Damaged brain accelerates bone healing by releasing small extracellular vesicles that target osteoprogenitors

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Clinical evidence has established that concomitant traumatic brain injury (TBI) accelerates bone healing, but the underlying mechanism is unclear. This study shows that after TBI, injured neurons, mainly those in the hippocampus, release osteogenic microRNA (miRNA)-enriched small extracellular vesicles (sEVs), which targeted osteoprogenitors in bone to stimulate bone formation. We show that miR-328a-3p and miR-150-5p, enriched in the sEVs after TBI, promote osteogenesis by directly targeting the 3′UTR of FOXO4 or CBL, respectively, and hydrogel carrying miR-328a-3p-containing sEVs efficiently repairs bone defects in rats. Importantly, increased fibronectin expression on sEVs surface contributes to targeting of osteoprogenitors in bone by TBI sEVs, thereby implying that modification of the sEVs surface fibronectin could be used in bone-targeted drug delivery. Together, our work unveils a role of central regulation in bone formation and a clear link between injured neurons and osteogenitors, both in animals and clinical settings.
The brain governs most physiological functions of the body, from complexities of cognition, learning, and memory, to regulation of basal body temperature, heart rate, and breathing. The brain is well established as a master regulator of breathing. The brain is well established as a master regulator of homeostasis in peripheral tissues; central regulation of bone remodeling represents a novel and expanding area of research. The brain is well established as a master regulator of homeostasis in peripheral tissues; central regulation of bone remodeling represents a novel and expanding area of research. The brain is well established as a master regulator of homeostasis in peripheral tissues; central regulation of bone remodeling represents a novel and expanding area of research.

To identify the specific miRNAs involved, we compared miRNA plasma sEVs from TBI patients and rats contribute to TBI-stimulated osteogenesis. To explore the mechanism by which TBI promotes bone formation, we first compared the osteogenic effects of plasma from patients or rats with TBI and controls. We found that plasma from TBI patients or rats promoted osteoblastic differentiation (Fig. 2a, b and Supplementary Fig. 5a) without affecting its proliferation (Supplementary Fig. 5b). Next, TBI plasma was separated into insoluble and soluble components to screen for factors responsible for TBI-stimulated osteogenesis. Unexpectedly, plasma sEVs from patients and rats with TBI had much greater potential to promote osteogenesis in vitro than soluble factors (Fig. 2c, d). The purified sEVs were verified by transmission electron microscopy (TEM), NanoSight analysis, and immunoblotting for protein markers (CD9, Alix, and TSG101), according to the proposal of the International Society of Extracellular Vesicles (Supplementary Fig. 6). Notably, the concentration of plasma sEVs in TBI rats was much higher than that in shams (Fig. 2e).

To confirm the role of sEVs in TBI-stimulated osteogenesis and bone healing in vivo, GW4869, an inhibitor of sEVs secretion, was intravenously injected into PTD rats with concomitant TBI. The significant reduction of sEVs isolated from the plasma of rats with GW4869 treatment was confirmed by NanoSight Analysis and western blotting (Supplementary Fig. 7a, b). Micro-CT analysis showed a significantly decreased callus volume, bone volume/tissue volumes, BV/TV%, and trabecular number (Tb.N), after GW4869 treatment on days 7 and 14 post-injury (P < 0.05) (Fig. 2f–h). Trabecular bone and OCN expression were also reduced dramatically after inhibition of sEV release by GW4869 (Fig. 2i–k). These results suggested that sEV release was required for TBI-stimulated osteogenesis and bone healing in rats.

Taken together, these results suggested that plasma sEVs from patients and rats with TBI had much greater potential to promote osteogenesis than soluble factors. sEVs contributed to TBI-stimulated bone formation in vitro and bone healing in rats.

Plasma sEVs released by TBI patients and rats carry potent osteogenic miRNAs, such as miR-328a-3p and miR-150-5p. Next, we performed miRNA sequencing and proteomics analysis to explore how TBI-derived sEVs promoted osteogenesis and bone healing. The miRNAs, which can be abundantly encapsulated in sEVs, are key regulators of bone remodeling in health and disease. We, therefore, evaluated the miRNA expression profiles in sham-sEV and TBI-sEV groups using miRNA sequencing. To identify the specific miRNAs involved, we compared miRNA expression profiles in sham-sEV and TBI-sEV groups using miRNA sequencing.
Fig. 1 TBI (traumatic brain injury) accelerates bone healing in patients and rats. a Fracture callus volume in fracture alone patients and TBI patients with concomitant fracture (n = 4 person per group per time point, Student’s two-sided unpaired t test). P(2w TBI+fracture vs. 2w Fracture) = 0.0787, P(4w TBI+fracture vs. 4w Fracture) = 0.0028, P(6w TBI+fracture vs. 6w Fracture) = 0.0082. b Scheme illustrating the fluid-percussion device for experimentally induced TBI. Representative micro-computed tomography (CT) 3D image (c) and BV/TV% analysis (d) of defect sites of PTD (proximal tibia defect) and PTD+TBI (PTD combined-TBI) groups on day 3, 7, and 14 after operation (n = 5 animals per group per time point; Student’s two-sided unpaired t test). P(3d PTD+TBI vs. PTD) = 0.5424, P(7d PTD+TBI vs. PTD) = 0.0001, P(14d PTD+TBI vs. PTD) = 0.0011. e Representative H&E staining of rat PTD sites show newly formed woven bone in the defect sites. Scale bars, 200 μm (n = 5 animals per group per time point). f, g Representative immunohistochemical staining and quantitation of OCN-positive cells in rat PTD sites at 7 and 14 days after surgery (n = 5; Student’s two-sided unpaired t test). P(7d PTD+TBI vs. PTD) = 0.0225, P(14d PTD+TBI vs. PTD) = 0.0014. Scale bars, 40 μm. The quantitation result were plotted as dot plots, showing the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01; ns = nonsignificant.
profiles of plasma sEVs derived from the TBI and the sham rats; the results are shown by heatmaps (Fig. 3a and Supplementary Table 2). Four of the maximally upregulated miRNAs and two of the downregulated miRNAs were validated by real-time quantitative polymerase chain reaction (qRT-PCR) (Fig. 3b).

To determine the effects of these miRNAs on osteoblast formation, MC3T3-E1 cells and primary rat BMSCs were transfected with miRNA mimics or negative controls. We found that miR-328a-3p and miR-150-5p significantly promoted osteoblast differentiation (Fig. 3c, d). Inhibitors of miR-328a-3p or miR-150-5p, on the contrary, prevented osteoblastic differentiation of osteoprogenitors (Fig. 3e, f). Interestingly, miR-328a-3p and miR-150-5p were also markedly increased in plasma sEVs from patients with TBI, as compared to healthy controls (Fig. 3g).

These data suggested that sEVs released by patients and rats with TBI carried potent osteogenic miRNAs, such as miR-328a-3p and miR-150-5p.
sEV miR-328a-3p and miR-150-5p promote osteogenesis by directly targeting FOXO4 and CBL 3′-UTR, respectively. To identify the targets of miR-328a-3p and miR-150-5p, first, we applied PicTar and TargetScan to search for putative miRNA targets. Based on the representation of miRNA sites in their 3′ UTRs, >100 mRNAs were predicted to be regulated by miRNAs. Among these candidates, eight genes were involved in the suppression of osteogenesis.

To determine whether miRNAs target these genes directly, we cloned the 3′UTRs of the putative targets into a dual luciferase assay system. Reporter assays using MC3T3-E1 cells revealed that miR-328a-3p repressed the FOXO4 3′UTRs and miR-150-5p repressed the CBL 3′UTRs, significantly (Fig. 4a–c). Mutations of the putative miRNA sites in these two 3′UTRs abrogated responsiveness to miRNA (Fig. 4c–e).
We next determined whether there was an association between expression levels of target genes in bone defect sites of TBI-TPD rats. We found that levels of FOXO4 and CBL were decreased at the bone defect site of TBI-TPD rats as compared with that of PTD rats, which suggests that FOXO4 and CBL may be involved in TBI-stimulated bone formation (Fig. 4f, g). Accordingly, transfection of miR-328a-3p or miR-150-5p mimics reduced endogenous FOXO4 or CBL protein levels in MC3T3-E1 cells. On the contrary, inhibition of miR-328a-3p or miR-150-5p significantly increased FOXO4 or CBL protein levels (Fig. 4h, i). We further found that knockdown of FOXO4 or CBL produced similar changes in osteogenesis to that of miR-328a-3p or miR-150-5p overexpression, respectively (Fig. 4j, k). Importantly, the osteogenic effect of miR-328a-3p or miR-150-5p could be partially rescued by overexpression of FOXO4 or CBL, indicating that FOXO4 and CBL were downstream targets of miR-328a-3p and miR-150-5p respectively, which promoted bone formation.

Taken together, these results suggested that sEV miR-328a-3p and miR-150-5p downregulated FOXO4 and CBL expression, respectively, by directly targeting their 3’UTR to promote bone formation.

Injured neurons produce sEVs that target bone and osteoprogenitors. We next explored where these osteogenic sEVs originated from during TBI. We speculated that these sEVs might originate from injured brain tissue. In situ hybridization showed that expression of miR-328a-3p in brain tissues of rats increased significantly after TBI, especially in the CA1 and DG regions of the hippocampus (Fig. 5a and Supplementary Fig. 8). Importantly, immunofluorescence staining showed that expression of CD63 in rat brains was intensively induced by TBI (Fig. 5b). Interestingly, most of the CD63 was expressed in microtubule-associated protein 2 (MAP2)-positive, especially in the CA1 region of the hippocampus, but not glial fibrillary acidic protein (GFAP)-positive cells, indicating that damaged neuronal cells, but not astrocytes secreted sEVs vigorously (Fig. 5b, c and Supplementary Figs. 9 and 10).

Primary traumatic injury generally initiates a cascade of secondary injury mechanisms such as edema, hemorrhage, and decreased cerebral blood flow resulting in ischemic injury to brain tissue. While the ischemic injury is a secondary consequence of TBI, ischemia is the primary mechanism of injury in cases that disrupt arterial cerebral blood flow leading to a lack of oxygen and glucose in brain tissue and oxygen-glucose deprivation (OGD) is an in vitro model commonly used for TBI18,19. The results of the western blot of RUNX2 and ALP staining showed that the osteoblastic differentiation of BMSC and MC3T3-E1 cells increased after cocultured with OGD treated primary hippocampal neurons (Supplementary Fig. 11d, e). Moreover, we found that the sEVs release and sEV miR-328a-3p and miR-150-5p levels were significantly elevated in cultured primary hippocampal neurons after OGD treatment (Fig. 5d and Supplementary Fig. 11b, c). And the miR-328a-3p content did not change significantly while the content of miR-150-5p was increased in sEVs of murine microglial cell line BV2 after OGD treatment (Supplementary Fig. 11a).

To determine whether these damaged neurons produced sEVs and their miRNA cargo could target bone and osteoprogenitors, rats were intravenously injected with PKH67-labeled sEVs (800 µg per rat) isolated from plasma of TBI or sham rats, and the distribution of PKH67-sEV was evaluated by biophotonic imaging after intracerebroventricular (ICV) injection or intravenous injection. We found that PKH67-labeled sEVs purified from rats with TBI, but not from rats with liver injury or sham rats, accumulated in bone, but not in visceral organs or brain (Fig. 5e and Supplementary Figs. 12 and 13c). Moreover, we compared the intake of PKH67-labeled sEVs by cultured BMSC, and MC3T3-E1 and MLO-Y4 cell lines (osteocyte cell lines) and found that BMSC and MC3T3-E1 cell lines absorbed more plasma sEVs from TBI rats than from sham rats, whereas no difference was observed in MLO-Y4 cells (Fig. 5f). Notably, in situ hybridization analysis of miR-328a-3p expression in rat PTD sites demonstrated that the abundance of miR-328a-3p was higher in PTD sites in TBI rats than the sham rats at 7 days after surgery (Fig. 5g).

Taken together, these data implied that sEVs and their miRNA cargo derived from injured neurons could target bone and osteoprogenitors.

Protein fibronectin 1 (FN1) directs sEVs to target osteoprogenitors and bone. It is interesting to note that TBI-induced sEVs accumulated in bone. We speculated that proteins expressed on sEVs surfaces or membranes may contribute to increased affinity of these sEVs to the bone; hence, we performed proteomic analysis and compared protein profiles of plasma sEVs from TBI and sham rats (Fig. 6a and Supplementary Data 1). It was of interest that the quantity of FN1, an extracellular matrix and surface glycoprotein, increased 1.32 times in amount in TBI sEVs as compared with control sEVs. Notably, A2M, a marker for neuronal injury20,21, also increased 3.099 times in amount, implying that these sEVs might originate from injured neurons. Further experiments results confirmed the increase of FN1 and
A2M protein in sEVs from TBI rats (Fig. 6b, c and Supplementary Fig. 14a, b). In addition, western blot results showed that MAP2 and UCHL1, two makers of neuron cells, were increased in sEVs isolated from TBI rat plasma (Supplementary Fig. 14a). Immunofluorescence staining showed that expressions of both FN1 and A2M in the brain were intensively induced by TBI (Fig. 6d, e). Furthermore, co-staining of A2M and OCN could be clearly observed at the PTD site of PTD-TBI rats but not PTD alone rats (Fig. 6f), thereby implying that uptake of sEVs might concurrently increase osteogenesis in PTD-TBI rats.

We further confirmed the FN1-directed targeting of sEVs to osteoprogenitors in vitro. As expected, FN1 expression was dramatically enhanced in OGD treated primary hippocampal neurons, HT22 cell lines and in its sEVs, as compared with controls (Fig. 6g–i and Supplementary Fig. 14c–g). In addition, the uptake sEVs isolated from primary hippocampal neurons and
Fig. 4 sEV miR-328a-3p and miR-150-5p promote osteogenesis by directly targeting FOXO4 and CBL 3′-UTRs, respectively. a The psiCHECK-2-SMAD3/FoxO4/SUFU/PBX1-1 3′-UTRs of wild-type genes were co-transfected with the NC or miR-328a-3p mimic into MC3T3-E1 cells. Cells were harvested for quantitation of dual luciferase activities 24 h post transfection (n = 6; Student’s two-sided unpaired t test). P(SMAD3 miR-328a-3p vs. NC) = 0.0684, P(FoxO4 miR-328a-3p vs. NC) = 0.0001, P(SUFU miR-328a-3p vs. NC) = 0.1952, P(PBX1-1 miR-328a-3p vs. NC) = 0.0795. b The psiCHECK-2-CBL/LEPR/ACVR1B/GSK3B 3′-UTR of wild-type genes were co-transfected with the NC or miR-150-5p mimic into MC3T3-E1 cells. Cells were harvested for quantitation of dual luciferase activities 24 h post-transfection (n = 6; Student’s two-sided unpaired t test). P(CBL miR-150-5p vs. NC) = 0.0002, P(LEPR miR-150-5p vs. NC) = 0.2135, P(ACVR1B miR-150-5p vs. NC) = 0.3862, P(GSK3B miR-150-5p vs. NC) = 0.0946. c Schematic diagram of putative miR-328a-3p and miR-150-5p binding sites in the FoxO4 and CBL 3′-UTR. Green letters denote mutation sites. Luciferase activity in the indicated MC3T3-E1 cells upon transfection of miR-328a-3p (d) and miR-150-5p (e) binding site mutant (Mut) of 3′-UTR-driven reporter constructs (n = 6; Student’s two-sided unpaired t-test). P(WT miR-328a-3p vs. NC) < 0.0001, P(MUT1 miR-328a-3p vs. NC) = 0.0233, P(MUT2 miR-328a-3p vs. NC) = 0.0063, P(MUT1+2 miR-328a-3p vs. NC) = 0.6581, P(WT miR-150-5p vs. NC) < 0.0001, P(MUT1 miR-150-5p vs. NC) = 0.0303. Representative immunohistochemical staining (f) and quantitation of the expression (g) of FOXO4 and CBL in rat PTD sites on day 7 after surgery (n = 5; Student’s two-sided unpaired t test). P(FoxO4 PTD-TBI vs. PTD) = 0.0073, P(CBL PTD-TBI vs. PTD) = 0.0011. Scale bars, 40 μm. Western blot analysis of the change in FOXO4 (h) and CBL (i) protein in MC3T3-E1 cells after transfection with NC, miR-328a-3p mimics, miR-150-5p mimics (50 nm), inhibitor-NC, inhibitor-miR-328a-3p, inhibitor-miR-150-5p (100 nm) for 72 h (n = 3; one-way analysis of variance with Turkey’s multiple comparisons test was performed). P(mimic-NC vs. mimic-328a-3p) < 0.0001, P(inhibitor-NC vs. inhibitor-328a-3p) = 0.9988, P(mimic-NC vs. mimics-150-3p) = 0.0007, P(inhibitor-NC vs. inhibitor-150-3p) = 0.8309. j, k The MC3T3-E1 cells were transfected with NC, miRNA mimic, siRNA against FOXO4/CBL, co-transfected with miRNA mimics and FOXO4/CBL or vector plasmid. ALP staining and western blotting were performed to analyze RUNX2, FOXO4, and CBL expression in MC3T3-E1 cells after transfection. GAPDH served as loading control (n = 3; one-way analysis of variance with Turkey’s multiple comparisons test was performed). P(WT NC vs. WT mimic) = 0.0002, P(WT NC vs. WT siRNA) = 0.0001, P(WT NC vs. FOXO4 siRNA) = 0.0005, P(WT+miR-328a-3p vs. WT+miR-328a-3p) < 0.0001, P(WT NC vs. FOXO4+miR-328a-3p) < 0.0001, P(CTRL NC vs. CTRL mimic) = 0.007, P(CTRL NC vs. CTRL siRNA) = 0.001, P(CTRL NC vs. CBL siRNA) = 0.0016, P(CTRL+miR-150-5p vs. CTRL+miR-150-5p) < 0.0001, P(CTRL NC vs. CTRL+miR-150-5p) < 0.0003. The quantitation result were plotted as dot plots, showing the mean ± SEM of three independent experiments. P < 0.05,” “P < 0.01,” “P < 0.001,” “P < 0.0001; ns not significant.

Hydrogels carrying miR-328a-3p-containing sEVs efficiently promote bone formation, and repair bone defects. To identify the therapeutic potential of osteogenic sEV and miRNAs released from the damaged brain to enable bone healing, miR-328a-3p/negative control mimics were electrotransferred into sEVs and embedded into the hydrogel. To load the sEVs with miR-328a-3p/negative control mimics, 100 μg of purified sEVs and nanoparticle-miRNA complexes (final concentration of 100 nM for miR-328a-3p/negative control mimics) were gently mixed in 100 μL of phosphate-buffered saline (PBS) at 4 °C. After electroporation at 200 V for 5 ms in electroporation cuvettes, the mixture was incubated at 37 °C for 30 min. We found that hydrogel carrying sEVs containing miR-328a-3p efficiently promoted osteoblast differentiation and mineralization in vitro, as manifested by a marked increase in ALP and Alizarin red staining in a hydrogel-embedded MC3T3-E1 3D model (Fig. 7a–d).

The hydrogel carrying sEV miR-328a-3p was then applied to the rat PTD model. Bone healing was detected at day 7 and day 14 post-surgery. The results showed that BV/TV%, Tb.N, OCN and FOXO4 protein levels were enhanced by sEV miR-328a-3p (Fig. 7e–g and Supplementary Fig. 15b–e). PTD treated with sEV mir-328a-3p hydrogel was replaced with newly formed bone, 14 days post-surgery, which was much faster than that of controls (Fig. 7e, h). No significant change in the number of osteoclasts TRAP-positive cells (Supplementary Fig. 15f, g) and osteoclast surface per bone surface (Supplementary Fig. 15h) was observed. These results suggested that sEV miR-328a-3p efficiently promoted bone healing in rats.

To determine the effects of hydrogel carrying sEV miR-328a-3p on the repair of calvarial defects, one of the most challenging orthopedic problems, a critical-sized rat calvarial defect model was generated and treated with the hydrogel. sEV release kinetics from hydrogel was showed (Supplementary Fig. 16a). Micro-CT analysis showed that the size of the remaining defect in rats treated with sEV-miR-328a-3p-embedded hydrogel was much smaller than that of controls, 1 (Supplementary Fig. 16b) and 3 (Fig. 7i) month post-surgery, and the BV/TV% and Tb.N increased up significantly (Fig. 7j, k and Supplementary Fig. 16c, d). HE staining showed that the calvarial defect was almost repaired by the hydrogel at 3 months post-surgery (Fig. 7l).

Taken together, these findings indicated that sEV miR-328a-3p containing hydrogel had potent potential to repair bone defects, without the involvement of any additional exogenous factors or cells.

Discussion

Although it has long been known in clinical medicine that TBI accelerates bone healing, the underlying mechanisms remain largely unknown. The present work showed that after TBI, injured neurons, especially those in the hippocampus, released osteogenic miRNAs-enriching sEVs, which are transferred to bone through the circulatory system, and target osteoprogenitors to promote osteogenesis and accelerate bone healing, suggesting a paradigm of brain-to-bone interaction during bone homeostasis. We found that miR-328a-3p and miR-150-5p enriched in plasma sEVs after TBI were strong osteogenic miRNAs, and hydrogel carrying miR-328a-3p-containing sEVs had potent potential to repair bone and treat delayed union and nonunion fractures. Moreover, we found that increased FN1 expression on sEVs surfaces contributed to the direction of TBI sEVs to the bone. Hence, it is plausible that modification of sEVs surface FN1 could be used in bone-targeted drug delivery, providing an avenue for bone-targeted therapy. Taken together, our findings have established a clear link between the damaged brain and bone formation both in animals and clinical settings (Fig. 8).

Clinical management of the bone fracture is oriented to attain bone healing with the best possible functional recovery in the shortest possible time frame. However, in about 10% of cases, fracture healing can go awry in the form of delayed or nonunion fractures, which has a significant effect on the quality of life of patients. There are currently no approved pharmacological...
agents for the treatment of established bone nonunion, or for the acceleration of fracture healing.

A better understanding of molecular mechanisms underpinning fracture healing and seeking innovative strategies that efficiently accelerate bone regeneration is essential for the treatment of delayed bone healing or bone non-union. The process of fracture healing is a complex process that requires many kinds of cells and factors. We studied bone repair with a monocortical defect model which has been used in many previous research.

Although this repair model recapitulates most steps of fracture healing, however, the majority of clinical fractures heal through secondary bone formation (endochondral ossification). Further studies using stabilized fracture model should be performed to verify its potential application. This study identified osteogenic miRNA-containing sEVs, which strongly accelerated bone healing, representing a potential strategy for the treatment of delayed or nonunion fractures.

Bone homeostasis relies on the transfer of active molecules between cells, and compelling evidence has emerged to show the...
regulatory activities of sEVs in bone remodeling by transfer-specific sEV miRNA and proteins.30,31 sEVs with their cargo miRNAs are key components of the fracture repair process, which is just beginning to be understood. During fracture healing, BMSC-derived sEVs contribute to bone remodeling in a mouse model.32 However, the network of regulatory activities of sEVs in bone homeostasis and their therapeutic potential in bone injury remain largely unknown. Furthermore, published reports have shown that sEVs are exchanged mainly among osteoblasts, osteoclasts, and osteocytes and their precursors in bone remodeling; the role of sEVs released by distant cells, such as neurons in this process is unclear.33–37 Here we demonstrated that miR-328a-3p and miR-150-5p in plasma sEVs secreted by damaged neurons promote osteogenesis and bone repair by targeting osteoprogenitors. Interestingly, previous studies have found that patients’ serum miR-328-3p level increased significantly in response to fracture,38 and upregulated miR-328-3p level was also detected in serum and cerebrospinal fluid samples of patients with TBI39, supporting our findings that miR-328a-3p is an osteogenic miRNA related to both bone fracture and TBI. FoxO4 is ubiquitously expressed in mammalian tissues, including bone.40 Mice with deletion of FoxO4 in osteoblast progenitors have high bone mass caused by increased osteoblast number and bone formation.41 The anti-osteogenic actions of FoxO4 result from the binding of FoxO4 to β-catenin and the prevention of the association between β-catenin and TCF/Lef transcription factors and, thereby Wnt/β-catenin transcriptional activity.41 Recent studies indicate that E3 ubiquitin ligases Cbl proteins regulate osteoprogenitor cell proliferation, differentiation, and survival through ubiquitination and degradation of receptor tyrosine kinases and other molecules.42,43 Our findings identified that FOXO4 and CBL are direct and functional target genes of miR-328a-3p and miR-150-5p, respectively, emphasizing the crucial role of these miRNAs in bone formation. Together, these observations are compelling, as circulating, miRNAs are not only biomarkers but also functional constituents in the intricate crosstalk between the injured bone and the remote bone healing processes.

Previous studies have found that extracellular matrix protein FN plays an important role in the targeting of sEVs.44,45 One of the unique features of FN is its ability to bind a large number of cell adhesion receptors, growth factors, and extracellular matrix proteins. Liver endothelial cells release SKI-containing sEVs, which engage with hepatic stellate cells via FN-integrin-dependent adhesion.45 Microvesicles released by human cancer cells can be taken up by fibroblasts in a FN-dependent manner.44 All these studies, however, were carried out in vitro, and, therefore, which tissues or organs the sEVs with FN will eventually reach in vivo is unknown. In this study, we found that following TBI, circulating sEVs and their cargo miRNAs were transferred selectively to peripheral organs and preferentially to the bone. Furthermore, the amount of FN1 on sEVs increased after TBI, which contributed to their osteogenic function. These could be, thus, used in bone-targeted drug delivery, providing an avenue for bone-targeted therapy.

Methods

Patients. From January 2016 to January 2019, 24 patients were admitted to the Third Affiliated Hospital of Southern Medical University. All patients were fully informed of the study, and they provided written consent prior to participation. Among the 24 patients, there were 12 patients with simple shaft fractures and 12 patients with shaft fractures and concomitant TBIs who were assigned to three groups (week 2, week 4, and week 6, four patients per group). On admission, patient inclusion criteria were severe cranial trauma with a Glasgow Coma Scale score ≤ 8 and head abbreviated injury score > 2. Exclusion criteria included all forms of prior neurological pathology or bone-related diseases, immunosuppression, rheumatoid arthritis, and diabetes, as well as steroid or bisphosphonate therapy. Plasma sEVs were collected from TBI patients (10 males) and healthy control (10 males) between the ages of 17 and 72 admitted to the Third Affiliated Hospital of Southern Medical University. Venous blood samples were collected from all patients immediately after hospital admission (always within 8 h after trauma). Healthy matched individuals were selected among donated plasma samples as control. They were trauma-free and matched for age and sex status. No subjects had histories of neurological disease, psychiatric illness, head injury, stroke, nor learning disabilities. We confirm that our study is compliant with the “Guidance of the Ministry of Science and Technology (MOST) for the Review and Approval of Human Genetic Resources”. Ethics approval was granted by the Human Research Ethics Committee of the Third Affiliated Hospital of Southern Medical University, and all patients or next of kin gave informed consent prior to the study. The study was conducted in accordance with the principles and guidelines of The Declaration of Helsinki.

All patients sustained an isolated shaft fracture of the femur. The long bone fractures were imaged by conventional radiographs in anteroposterior and lateral projections. The fractures in all patients were treated. All femoral fractures were stabilized with an intramedullary nail with the use of a reamed technique. Two groups were not significantly different in terms of the time interval of surgical treatment for the bone fracture.

Rat models. All animal experimental protocols were approved by the Animal Care and Use Committee of the Southern Medical University, Guangzhou, China (SYXX (Guangdong) 2016-0167). None of the authors are members of this committee. The care of animals was in accordance with the guidelines of the US National Institutes of Health (NIH) and the Chinese National Institute of Health. Male SD rats (body weights, 220–250 g) were housed individually under controlled environmental conditions with a 12 h light/dark cycle and were given unrestricted access to pellet food and water throughout the study. The animals were purchased from the Experimental Animal Center of the Southern Medical University in Guangzhou, China. All surgical interventions were performed under anesthesia with a mixture of 13.3% urethane and 0.5% chloralose (0.65 mL/100 g body weight, ip), using a standardized protocol established in our laboratory. All efforts were made to reduce the number of animals used to maintain animal discomfort.

Cell lines. The BMSCs were harvested from the femora and tibiae of SD rats. Femurs and tibiae were aseptically removed, and bone marrow was flushed with PBS using a sterile syringe. Bone marrow samples were centrifuged for 5 min at
1000 × g and then placed in minimum essential medium alpha (α-MEM; Gibco, Gaithersburg, MD, USA) and replenished with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin and streptomycin (Thermo), then incubated at 37 °C in an incubator containing 5% CO2 for 48 h. Subsequently, the nonadherent cells were discarded, and the adherent cells were allowed to grow to 80% confluence. These cells were defined as passage one cells (P1). P3 cells were used for all experiments. The MC3T3-E1 cells were purchased from American Type Culture Collection and grown in α-MEM (Invitrogen, Waltham, MA, USA) containing 10% FBS and 1% penicillin and streptomycin. For induction of osteoblastic differentiation, MC3T3-E1 cells were incubated in an osteogenic medium (α-MEM, 10% FBS, 10 mM β-glycerophosphate, and 50 µg/mL ascorbic acid) and treated with 1 µM dexamethasone (DEX) (Sigma-Aldrich, St. Louis, MO, USA) or vehicle control. The HT22 cells were purchased from American Type Culture Collection and were cultured in DMEM (Invitrogen, Waltham, MA, USA) with 10% FBS. The cell lines were not authenticated after purchase but routinely tested negative for mycoplasma contamination.
Oxygen-glucose deprivation (OGD). Primary cultured hippocampal neurons and mouse neuron HT22 cells were subjected to OGD in vitro to simulate ischemia-like conditions. Briefly, the culture medium was replaced by glucose-free EBSS, and then cells were placed into an oxygen deprived (94% N2, 1% O2, and 5% CO2) incubator for 2 h at 37 °C. The EBSS was then replaced with a regular medium and placed back into an incubator at normal conditions to undergo reperfusion for 24 h.

Lateral fluid-perfusion (LFP) brain injury and GW4869 administration. LFP was performed as previously described. Briefly, anesthetized rats were placed in a stereotaxic frame. After incision of the scalp, the temporal muscles were reflected, and a burr hole was drilled (2.5 mm lateral to the sagittal sinus and centered between the bregma and lambda), keeping the dura mater intact. A hollow diamond saw (Leica SP1600) was used to create a 5 mm full-thickness calvarial defect, 2 mm in diameter, was created in the anteromedial surface of the proximal metaphysis using a burr drill. The cortical bone surface. A circular unicortical defect, 2 mm in diameter, was created overlying muscle and skin were closed in layers. A 5 mm full-thickness calvarial defect was created in male SD rats on the right side of the parietal bone with a trephine drill. Each defect was syringed with sterile saline solution to remove bone debris and then implanted with methacrylated glycol chitosan (MeGC) hydrogels containing various nanoparticles (200 µg/mL) or left empty. The hydrogels were polymerized with sodium at week 4, the defected tibia was harvested. Then, the tibia was embedded in polymethylmethacrylate, and undecalciﬁed sections were acquired using a Leica diamond saw (Leica SP1600). A confocal laser scanning microscope (Leica CLSM) was used to observe calcin-labeled bone.

Cell counting kit-8 assay. Cell Counting Kit-8 (CCK-8) was used to determine cell viability. Totally 4 × 103 cells were seeded in 96-well plates and allowed to adhere overnight. After incubation with compounds under normal conditions, 20 µl of CCK-8 solution were added to each well, and cells were incubated for 1 h at 37 °C. Subsequently, the absorbance was determined at 450 nm. The cell viability was calculated by Eq

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\text{Cell viability} = \left( \frac{A_{600}}{A_{0}} \right) \times 100 \%
\]

where \(A_600\) is the absorbance of the well-containing cell, control medium, CCK-8 solution, and sample; \(A_0\) is the absorbance of the well-containing cell, culture medium, and CCK-8 solution; \(A_{600}\) is the absorbance of well without cell, culture medium, CCK-8 solution, and sample. The results were assayed in triplicate experiments.

Primary culture of rat hippocampal neurons. SD rats (purchased from Southern Medical University (Guangzhou, China)) younger than 12 h old were sterilized with 75% ethanol, and the hippocampus tissues were separated under aseptic conditions after they were anesthetized by intraperitoneal injection with 10% chloral hydrate (dose: 5 ml/kg). The study was approved by the Animal Care and Use Committee of the Southern Medical University (Guangzhou, China). Briefly, the hippocampus was removed from the brain under sterile conditions and minced into 0.2–0.5 cm size. Then the tissue was digested by 0.125% (w/v) trypsin (Invi-

Plasma samples. Twenty-four-hour post TBI surgery, all rats were anesthetized and peripheral blood was collected in a tube containing EDTA as an anticoagulant. The samples were stored at 4 °C for a short time and immediately centrifuged at 4 °C and 3000 rpm for 10 min. The supernatant was carefully collected and transferred to a new tube without disturbing the intermediate buffy coat layer. In total, 20 samples from the TBI group and 10 from the sham group were used to isolate plasma. All samples were analyzed in triplicate under all conditions.

sEV labeling and cellular uptake. sEVs were labeled with PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) by following the manufacturer’s protocol.
The isolated sEVs diluted in PBS was added to 0.5 mL of Diluent C. Two µL of PKH67 dye was added and incubated for 4 min at room temperature. Two mL of 1% BSA/PBS was added to bind excess dye. The synthetic peptide, GRGDNP (300 µg/mL) was incubated with labeled sEVs (50 µg/mL) for 30 min. The labeled sEVs were washed at 100,000 × g for 70 min, and the sEV pellet was suspended in PBS and used for uptake experiments. We then cocultured these PKH67 sEVs with BMSC and MC3T3-E1. After the indicated time of co-culture, we stained BMSC, MC3T3-E1 with DAPI (Sigma-Aldrich) and observed them with confocal microscopy.

RNA isolation. RNA was extracted from cells using the QIAzol Lysis Reagent (QIagen, Hilden, Germany) according to the user guidelines. Briefly, the cells were collected in a reaction tube, lysed with QIAzol, and mixed with chloroform. After being centrifuged at 12,000 × g for 15 min at 4 °C, the upper aqueous phase was transferred to an RNeasy Mini spin column in a 2 mL collection tube and mixed with 100% ethanol. After being washed at 7500 × g for 5 min at 4 °C, the total RNA was collected for qRT-PCR analysis. RNA was extracted from plasma and sEVs.
Fig. 7 Hydrogel carrying miR-328a-3p-containing sEVs efficiently promotes bone formation and repairs bone defects. a Schematic representation of sEV loading and treatment model. Purified sEVs were loaded with miR-328a-3p through electroporation and encapsulated into hydrogels. Then, the hydrogels were injected into bone defect sites for treatment. b Alizarin Red staining of MC3T3-E1 in hydrogel culture with blank (no cell group), cell (MC3T3-E1 cell group), Lipo + cell NC (transfected with negative control in MC3T3-E1 cell group by liposome), Lipo + cell + miR-328a-3p (transfected with miR-328a-3p mimics in MC3T3-E1 cell group by liposome), sEV + cell NC (electroporated with negative control in MC3T3-E1 cell group by sEVs), sEV + cell + miR-328a-3p (electroporated with miR-328a-3p mimics in MC3T3-E1 cell group by sEVs) were carried out on day 28 for mineralization analysis and ALP staining on day 7. The OD values of ALP activity (c) and mineralization (d) were evaluated for quantitation (n = 3; one-way analysis with Turkey’s multiple comparisons test was performed). P(AlP Lipo+NC vs. Lipo+miR-328a-3p) = 0.0023, P(AlP Control vs. Lipo+miR-328a-3p) = 0.0003, P(AlP sEV+NC vs. sEV+miR-328a-3p) = 0.015, P(Alizarin Red sEV+NC vs. sEV+miR-328a-3p) < 0.0001, P(Alizarin Red sEV+NC vs. sEV+miR-328a-3p) = 0.0001, P(Alizarin Red Control vs. sEV+miR-328a-3p) < 0.0001. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. 8 Scheme of the mode of damaged brain accelerates bone healing by releasing sEVs that target osteoprogenitors. After TBI, the injured neurons released osteogenic miRNA-enriching sEVs, which were transferred to bone through the circulatory system, and target osteoprogenitors to promote osteogenesis and accelerate bone healing. The increased FN1 expression on sEVs surface contributed to direct sEVs to target osteoprogenitors. miR-328a-3p and miR-150-5p enriched in plasma sEVs after TBI were strong osteogenic miRNAs, which had potent potential to repair bone by directly targeting FOXO4 and CBL 3′-UTR, respectively.
fractions using Qiagen miNeasy Mini kit (Qiagen), according to the manufacturer’s instructions, with a final elution volume of 50 μL.

**RT-PCR and qRT-PCR.** RNA samples were quantitated and qualified using NanoDrop analysis (ThermoFisher Scientific). Equal quantities (5 μg) of total RNAs from each sample were used for cDNA synthesis using the PrimeScript® RT reagent kit (TaKaRa, Tokyo, Japan). The reverse transcriptions of miRNAs were performed by looped miRNA-specific RT primers for miRNAs. qRT-PCR was performed using an Applied Biosystem StepOnePlus, using a SYBR Green 1 Real-Time PCR Master Mix (ThermoFisher Scientific) at 150-3p and miR-328a-3p. Dissociation curves were generated to ensure the specificity of each qRT-PCR reaction. The relative expression levels of miRNAs in each sample were calculated and quantified using the 2−ΔΔCt method after normalization for expression of the positive control.

**TEM and nanosight tracking analysis.** The morphology of the sEVs was observed by TEM. Briefly, the sEVs suspension was mixed with an equal amount of 4% paraformaldehyde. After washing with PBS, 4% uranyl acetate was added for chemical staining of sEVs, and images were captured using a Hitachi H-7650 TEM (Hitachi, Tokyo, Japan). Nanosight tracking analysis (NTA) was performed to measure the size and the concentration of the isolated sEVs using NanoSight NS500 (Malvern, Westborough, MA, USA), according to the operating instructions, without any changes. The sEVs were diluted to be within the recommended concentration range. 60 s videos were captured for each sample during flow mode (camera settings: slider shutter 890, slider gain 146). Plasma-derived sEVs were diluted and loaded onto the NS500 instrument by a syringe at a constant flow of 20. The videos were analyzed with the NTA 3.2 software (Malvern). All measurements were performed at room temperature.

**miRNA sequencing.** Total RNA extraction of the samples was conducted using the miNeasy Serum/Plasma Kit (Qiagen), according to standard operating procedures provided by the manufacturer. The quality and integrity of the total RNA were examined using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip Kit provided by the manufacturer. The quality and integrity of the total RNA were measured using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip Kit provided by Illumina that performed real-time data analyses. The RAW data files were analyzed using Proteome Discoverer (Thermo Fisher Scientific, Version 2.4) against Uniprot Rattus norvegicus database (version 20200617; 35,779 sequences). The MS/MS search criteria were as follows: mass tolerance of 20 ppm for MS and 0.02 Da for MS/MS Tolerance, trypsin as the enzyme with two missed cleavage sites, carbamidomethylation of cysteine, and the TMT of N-terminus and lysine fixed modifications. The thresholds of fold changes (>1.2 or <0.83) and low intensity cations were ignored, and high abundance cations by X-correlation were generated. The MS/MS search was performed using the single-random program. The sequencing process was controlled by the data collection software provided by Illumina that performed real-time data analyses.

**LC-MS/MS analysis.** The labeled peptides were dissolved in solvent A (2% ACN with 0.1% formic acid), and then centrifuged at 4°C, 12,000 × g for 20 min. After centrifugation at 12,000 × g for 30 min at 4°C, the protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific).

Proteins were digested as follows. A hundred microliter of protein sample was taken and the volume was replenished to 90 μl with lysate. Tris(2-carboxyethyl) phosphonium (TCEP) (final concentration of 10 μM) was added and the mixture was incubated at 37°C for 60 min. Then, 40 μM iodoacetamide was added to the mixture, and the mixture was incubated at room temperature in the dark for 40 min. Prechilled aceton was added and incubated for 4 h at −20°C before centrifugation at 10000 × g for 20 min. All collected proteins were transferred to a new tube, followed by trypsin digestion with a substrate ratio of 1:50 (w/w) at 37°C overnight according to the manufacturer’s protocols. The digested peptides were labeled with a TMT Reagent kit according to the manufacturer’s protocol (Thermo Fisher Scientific). After tagging for 2 h at room temperature, hydroxyamine was added for 15 min. Finally, all samples were pooled, desalted, and vacuum-dried.

**Reversed-phase chromatography with high separation.** The TMT-tagged peptides were mixed and reconstituted in UPLC loading buffer, followed by loading on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 150 mm; Waters, Milford, MA, USA). The peptides were eluted at a flow rate of 200 μl/min with a linear gradient of 0–5% solvent B (80% acetonitrile (ACN) 20 mM NH₄HCO₃, pH 10) for 2 min; 5% solvent B for 15 min, 5–30% solvent B for 8 min; 30–36% solvent B for 3 min; 36–42% solvent B for 1 min; 42–100% solvent B for 1 min and hold for 8 min (total of 20 fractions were collected based on the LC gradient time) which were combined into 10 fractions per sample. Each of them was dried by vacuum centrifugation.

**Coculture experiments.** The well inserts with a 0.4 mm pore size filter (BD Falcon, Corning, NY, USA) for 6-well plates were used following the manufacturer’s instructions. Primary hippocampal neurons were seeded into the well inserts with DMEM/F12 medium or after OGD treatment. BMSC and MC3T3-E1 cells were seeded into 6-well plates and induced into osteoblasts using complete α-MEM. After differentiation, osteoblasts were washed with PBS, and then co-cultured for 7 d according to the experimental protocol. All co-culture experiments were conducted in α-MEM with sEV-free FBS (Life Technologies, Carlsbad, CA, USA).

**Luciferase assays.** MC3T3-E1 and BMSC cells were cultured at 1 × 10⁵ cells/well in 12-well plates. The cells were co-transfected with miR-328a-3p and miR-150-5p mimic (50 nM) or miRNA mimic negative control (50 nM) and 0.2 μg of psiCHECK−1-UTR. Transfection was performed using Lipofectamine 3000. After 48 h, cells were collected and luciferase activity was determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) with the dual luciferase assay reporter-ready luminometer (Promega). The assays were performed in triplicate.

In **situ hybridization.** In situ hybridization was performed to determine the expression of miR-328a-3p using miRCURY LNA miRNA Detection, Optimization Kit 2.0 (miR-328a-3p) (Exiqon, Vedbæk, Denmark), according to the manufacturer’s protocol. Sections were serially cut to 4 μm thicknesses, and after deparaffinization of coronal sections of rat brain from the TBI and sham group, the tissues were subjected to pepsin (1.3 mg/mL, Sigma-Aldrich) for 30 min. After washing in PBS, the slides were submerged in 99.7% ethanol and air-dried. Hybridization with miR-328a-3p LNA was performed in a hybridization chamber at 37°C for 16–18 h with stringent washes with saline-sodium citrate buffer at 37°C. For immunode-tection, sections were blocked with digoxigenin blocking reagent (Roche, Mannheim, Germany) in maleic acid buffer containing 2% sheep serum at ambient temperature for 30 min, and then incubated with sheep anti-mouse conjugated to ALP (diluted 1:800 in blocking reagent) at ambient temperature for 60 min, resulting in dark-blue staining. The NBT/BCIP reaction mixture1 was incubated within a humidified chamber at 37°C for 2 h and the reaction was stopped with KTB buffer, and then washed in PBS three times, dehydrated, and mounted for microscopy. Five random microscopic fields per section were evaluated at 20× magnification and the integral optical density of each visual field was calculated.

**Protein extraction, digestion, and TMT labeling.** The sEVs proteins of samples were suspended in lysis buffer (8 M urea, 1% SDS with a protease inhibitor cocktail) and treated by ultrasound at 40 kHz, 40 W for 2 min, incubated on ice for 60 min. After centrifugation at 12,000 × g for 30 min at 4°C, the protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific).

Proteins were digested as follows. A hundred microliter of protein sample was taken and the volume was replenished to 90 μl with lysate. Tris(2-carboxyethyl) phosphonium (TCEP) (final concentration of 10 μM) was added and the mixture was incubated at 37°C for 60 min. Then, 40 μM iodoacetamide was added to the mixture, and the mixture was incubated at room temperature in the dark for 40 min. Prechilled aceton was added and incubated for 4 h at −20°C before centrifugation at 10000 × g for 20 min. All collected proteins were transferred to a new tube, followed by trypsin digestion with a substrate ratio of 1:50 (w/w) at 37°C overnight according to the manufacturer’s protocols. The digested peptides were labeled with a TMT Reagent kit according to the manufacturer’s protocol (Thermo Fisher Scientific). After tagging for 2 h at room temperature, hydroxyamine was added for 15 min. Finally, all samples were pooled, desalted, and vacuum-dried.

**Database search and quantitative proteomics analysis.** The RAW data files were analyzed using Proteome Discoverer (Thermo Fisher Scientific, Version 2.4) against Uniprot Rattus norvegicus database (version 20200617; 35,779 sequences). The MS/MS search criteria were as follows: mass tolerance of 20 ppm for MS and 0.02 Da for MS/MS Tolerance, trypsin as the enzyme with two missed cleavage sites, carbamidomethylation of cysteine, and the TMT of N-terminus and lysine side chains of peptides as fixed modification, and methionine oxidation as dynamic modifications. High confidence peptides were used for protein identifications by setting a target false discovery rate threshold of 1% at the peptide level. Identified proteins that had at least one unique peptide were used for protein identifications. The thresholds of fold changes (>1.2 or <0.83) and P-value < 0.05 were used to identify differentially expressed proteins. A total of 273 expressed proteins were
identified as belonging to the proteome of Solanum tuberosum. Then we found 36 upregulated and 68 downregulated proteins in group TBI compared with the sham group.

Micro-CT scanning. After the animals were euthanized, the tibiae of the animals in the experimental groups were fixed in 4% paraformaldehyde and analyzed at a resolution of 12 µm on a micro-CT Scanner (Viva CT40; Scanco Medical AG, Bassersdorf, Switzerland). The area used for analysis began at the defect site close to the femoral head and extended through the whole defect area proximally for 500 slices. Using two-dimensional data from scanned slices, three-dimensional structure and morphology were conducted to calculate morphometric parameters defining microarchitecture, including bone volume/tissue volume (BV/TV) and trabecular number (TB.N 1/ mm). 3D images of the ROI were created using Skyscan CTvol software (Bruker microCT, Kontich, Belgium).

Electroporation. To load plasma sEVs with miR-328a-3p, a total protein concentration of purified sEVs (50 µg) and miR-328a-3p mimic (400 nanomoles) were gently mixed in 400 µL PBS at 4 °C. The mixtures were transferred into ice-cold electroporation cuvettes and electroporated at 200 V for 5 ms, and then the mixture was incubated at 37 °C for 30 min. Non-electroporated samples were used as negative controls. All electroporations procedures were performed on a NEPA21 Type II Electroporator (NEPA GENE, Chiba, Japan).

 Nanoscale flow cytometry analysis of sEVs. Samples were analyzed using an A 50 Micro-PLUS flow cytometer (Apogee Flow Systems, Hertfordshire, UK). The sample flow rate was 1.5 µL/min and the time of acquisition was 60 s for all measurements. An illumination wavelength of 405 nm (70 mW) was used to detect scattered light by microparticles. Before sample analysis, calibration of flow cytometer was performed using a reference head mix (ApogeeMix, Apogee Flow Systems), composed of a mixture of silica nanoparticles with diameters of 110, 180, 240, 300, 590, 880, and 1300 nm with a refractive index (RI) of 1.42, and 110 and 500 nm green fluorescent (excited by blue laser) polystyrene nanoparticles with an RI of 1.59 (latex) were used. The upper limit of the microvesicles gate was determined using size-calibrated green fluorescent silica beads of size 200 nm, and then applied to all samples. In order to exclude excessive background noise, a combined threshold on light scatter and green fluorescence was set above the background using PBS and an unlabeled sEV sample, respectively. Briefly, 50 µg of sEVs were incubated at 37 °C for 30 min with PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich), in the presence or absence of 10 µg/mL of PE-labeled FN1 (Novus Biologicals, Littleton, CO, USA). Titration of all detecting antibodies was performed using size-calibrated green fluorescence, using PBS and an un-labeled sEV sample, respectively. Briefer, we used HRP-labeled secondary antibodies (1:200 in 1% bovine serum albumin [BSA], 1 h) at 37 °C. Anti-rabbit IgG (cat#7074, dilution 1:200) from Cell Signaling Technology (MA, USA), anti-mouse IgG (cat# A9044, dilution 1:200) from Sigma Aldrich (MO, USA). DAB was used as chromogen; hematoxylin was used as counterstain. Brains were fixed in situ by intra-aortic perfusion with 350 mL of 4% paraformaldehyde in PBS, and post-fixed in the same fixative overnight at 4 °C. Vibration coronal brain sections, 50 μm in thickness, were stored at −80 °C in the Optimal Cutting Temperature Compound. The free-floating section procedure was used.

For immunofluorescence staining, we incubated primary antibodies that recognized CN1 (Abcam, Cambridge, UK, #ab13420, 1:200), FoxO4 (Proteintech, Wuhan, China, #A21535-1-AP, 1:100), and βIII (Abclonal, Wuhan, China, #A7861, 1:100) overnight at 4 °C. Under high power magnification, the new matrix formation was measured and compared among all samples and the control.

Histological evaluation. The fixed tissues were decalcified using 10% EDTA solution with gentle shaking for 1 month. The EDTA solution was changed every day. Decalcified samples were embedded in paraffin and cut into sections of 5 μm thickness. The defect area around the grafted bone was measured and the percentage of the bone matrix within the callus was calculated by Image Pro Plus 6.0 software (Media Cybernetics, MD, USA) and compared with the control group. Under high power magnification, the new matrix formation was measured and compared among all samples and the control.

Immunofluorescence staining and immunohistochemistry. The injured-side cortices of the tibia from rats were harvested at different times and were fixed using 4% paraformaldehyde at 4 °C for 48 h and decalcified in 0.5 M EDTA, pH 7.4, on a shaker for 1 month. The bone tissues were embedded in paraffin and 2-μm sagittal sections were prepared for histological analyses. After deparaffinization and rehydration, sections were incubated in citrate buffer (10 mM citric acid, pH 6.0) for 30 min at 90 °C or treated with 200 mg/mL protease K (Sigma-Aldrich) for 10 min at 37 °C to unmask antigen. Sections for immunohistochemistry were treated with 3% hydrogen peroxide for 15 min. After that, the sections were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature and then blocked with 1% sheep serum at room temperature for 1 h. For immunohistochemistry, we incubated primary antibodies that recognized OCN (Abcam, Cambridge, UK, #ab13420, 1:200), FoxO4 (Proteintech, Wuhan, China, #A7861, 1:100) overnight at 4 °C. The sections were permeabilized with 0.1% Triton X-100 in PBS and incubated in 2% Alizarin Red S solution for 5 min. Then, the gels were washed with PBS with gentle shaking for 1 h, and the PBS was changed at least three times. The images were acquired using an Olympus SZX16 stereomicroscope (Tokyo, Japan).

IVC injection. For IVC injection of sEVs, rats were anesthetized with 2.5% isoflurane in a stereotaxic apparatus. A portion of the parietal skull was carefully removed, and a guide cannula was then inserted into the right lateral ventricle (coordinates from the bregma: −1.5 mm; medial/lateral, ±1.0 mm; dorsal/ventral, −3.2 mm) and fixed with dental cement. To verify the injected location, the guide cannula was connected to a polyethylene (PE) pipe that was filled with an intraventricular fluid. To visualize the PE pipe was vertically inserted into the right lateral ventricle through the guide cannula. When the liquid level in the PE pipe was dropped down, it implied that the PE pipe was successfully inserted into the lateral ventricle. Then, after the rat recovered from surgery, 10 µL of either PKH-67-labeled sEVs (200 µg/mL) or PBS (0.01 M) was micro-infused into the lateral ventricle using a 33-gauge infusion cannula connected to a microsyringe (Gaoge, Shanghai). The sEVs were injected with the use of an electric micropipet injector at a flow rate of 0.5 µL/min. The cannula remained in situ for at least 5 min after infusion and was then slowly withdrawn.

Fluorescence imaging analysis of organ distribution of sEVs. To facilitate tracking in vivo, we injected PKH67-labeled sEVs into SD rats. A total of 200 µg of sEVs was injected intravenously into rats. After the OGs were injected intravenously, the rats were killed at 24 h (IVC) and 8 h (intra-venous) after the injection and subjected to biophotonic imaging. Fluorescence imaging for PKH67-labeled sEVs in...
these organs was performed using the optical imaging system, Fx Pro (Bruker BioSpin MRI GmbH, Ettlingen, Germany). For anatomical orientation, a white light/grayscale picture was performed and used with the fluorescent signal (Cy3.5: excitation = 570 nm; emission = 620 nm). For data analysis, molecular imaging software 7.1.3 was used. Regions of interest (ROI) were defined around the bone defect parts and analyzed compared to the background of injection of PBS for calculation of the signal to noise ratio.

**Western blot analysis.** We lysed cells and sEV with 2% SDS, 2 M urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 10 mM dithiothreitol, and 1 mM phe-

**Statistical analysis.** All data were analyzed for statistical significance using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). The miRNA-seq Illumina reads for all samples data generated in this study have been deposited in the Sequence Read Archive at the National Center for Biotechnology Information database under accession code PRJNA670580. The mass spectrometry data (48 samples) was calculated using an A-50 Micro-PLUS and incubated at 37 °C in 1 mL PBS in a 12-well plate. At each time point, PBS was exchanged. To examine sEV release from hydrogels, PKH67-labeled sEVs were loaded in hydrogels at a concentration of 50 µg/hydrogel and incubated at 37 °C in 1 mL PBS in a 12-well plate. At each time point, PBS was exchanged.

**Data availability.** The miRNA-seq Illumina reads for all samples data generated in this study have been deposited into the Sequence Read Archive at the National Center for Biotechnology Information database under accession code PRJNA670580. The mass spectrometry proteomics data used in this study are available in the PRIDE database under accession code PXD002126. All other relevant data are available from the authors upon reasonable request. We use WPS 11.1 software to collect data. Source data are provided with this paper.

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**Author contributions**

Q.-C.S. and X.-C.B. conceptualized the research. W.X., Q.-C.S. and X.-C.B. designed the experiments; W.X. conducted the experiments. Z.-Q.C., J.X. and J.W. assisted in rat experiments. Z.-K.C assisted in 3D gel experiments. W.X., R.Z., X.-M.Z. and Z.-P.Z. performed cell experiments; J.-H.C. and X.-R.M. provided clinical samples. Z.-T.G., Z.-M.Z. and Mac Maegele contributed to developing the fluid percussion injury model. Funding Acquisition, W.X., Q.-C.S., X.-C.B., Q.-C.S. and X.-C.B. supervised the study, interpreted the data and wrote the manuscript. Q.-C.S. and X.-C.B. conceptualized the research. W.X., Q.-C.S. and X.-C.B. designed the experiments; W.X. conducted the experiments. J.X., Z.-Q.C., X.-H.L. and J.W. assisted in rat experiments. Z.-K.C. and Y.Z. assisted in 3D gel experiments. W.X., R.Z., X.-M.Z. and Z.-P.Z. performed cell experiments; J.-H.C. and X.-R.M. provided clinical samples. Z.-T.G., Z.-M.Z., M.Z. and Mac Maegele contributed to developing the fluid percussion injury model. Funding Acquisition, W.X., Q.-C.S., X.-C.B.; Q.-C.S. and X.-C.B. supervised the study, interpreted the data and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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