Exosomes/tricalcium phosphate combination scaffolds can enhance bone regeneration by activating the PI3K/Akt signalling pathway

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Background

- Effective reconstruction of bone defects challenging
- Most widely used treatments:
  autologous and allogenic bone grafting
- Negatives:
  autologous: limited availability
  extra damage to harvest site
  need for a second operation

  allogenic: significant failure rates
  poor mechanical stability
  immunological rejection

Background

- Bioactive materials provide an alternative

  -> Tricalcium phosphate (β-TCP)

- same ions as bone
  well characterized osteoconducitve biomaterial
  already in clinical use for repair and regeneration

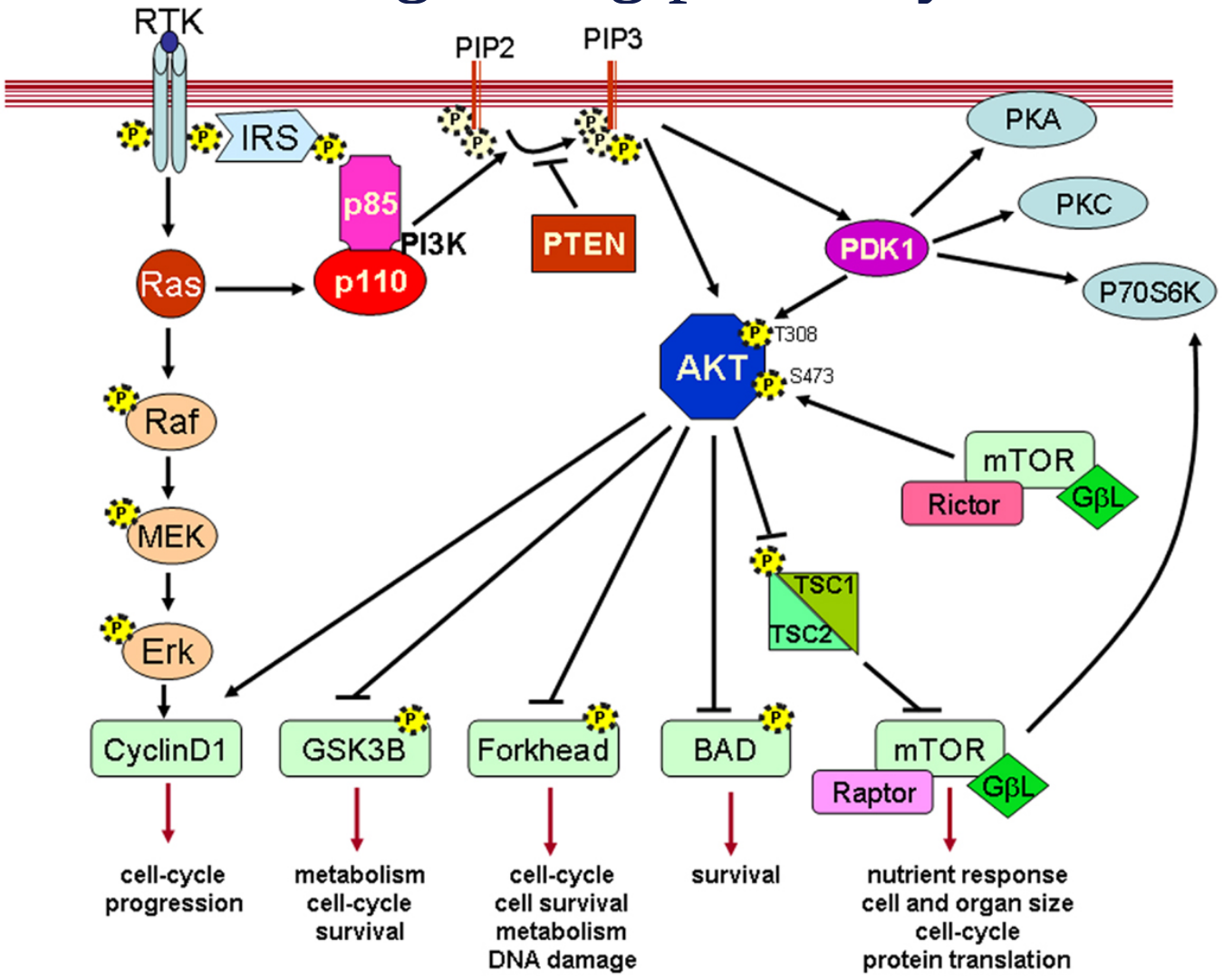
- Lack of osteoinductive activity

human induced Pluripotent Stem-Mesenchymal Stromal Cells (hiPS-MSCs)

- MSCs- Positive effects on tissue repair due to stimulation of the tissue resident recipient cells via paracrine mechanisms
- Exosomes as nanocarriers
  no apparent adverse effects
- iPS-MSCs showed bone regenerating ability when directly transplanted into calvarial defects in immuno-compromised mice

- hiPS-MSCs-Exos -> To improve the osteogenesis ability of the β-TCP

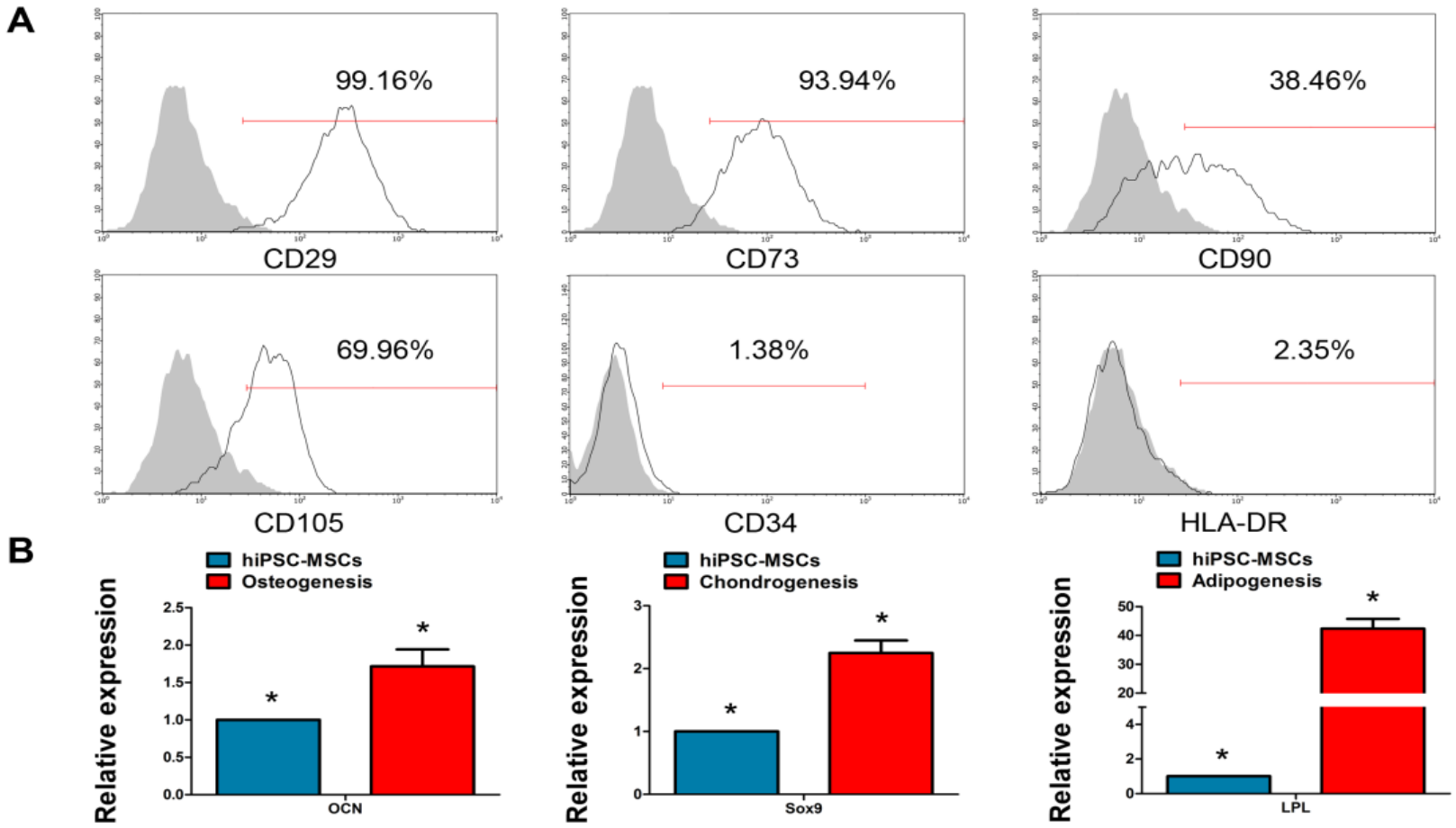
The PI3K/Akt signaling pathway



Methods

Generation and characterization of hiPS-MSCs

- hiPS were cultured and expanded on ESC-Qualified BD Matrigel in six-well plates in mTESR1
- When 90% confluent, mTESR1 replaced by low-glucose Dulbecco‘s modified Eagle medium containing 10% fetal bovine serum and 2mM L-Glu
- After 14 days in culture, into 0,1% gelatin-precoated dishes until 80-90% confluency
- MSC cell surface identification via flow cytormetry



- **Fig. S1** Characterization of hiPS-MSCs: (A) Flow cytometry analysis of the cell surface markers in hiPS-MSCs. (B) The qRT-PCR results for OCN, Sox9, and LPL after 7 days in culture with osteo-, chondro-, and adipogenic mediun. (ANOVA, *$p < 0.05$ compared with the control group)

Methods
Isolation and identification of hiPS-MSC-Exos

- 48h cultivation in MGro-500 chemically defined serum-free MSC medium
- Centrifuged to remove dead cells and cellular debris
- The supernatant was filtered through 0.22-μm filter sterilizer to remove the residual cellular debris
- Supernatant was centrifuged at 4000 x g to about 200μL by ultra filtration in a 15mL Amicon Ultra -15 Centrifugal Filter Unit, washed twice with PBS and re-ultra-filtrated
- For exosome purification the liquid was overlaid onto a 30% sucrose-D_2O cushion in a sterile Ultra-Clear™ tube and ultra-centrifuged at 100,000 × g for 2 h to pellet the small vesicles that correspond to exosomes
- The pelleted exosomes were resuspended in 15 mL PBS and centrifuged at 4000 × g in Centrifugal Filter Units until the final volume was reduced to approximately 200 μL.
- All procedures were performed at 4 ˚C. Exosomes were stored at −80 ˚C or used for downstream experiments.
- Transmission electron microscopy (TEM), tunable resistive pulse sensing (TRPS) analysis, and western blotting were used to identify hiPS-MSC-Exos.

In vivo animal experiments
Surgical procedures

- β-TCP scaffolds with a dimension of 5 mm in diameter and 2 mm in depth with an average pore size of 500 μm and 75 % porosity were used as exosome carriers in the in vivo studies
- Five $\times 10^{11}$ particles/mL or 1×10^{12} particles/mL of exosomes (100 μL) or an equal volume PBS were blotted onto each β-TCP scaffold and left for 4 h for the exosomes to be totally absorbed
- Sprague Dawley rats got two critical-sized calvarial defects with a diameter of 5 mm on each side of the cranium using dental trephine followed by implantation of the scaffolds into the defects.

In vivo experimental animal experiments

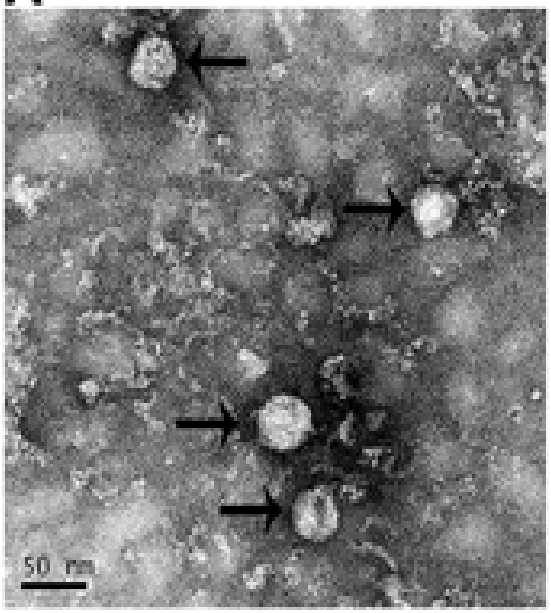
- At 2,4, and 6 weeks after the operation the rats were injected with tetracycline, alizarin red and calcein for fluorescent labeling
- 8 weeks after the OP rats were sacrifized
- The craniums were scanned by micro-CT and new bone volume was calculated
- Histological analysis with the fluorochromes using CLSM and van Gieson‘s staining
  osteogenesis was evaluated by IHC analysis for Osteocalcin

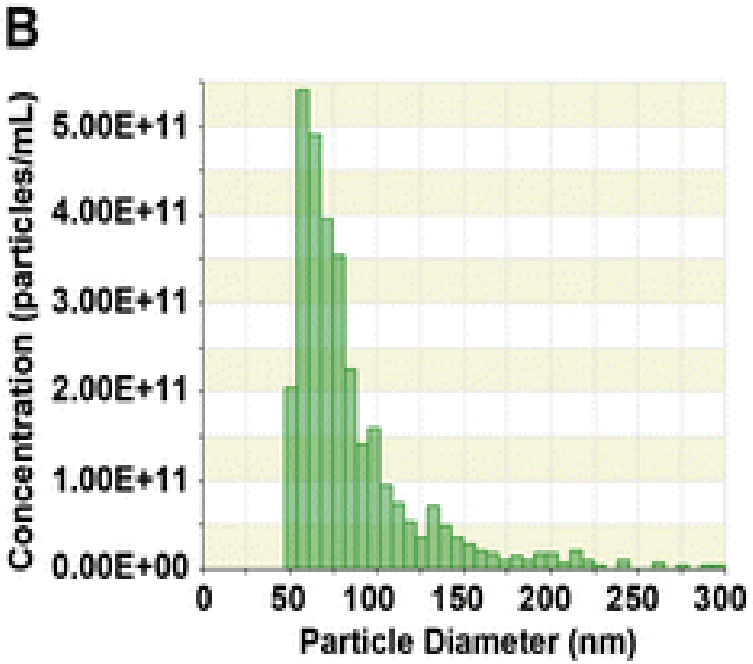
In vitro studies

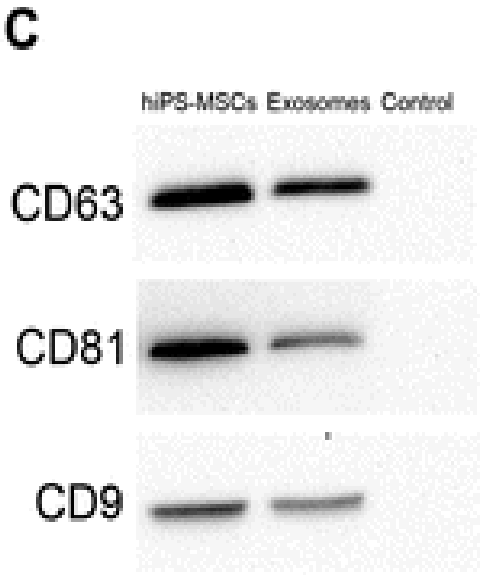
- Exosome release from β-TCP (1day and 5days)
- Cell proliferation assay
- Migration assay (using a scratch wound healing assay)
  hBMSCs with Exosomes 5×10^{11} or 1×10^{12} particles/mL
- Gene expression profiling and bioinformatics analysis by using the GeneChip® PrimeView™ Human Gene Expression Array
  hBMSCs with Exosomes 5×10^{11} particles/mL
- qRT-PCR
- Western Blot analysis
- PI3K/Akt signaling inhibition to confirm the involment
- Alkaline phosphatase (ALP) assay and Alizarin red S (ARS) staining

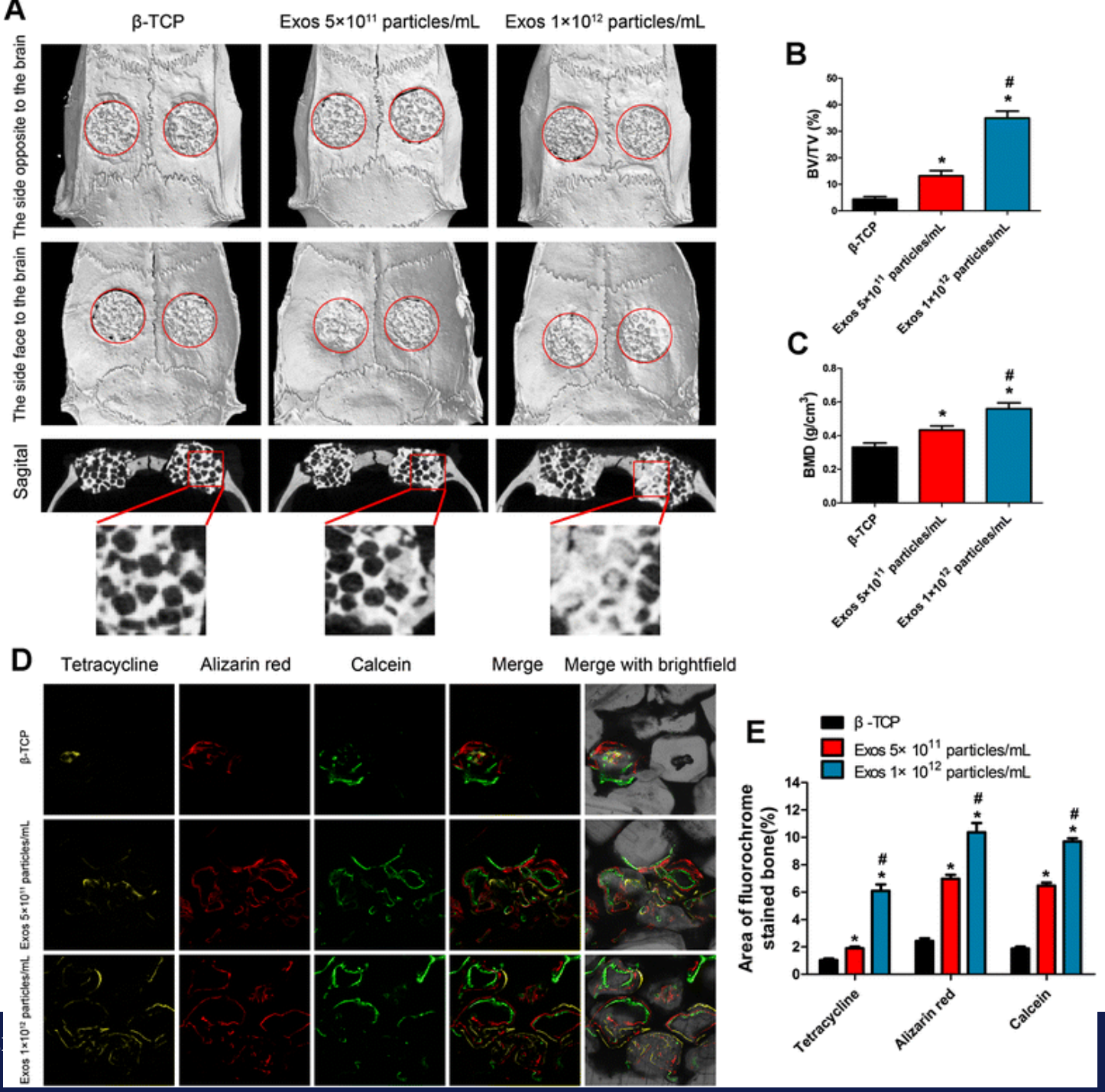
Results

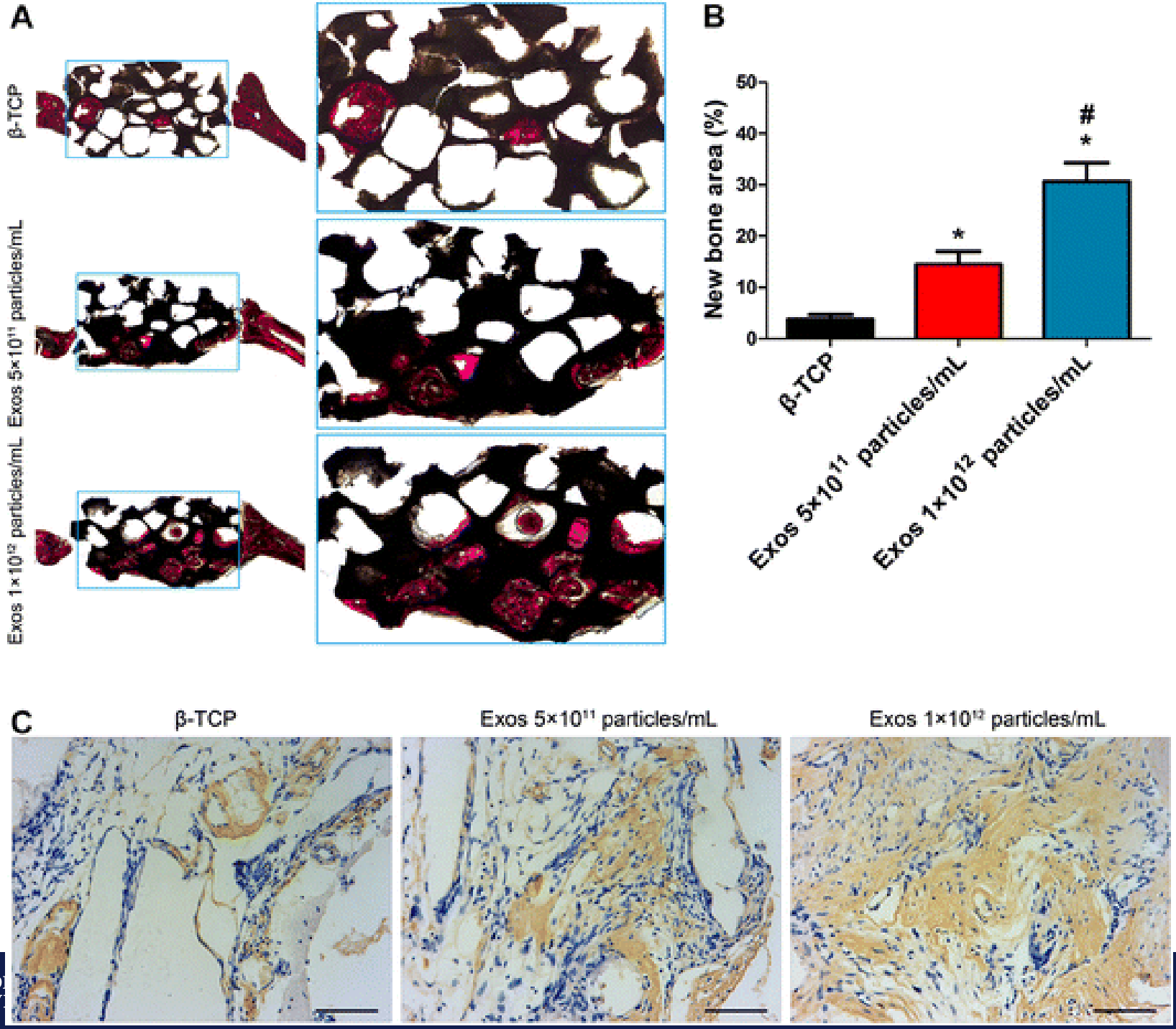
- Characterization suggest that the vast majority of the nanocarriers derived from hiPS-MSCs were exosomes
- In vivo experiment showed a dose-dependent increase in new bone formation
- DiO-labeled exosomes can be found in the perinuclear region of hBMSCs which surround β-TCP
- Exosomes enhanced the hBMSCs proliferation
  Scratch wound assay showed that, after being cultured with exosomes, the migration ability of hBMSCs was remarkably enhanced

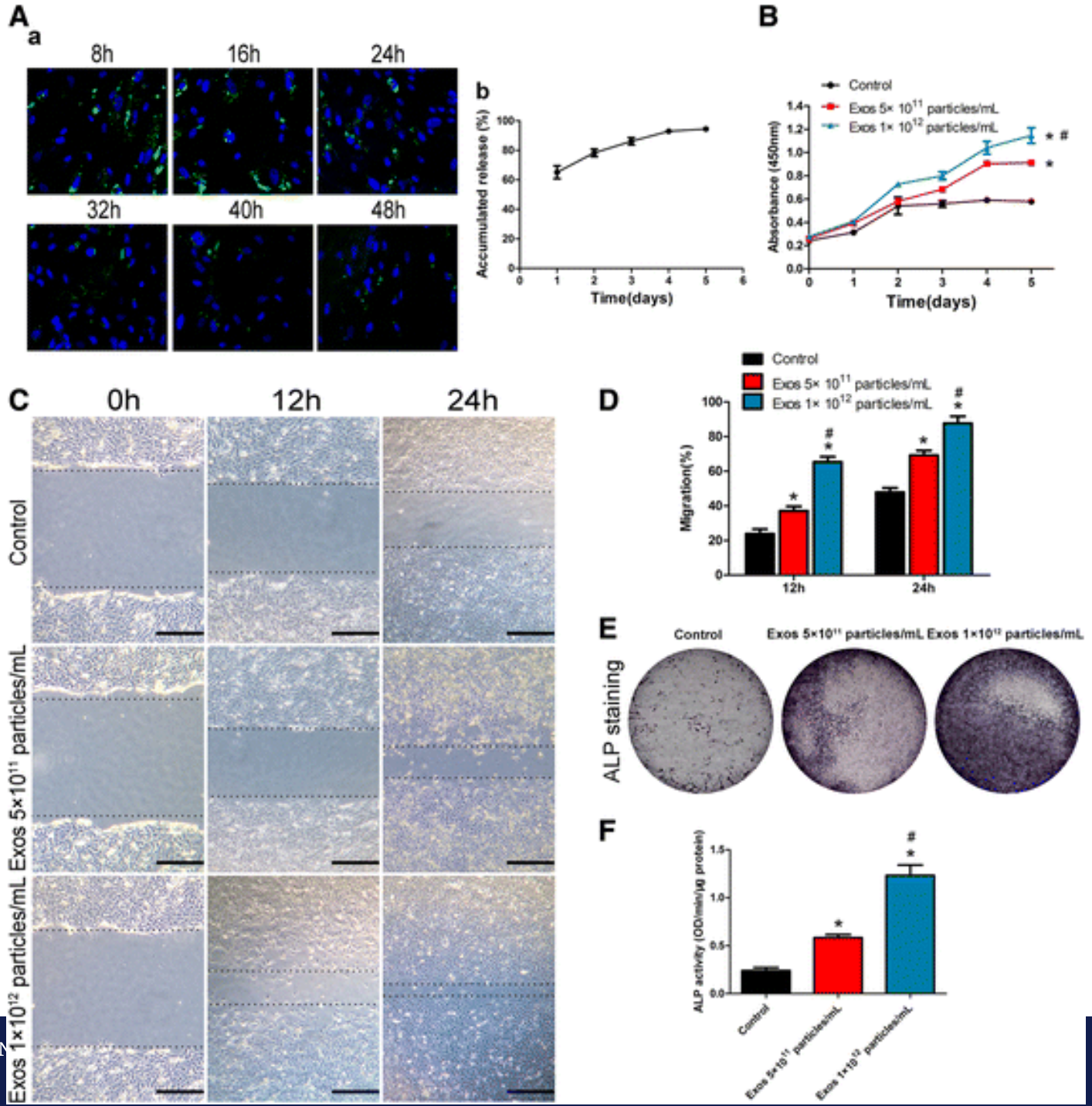

A
50 nm


B
Concentration (particles/mL)
5.00E+11
4.00E+11
3.00E+11
2.00E+11
1.00E+11
0.00E+00
0
50
100
150
200
250
300
Particle Diameter (nm)


C
hiPS-MSCs Exosomes Control
CD63
CD81
CD9

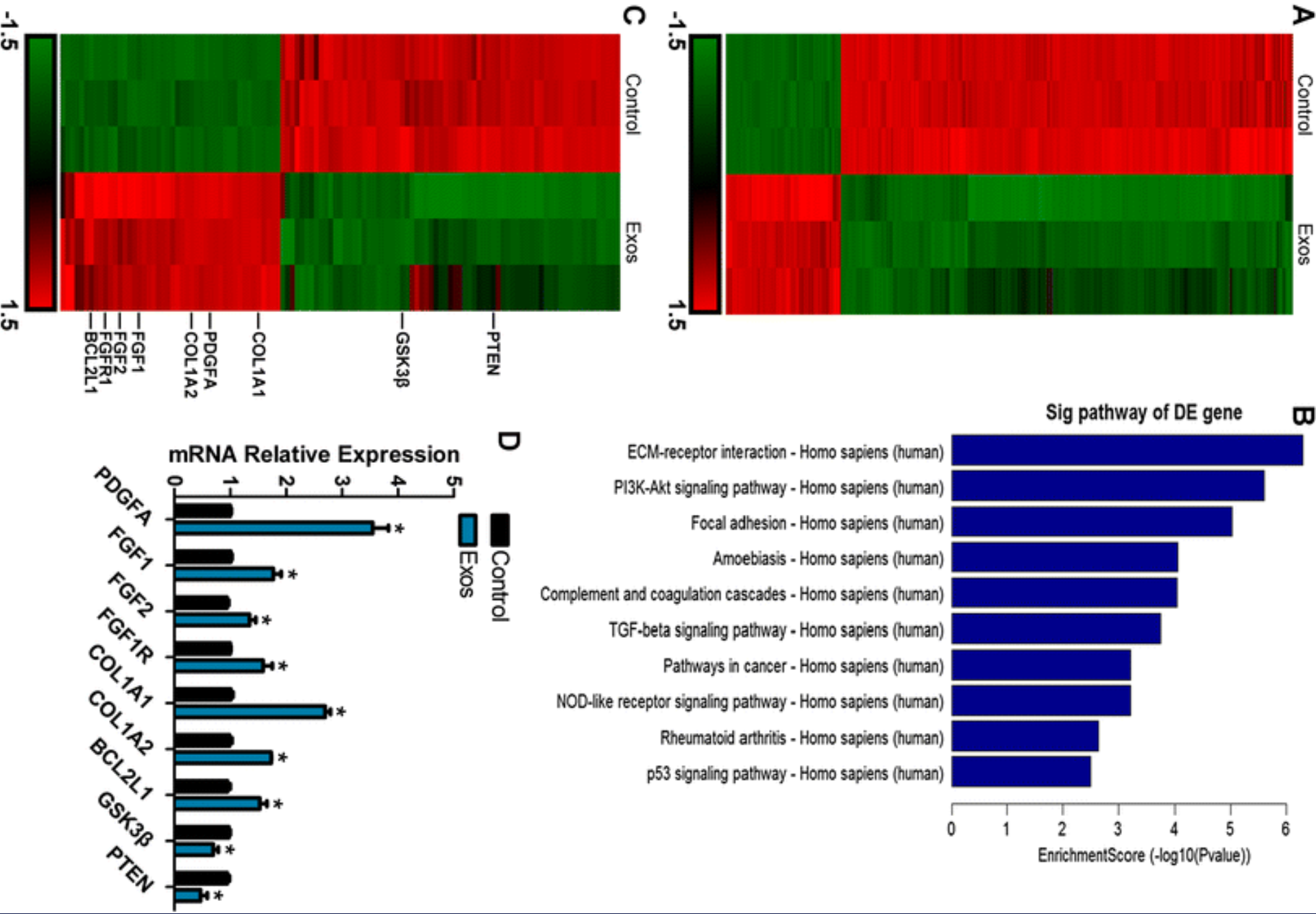

A
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL
The side opposite to the brain
The side face to the brain
Sagital
B
BV/TV (%)
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL
C
BMD (g/cm³)
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL
D
Tetracycline
Alizarin red
Calcein
Merge
Merge with brightfield
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL
E
β -TCP
Exos 5× 10¹¹ particles/mL
Exos 1× 10¹² particles/mL
Area of fluorochrome stained bone(%)
Tetracycline
Alizarin red
Calcein

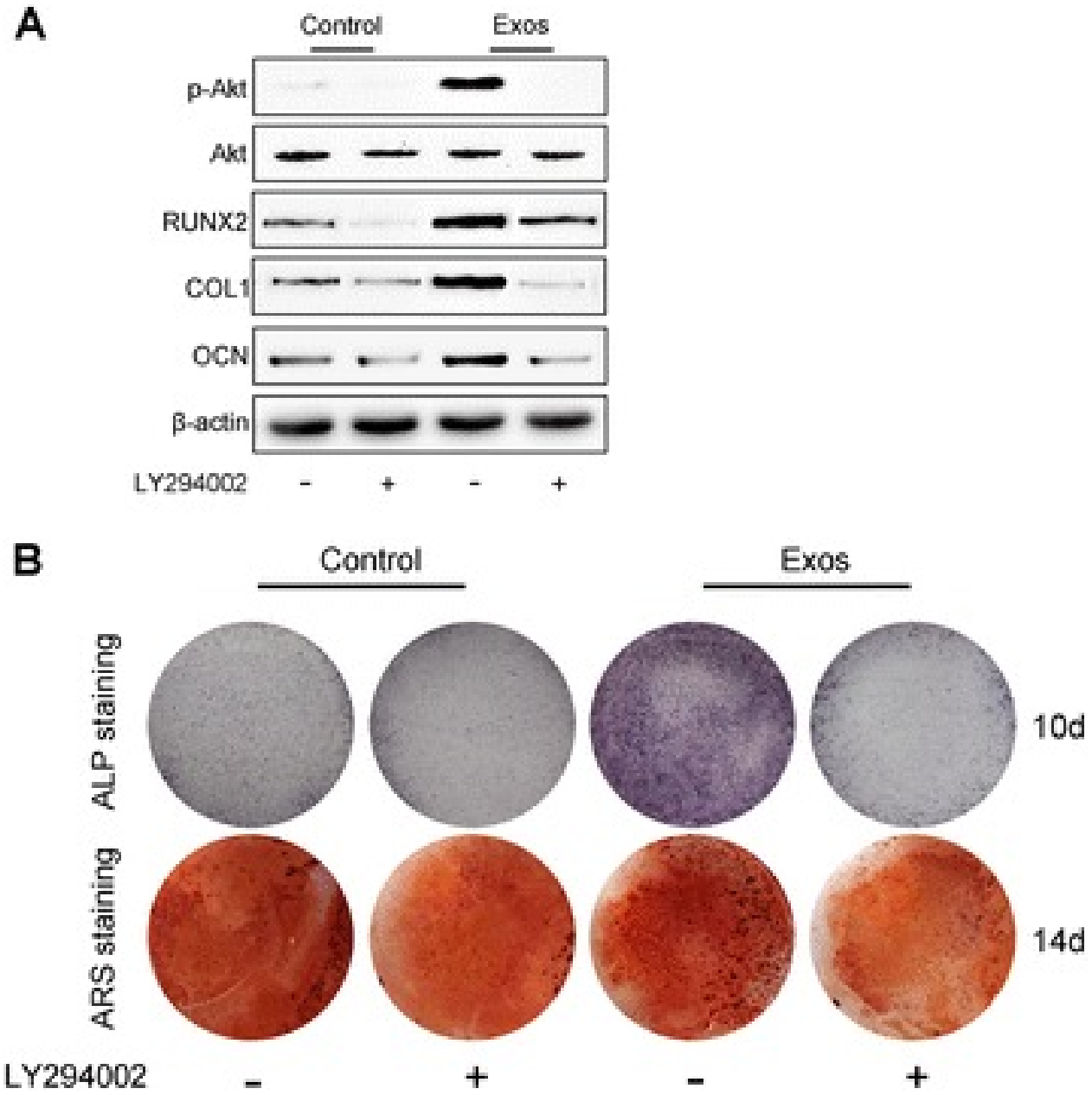

A
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL
B
New bone area (%)
50
40
30
20
10
0
*
#
*
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL
C
β-TCP
Exos 5×10¹¹ particles/mL
Exos 1×10¹² particles/mL


A
a
8h
16h
24h
32h
40h
48h
b
Accumulated release (%)
Time(days)
B
Control
Exos 5× 10^11 particles/mL
Exos 1× 10^12 particles/mL
Absorbance (450nm)
Time(days)
C
0h
12h
24h
Control
Exos 5×10^11 particles/mL
Exos 1×10^12 particles/mL
D
Control
Exos 5× 10^11 particles/mL
Exos 1× 10^12 particles/mL
Migration(%)
12h
24h
E
Control
Exos 5×10^11 particles/mL
Exos 1×10^12 particles/mL
ALP staining
F
ALP activity (OD/min/μg protein)
Control
Exos 5×10^11 particles/mL
Exos 1×10^12 particles/mL

Genexpression and PI3K/Akt pathway

- Significant differential expression of 1447 candidate genes
- Among these 293 genes were upregulated and 1154 genes were downregulated with exosome treatment
- Multiple pathways were prominently enriched
  PI3K/Akt-pathway
  ECM-receptor interaction
  focal adhesion
- The PI3K/Akt signaling pathway
  positive effector genes upregulated
  negative effector genes downregulated
- Control with LY294002 = PI3K-Inhibitor


A
Control
Exos
-1.5
1.5
B
Sig pathway of DE gene
ECM-receptor interaction - Homo sapiens (human)
PI3K-Akt signaling pathway - Homo sapiens (human)
Focal adhesion - Homo sapiens (human)
Amoebiasis - Homo sapiens (human)
Complement and coagulation cascades - Homo sapiens (human)
TGF-beta signaling pathway - Homo sapiens (human)
Pathways in cancer - Homo sapiens (human)
NOD-like receptor signaling pathway - Homo sapiens (human)
Rheumatoid arthritis - Homo sapiens (human)
p53 signaling pathway - Homo sapiens (human)
0 1 2 3 4 5 6
EnrichmentScore (-log10(Pvalue))
C
Control
Exos
-1.5
1.5
FGF1
FGF2
FGFR1
BCL2L1
PDGFA
COL1A2
COL1A1
GSK3β
PTEN
D
mRNA Relative Expression
0 1 2 3 4 5
PDGFA
FGF1
FGF2
FGF1R
COL1A1
COL1A2
BCL2L1
GSK3β
PTEN
Control
Exos
*


A
Control
Exos
p-Akt
Akt
RUNX2
COL1
OCN
β-actin
LY294002 - + - +
B
Control
Exos
ALP staining
ARS staining
10d
14d
LY294002 - + - +

Discussion

- Currently a growing body of evidence indicates that MSC paracrine action may be applied as a novel strategy for tissue repair
- The exosome/β-TCP combination scaffolds can be potentially used as a promising graft for bone defect repair
- exosomes in perinuclear regions of hBMSCs indicate that the exosomes are released by the exosome/β-TCP scaffold and then be internalized by hBMSCs, which stimulates their proliferation, migration osteogenic differentiation
- PI3K-inhibitor markedly, but not completely, abolished the pro-osteogenic effect of hiPS-MSC-Exos
  -> exosome induced effect not fully attributed to PI3K/Akt

Conclusions

- hiPS-MSCs-Exos- functionalized β-TCP scaffold can effectively promote bone repair and regeneration in a rat model
- bone regeneration through activation of endogenous BMSCs in the bone defect site
- The PI3K/Akt signaling pathway plays a critical role in the pro-osteogenesis effects of the exosome/β-TCP scaffold on BMSCs
- Further studies are needed to assess the long-term effects

Thank you for your attention