Science Advances
AAAS

Supplementary Materials for

Efficient healing of large osseous segmental defects using optimized chemically modified messenger RNA encoding BMP-2

Rodolfo E. De La Vega, Martijn van Griensven, Wen Zhang, Michael J. Coenen, Christopher V. Nagelli, Joseph A. Panos, Carlos J. Peniche Silva, Johannes Geiger, Christian Plank, Christopher H. Evans, Elizabeth R. Balmayor*

*Corresponding author. Email: e.rosadobalmayor@maastrichtuniversity.nl

Published 16 February 2022, *Sci. Adv.* **8**, eabl6242 (2022)
DOI: 10.1126/sciadv.abl6242

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplementary Materials and Methods

Groups of animals (n=5/group) were euthanized at days 3 and 10 post-treatment with 50 µg BMP-2 cmRNA or 11 µg rhBMP-2 to evaluate *in vivo* expression of RANKL, BMP-1, -2, and -3 as well as the local production of IL-17A and IL-1β in the femoral defects (Supplemental Fig. S1D).

Early, local expression of RANKL, BMP-1, -2, and -3 by qPCR

Bone specimens were kept frozen in TRIzol (ThermoFisher) until further processing. Frozen samples were homogenized with steel beads in TRIzol using a TissueLyser II (Qiagen; 3 min at 30 Hz). Samples were then immediately processed following a standard phenol/chloroform method, saving the organic phase obtained for later protein isolation. For this, the standard protocol provided by ThermoFisher for working with TRIzol was carefully followed. Total RNA was reverse-transcribed into cDNA by using a First-Strand cDNA Synthesis Kit (ThermoFisher). Real-time qPCR was performed with SsoFast Eva Green Supermix (Bio-Rad) on a Bio-Rad CFX96 thermal cycler (Bio-Rad). The sequences of primers used to amplify RANKL, BMP-1, -2, and -3 as well as rat tubulin beta 2A class IIa, (Tubb2a) can be found in the Dataverse repository (https://dataverse.nl/privateurl.xhtml?token=73db97df-5749-44aa-83d6-e8b0a123aa9e).

ELISA for IL-17A and IL-1β

Proteins were precipitated with isopropanol using the organic phase saved from the RNA isolation. The instructions from ThermoFisher for this purpose were carefully followed. Protein concentration was measured by Bradford assay, then proceed directly to the ELISA analyses. A commercial DuoSet® ELISA Development System was used for IL-1β (R&D Systems). A SimpleStep ELISA® kit was used for IL-17A (Abcam).

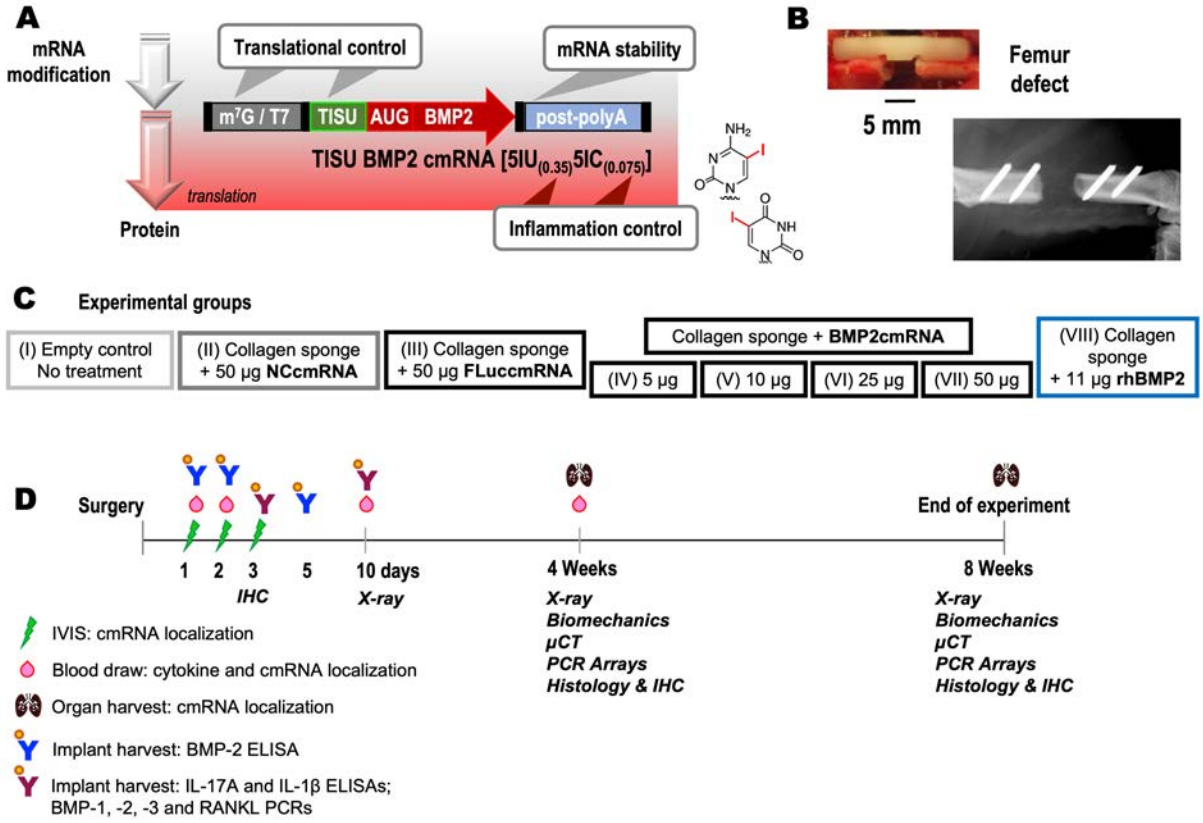

Fig. S1. Experimental design. (**A**) Structure of the osteogenic, BMP-2 cmRNA administered in a collagen sponge. (**B**) Critical-sized defect created in the rat femur featuring 5 mm gap. A macroscopic image and X-ray visualization of the defect also show the fracture stabilization placed during surgery. (**C**) Experimental groups evaluated included empty control, non-coding (NC) cmRNA control, and FLuc cmRNA for bioluminescence imaging. As osteogenic BMP-2 cmRNA was evaluated at doses of 5 µg, 10 µg, 25 µg, and 50 µg; rhBMP-2 was used at a dose of 11 µg, equivalent to the dose of recombinant protein used clinically. (**D**) Time line of experiments. The type of specimen and relevant assays are indicated for each time point.

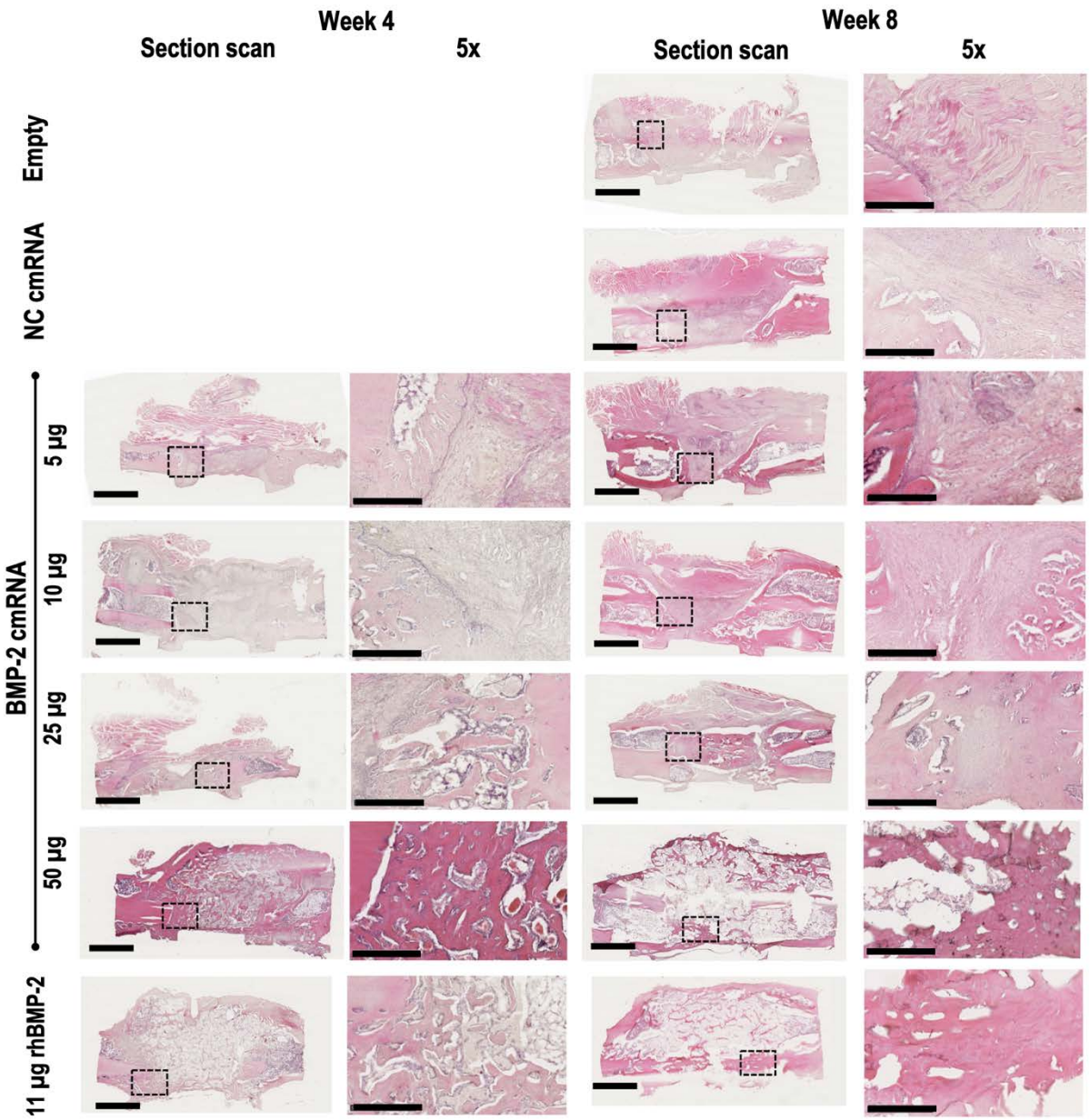

Fig. S2. H&E staining shows newly formed bone tissue (stained in dense pink) as result of BMP-2 cmRNA treatment. A clear dose dependency is apparent. H&E staining was performed on harvested explants from all groups 4- and 8-weeks after surgery. From left to right, a section scan illustrates the entire defect area while a 5x magnification image shows detail. Insets in section scans indicate the area where the 5x magnification picture was taken. Representative images from n=6 rats per group are shown. Scale bar on section scans = 2.5 mm. Scale bar on 5x magnification images = 500 µm.

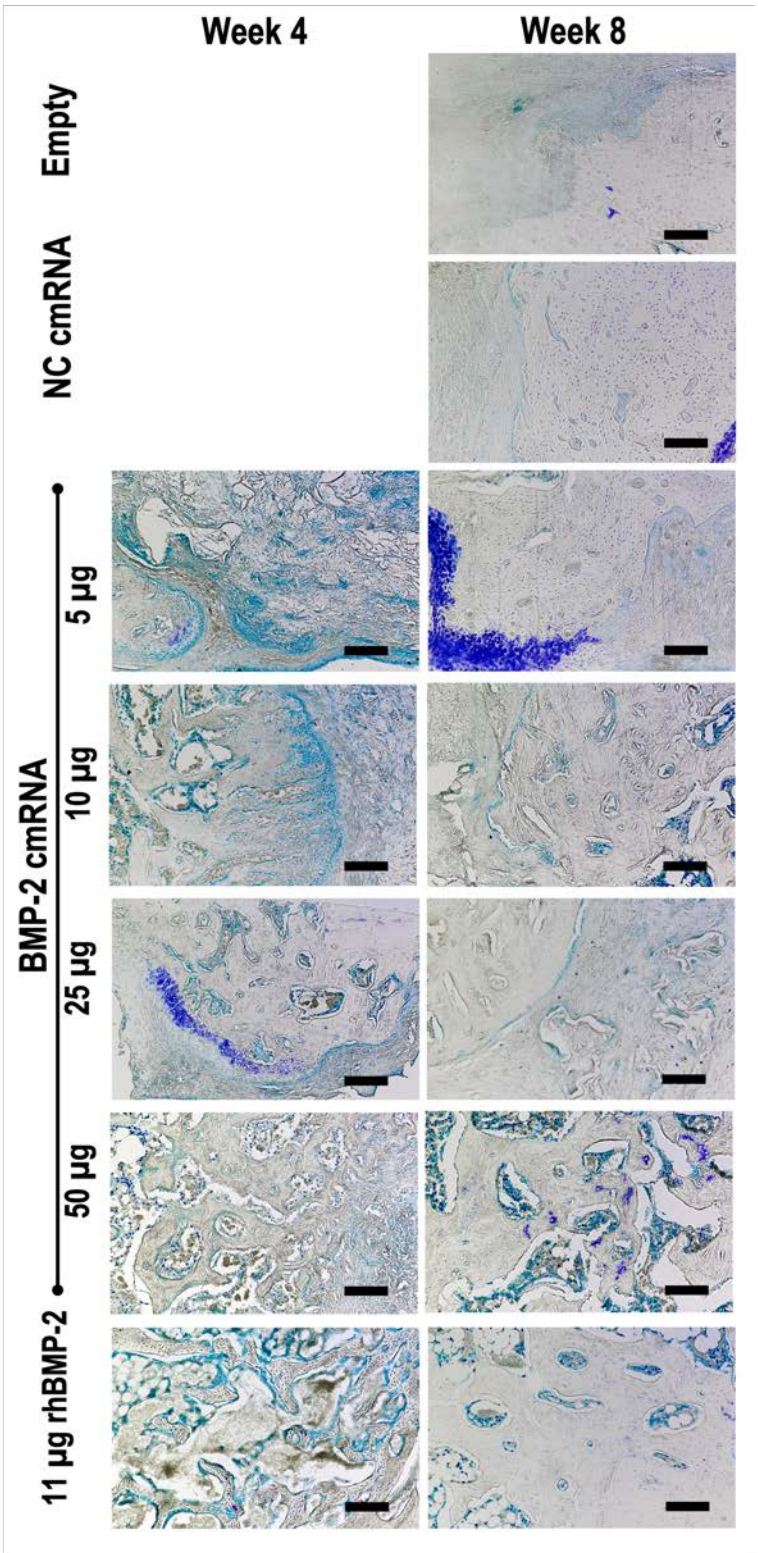

Fig. S3. Toluidine Blue staining shows bone fibrous matrix (osteoid) as result of BMP-2 cmRNA treatment. At 8 weeks post treatment, the staining seemed to be less prominent, which may indicate mineralization of the osteoid. Scale bar = 200 µm.

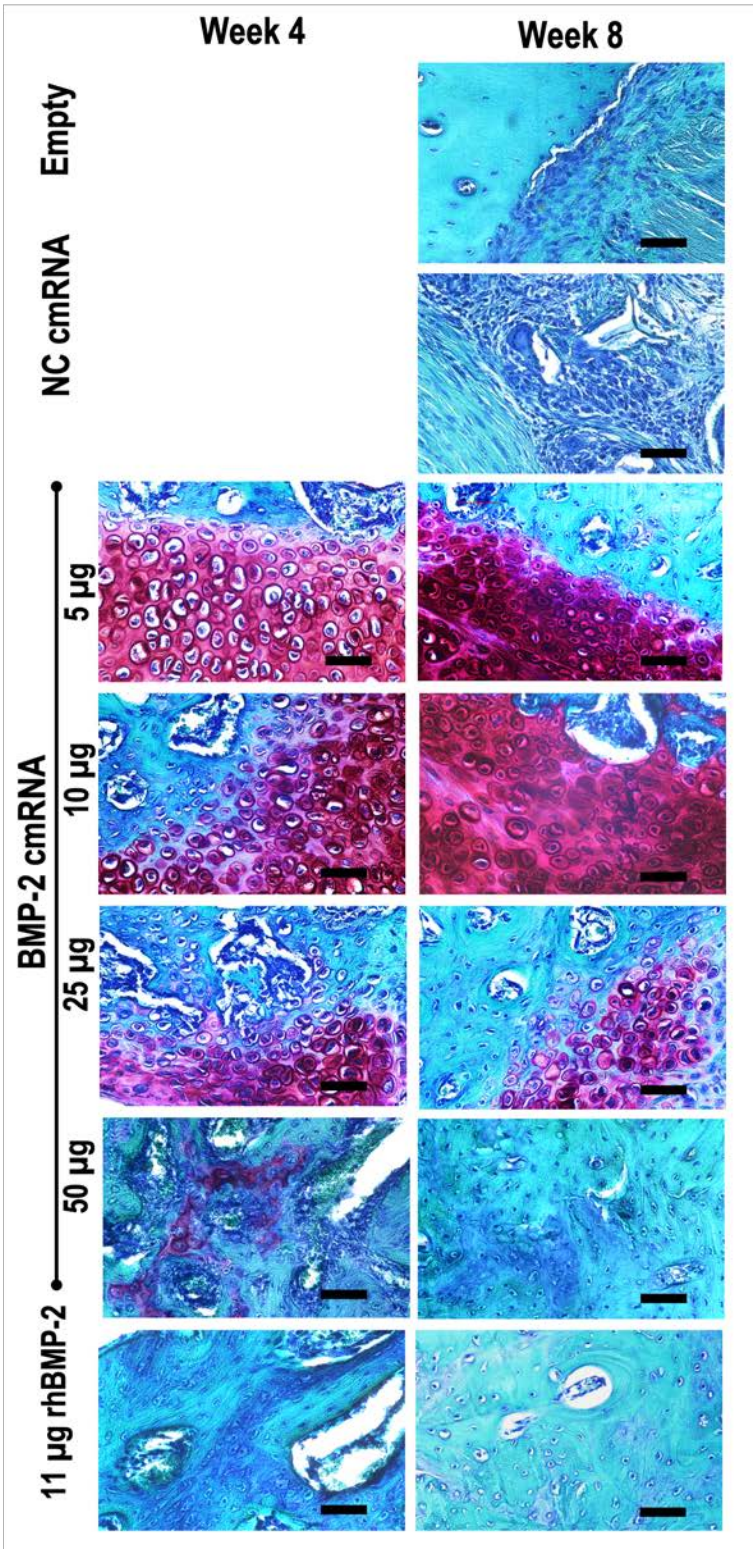

Fig. S4. Safranin O staining shows chondrocytic cells in the BMP-2 cmRNA groups. Scale bar = 50 µm, magnification 40x.

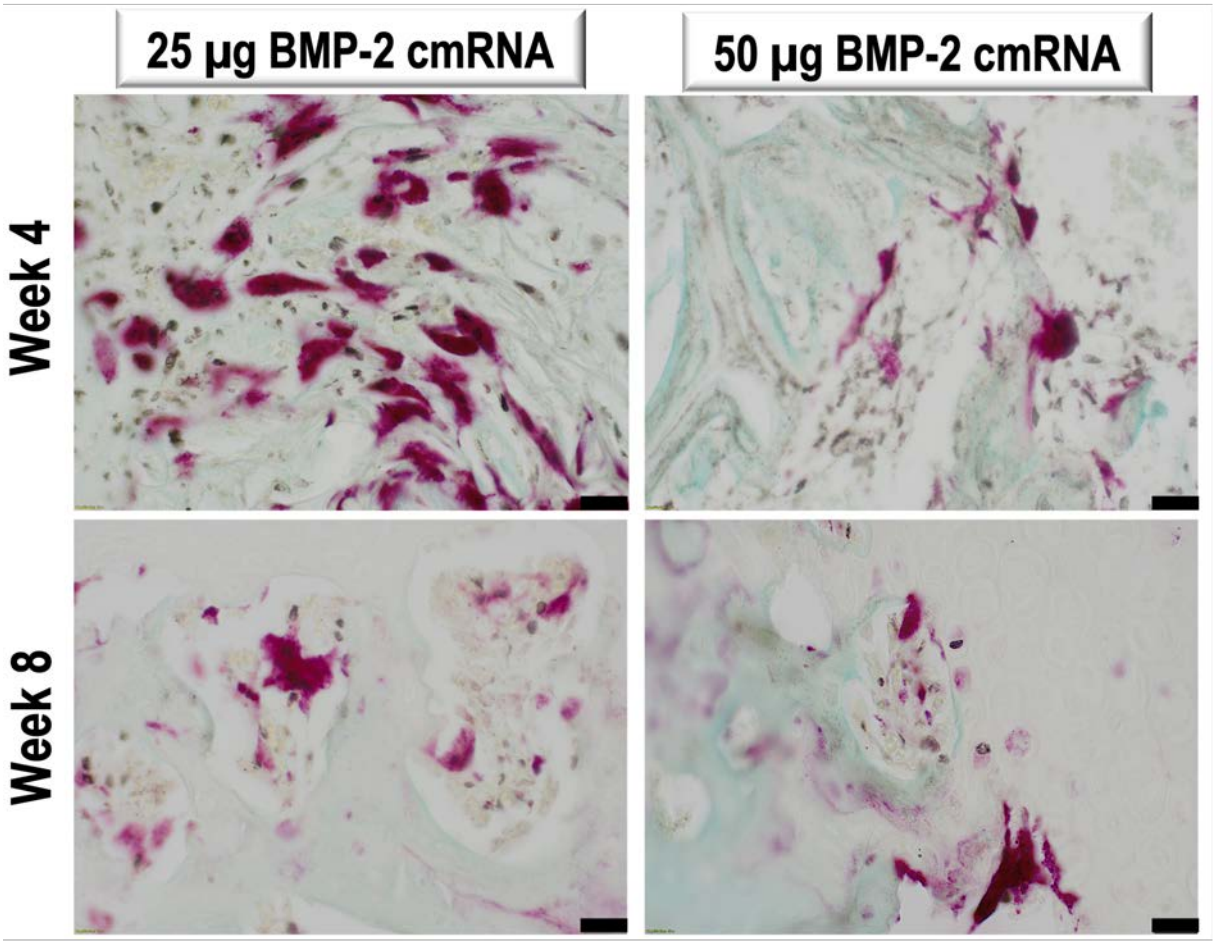

Fig. S5. TRAP staining shows multinucleated osteoclast-like cells populating the newly formed tissue in the 25 µg and 50 µg BMP-2 cmRNA treated groups. Scale bar = 20 µm, magnification 60x.

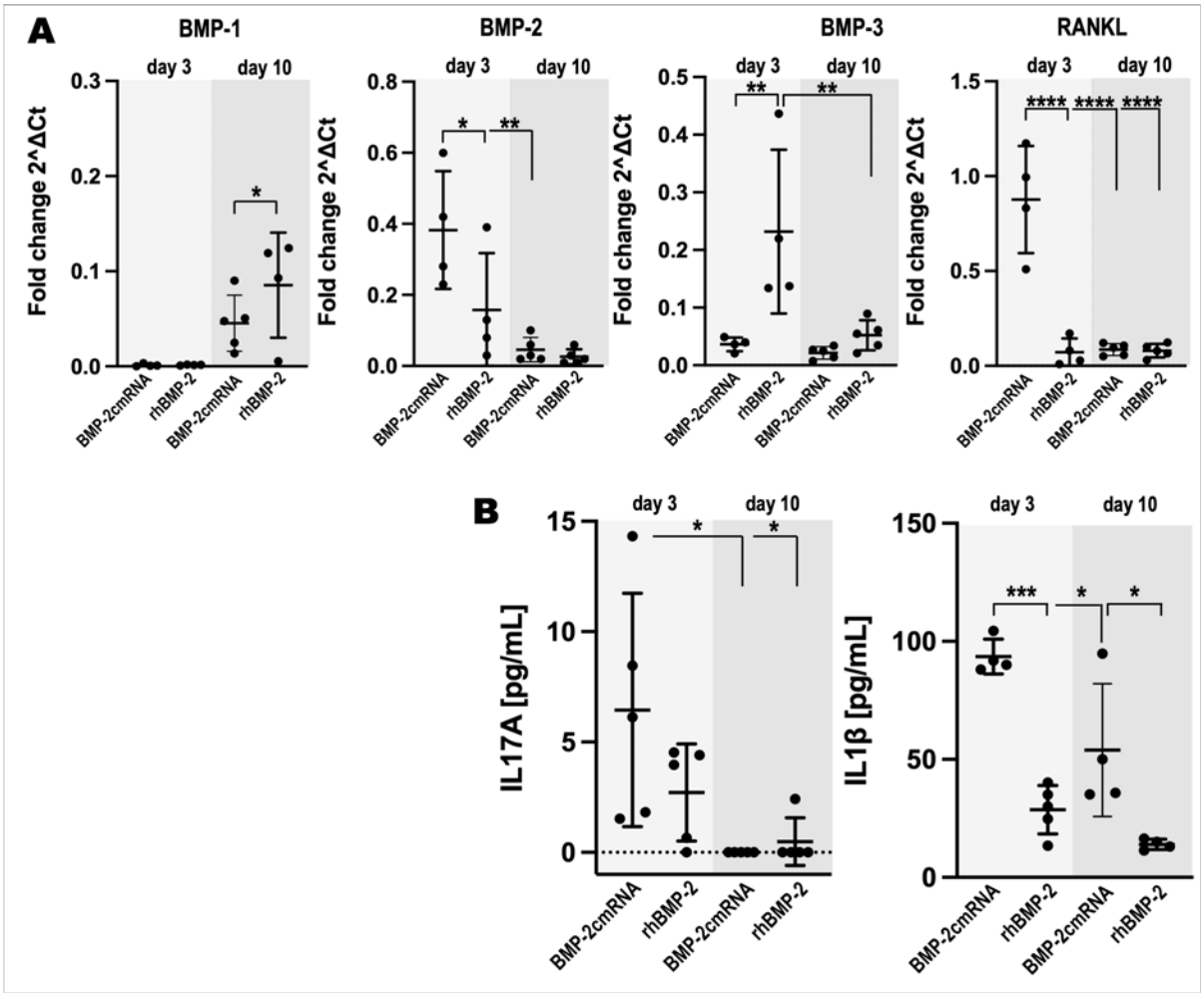

Fig. S6. (A) Local expression of BMP-1, -2, -3, and RANKL, as well as (B) Local IL-17A and IL-1β levels in the newly formed tissue at 3- and 10-days after BMP-2 cmRNA or rhBMP-2 administration.

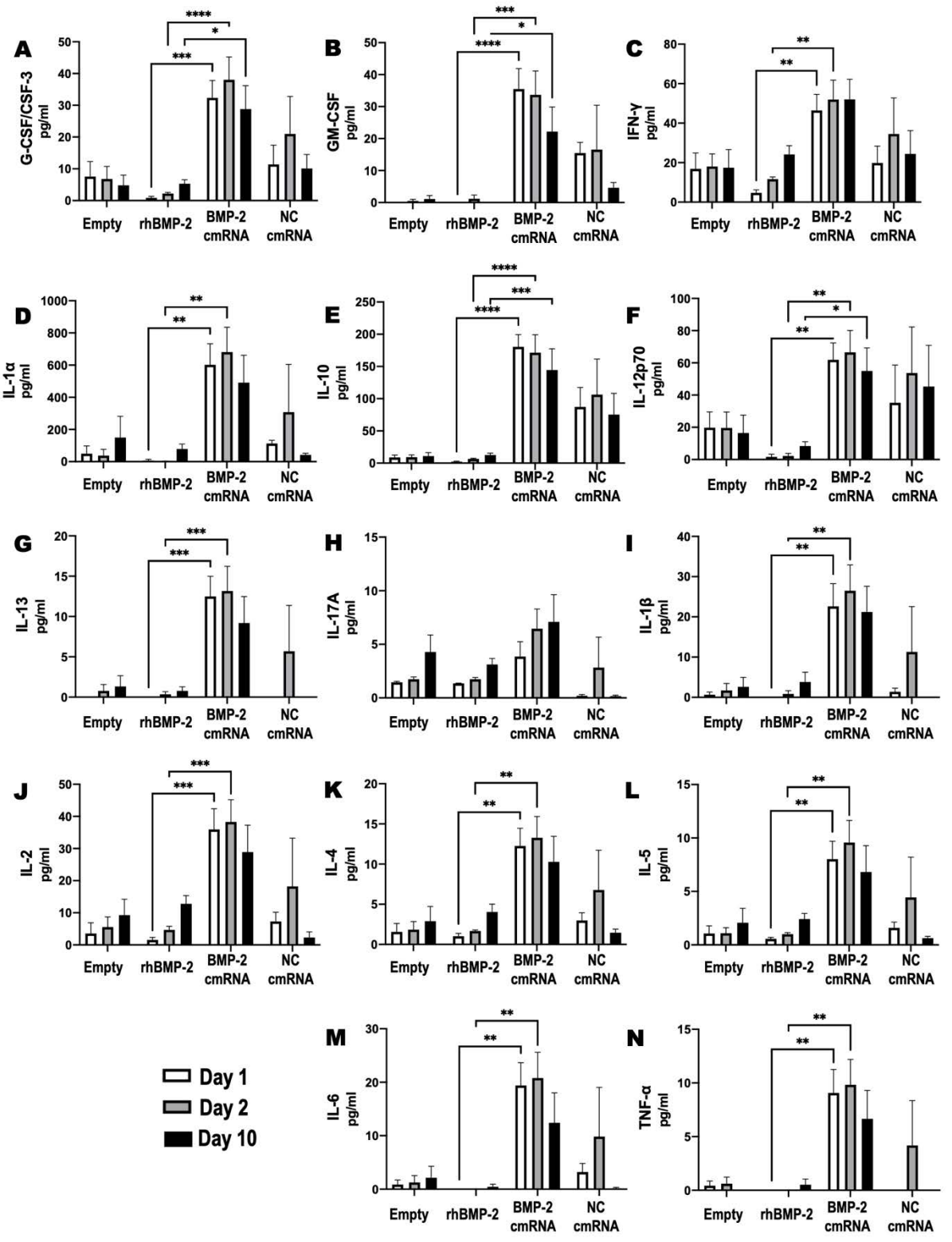

Fig. S7. Circulating cytokines elevated as a result of cmRNA application. Cytokine concentrations (pg/ml) 1, 2, and 10 days after surgery in the empty, NC cmRNA, BMP-2 cmRNA, and rhBMP-2 groups (**A**) G-CSF/CSF-3, (**B**) GM-CSF, (**C**) IFN-γ, (**D**) IL-1α, (**E**) IL-

10, (**F**) IL-12p70, (**G**) IL-13, (**H**) IL-17A, (**I**) IL-1β, (**J**) IL-2, (**K**) IL-4, (**L**) IL-5, (**M**) IL-6, and (**N**) TNF-α. Six plasma samples, each corresponding to an independent treated animal were used for analysis. Data were analyzed using two-way ANOVA corrected by Tukey for multiple comparisons. Statistical significance is shown only for the comparison between the BMP-2 cmRNA and the rhBMP-2 treated groups. A full report on the two-way ANOVA analysis is presented in Supplemental Table S8. In the graphs, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, and **** = $p \leq 0.0001$.

Legends for tables S1 to S9:

Table S1 (Microsoft Excel format). Fold change in expression of the most upregulated genes for each treatment and timepoint using the osteogenic and the ECM PCR arrays. Genes are listed alphabetically. The top 10 upregulated genes are highlighted in green for each group, in both arrays. "nd" indicates a gene whose expression was undetectable.

Link: https://surfdrive.surf.nl/files/index.php/s/sw2ZSoUfeP500yE

Table S2 (Microsoft Excel format). Fold change in expression of selected transcription factors (Runx2, Sp7, Smad2-5, and Nfkb1) using the osteogenic PCR array. Selection is based on relevance to the study aims; n.d. = not detected

Link: https://surfdrive.surf.nl/files/index.php/s/PiRFVGu1NByYpmX

Table S3 (Microsoft Excel format). Fold change in expression of selected growth factors and receptors (Bmp1-4, Bmp7, Bmpr1a and b, Bmpr2, Fgfr1 and 2, Flt1, Tgfb1-3, Tgfbr1 and 2, Vegfa and b) using the osteogenic PCR array. Selection is based on relevance to the study aims; n.d. = not detetcted.

Link: https://surfdrive.surf.nl/files/index.php/s/3AyOSRYsso4pWc9

Table S4 (Microsoft Excel format). Fold change in expression of selected genes relevant to osteogenesis (Alpl, Bglap, Bgn, Comp, Spp1, and Sparc) using the osteogenic and the ECM PCR arrays. Selection is based on relevance to the study aims; n.d. = not detected.

Link: https://surfdrive.surf.nl/files/index.php/s/3hEnzmdSFkf5Nrb

Table S5 (Microsoft Excel format). Fold change in expression of selected genes relevant to ECM (Col1a1 and a2, Col2a1, Col3a1, Col4a1 and a2, Col5a1, Col6a1, Col8a1, Col10a1, Col14a1, Lama1-3, Lamb2 and 3, Lamc1, and Postn) using the osteogenic and the ECM PCR arrays. Selection is based on relevance to the study aims; n.d. = not detected.

Link: https://surfdrive.surf.nl/files/index.php/s/Vig3sZKKI5GBq9u

Table S6 (Microsoft Excel format). Fold change in expression of selected metalloproteinases and inhibitors (Mmp2 and 3, Mmp8-14, Mmp16, Timp1-3, Adamts1 and 2, Adamts5, and Ctsk) using the osteogenic and the ECM PCR arrays. Selection is based on relevance to the study aims; n.d. = not detected.

Link: https://surfdrive.surf.nl/files/index.php/s/VEAlJr4PeU1JzSf

Table S7 (Microsoft Excel format). Fold change in expression of selected adhesion molecules (Vcam1, Cdh1 and 2, Cdh11, Itga4, Itgad, and Sele) using the osteogenic and the ECM PCR arrays. Selection is based on relevance to the study aims; n.d. = not detected.

Link: https://surfdrive.surf.nl/files/index.php/s/agbmXO2iRj5N3KO

Table S8 (Microsoft Excel format). Statistics Figure S7: Results of the two-way ANOVA corrected by Tukey for multiple comparisons performed for cytokine production 1, 2, and 10 days after surgery. Results are displayed in Figure S7. Groups evaluated are empty, NC cmRNA, BMP-2 cmRNA, and rhBMP-2.

Link: https://surfdrive.surf.nl/files/index.php/s/4Nm60SWte4nsDWw

Table S9 (Microsoft Excel format). Gene list for the Osteogenic (PARN-026Z) and Extracellular Matrix (PARN-013Z) Qiagen PCR arrays used in the study.

Link: https://surfdrive.surf.nl/files/index.php/s/PplKrINiI2CRboT

Legends for movies S1 and S2:

Movie S1 (mp4 movie format). Torsional test performed on a native femur using the custom-made material testing system (Mayo Clinic, USA).

Link: https://surfdrive.surf.nl/files/index.php/s/eka82hm3qXNPtx5

Movie S2 (mp4 movie format). Torsional test performed on a BMP-2 cmRNA treated femur using the custom-made material testing system (Mayo Clinic, USA).

Link: https://surfdrive.surf.nl/files/index.php/s/QwGVZPpbkNzUQ4o