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### ► To cite this version:

Pinpin Wang, Delphine Logeart-Avramoglou, Hervé Petite, Cristine Gonçalves, Patrick Midoux, et al. Co-delivery of NS1 and BMP2 mRNAs to murine pluripotent stem cells leads to enhanced BMP-2 expression and osteogenic differentiation. *Acta Biomaterialia*, 2020, 108, pp.337-346. 10.1016/j.actbio.2020.03.045 . hal-02553030

**HAL Id: hal-02553030**

**<https://hal.science/hal-02553030v1>**

Submitted on 24 Nov 2020

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1 **Co-delivery of NS1 and BMP2 mRNAs to murine pluripotent stem cells leads to enhanced BMP-2**  
2 **expression and osteogenic differentiation**

3  
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14

15 **Abstract**

16 Application of messenger RNA (mRNA) for bone regeneration is a promising alternative to DNA,  
17 recombinant proteins and peptides. However, exogenous *in vitro* transcribed mRNA (IVT mRNA) triggers  
18 innate immune response resulting in mRNA degradation and translation inhibition. Inspired by the ability  
19 of viral immune evasion proteins to inhibit host cell responses against viral RNA, we applied non-structural  
20 protein-1 (NS1) from *Influenza A virus (A/Texas/36/1991)* as an IVT mRNA enhancer. We evidenced a dose-  
21 dependent blocking of RNA sensors by NS1 expression. The co-delivery of NS1 mRNA with mRNA of  
22 reporter genes significantly increased the translation efficiency. Interestingly, unlike the use of nucleosides  
23 modification, NS1-mediated mRNA translation enhancement does not dependent to cell type. Dual  
24 delivery of NS1 mRNA and BMP-2 mRNA to murine pluripotent stem cells (C3H10T1/2), promoted  
25 osteogenic differentiation evidenced by enhanced expression of osteoblastic markers (e.g. alkaline  
26 phosphatase, type I collagen, osteopontin, and osteocalcin), and extracellular mineralization. Overall,  
27 these results support the adjuvant potentiality of NS1 for mRNA-based regenerative therapies.

28 **Keywords:** mRNA delivery, non-structural protein 1, RNA sensors, bone morphogenetic protein 2,  
29 osteogenesis

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## 1 **1. Introduction**

2 Bone defects affect millions of people every year [1]. The treatment of large bone defects resulting from  
3 trauma, nonunion fractures, tumor resections or craniofacial malformations remains challenging. The  
4 situation is more severe with the increase of aging population.

5 Many strategies have been proposed to improve the regeneration of damaged bone tissue. Among them,  
6 the delivery of osteoinductive growth factors (or derivatives), mostly bone morphogenetic protein-2 (BMP-  
7 2), to the lesion site remains a promising approach to promote bone healing [2, 3]. However, delivery of  
8 supraphysiological dose induced deleterious side effects, including significant inflammation, swelling, and  
9 heterotopic ossification, that had limited the extent of their clinical use [4].

10 DNA and messenger RNA (mRNA)-based gene therapies represent alternative approaches for locally  
11 delivering growth factors [5]. They allow an in-situ expression of growth factors in host cells, and undergo  
12 precise post-translational modifications required for optimal activity. Furthermore, gene therapy is  
13 preferable for delivering products that exert their function intracellularly, such as transcription factors and  
14 signaling transduction molecules [6].

15 DNA delivery to produce osteogenic proteins has demonstrated an enhancement of osteogenesis both *in*  
16 *vitro* and *in vivo*, proving the great potential of this strategy for bone healing and regeneration [7, 8]. The  
17 main limitation of DNA delivery, however, is the requirement of nuclear transport of the transgene for its  
18 transcription leading to low transfection efficiency and risk of genome insertion. Despite the high gene  
19 transfer efficiency obtained with viral vectors, the random genomic integration and the immunogenicity  
20 that are hard to handle and should not be ignored [9, 10]. In contrast, mRNA molecules are directly  
21 translated inside the cytoplasm, making this technology more efficient, and suitable in non-dividing cells.  
22 This aspect is especially useful when inducing cell differentiation since terminal differentiation usually  
23 coincides with proliferation arrest [11]. Although mRNA therapy demonstrates clear advantages, its broad  
24 application has been so far limited to vaccination because of its instability and immunogenicity [12, 13].  
25 After internalization, IVT mRNA molecules mainly accumulate into endosomes, where they are recognized  
26 by Toll-like receptors (TLRs), specifically TLR3, 7, and 8, located in the endosome membrane [14-16]. TLRs  
27 engagement induces type I interferon (INF $\alpha$ / $\beta$ ) expression and associated anti-viral responses resulting in  
28 IVT mRNA translation suppression and degradation, and even host cell apoptosis [17, 18]. To reduce such  
29 immune effects, chemically modified nucleotides have been developed; the synthesis of such nucleotides,  
30 however, is expensive and the type and percentage of modified nucleotides in the mRNA sequence must  
31 be tuned as a function of mRNA and cell type for maximum activity [19]. Recently, other strategies have  
32 been developed based on RNA-virus mimicry [20, 21].

1 During millions of years of coexisting with their hosts, viruses have acquired the ability to manipulate host  
2 immune mechanisms [22, 23]. Viruses express various immune evasion proteins (e.g. protein K3 (K3L),  
3 protein E3 (E3L), soluble interferon  $\alpha/\beta$  receptor B18 (B18R), NS1) which sequester the host immune-  
4 mediating proteins (e.g. interferons (IFNs), retinoic acid-inducible gene I protein (RIG-I), interferon-  
5 induced, double-stranded RNA-activated protein kinase (PKR)) induced by detecting immune triggers (e.g.  
6 dsRNA). Mimicking this viral immune-evasion process could, therefore, be an interesting strategy to bypass  
7 the IVT mRNA-triggered immune responses and, therefore to increase the transfection efficiency [24].  
8 Specifically, B18R is a secreted protein that exerts its function as a soluble receptor for type I IFNs [25]; the  
9 use of recombinant B18R from Western Reserve strain of vaccinia virus as cell culture medium supplement  
10 during *in vitro* transfection is known to maintain cell viability and to enhance the IVT mRNA translation  
11 efficacy [26-28]. In contrast to B18R, NS1 is a multifunctional viral protein that participates in every step  
12 of host immune reaction [29]. During the viral replication cycle, NS1 completely interacts with dsRNA,  
13 thereby inhibiting 2'-5'-oligoadenylate synthase (OAS)/RNase L-mediated RNA degradation, and PKR-  
14 mediated protein synthesis reduction [30, 31]. NS1 forms a complex with RIG-1 that blocks the activation  
15 of NF- $\kappa$ B (nuclear factor kappa B) and IRF3 (interferon regulatory factor 3) and, subsequently, reduces IFN  
16  $\alpha/\beta$  expression [32, 33]. Co-delivery of NS1 mRNA with mRNA of interest was able to enhance mRNA  
17 translation to an extent superior to the incorporation of modified nucleotides [20, 21]. In addition, we  
18 found that NS1-mediated translation enhancement is independent of gene context and cell type which is  
19 not the case for chemically modified mRNA [19, 34].

20 Here, we propose to combine NS1 mRNA with BMP-2 mRNA to improve the efficiency of mRNA therapy  
21 for bone regeneration. The hypothesis is that the co-delivery of both NS1 mRNA and BMP-2 mRNA into  
22 osteoprogenitor cells enhances the BMP-2 expression and ultimately promotes osteogenesis. In this study,  
23 a murine pluripotent stem cell line (C3H10T1/2) was used to evaluate the *in vitro* osteogenic potential of  
24 this dual mRNA (BMP2/NS1 mRNAs) strategy. The stem cells transfected with the dual mRNAs, under  
25 optimized mass ratio, expressed significantly higher BMP-2 than cells transfected with BMP-2 mRNA alone,  
26 which led to increased induction of osteogenic genes and extracellular calcium deposits.

27 Through this proof-of-concept study, we successfully validated the application of NS1 mRNA as a  
28 supplement for enhanced therapeutic mRNA transfection. To the best of our knowledge, this is the first  
29 study demonstrating the therapeutic potential of non-modified mRNAs in regeneration medicine. We  
30 hope that through this strategy, a new horizon of mRNA therapy could be developed.

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## 1 **2. Materials and methods**

### 2 **2.1. Preparation of plasmids and mRNAs**

3 Mouse BMP-2 ORF was PCR amplified from pCMV3-mBMP2-GFPSpark (Sino Biologicals) with 5'-  
4 TATGGATCCACTTAAGATGGTGGCCGGGACCCGCTGT-3' as forward primer and 5'-  
5 TATTGCGGCCGCTTAACGACACCCGACCCCTC-3' as reverse primer (Eurogentec). NS1 (A/Texas/36/1991)  
6 ORF was PCR amplified from pUC57-NS1 (Genscript) with 5'-tgtacggatcctcctatggattccaactgtgtc-3' and 5'-  
7 atttgggccgctcaaacttctgacct-3' as the forward primer and reverse primer (Eurogentec), respectively. The  
8 BMP2 and NS1 segments were inserted into the previously described pGEM4Z-luc-A64 plasmid [35],  
9 between BamHI and NotI, respectively. Plasmid DNAs were extracted, purified with NucleoBond® Xtra  
10 Maxi EF kit (Macherey-Nagel) and verified by sequencing (Eurofins). The maps of pGEM4Z-BMP2-A64 and  
11 pGEM4Z-NS1-A64 were shown in Supplementary Fig.1.

12 Plasmids were linearized for mRNA preparation with a mMessage mMachine T7 kit (Ambion) according to  
13 the manufacturer's instructions. The poly(A) tail was extended with PolyA polymerase (Ambion) to  
14 generate at least 150nt guaranteed by the manufacturer. To produce chemically modified mRNAs  
15 (cmRNAs), 50% uridine-5'-triphosphate were replaced with pseudouridine-5'-triphosphate (TriLink).  
16 Synthesized mRNAs were purified with phenol: chloroform and isopropanol precipitation. Sizes and  
17 integrity of the mRNAs were verified by denatured agarose gel electrophoresis (Supplementary Fig. 2).

### 18 **2.2. Cell culture**

19 C2C12 murine myoblasts, 4T1 murine breast cancer cells, A549 human lung cancer cells, U87-MG human  
20 glioblastoma cells, HepG2 human hepatocellular carcinoma cells and, C3H10T1/2 murine pluripotent stem  
21 cells were purchased from the American Type Culture Center (ATCC). Murine DC 2.4 cells were a kind gift  
22 from Pr. Kenneth Rock [36]. C2C12-BRE/Luc cells, which contain a BMP-2 sensitive reporter gene were  
23 obtained after stably transfection with the BMP-responsive element (BRE) fused to the luciferase gene as  
24 previously described [37]. Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells  
25 were mycoplasma-free as evidenced by MycoAlert Mycoplasma Detection Kit (Lonza).

### 26 **2.3. *In vitro* transfection**

27 All transfections were mediated by Lipofectamine MessengerMax (LFM) (ThermoFisher) transfection  
28 reagent according to the manufacturer's protocol. Briefly, to prepare mRNA-LFM complexes (lipoplexes),  
29 0.5µg mRNA and 0.75µl LFM were diluted in 25µl opti-MEM (Gibco). Ten min after, the mRNA solution was  
30 added into LFM suspension, followed by 15min incubation. The final volume of LFM and opti-MEM were  
31 scaled up/down according to mRNA dose.

32

#### 1 **2.4. Evaluation of translation of BMP2 mRNA and NS1 mRNA containing different 3' UTR**

2 The day before transfection, C2C12-BRE/Luc cells were seeded at  $5 \times 10^4$  cells/well in 24-well plate. The  
3 next day, 0.5  $\mu\text{g}$  BMP2-NotI mRNA (transcribed from NotI linearized BMP2 plasmid) or 0.5  $\mu\text{g}$  BMP2-XbaI  
4 mRNA (transcribed from XbaI linearized BMP2 plasmid) both complexed with LFM were delivered to  
5 C2C12-BRE/LUC cells. One day post-transfection, cells were lysed, and the luciferase activity was  
6 quantified as previously reported [35]. Luminescence from cell lysates was measured with the LB9075  
7 illuminometer (Biorad) and, expressed as relative light unit (RLU) per mg of proteins.

8 NS1-NotI and NS1-XbaI mRNA were delivered using the same procedure as BMP2 mRNAs, and semi-  
9 quantified by Western blot. Briefly, 24h post-transfection, the cells were collected and lysed in 100 $\mu\text{l}$   
10 Pierce<sup>TM</sup> RIPA buffer (ThermoScientific). Cell lysate containing 15 $\mu\text{g}$  total protein was loaded into each well  
11 of the SDS-PAGE gel (5% for concentration gel, and 12% for separation gel). The NS1 and beta-actin  
12 contents were observed with ChemiDoc<sup>TM</sup> imaging system (Bio-Rad) after labeling with primary antibodies  
13 (NS1 mouse monoclonal antibody (Santa Cruz); actin rabbit polyclonal antibody (Sigma-Aldrich) and  
14 secondary antibodies (anti-mouse and -rabbit IgG polyclonal antibodies (Enzo life sciences), respectively.

#### 15 **2.5. Assessment of NS1 cytocompatibility**

16 C3H10T1/2 cells ( $5 \times 10^4$  cells/well in 24-well plate) were transfected with increasing doses of NS1 mRNA (0  
17 .1 $\mu\text{g}$ , 0.3 $\mu\text{g}$ , and 0.5 $\mu\text{g}$  per well) complexed with LFM. Same amount of GFP mRNA was used as control.  
18 24h and 48h later, cytotoxicity level was evaluated by an XTT assay (Cell Proliferation Kit II, Sigma-Aldrich)  
19 following the manufacturer's instruction.

#### 20 **2.6. Validation of NS1-mediated IVT mRNA translation enhancement**

21 One day after cell plating, 0.2 $\mu\text{g}$  GFP mRNA was co-delivered with either 0.1 $\mu\text{g}$ , or 0.3 $\mu\text{g}$ , or 0.5 $\mu\text{g}$  NS1  
22 mRNA into C3H10T1/2 cells. Twelve hours post-transfection, the GFP expression was evaluated by flow  
23 cytometry (FACSort, Becton Dickinson), and the intracellular NS1 from each condition tested was semi-  
24 quantified by Western-blot.

25 To measure the kinetic of GFP expression, 0.2 $\mu\text{g}$  GFP mRNA was co-delivered with either 0.2 $\mu\text{g}$  Gluc mRNA  
26 or 0.2 $\mu\text{g}$  NS1 mRNA. The GFP expression was measured from 6h to 168 h post-transfection.

27 To study the immune activation post-transfection, C3H10T1/2 cells were transfected with 1  $\mu\text{g}$ /well of  
28 total mRNA comprising different weight ratios of GFP mRNA and NS1 mRNA: 1 $\mu\text{g}$  GFP mRNA (1 GFP);  
29 0.75 $\mu\text{g}$  GFP mRNA and 0.25 $\mu\text{g}$  NS1 mRNA (0.75 GFP/0.25 NS1); 0.5 $\mu\text{g}$  GFP mRNA and 0.5 $\mu\text{g}$  NS1 mRNA (0.5  
30 GFP/0.5 NS1); 0.25 $\mu\text{g}$  GFP mRNA and 0.75 $\mu\text{g}$  NS1 mRNA (0.25 GFP/0.75 NS1); 1 $\mu\text{g}$  NS1 mRNA (1 NS1) Cells  
31 transfected with  $\Psi$ -modified GFP mRNA (GFP(50% $\Psi$ )) were set as controls. Interferon alpha (IFN  $\alpha$ )  
32 secreted in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA, PBL Assay

1 Science) according to the manufacturer's instructions. Gene expression level of IFN  $\alpha/\beta$ , PKR, RIG-1, OAS1,  
2 Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) were determined by real-time  
3 quantitative reverse-transcription polymerase chain reaction (RT-qPCR) according to a previous report [38].  
4 Briefly, total RNA were extracted using RNeasy Mini kit (Qiagen), RNA concentration and purity was  
5 measured using Nanodrop (ThermoFisher). First strand cDNA synthesis kit (Thermo Scientific) was used  
6 for reverse transcription following manufacturer's protocol. qPCR was performed using the Luna qPCR  
7 Master mix (NEB) with a Light Cycler<sup>®</sup> 480 PCR system (Roche). The qPCR data were analyzed by the  
8 comparative  $\Delta\Delta C_t$  method using the GAPDH RT-qPCR signal as the internal control for normalization.  
9 Primers for RT-qPCR were listed in Supplementary Table 1.

## 10 **2.7. Screening of NS1 function as a function of mRNA and cell context**

11 C3H10T1/2, C2C12, DC 2.4, 4T1, A549, U87MG, HepG2 cells were seeded and cultured into 24-well plate  
12 in order to obtain 70-80% confluence at the time of transfection. These cells were transfected with  
13 lipoplexes containing 0.25  $\mu\text{g}$  mRNA of reporter genes (i.e., GFP, Fluc, and Gluc) with either 0.25  $\mu\text{g}$  NS1  
14 mRNA or 0.25  $\mu\text{g}$  noncoding mRNA. The GFP and Fluc expression were evaluated as described before. Gluc  
15 expression, both secreted and intracellular, was evaluated with the Pierce Gaussia Luciferase Glow assay  
16 kit (ThermoScientific). The luminescence obtained from secreted and intracellular Gluc were normalized  
17 to total cellular protein and per mg cellular protein, respectively.

## 18 **2.8. Optimization of BMP2 mRNA and NS1 mRNA ratio**

19 C2C12-BRE/LUC cells were seeded into a 24-well plate at the density of  $1 \times 10^5$  cells/well. One day later,  
20 cells were transfected with 1  $\mu\text{g}$ /well total mRNA with different weight ratio of BMP2 mRNA and NS1  
21 mRNA: 1 $\mu\text{g}$  BMP2 mRNA (1 BMP2); 0.75 $\mu\text{g}$  BMP2 mRNA and 0.25 $\mu\text{g}$  NS1 mRNA (0.75 BMP2/0.25 NS1);  
22 0.5 $\mu\text{g}$  BMP2 mRNA and 0.5 $\mu\text{g}$  NS1 mRNA (0.5 BMP2/0.5 NS1); 0.25 $\mu\text{g}$  BMP2 mRNA and 0.75 $\mu\text{g}$  NS1 mRNA  
23 (0.25 BMP2/0.75 NS1). Luciferase activity was measured one day later as described above.

## 24 **2.9. Quantification of BMP-2 secreted by transfected C3H10T1/2**

25 C3H10T1/2 cells were seeded into 4-well plate at a density of  $5 \times 10^4$  cells/well and cultured until  
26 confluency. Then, 1  $\mu\text{g}$  total mRNA with optimized BMP2 and NS1 mRNA ratio were used to transfect the  
27 cells. Every 12 h, half of the culture medium was collected and replaced with fresh medium. Collected  
28 media were stored at  $-80^\circ\text{C}$  before BMP-2 content quantification. BMP-2 contents were determined by  
29 ELISA (Abcam) following the manufacturer's instruction. The absorbance was measured at 450 nm and  
30 BMP2 contents were calculated based on a standard curve (range: 0-4000 pg/ml mouse BMP2).

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## 1 **2.10. *In vitro* osteogenesis**

2 C3H10T1/2 cells were seeded as indicated in 2.9. Prior to transfection, cells were cultured in osteogenic  
3 medium containing 25ng/ml rhBMP-2 (R&D system), 50µg/ml ascorbic acid-2-phosphate (Sigma) and  
4 10mM β-glycerophosphate (Sigma) for seven days and half of the medium was changed every other day.  
5 Then, cells were transfected twice with 1 µg/well mRNA at day 0 and day 7 (experimental scheme shown  
6 in Fig. 7. A), and maintained in osteogenic medium (without rhBMP-2) throughout the whole osteogenic  
7 induction process. From day 0, half of the medium was changed every 3 days during the first 10 days and  
8 then every 2 days.

9 At day 3, 7, and 14 post-transfection, ALP activity was quantified with ALP activity colorimetric assay kit  
10 (Biovision) following the manufacturer's protocol. Intracellular ALP expression was visualized by Nitro Blue  
11 Tetrazolium (NBT)/5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) (Sigma) staining as  
12 previously reported [39] with slight modification. Briefly, at day 14 post-transfection, cells were washed  
13 with PBS and then fixed in 4% formaldehyde for 2 min. BCIP/NBT solution was added to react with ALP for  
14 30 min in dark at RT. The staining solution was washed out with PBS.

15 At day 1, 3, 5, 7, 14, and 21 post-transfection, the expression of Runx2, ALP, collagen type 1, OCN, OPN  
16 were quantified by RT-qPCR. The process was the same as described before, except that total RNA was  
17 isolated and purified by TRIzol (Life technology)-chloroform method. Primers for RT-qPCR were listed in  
18 Supplementary Table 1.

19 Alizarin Red staining was performed 28 days post-transfection to evaluate calcium deposition. Cells were  
20 washed with D-PBS and then fixed in 4% p-formaldehyde for 30 min at room temperature (RT) followed  
21 by 2 times washes with distilled water (dH<sub>2</sub>O). Extracellular calcium was stained by incubating the cell  
22 layers in Alizarin red S (Sigma) solution (saturated in dH<sub>2</sub>O, pH 4.1). Nonspecific staining was removed by  
23 washing the wells with dH<sub>2</sub>O five times under gentle agitation. To quantify the mineralization, the Alizarin  
24 red dye was subsequently extracted with cetylpyridinium chloride (Sigma) solution (10% in 10mM sodium  
25 phosphate) for 30 min at RT. Absorbance was then measured at 560nm using the Victor <sup>3</sup>V  
26 spectrophotometer (PerkinElmer).

## 27 **2.11. Statistical analysis**

28 Unless otherwise indicated, each experiment was performed in triplicates with two independent repeats.  
29 All numerical data are expressed as mean ± standard deviation. Statistical analysis was performed using  
30 GraphPad Prism version 6.07 (GraphPad software). Any p-value less than 0.05 was considered statistically  
31 significant. Specifically, \* and # represent P < 0.05; \*\* and ### represent P < 0.01; \*\*\* and #### represent P <  
32 0.001; \*\*\*\* and ##### represent P < 0.0001.



## 1 **3. Results**

### 2 **3.1. 3' UTR affects IVT mRNA translation efficiency**

3 The production of mRNA by *in vitro* transcription requires the linearization of the plasmid DNA template.  
4 In our construction, this linearization was done by digesting the template with either NotI or XbaI  
5 endonuclease enzymes, resulting in the production of mRNAs with different 3' UTRs (Fig. 1. A).  
6 We first assessed which of those two mRNA types has the best translation efficiency. As shown in Fig. 1. B,  
7 the amount of NS1 produced from NS1-NotI mRNA was 1.5-fold higher than that obtained with NS1-XbaI  
8 mRNA. The same observation was made with BMP2 mRNA, which was evaluated in C2C12-BRE/LUC cells  
9 that express the luciferase upon BMP-2 activation. These data indicate that mRNA-NotI gave a higher  
10 protein expression than mRNA-XbaI regardless of the gene type. The mRNA-NotI was chosen for the next  
11 experiments.

### 12 **3.2. NS1 promotes IVT mRNA translation by suppressing cellular innate immune responses**

13 We first checked the impact of NS1 expression on cell viability. Results shown in Fig. 2 indicate that the  
14 cell viability decreased in a dose-dependent manner. The highest cytotoxic effect was observed with  
15 0.5µg/well, which results in 70% of viable cells compared to untreated cells 48h post-transfection. No  
16 effect on the viability of cells transfected with 0.1µg/well of either NS1 or GFP mRNAs was observed. At  
17 0.3µg/well and 0.5µg/well, we found that NS1 mRNA expression did not lead to a significant cell viability  
18 decrease at 24h post-transfection, whilst GFP mRNA did. For the next 24h, the trend was reversed. No  
19 significant difference either between GFP and NS1 transfected cells or between 24h and 48h post-  
20 transfection time points were observed.

21 Next, we validated the positive effect of NS1 expression on the translation of GFP mRNA (Fig. 3). Different  
22 amounts of NS1 mRNA (0.1µg to 0.5µg/well) were co-delivered with a fixed amount of GFP mRNA  
23 (0.2µg/well). Compared to cells transfected with GFP mRNA alone, the co-delivery with NS1 mRNA  
24 resulted in a significant dose-dependent enhancement of GFP expression. By contrast, cells co-transfected  
25 with GLuc mRNA did not lead to an improved GFP translation. Note that the percentage of GFP positive  
26 cells in each group remains the same. Fig.3. B confirmed the increased amount of NS1 proteins produced  
27 as a function of mRNA used for the transfection. The kinetic profiles of GFP produced in cells transfected  
28 with either GFP mRNA/NS1 mRNA or GFP mRNA/GLuc mRNA were similar with a peak of expression  
29 occurring between 12- and 24-hours post-transfection, and followed by a dramatic drop of the protein  
30 expression after 72 hours in both conditions. However, in the presence of NS1, the number of produced  
31 GFP copies was increased as evidenced by the 1.4-fold increase of the MFI. Such NS1-mediated  
32 enhancement lasted for 72h.

1 Cell transfection with non-chemical modified IVT mRNA is known to activate host cell immune response.  
2 Therefore, we assessed the effect of NS1 expression on the IVT mRNA-induced host immune responses in  
3 comparison with the use of chemically modified mRNA (GFP (50% $\psi$ )) (Fig.4). As expected, non-modified  
4 GFP mRNA (1 GFP) transfection significantly induced IFN $\alpha$  expression as well as other RNA sensors, i.e.  
5 IFN $\beta$ , PKR, RIG 1, IFIT 1, and OAS 1, whereas the presence of NS1 led to opposite effects (Fig. 4. B, C). It is  
6 worth to note that the NS1 mRNA was non-modified as well. In addition, when cells were transfected with  
7 mRNA mix comprising of 75% GFP and 25% NS1 mRNA (0.75 GFP/0.25 NS1), the expression level of RNA  
8 sensors was comparable to that obtained from GFP (50% $\psi$ ) transfection. Consequently, the co-delivery  
9 with NS1 mRNA enhanced GFP expression as observed in Fig. 4. D.

### 10 **3.3. NS1 promotes IVT mRNA translation regardless of gene context and cell type**

11 GFP, Fluc and Gluc reporter mRNAs were co-delivered with equal amount of NS1 mRNA in 4 murine (i.e.  
12 C2C12, 4T1, DC 2.4, C3H10T1/2) and 3 human (A549, U87-MG, HepG2) cell types (Fig. 5). The translation  
13 of GFP mRNA was enhanced only 1.2-fold in DC 2.4 and HepG2 cells, and 4.2-fold in A549 cells (Fig. 5. A),  
14 while the translation of Fluc mRNA was higher increased i.e. 10.5-fold in DC 2.4 cells and 175.3-fold in  
15 HepG2 cells (Fig. 5. B). The effect on Gluc mRNA translation was evaluated by measuring the Gluc activity  
16 both in the medium and inside the cells. The improvement ranged from 1.2-fold (A549 cells) to 3-fold (4T1,  
17 U87MG, C3H10T1/2 and C2C12 cells) for Gluc released in the medium (Fig. 5. C). Concerning Gluc activity  
18 inside the cells, the range of enhancement was from 2.0-fold (A549 and HepG2 cells) to 21.1-fold (C2C12  
19 cells) (Fig.5. D). Overall, those data indicated that the co-delivery of NS1 mRNA enhanced the level of  
20 translation of all the reporter genes tested and no inhibition was observed; the level of enhancement,  
21 however, was dependent on the cell type.

### 22 **3.4. Co-delivery of BMP2 mRNA with NS1 mRNA generates a high level of BMP-2**

23 C2C12-BRE/Luc cells were transfected with both BMP2 and NS1 mRNAs at different ratio (1 BMP2, 0.75  
24 BMP2/0.25 NS1, 0.5 BMP2/0.5 NS1, 0.25 BMP2/0.75 NS1 ), and the luciferase activity, which can be  
25 correlated with the production of BMP-2, was measured after 24, 48 and 72 hours post-transfection. Fig.  
26 6. A shows a clear benefit of 25% NS1 mRNA substitution (0.75 BMP2/0.25 NS1) throughout the measuring  
27 time. The luciferase activity from cells transfected with 0.75 BMP2/0.25 NS1 was significantly higher (2.7-  
28 fold at 24h; 3.5-fold at 48h; 4.7-fold at 72h) than in cells transfected with BMP2 mRNA alone (1 BMP2).  
29 The decay of luciferase activity obtained from the BMP2 group was quite drastic after 48h (65%) and 72h  
30 (91%) which was not the case for 0.75 BMP2/0.25 NS1 group (48h-54%; 72h-85%). Notably, even at one-  
31 third BMP2 mRNA dose (0.25 BMP2/0.75 NS1), the luciferase expression remained higher than full dose

1 of BMP2 mRNA alone at 48h and 72h post-transfection. Thus, the BMP2 mRNA to NS1 mRNA mass ratio  
2 of 3: 1 was used in further BMP-2 quantification and osteogenesis experiments.

3 Fig. 6. B shows the quantification of BMP-2 production from C3H10T1/2 pluripotent cells transfected with  
4 either BMP2 mRNA or BMP2/NS1 mRNAs. For both groups, BMP2 was extensively secreted in the first 24h  
5 post-transfection. Compared to BMP2 mRNA alone, BMP2/NS1 mRNAs generated 8.5-fold higher BMP2  
6 production in the first 24h, and further to 10.5-fold in the next 24 h.

### 7 **3.5. Dual delivery of BMP2 mRNA with NS1 mRNA favors osteogenic commitment of C3H10T1/2** 8 **pluripotent stem cells**

9 The osteogenic differentiation of C3H10T1/2 cells was evaluated from multiple aspects, i.e. osteoblastic  
10 gene expression, alkaline phosphatase (ALP) activity, and extracellular mineralization. For osteogenic  
11 induction, the C3H10T1/2 cells were transfected twice (Fig. 7. A), as single transfection did not induce the  
12 visible calcium deposition (data not shown). As shown in Fig. 7. C, cellular ALP activity gradually increased  
13 with time compared to non-transfected cells (NTrans). In cells transfected with NS1 mRNA, the increase  
14 was more significant at day 7 and day 14 compared to cells transfected with BMP2 mRNA alone. The ALP  
15 staining by BCIP/NBT at day 14 revealed that BMP2/NS1 transfected cells exhibited more intense blue-  
16 violet color than BMP2 mRNA transfected cells and NTrans cells confirming ALP quantification results (Fig.  
17 7. B). Twenty-eight days after the first transfection, the deposited calcium was stained with Alizarin red S  
18 (ARS). BMP2/NS1 mRNA transfected cells exhibited more calcium nodules than BMP2 mRNA transfected  
19 cells (Fig. 7. D). These qualitative images were validated with the quantification of ARS-stained calcium  
20 following dissolution of aggregates (Fig. 7. E). Both transfected groups showed a significantly higher  
21 calcium deposition in comparison to not transfected group (NTrans).

22 From the gene expression aspect, the osteogenic-related transcripts were quantified at different time  
23 points by RT-qPCR (Fig. 7. F). Compared to controls, BMP2/NS1 mRNAs transfection induced Runx2 and  
24 ALP expressions peaked at day 7, which were significantly higher than that induced by BMP2 mRNA  
25 transfection (Fig. 7. F-a and b). On day 14, ALP in BMP2/NS1 group still maintained a high expression and  
26 in line with the results shown in Fig. 7. C. For Runx2, no significant difference was observed at day 14 and  
27 day 21. In contrary to Runx2 and ALP transcripts, the transcripts of Collagen type 1 started only to increase  
28 on day 7, and were steady up to day 21 (Fig. 7. F-c). For OPN transcripts, the expression trend was similar  
29 to that of Runx2 and ALP. However, no significant differences were found between BMP2/NS1 group and  
30 BMP2 group (Fig. 7. F-d). OCN expression gradually increased over time, and same as OPN expression, the  
31 differences between BMP2/NS1 mRNAs and BMP2 mRNA transfected cells were not significant.

32

#### 1 4. Discussion

2 Different reports have shown the use of mRNA as a potent therapeutic for improving osteogenesis, but  
3 only using chemically modified equivalents [5, 40-42]. The goal of our study is to demonstrate that non-  
4 modified mRNA can promote osteogenic effects when combined with NS1 mRNA, encoding for an immune  
5 evasion protein derived from influenza A virus. Here, we adopted NS1 from A/Texas/36/1991 strain as IVT  
6 mRNA translation enhancer, which was proved to be the most potent than NS1 from other strains, i.e.  
7 A/Hong Kong/156/1997; A/Vietnam/1203/2004; A/Puerto Rico/8/1934; A/Texas/36/1991;  
8 A/California/04/2009; A/Shanghai/2013) [20].

9 Building the mRNA structure is one of the critical steps for mRNA therapy. A classical eukaryotic mRNA  
10 contains the 5' methylated guanosine cap, the 5' and 3' untranslated region (5'- and 3'-UTR), the open  
11 reading frame (ORF), and the poly(A) tail. It was shown that all parts of this structure have an impact on  
12 mRNA translation efficiency to some extent [5, 43]. For instance, we and other groups reported that mRNA  
13 containing longer poly(A) (e.g. 120nt) showed higher translation efficiency than that containing shorter  
14 poly(A) tail (e.g. 67nt) [44-46]. In line with this, we observed that BMP2-A150 mRNA resulted in higher  
15 protein expression than BMP2-A64 mRNA (data not shown). To optimize the protein expression, we  
16 generated mRNAs with different 3' UTRs (Fig. 1. A), and found that the mRNA with shorter 3' UTR resulted  
17 in higher protein expression (Fig. 1. B and C). This phenomenon was probably caused by differences in the  
18 stability of mRNA secondary structures. Indeed, *via* computation (RNAfold 2.4.13), BMP2-NotI mRNA  
19 forms a secondary structure different from BMP2-XbaI, wherein less base-pair probabilities at 3' of the  
20 mRNA (not shown).

21 As NS1 is a virus-derived protein, we explored whether its expression could induce a cytotoxic effect (Fig.  
22 2). We did not find significant cell viability differences between NS1 mRNA transfected cells and GFP mRNA  
23 transfected cells with the dose range used during this study. Interestingly, XTT results indicated that GFP  
24 mRNA-transfected cells started to recover at 48h post-transfection, while getting worse for NS1 mRNA  
25 transfected cells. However, we did not observe a significant increase of detached cells in NS1-treated wells.  
26 It was reported that during virus infection, NS1 could arrest the host cell at G0/G1 cycle to benefit virus  
27 replication [47]. Thus, we hypothesized that the reverse trend of XTT data could be due to NS1 cytostatic  
28 effect. Subsequently, we checked the cell cycle of treated cells, and found that the cell population in G0/G1  
29 state was greater in NS1 mRNA transfected cells than in GFP mRNA transfected cells (Supplementary Fig.  
30 3). So, the cell number decrease in NS1 transfected cells could be caused, at least partly, by this cytostatic  
31 activity.

1 The co-delivery of NS1 mRNA with GFP mRNA into C3H10T1/2 cells resulted in enhanced protein  
2 expression, thanks to RNA sensors inhibition (Fig. 3 and Fig. 4). Besides, this function is evidenced by the  
3 variation of intracellular GFP mRNA after co-delivery of GFP mRNA with NS1 mRNA or Gluc mRNA to  
4 C3H10T1/2 cells (S. Fig. 4). In the early stage (from 6h to 60h post-transfection), GFP/NS1 group preserved  
5 a higher GFP mRNA copy number than GFP/Gluc group. This is probably due to the inhibition of OAS/RNase  
6 L-mediated RNA degradation by NS1 (Fig. 4C-OAS1) [30]. While, from 60h to 160h, the trend is reversed.  
7 It is known that mRNA poly(A) tail is shortened along with mRNA translation leading to mRNA degradation  
8 [48, 49]. The lower GFP mRNA copy number in GFP/NS1 group could be a consequence of GFP mRNA  
9 extensive translation (Fig. 3C).

10 The incorporation of chemically modified nucleosides is also known to abrogate the IVT mRNA-induced  
11 immunogenicity. However, the effect is highly dependent on modification type/ratio, mRNA context, and  
12 cell type [19, 50]. For instance, Li *et al.*, reported that, in THP-1 macrophages, 5meC/ $\psi$  modified Fluc mRNA  
13 resulted in significantly higher Fluc expression, while 5meC/ $\psi$  modified eGFP mRNA resulted in a  
14 decreased GFP expression; me1 $\psi$  modified Fluc in THP-1 cells generated 8-fold more Fluc than that in  
15 hepatocellular carcinoma Hep 3B cells [34]. To check if NS1 mRNA could act as a versatile enhancer of  
16 mRNA translation, we co-delivered NS1 mRNA with different well-known reporter genes into several cell  
17 types (Fig. 5). By contrast to chemically modified mRNA, no negative effect was found in all tested  
18 conditions. Nevertheless, there were still variations in the translation efficacy depending on the gene of  
19 interest and the cell type. Specifically, Firefly luciferase mRNA expression was the most sensitive to the  
20 NS1 effect and, dendritic cells seemed to be more refractory likely because of its immune feature. But this  
21 must be studied more deeply. For the majority of cell types tested including mesenchymal cells, the  
22 enhancement was at least 2-fold which is already significant in terms of the amount of proteins produced.  
23 The opportunity cost of replacing BMP2 mRNA with NS1 mRNA was measured (Fig. 6). The mass ratio of 3  
24 to 1 of BMP2 mRNA to NS1 mRNA resulted in the highest BMP-2 expression. As for gene delivery, the  
25 mRNA dose is correlated with the amount of carrier, and to avoid the cytotoxicity, a dose limit of the  
26 carrier must be respected. In this strategy, the replacement of part of mRNA of interest with NS1 gave a  
27 higher transgene expression indicating no opportunity cost as described previously [20].

28 In our study, NS1 increased BMP-2 expression for up to 8-fold. Interestingly, even the expression of BMP-  
29 2 mRNA alone was higher (10 times more) compared to studies based on chemical modified BMP-2 mRNA  
30 [40, 41]. This phenomenon could be explained by the type of cell, the transfection reagents used and the  
31 difference in BMP2 mRNA sequences, i.e. UTRs and length of poly(A) tail, which could also dramatically  
32 influence mRNA performance [51]. The level of secreted BMP-2, however, remarkably decreased within 3

1 days due to the transient expression of mRNA. This finding is aligned with the GFP expression kinetic shown  
2 in Fig. 3C, and the data reported in the literature [28, 52], due to the inherent transient expression profile  
3 of mRNA.

4 As expected, secreted BMP-2 induced the expression of osteogenesis-related genes since this molecule is  
5 a potent osteogenic growth factor. The expressions of both early and late osteogenic genes were  
6 significantly enhanced following BMP2 mRNA transfection and were further improved when cells were co-  
7 transfected with both BMP2 mRNA and NS1 mRNA. Our data indicated that Runx2, the master osteoblastic  
8 transcriptional factor, was firstly upregulated under BMP-2 stimulation with a peak of expression at day 7  
9 post-transfection (Fig. 7. F-a). Then, there is a production of bone matrix proteins expressed at middle-to-  
10 late stages of osteoblastic maturation, i.e. ALP, Col1 $\alpha$ 1, OPN, and OCN (Fig. 7. F-b, c, d, e), all of them  
11 regulated by Runx2 through binding to their gene promoters [53]. Notably, osteocalcin was continuously  
12 expressed after 14 days (Fig. 7. F-e), which is known to promote the deposition of mineral substance (Fig.  
13 7. D-E) due to the presence of calcium-binding residues (di-carboxylic glutamyl (gla)). Outcomes from this  
14 study were comparable to those reported previously by others using chemically modified BMP2 mRNA  
15 (cmBMP2 mRNA) in terms of expression kinetics of osteogenic genes. It worth to note that the osteogenic  
16 commitment of C3H10T1/2 stem cells could benefit from the cytostatic effect of NS1 expression. We are  
17 aware that the calcium deposits obtained were not as high as expected (Fig. 7. D-E), indicating that the  
18 transient BMP-2 enhancement was not sufficient to generate a greater osteogenesis. However, these  
19 results are valuable considering the fact that they were obtained with C3H10T1/2 cells. Indeed, it is known  
20 that C3H10T1/2 hardly differentiate toward osteoblasts compared to murine tissue-derived primary  
21 mesenchymal stem cells (MSCs) which could spontaneously differentiate toward osteoblasts when  
22 cultured under osteogenic medium [54]. Compared to murine MSCs, human MSCs have lower  
23 responsiveness under BMP-2 stimulation [55, 56]. Previously, delivery of cmBMP2 mRNA demonstrated the  
24 ability to induce osteoblastic differentiation of human bone marrow derived MSCs (BMSCs) [40, 57]. The  
25 secreted BMP-2 from BMP2/NS1 mRNAs transfected cells is much higher than that from cmBMP2 mRNA  
26 transfected cells [40], indicating the the dual mRNA system has the potential to direct cell fate of human  
27 BMSc. Our effort is now focused on developing mRNA-based platform enabling long-term BMP-2  
28 expression (e.g. mRNA activated matrix) and, applying this strategy *in vivo* to heal non-union bone fracture,  
29 which is a challenging public health issue with the aging of human population.

30  
31  
32

## 1 **5. Conclusion**

2 Overall, results from this study demonstrated for the first time that dual delivery of non-modified BMP2  
3 mRNA with NS1 mRNA as translation enhancer is a potential therapeutic since it allows a high expression  
4 of BMP-2 that results in the improvement of osteogenic genes expression. The strategy could be used as  
5 an alternative of chemically modified mRNA as the production of BMP-2 is higher than obtained with  
6 reported previously.

## 8 **Acknowledgments**

9 We are grateful to David Gosset of the P@CYFIC platform (CBM, CNRS Orléans) for technical help in flow  
10 cytometry experiments. We would like to thank Dr. Kyle K.L. Phua for his helpful discussion about the NS1  
11 strategy. We are indebted to by Region Centre Val de Loire, CNRS and University of Orléans that supported  
12 this work. PW is a recipient of a CSC grant from China.

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