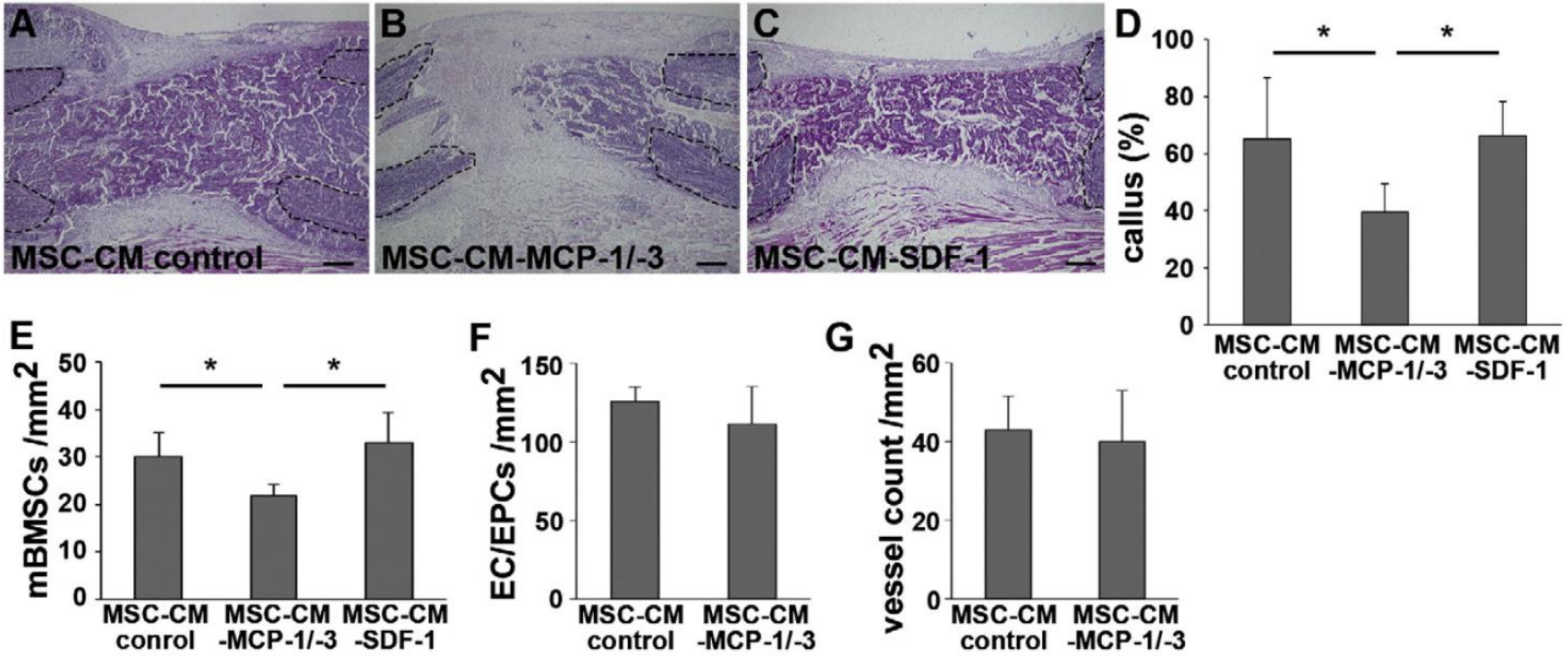


Fig. 6. MSC-CM depleted of MCP-1/-3 failed to recruit mBMSCs or to restore callus formation in the H-DO gap. (A–C) Representative micrographs of H-DO gap sections stained with hematoxylin–eosin (HE). Bar = 300 μm. Neocallus formed in H-DO gaps treated with MSC-CM control (A) or MSC-CM depleted of SDF-1 (MSC-CM-SDF-1) (C), but not in those treated with MSC-CM depleted of MCP-1/3 (MSC-CM-MCP-1/3) (B). (D–G) Quantification of callus formation (n = 8), the migration of Sca-1$^+$/PDGFRα$^+$ mBMSCs (n = 5) and CD31$^+$ EC/EPCs (n = 5), and vessel formation (n = 5). The specific depletion of MCP-1/3 significantly reduced the MSC-CM-mediated callus formation and mBMSC migration (D, E), but did not affect the EC/EPSC migration or vessel formation (F, G). Data represent the mean ± SD; $^*p < 0.05$.

Discussion

Discussion

- Distal end tissue regeneration improved by MSC-CM in H-DO
  - In H-DO alone, the distal end failed to generate neocallus
- H-DO supresses recruitment of EC/EPCs ~ neovessel formation
- H-DO supresses recruitment of mBMSCs ~ osteogenic progenitors
- MSC-CM treatment recovers these mechanisms in H-DO
- The authors suspect, that immunosuppressive factors in the MSC-CM may counteract pro-inflammatory properties of of MCP-1/-3 and IL-3/-6 and enhance their regenerative activities, -> enhancing callus formation in the H-DO gap.
- Poor MSC survival indicates that effects are primarily through paracrine mechanisms.

Discussion

- C-DO sacrificed after 15 days vs H-DO after 5/7/11 days – comparability?
- Callus measurement – no micro ct used, only 3 sections (the center and to each side of the center) have been done & measurement of digital images
- Small groups of 10 mice (H-DO – MSC-CM, FB-CM, DMEM) or 5 mice (immunohystochemical analysis MHC I) - representative?
- Callus formation appeared outside of the distraction zone – not measured – further research necessary

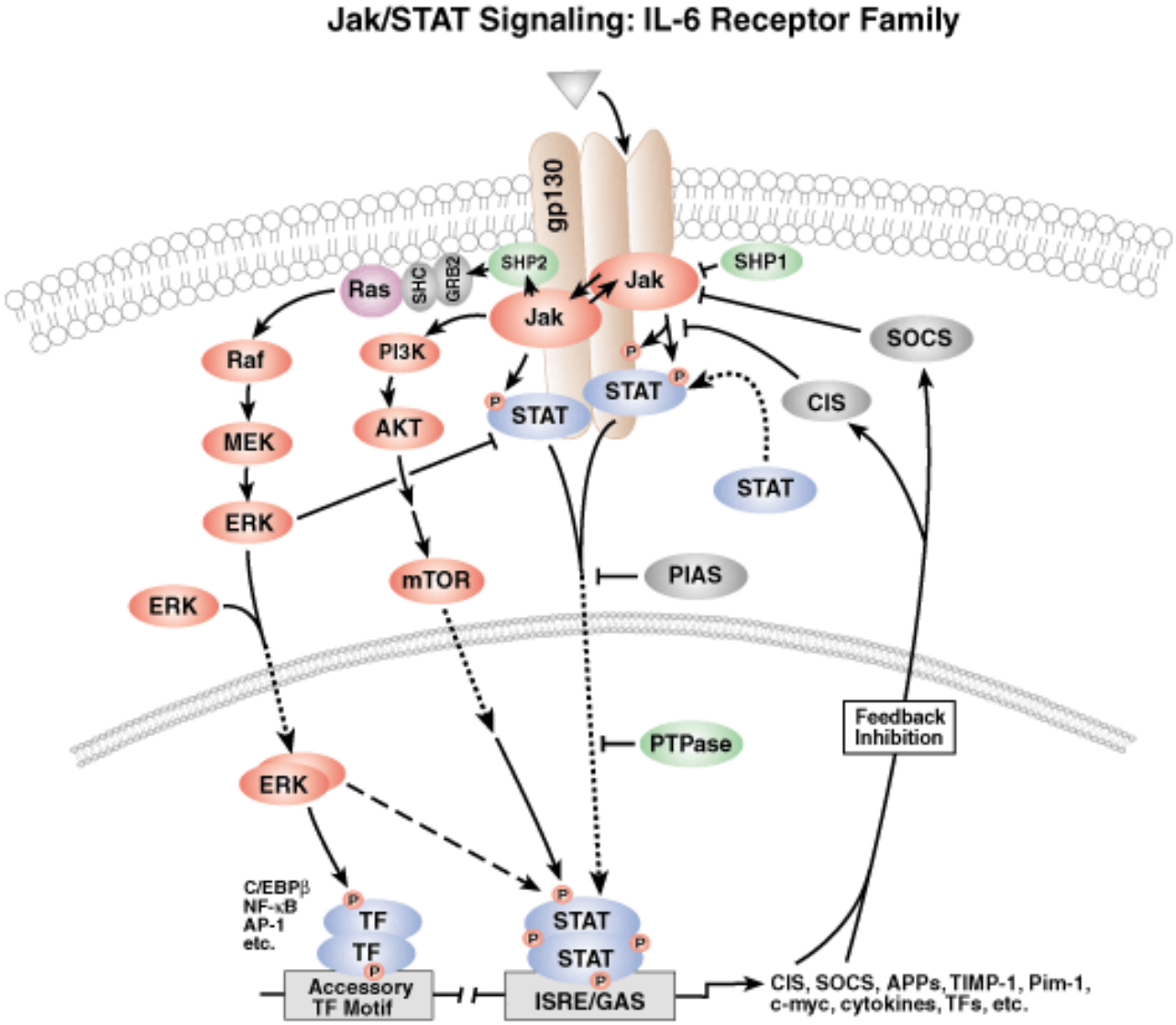
Thank you for your attention!

Take home message

- MSC-CM accelerates callus formation in the DO gap.
- MSC-CM promotes the recruitment of endogenous mBMSCs and EC/EPCs.
- MSC-CM contains multiple tissue-regenerative factors.
- MCP-1 and MCP-3 contained in MSC-CM recruit mBMSCs to the DO gap.
- IL-3/IL-6 in MSC-CM recruits EC/EPCs and promotes mBMSC osteogenic differentiation.

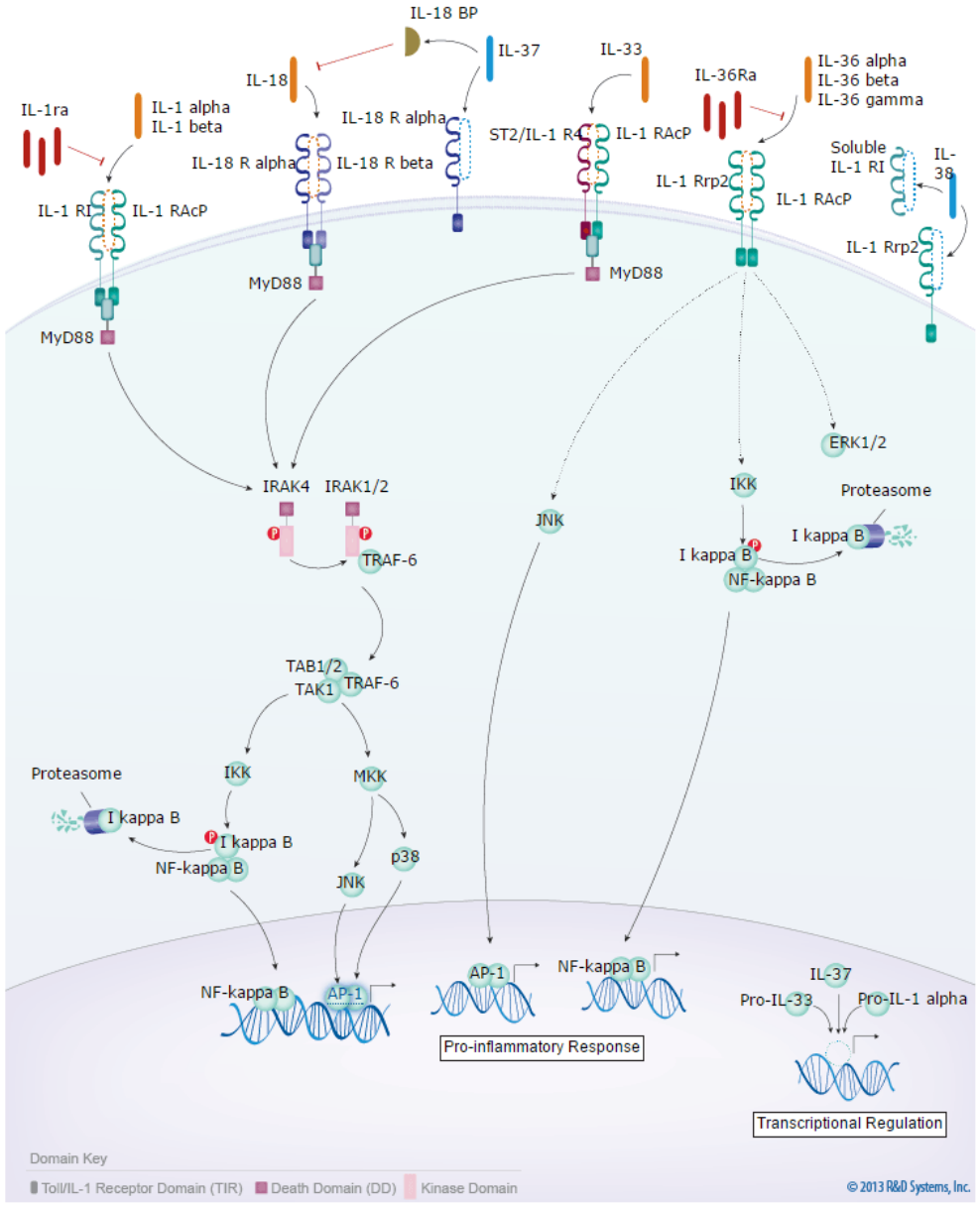
Further information

IL-6

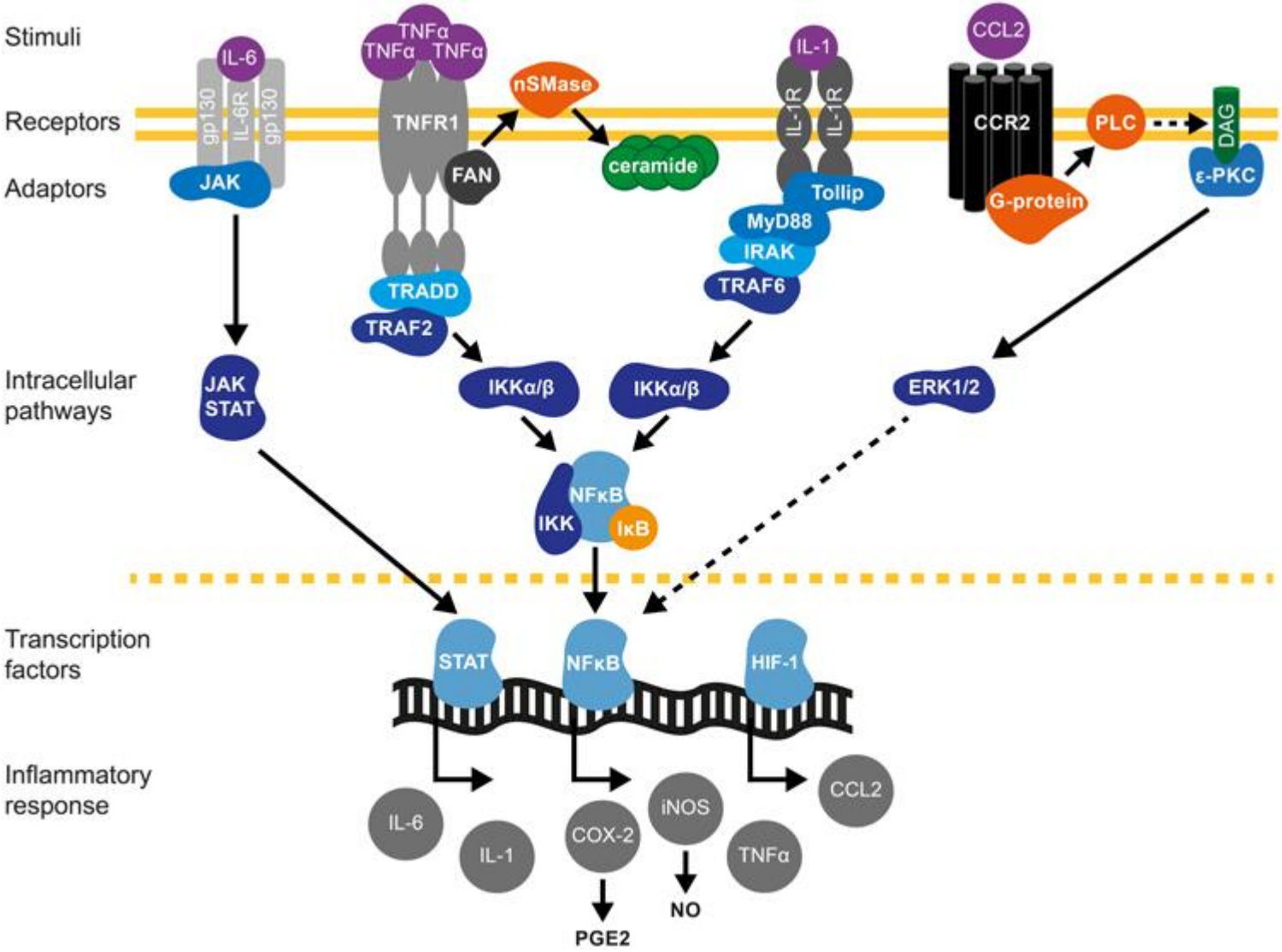


Further information

IL-1



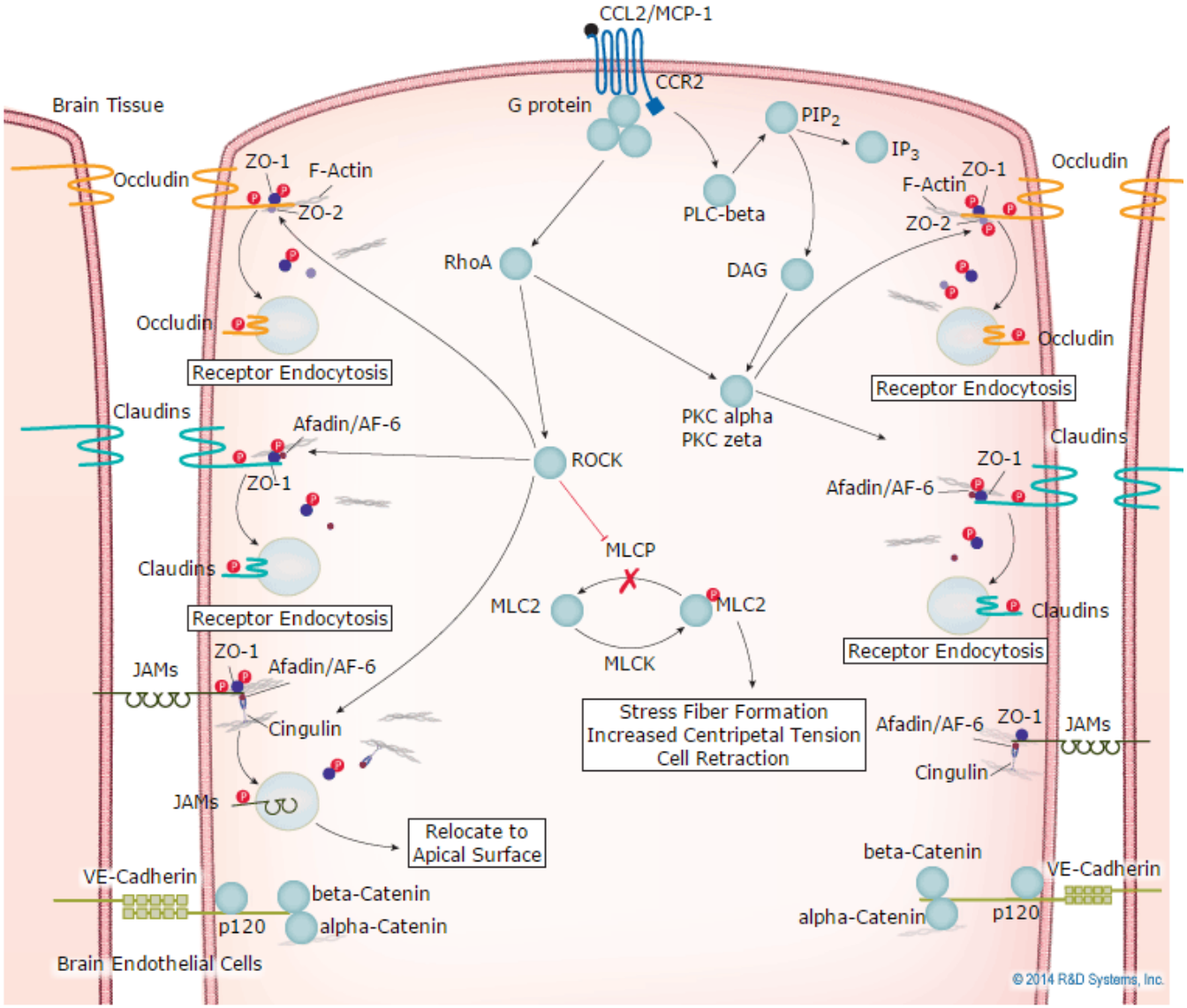
Further information



https://www.researchgate.net/figure/Cytokine-signaling-pathways-in-brain-preconditioning-Following-preconditioning-PC-the_fig3_260761438

Fu

Blood-Brain Barrier and Immune Cell Transmigration: CCL2/MCP-1 Signaling Pathways



Further information

- Cytotherapy. 2015 Aug;17(8):1119-29. doi: 10.1016/j.jcyt.2015.04.009. Epub 2015 May 29.
- **Factors secreted from dental pulp stem cells show multifaceted benefits for treating acute lung injury in mice.**
- Wakayama H[1], Hashimoto N[2], Matsushita Y[1], Matsubara K[1], Yamamoto N[1], Hasegawa Y[2], Ueda M[1], Yamamoto A[3].

Further information

- Bone. 2011 Oct;49(4):693-700. doi: 10.1016/j.bone.2011.06.024. Epub 2011 Jun 29.
- **Stromal cell-derived factor-1 enhances distraction osteogenesis-mediated skeletal tissue regeneration through the recruitment of endothelial precursors.**

- We then tested the ability of a local application of stromal cell-derived factor-1 (SDF-1), a major chemo-attractant for BM-ECs/EPCs, to accelerate the bone regeneration in H-DO. Our data showed that, in H-DO, SDF-1 induced callus formation in the gap through the recruitment of BM-ECs/EPCs, the maturation of neo-blood vessels, and increased blood flow. These results indicate that the active recruitment of endogenous BM-ECs/EPCs may provide a substantial clinical benefit for shortening the treatment period of DO.

Further information

More conditioned Medium related papers of the authors

- Spinal cord injury recovery improved
- Improved function in alzheimers desease model mice
- Protect against hypoxic ischemic brain injury in neonatal mice
- Cardiac injury
- Rheumathoid arthritis

Further information

Further information