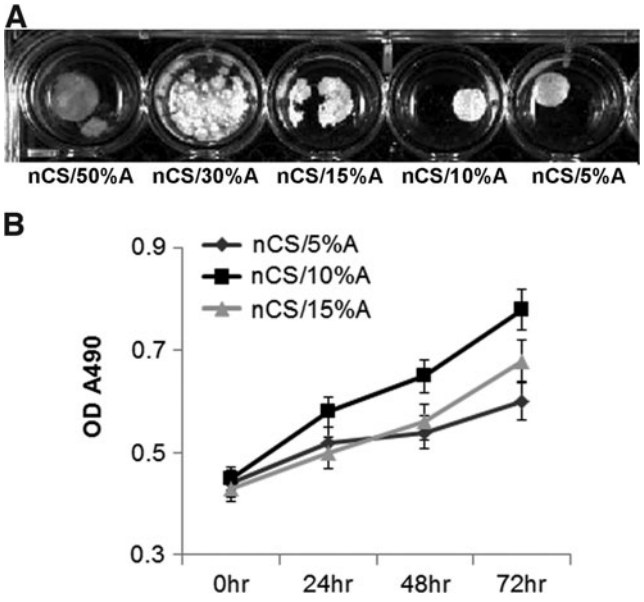
Supplementary Data

Supplementary Materials and Methods

Preparation of mesenchymal stem cells

Animal procedures are conducted in accordance with the protocol approved by IACUC of the University at Buffalo. Sprague–Dawley (SD) rats, 6–8 weeks old, were euthanized by CO_2. In a sterile hood, the rat femurs and tibias were dissected free from the surrounding soft tissue. Metaphysis from both ends were resected, and bone marrow cells were collected by flushing the diaphysis with phosphate-buffered saline (PBS) and separated by Histopaque-1083 (Sigma) density gradient centrifugation at 400 *g* for 20 min. The mesenchymal stem cells (MSC) expansion was performed as previously described.[1] Briefly, the light-density bone marrow-derived mononuclear cells (BMNCs) were collected, washed twice with Dulbecco's PBS supplemented with 2 mM ethylenediaminetetraacetic acid, and counted manually. Then, BMNCs were cultured in the basal media (Dulbecco's Modified Eagle Medium [DMEM] [Gibco], supplemented with 10% fetal bovine serum [FBS; Gibco], L-glutamine [2 mM], and penicillin [100 U/mL]). Non-adherent cells were removed with each medium change. After 5–7 days, the adherent cells were released using 1×TrypLE™ (Invitrogen) and reseeded onto tissue culture flasks for subsequent passages.



SUPPLEMENTARY FIG. S1. Properties of injectable nano calcium sulfate/alginate (nCS/A) pastes. **(A)** Dissolution behavior of nCS/A pastes after 48 h incubation in water. **(B)** Cell viability of mesenchymal stem cells (MSCs) in nCS/A pastes. MSCs were, respectively, mixed with nCS/5%A, nCS/10%A, and nCS/15%A as described in Materials and Methods section; then induced with osteogenic (OS) media for 0, 24, 48, and 72 h for MTS cell viability assay.

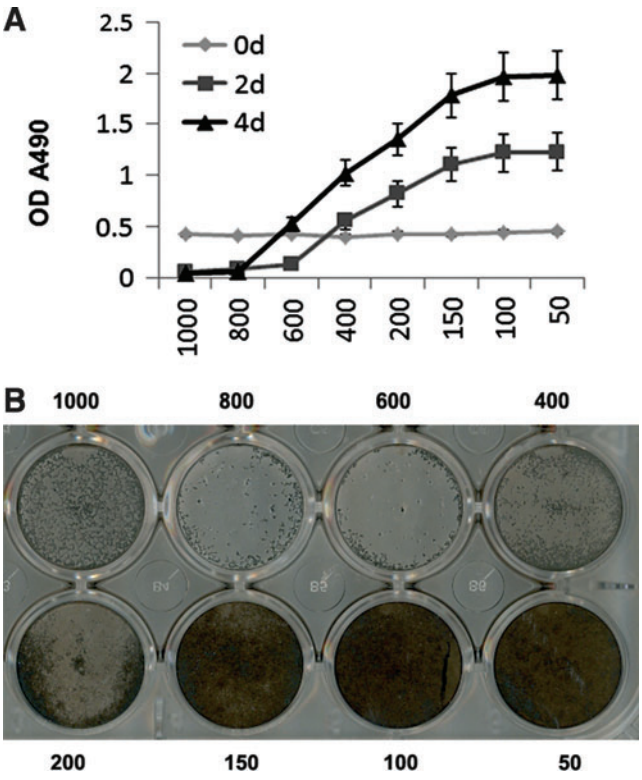
Characterization of MSCs

MSCs were characterized by fluorescence-activated cell sorting (FACS) analysis.[2] Briefly, MSCs were detached from the culture dish using 1×TrypLE at passage 3, and 1×10^6 cells were washed by 10% FBS/PBS and centrifuged for 10 min at 1000 rpm to gather the pellet. MSCs were then, respectively, stained with fluorescein isothiocyanate-conjugated rat anti-CD44, Cy5.5-conjugated rat anti-CD90, PE-conjugated rat anti-CD31, and Alexa Fluro 647-conjugated rat anti-CD34 antibodies at a concentration of 2 μg/mL at 4°C for 30 min. The cells stained with corresponding mouse immunoglobulin G served as negative controls. The excess and nonspecifically-bound antibodies were removed by multiple washes with PBS buffer containing 2% (v/v) serum. The labeled cells were analyzed using a BD FACS Calibur system (BD Bioscience) with 10,000 events being recorded for each condition. The results were analyzed by FACS Express software to create the histograms. For immunostaining, MSCs were plated in 24-well plates at 40,000 cells/well and stained with rat anti-CD44 and rat anti-CD90 antibodies as described in Yang *et al.*[3]

To test the multi-potency of the cells, P3 MSCs were seeded in the six-well plates of $2.5\times10^4/cm^2$. After 2 days, the growth medium was replaced with osteogenic (OS) medium (growth medium supplemented with 100 nM dexamethasone, 50 μg/mL ascorbate-2-phosphate and 10 mM β-glycerol phosphate), adipogenic medium (growth medium supplemented with 1 μM dexamethasone, 50 μg/L insulin, 0.5 mM methyl-isobutylxanthine, and 100 μM indomethacin), or the chondrocyte media (Promocell) comprising the basic media consisted of a 1:1 mixture of DMEM and Ham's F-12 growth medium supplemented with 5% FBS, 10 μg/mL human transferrin, 3×10^{-8} M sodium selenite, and 10 μg/mL human recombinant insulin. The medium was changed twice a week. After 18 days, MSCs Standard alizarin red, oil red O, and alcian blue stains were used to identify adipocyte-, chondrocyte-, and osteoblast-like cells, respectively.[1] Briefly, for osteogensis, the calcification was visualized by staining the cells with 2% Alizarin Red S solution for 5 min after the fixation with 10% buffered formalin (v/v) for 30 min. For adipogenesis, the culture cells were rinsed twice with PBS and fixed with 10% buffered formalin (v/v) for 10 min. The fixed cells were washed and stained with 0.2% Oil Red O-isopropanol for 15 min.[4] Excessive stains were removed thrice by distilled water. For chondrogenesis, the cells were washed twice with PBS, fixed with methanol at −20°C for 2 min, stained with 0.1% Alcian blue 8Gx (Sigma) in 0.1 N HCl overnight, and rinsed repeatedly with distilled water.[5] Photomicrographs were taken with an inverted microscope.

BMP2 gene expression

Bone morphogenetic protein 2 (BMP2) adenovirus was produced with an AdEasy system (Strategene). Full-length



SUPPLEMENTARY FIG. S2. Safety of Ad-BMP2 to the MSCs. MSCs were first infected with Ad-*BMP2* with a multiplicity of infection (MOI) of 1000, 800, 600, 400, 200, 150, 100, and 50; then induced with OS media for 0, 2, and 4 days for MTS analysis **(A)**; and for 18 days for cell mineralization analysis by using Von Kossa staining **(B)**. The cells infected with MOI 100 and 50 showed normal cell growth and stronger cell mineralization compared with other groups.

human *BMP2* cDNA (GenBank accession no. NM_001200.1) was inserted into AdTrack-CMV vector (Stratagene). The resultant plasmid was linearized and co-transformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid pAdEasy-1. The recombinants were selected for kanamycin resistance and confirmed by multiple restriction endonuclease analyses. Then, the linearized recombinant plasmid was transfected into 293A cells to package adenovirus (Ad-*BMP2*) according to the manufacturer's protocol. Viral titers were estimated by optical density and the standard plaque assay as described in Yang *et al.*[3]. These preparation methods obtained 1.8×10^{10} particles/mL. For the transduction of MSCs, Ad-LacZ (as a control) or Ad-*BMP2* adenovirus at the desired titer was added to cells in a serum-free medium. After 4 h, serum was added to a final concentration of 2%, and cells were grown for an additional 24 h. The cells were then transferred to an OS medium and fed every 2 days unless indicated otherwise. To determine the BMP2 level, the infected cells were induced with OS media for 0, 2, 4, 7, 14, and 21 days. Then, the medium was collected for the determination of BMP2 levels by the ELISA kit according to the manufacturer's protocols (R&D System). For alkaline phosphatase and Von Kossa assays, the infected cells were induced with OS media at the indicated time. For *in vivo* study, the infected cells were directly mixed with nano-calcium sulfate/alginate (nCS/A) for implantation.

Injectability testing

A 10-mL syringe with a barrel diameter of 10 mm and a needle opening of 2.8 mm was used. After mixing the nCS with 15%, 10%, or 5% alginate for 1 min, the as-prepared paste was poured into the syringe. The syringe was placed between the compression plates of a computer-controlled Universal Testing Machine (5500R; MTS Systems Corp.). 1.5 min after the commencement of mixing, the compression was started and the paste was extruded at a crosshead speed of 15 mm/min. This was continued until either the entire paste was extruded, or a maximum force of 300 N was reached (at which point the test was stopped).

Scanning electron microscopy

The samples were rinsed in 0.1 M phosphate buffer twice every 3 min and then placed in 0.1 M sodium cacodylate (Sigma) for 30 min. The samples were then dehydrated in ethanol (70% for 10 min, 95% for 10 min, and 100% for 20 min), treated with hexamethyldisilizane twice every 5 min, and air dried. The specimens were sputter coated with carbon and observed under a scanning electron microscope in a high-vacuum mode at 20 kV.

Osteoblast cell marker gene expression

MSCs were infected with or without Ad-*BMP2* for 24 h. Then, the cells were mixed with nCS/10%A paste and induced with OS media for the indicated times. Then, total RNAs were isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of total RNA was carried out for 50 min at 42°C and then 15 min at 70°C, using the SuperScriptTM first-strand synthesis system for reverse transcription–polymerase chain reaction (RT-PCR) (Invitrogen). Synthesized cDNA was used to perform real time-PCR reactions. PCR amplifications were performed using the specific primers for analyzing the expression of osteoblast marker genes: *osteocalcin* (*OCN*), *collagen Type I, Alpha 1* (*Col1α1*), *BMP2*, and *vascular endothelial growth factor* (*VEGF*).[3,6] The primers for different genes are as follows: *OCN* (forward primer, 5′-ATGAGGACCCTCTCT CTGCT-3′; reverse primer, 5′-GGAGC TGCTGTGACATCC AT-3′); *Col1α1* (forward primer, 5′-AAAGAAGCACGTCT GGTTTG-3′; reverse primer, 5′-GAGGGAGTTTACACGAA GCA-3′); *BMP2* (forward primer, 5′-GTTTGGCCTGAAG CAGAGAC-3′; reverse primer, 5′-TCTAAATGGGCCACTTC CAC-3′); *VEGF* (forward primer, 5′-AGGCTTCAGTGTG GTCT GAG-3′; reverse primer, 5′-TGATCACACAAGGT CCTCCT-3′.); and *GAPDH* (forward primer, 5′-ACCAC AGTCCATGCCATCAC-3′; reverse primer, 5′-TCCACCACC CTGTTGCTGTA-3′). Real-time PCR was performed on an ABI PRISM 7500 sequence detection system with SYBR GREEN PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The PCR conditions were 94°C for 1 min followed by 95°C for 30 s, then 58°C for 40 s with a total of 35 cycles. All the reactions were run in triplicate and were normalized to GAPDH. The relative differences in PCR results were calculated by using the comparative cycle threshold method.

Rat critical-sized calvarial bone defect model

Twenty-four male SD rats at 8 weeks old were used in this study. The *in vivo* experimental protocol was reviewed and approved by the University at Buffalo Animal Care and Use Committee. Rats were operated under general anesthesia, achieved with the 5% isoflurane/O_2 gas inspiration for induction and 1%–2% isoflurane/O_2 gas for maintenance by using a facial mask. Buprenorphine was administered as an analgesic presurgery. The scalps covering the calvarial vault were shaved and scrubbed with betadine solution and infiltrated with 0.1–0.5 mL of a local anesthetic agent of 2% lidocaine (20 mg/mL) with 1:100,000 epinephrine (0.01 mg/mL). An incision was made along the midline. Full-thickness skin and the periosteum were raised to expose the calvarial bone surface. An 8-mm-diameter trephine bur was used to drill a standardized, round, segmental defect around the sagittal suture. During drilling, the area was irrigated with saline solution, and the underlying dura mater was maintained intact. A single transplant of 1×10^6 cells mixed with nCS/10%A was injected into the defect. The periosteum (pericranium) and skin were closed in layers with nonabsorbable 4-0 prolene sutures, respectively. After surgery, the rats were treated with carprofen for 2 days to minimize pain or discomfort according to the protocol. Animals were divided into four groups: group 1, blank control; group 2, nCS/A; group 3, nCS/A + MSC (nCA/A + M); and group 4, nCS/A + *BMP2*-gene-modified MSC (nCS/A + M/B2). At the end of the 7-week period, the animals were euthanized using CO_2. and all implants were harvested for a further analysis.

References

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