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Original Article

Mesenchymal stem cell engineering by ARCA analog-capped mRNA

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We previously have shown that mRNA-based engineering may enhance mesenchymal stem cell (MSC) trafficking. However, optimal conditions for *in vitro* mRNA engineering of MSCs are unknown. Here, we investigated several independent variables: (1) transfection factor (Lipofectamine 2000 vs. TransIT), (2) mRNA purification method (spin column vs. high-performance liquid chromatography [HPLC] column), and (3) mRNA capping (ARCA vs. β-S-ARCA D1 and β-S-ARCA D2). Dependent variables included protein production based on mRNA template (measured by the bioluminescence of reporter gene luciferase over hours), MSC metabolic activity corresponding with their wellbeing measured by CCK-8 over days, and endogenous expression of genes by RT-qPCR related to innate intracellular immune response and decapping at two time points: days 2 and 5. We have found that Lipofectamine 2000 outperforms TransIT, and used it throughout the study. Then, we showed that mRNA must be purified by HPLC to be relatively neutral to MSCs in terms of metabolic activity and endogenous protein production. Ultimately, we demonstrated that β-S-ARCA D1 enables higher protein production but at the cost of lower MSC metabolic activity, with no impact on RT-qPCR results. Thus Lipofectamine 2000-based *in vitro* transfection of HPLC-purified and ARCA- or β-S-ARCA D1-capped mRNA is optimal for MSC engineering.

INTRODUCTION

Aging and a sedentary lifestyle contribute to the epidemic of a number of diseases that are thought to be related to tissue wear and malfunction and are not easily managed by pharmacology and surgery. Recent advances in stem cell biology have made tissue regeneration a realistic and approachable target. Cell therapy can be applied independently or to enhance existing treatments. Mesenchymal stem cells (MSCs) are known for their universally supportive role and participation in reparative processes; thus, they are well suited for regenerative medicine.[1–3]

Despite the enormous amount of scientific work showing the positive effect of MSCs on regeneration processes, their action often turns out to be insufficient to obtain a clinical effect.[4] Therefore, emboldening their function using clinically applicable cell engineering methods may be a promising approach. Furthermore, we and others showed MSCs to be particularly resistant to plasmid-based modification, whereas virus-based methods are burdened by ambiguous safety profiles.[5] On the contrary, the mRNA-based modification of MSCs seems to meet cell modification's needs for clinical applications perfectly. mRNA-based cell modification is an integration-free method that can activate immune cells' response, restore cells' physiological functions, and even provide them with new functionalities.[6] In MSC-related research, mRNA has been used to stimulate their reprogramming, e.g., a cell with cardiac-like phenotype,[7] or to enhance its delivery to the target organ, as shown for CXCR2 mRNA-engineered MSC transplantation in a mouse model of inflammatory bowel disease.[8] In our previous research, we used mRNA-ITGA4 to induce the overexpression of integrin-α4 in MSCs. Modified cells were characterized by increased docking rate in an *in vitro* model of inflamed endothelium, as well as by enhanced accumulation in the blood vessels crossing the area of brain lesion *in vivo* after intra-arterial transplantation in a rat model of stroke.[9] This shows that, using an mRNA-based modification of MSCs, we can obtain the overexpression of a functional protein product. However, in our study, both MSC conditions and immunogenicity were compromised by mRNA transfection. Interestingly, recent research has shown that the mRNA cap's structure can considerably impact transfected cells' cellular response.[10]

Physiologically, nascent pre-mRNA undergoes a set of post-transcriptional modifications. Their successful completion is indispensable for generating a functional, mature mRNA that can then engage in translation—the ultimate stage of gene expression. Such changes include splicing, polyadenylation, and capping. Biosynthesis of the

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7-methylguanosine constituent linked by a 5′5′-triphosphate bridge on the 5′ end of maturating mRNA, or capping, is crucial for protecting mRNA against degradation, identifying it as self and thereby preventing the host's immune response and further recruitment of translation factors.[11]

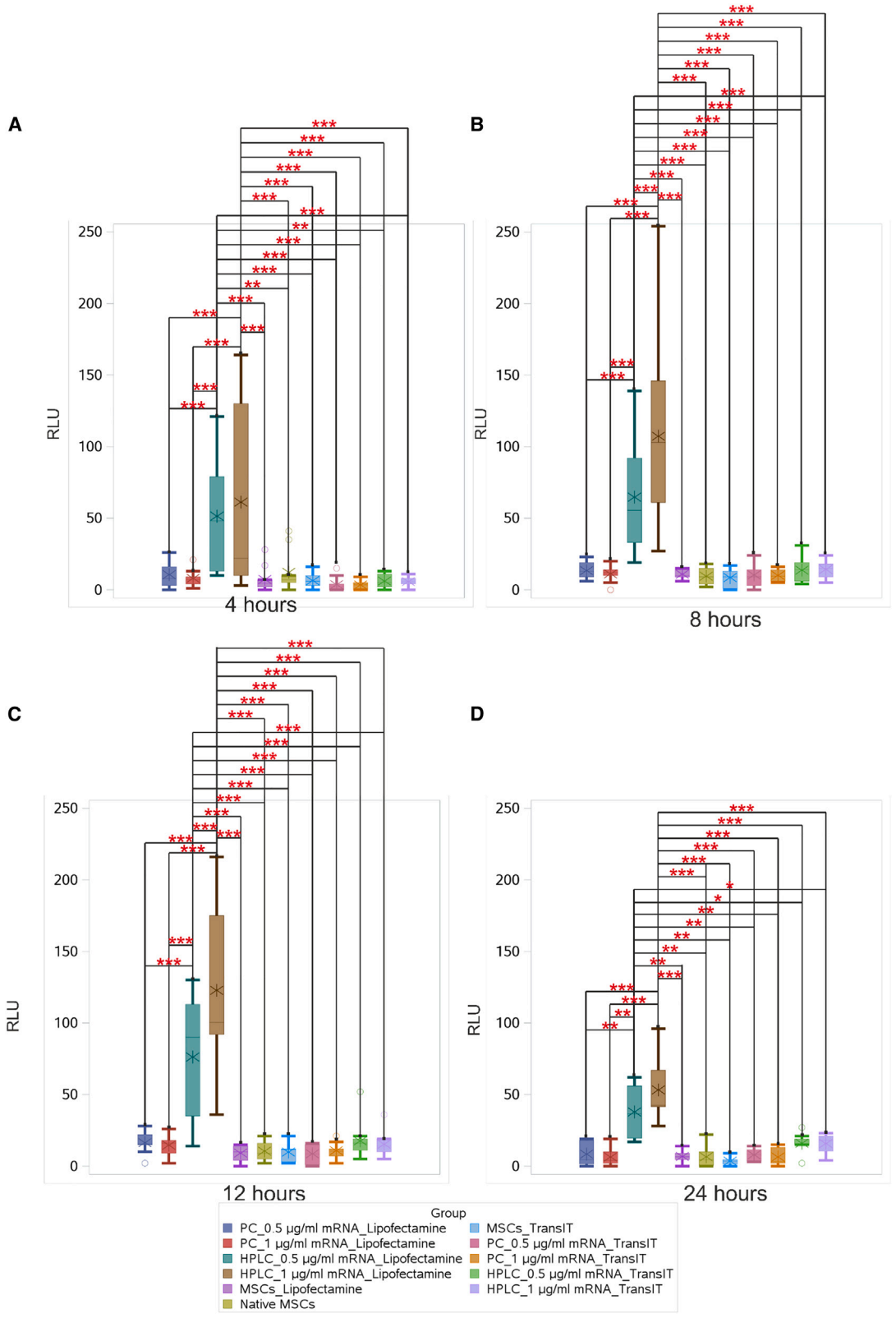
During the *in vitro* mRNA synthesis process, in only 50% are standard cap analogs incorporated in the correct orientation. Because of polymerase nucleophilic attack by the 3′-OH of the Guo and m^7Guo moieties of the cap analog, one-half of the mRNA particles undergo elongation in the undesired direction, which makes them non-functional. Therefore, in our previous work, to improve the efficiency of the MSCs transfection process, we used an analog of the cap, $m_2^{7,3'-O}$GpppG (anti-reverse cap analogs [ARCA]), which guarantees its incorporation only in the physiological orientation during mRNA synthesis. Consequently, it enables the correct recognition and initiation of the protein translation process.[12] Interestingly, methylation at the 3′-*O* position of m^7Guo also affects interactions with other proteins binding the 5′ end of mRNA in the cell. Presence of this modification weakens binding to the interferon-induced protein with tetratricopeptide repeats (IFIT1), which makes mRNA less sensitive to translational blocking by the innate immune system. Kinetic analysis of IFIT1 and IFIT5 interactions with different native and engineered RNAs had its consequences for designing mRNA-based therapeutics.[13] ARCA cap analog was a critical modification that allowed the induction of ITGA4 mRNA expression in MSC. Also the mRNA used in coronavirus disease 2019 (COVID-19) prophylactic vaccines was methylated at the 3′-*O* position of m^7Guo, further confirming the benefit of this modification. Currently, a much more comprehensive range of mRNA cap analogs is available. The introduction of additional changes in cap structure can provide superior translation efficiency, higher stability of mRNA, protection from digestion by endonuclease, and decreased immunogenicity.[14–16] Some of the most promising ARCA cap analogs were created by substitutions of oxygen at either the α, β, or γ position of the triphosphate chain by single phosphorothioate modification. In this reaction, each analog is obtained as a mixture of two diastereomers, D1 and D2, which can be separated by high-performance liquid chromatography (HPLC). This modification stabilizes the connection between eukaryotic translation initiation factor 4E and the cap and increases the resistance of mRNA to hydrolysis by decapping enzymes.[17] In eukaryotes, m7GpppN-mRNA hydrolase (DCP2), m7GpppX diphosphatase (DCPS), and diphosphoinositol polyphosphate phosphohydrolase 1 are the most commonly investigated enzymes known to decap mRNA. When improperly caped mRNA is present inside cells, they activate signaling pathways analogous to those induced by viral infection. The procedure of preparing mRNA *in vitro* is fraught with formation of various types of by-products. These include impurities such as double-stranded RNA (dsRNA), which are highly immunogenic and must be removed for the molecule to fulfill its function inside the cell and prevent immunogenicity of *in vitro*-transcribed RNA.[18] The presence of dsRNA in the preparation as well as the lack of a full set of modifications at the 5′ end of the RNA activate pattern recognition receptors like Melanoma differentiation-associated protein 5 (MDA5), Nucleotide binding oligomerization domain containing 2 (NOD2), or Retinoic acid-inducible gene 1 protein, expression of proteins induced by interferon (IFIT-1, Interferon-induced GTP-binding protein Mx1, and 2′-5′-oligoadenylate synthase 1 [OAS1], and Interferon-induced, dsRNA-activated protein kinase [PKR]), and enzymes degrading RNAs (e.g., 2-5A-dependent ribonuclease).[11,19] All these elements are characteristic of the innate immune response of cells. However, MSCs have not been studied before for their decapping process and immune responses upon transfection with *in vitro* transcription (IVT) mRNAs and HPLC-purified mRNAs.

Thus, in this study, our goal was to develop a robust, well-tolerated by cells and low-immunogenic method of MSC engineering by mRNA. To accomplish this task, we used different protocols for mRNA purification, concentrations, and transfection, as well as compared the effects of a few cap analogs, including $m_2^{7,3'-O}$GpppG (ARCA),[15] β-S-ARCA D1, and β-S-ARCA D2[20] on the level of transcript translation, MSCs metabolic activity, and expression of genes involved in the process of decapping and immune response.

RESULTS

The impact of mRNA preparation, concentration, and transfection method on protein production efficiency in transfected MSCs

To maximize protein expression in transfected MSCs, initially we compared the two protocols for mRNA purification, two different mRNA concentrations, and two transfection methods. All experiment variants used mRNA containing "anti-reverse" 3′ARCA cap analog (ARCA) with pseudouridine moieties. ARCA only incorporates cap analogs into mRNA in the correct orientation.[15] We used reporter mRNA coding firefly luciferase enzyme to allow exact measurements of protein expression. Luciferase catalyzes luciferin oxidation leading to bioluminescence, which is easily measurable and directly correlates with luciferase expression.[20] Luminescence readouts were performed across four time points: 4, 8, 12, and 24 h after mRNA transfection. Next, the purification of mRNA was performed using either NucleoSpin RNA Clean-Up purification columns (PC) or HPLC with a dedicated reversed-phase column. The mRNA was tested in two concentrations: 0.5 and 1 μg/mL. Finally, mRNA transfection was performed based on either TransIT-mRNA ("TransIT") or Lipofectamine 2000 reagent ("Lipofectamine"). To facilitate cross-comparison between groups, each group received a unique identifier, consisting of (1) readout time point (4, 8, 12, or 24 h), then an underscore, followed by (2) mRNA purification protocol (PC or HPLC), and subsequently by an underscore, followed by (3) mRNA concentration (0.5 or 1 μg/mL mRNA), and by an underscore, followed by (4) transfection method (TransIT or Lipofectamine). Native, non-transfected MSCs and MSCs with sole transfection reagents (TransIT-mRNA or Lipofectamine 2000), hereinafter referred to as "native MSCs," "MSCs_TransIT," and "MSCs_Lipofectamine," respectively, with



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each name preceded by the readout time point and an underscore, were treated as controls for the above experiments.

Our studies showed that luminescence was significantly enhanced at each time point when mRNA was purified by HPLC, in comparison with PC and regardless of mRNA concentration, albeit only when Lipofectamine 2000 was used for transfection (4h_HPLC_0.5 μg/mL mRNA_Lipofectamine vs. 4h_PC_0.5 μg/mL mRNA_Lipofectamine: $p < 0.001$; 8h_HPLC_0.5 μg/mL mRNA_Lipofectamine vs. 8h_PC_0.5 μg/mL mRNA_Lipofectamine: $p < 0.001$; 12h_HPLC_0.5 μg/mL mRNA_Lipofectamine vs. 12h_PC_0.5 μg/mL mRNA_Lipofectamine: $p < 0.001$; 24h_HPLC_0.5 μg/mL mRNA_Lipofectamine vs. 24h_PC_0.5 μg/mL mRNA_Lipofectamine: $p = 0.0049$; 4h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 4h_PC_1 μg/mL mRNA_Lipofectamine: $p < 0.001$; 8h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 8h_PC_1 μg/mL mRNA_Lipofectamine: $p < 0.001$; 12h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 12h_PC_1 μg/mL mRNA_Lipofectamine: $p < 0.001$; 24h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 24h_PC_1 μg/mL mRNA_Lipofectamine: $p < 0.001$; Figures 1 and 2A–2D). Transfection using TransIT-mRNA had no significant effect on luminescence intensity each time, regardless of the purification protocol and mRNA concentration, in comparison with the control groups (Figures 1 and 2H–2K). In comparison with 0.5 μg/mL, the 1 μg/mL mRNA concentration enhanced luminescence readouts at the 8- and 12-h time points, although only when HPLC was used for purification and Lipofectamine 2000 was used for transfection (8h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 8h_HPLC_0.5 μg/mL mRNA_Lipofectamine: $p < 0.001$; 12h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 12h_HPLC_0.5 μg/mL mRNA_Lipofectamine: $p < 0.001$). Most importantly, transfection of HPLC-purified mRNA at 1 μg/mL concentration using Lipofectamine 2000 generated consistent, strong luminescence readouts across the experiment, which increased from the 4-h time point (4h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 8h_HPLC_1 μg/mL mRNA_Lipofectamine: $p < 0.001$), reached a plateau between the 8- and 12-h time points (8h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 12h_HPLC_1 μg/mL mRNA_Lipofectamine: $p = 0.0966$), and decreased beyond the 12-h time point (12h_HPLC_1 μg/mL mRNA_Lipofectamine vs. 24h_HPLC_1 μg/mL mRNA_Lipofectamine: $p < 0.001$). Native MSCs, MSCs_TransIT, and MSCs_Lipofectamine generated comparable, weak luminescence signals at each time point (Figures 1 and 2E–2G).

To summarize, we found that the luminescence signal was strongest when mRNA was purified by HPLC and transfected into MSCs at 1 μg/mL concentration using Lipofectamine 2000 Transfection Reagent. Therefore, we followed the same protocol in further experiments.

The influence of different 5′ cap analogs on protein expression pattern and efficiency in transfected MSCs

Next, we used the same firefly luciferase mRNA-based reporter expression system to choose between ARCA and two derivatives of ARCA analogs: $m_2^{7,2'-O}$GppSpG D1 (β-S-ARCA D1) and $m_2^{7,2'-O}$GppSpG D2 (β-S-ARCA D2) that not only provide incorporation of cap analogs into mRNA only in the correct orientation,[12] but also provide resistance to decapping enzymes prolonging mRNA half-life.[17] Finally, we tested HPLC-purified mRNAs capped with the ARCA mentioned above caps containing pseudouridine moiety. Depending on the cap analog used—β-S-ARCA D1 or β-S-ARCA D2—and the purification process applied to the mRNA molecule hereafter are referred to in this work as "β-S-ARCA_D1_HPLC" and "β-S-ARCA_D2_HPLC," respectively. All mRNAs were transfected into MSCs at 1 μg/mL concentration using Lipofectamine 2000 Transfection Reagent, as described above. Native, non-transfected MSCs ("native MSCs") and MSCs with sole Lipofectamine 2000 Transfection Reagent ("MSCs_Lipofectamine") were treated as controls for the above experiments. Luminescence readouts were performed across six time points: 4, 8, 12, 24, 36, and 48 h after mRNA transfection. To facilitate cross-comparison between groups, the name of each group was preceded by the name of a relevant readout time point (4, 8, 12, 24, 36, or 48 h) and an underscore.

Consistent with our previous results, we again observed a markedly stronger luminescence signal when mRNA was subjected to HPLC-based purification, in comparison to PC (4h_ARCA_HPLC vs. 4h_ARCA_PC: $p < 0.001$; 8h_ARCA_HPLC vs. 8h_ARCA_PC: $p < 0.001$; 12h_ARCA_HPLC vs. 12h_ARCA_PC: $p < 0.001$). However, transfection using ARCA_PC had no significant effect on luminescence readouts, even compared with the control groups, regardless of the time point (Figures 3 and 4A). Meanwhile, we noticed a consistent, vigorous luminescence intensity across the 4-, 8-, and 12-h time points for β-S-ARCA_D1_HPLC, β-S-ARCA_D2_HPLC, and ARCA_HPLC, in comparison with control groups (Figures 3 and 4B–4D). Interestingly, at the 8-h time point, we observed a significant signal increase in favor of β-S-ARCA_D1_HPLC (8h_β-S-ARCA_D1_HPLC vs. 8h_ARCA_HPLC: $p < 0.001$; 8h_β-S-ARCA_D1_HPLC vs. 8h_β-S-ARCA_D2_HPLC: $p = 0.0089$; 8h_β-S-ARCA_D2_HPLC vs. 8h_ARCA_HPLC: $p = 0.2609$). At the 12-h time point, β-S-ARCA_D1_HPLC continued to generate a stronger signal than ARCA_HPLC (12h_β-S-ARCA_D1_HPLC vs. 12h_ARCA_HPLC: $p < 0.001$), but the previous difference between β-S-ARCA_D1_HPLC and β-S-ARCA_D2_HPLC became insignificant (12h_β-S-ARCA_D1_HPLC vs. 12h_β-S-ARCA_D2_HPLC: $p = 0.1159$). Most notably, however, β-S-ARCA_D1_HPLC was the only tested analog providing a consistent, statistically significant

Figure 1. The impact of mRNA preparation, concentration, and transfection method on protein expression efficiency in transfected MSCs, groups within time points

MSCs were transfected with mRNA coding firefly luciferase. Luminescence readouts were performed across four time points: 4, 8, 12, and 24 h after mRNA transfection (A, B, C, D, respectively). Purification of mRNA was performed using either PC or HPLC. The mRNA was tested in two concentrations: 0.5 μg/mL or 1 μg/mL. Transfection was performed based on either TransIT-mRNA or Lipofectamine 2000 reagent. The following symbols of the level of statistical significance were adopted: $p < 0.05$*; 0.01**; <0.001***; absence of symbol indicates no statistical significance ($p > 0.05$). RLU, relative light units.

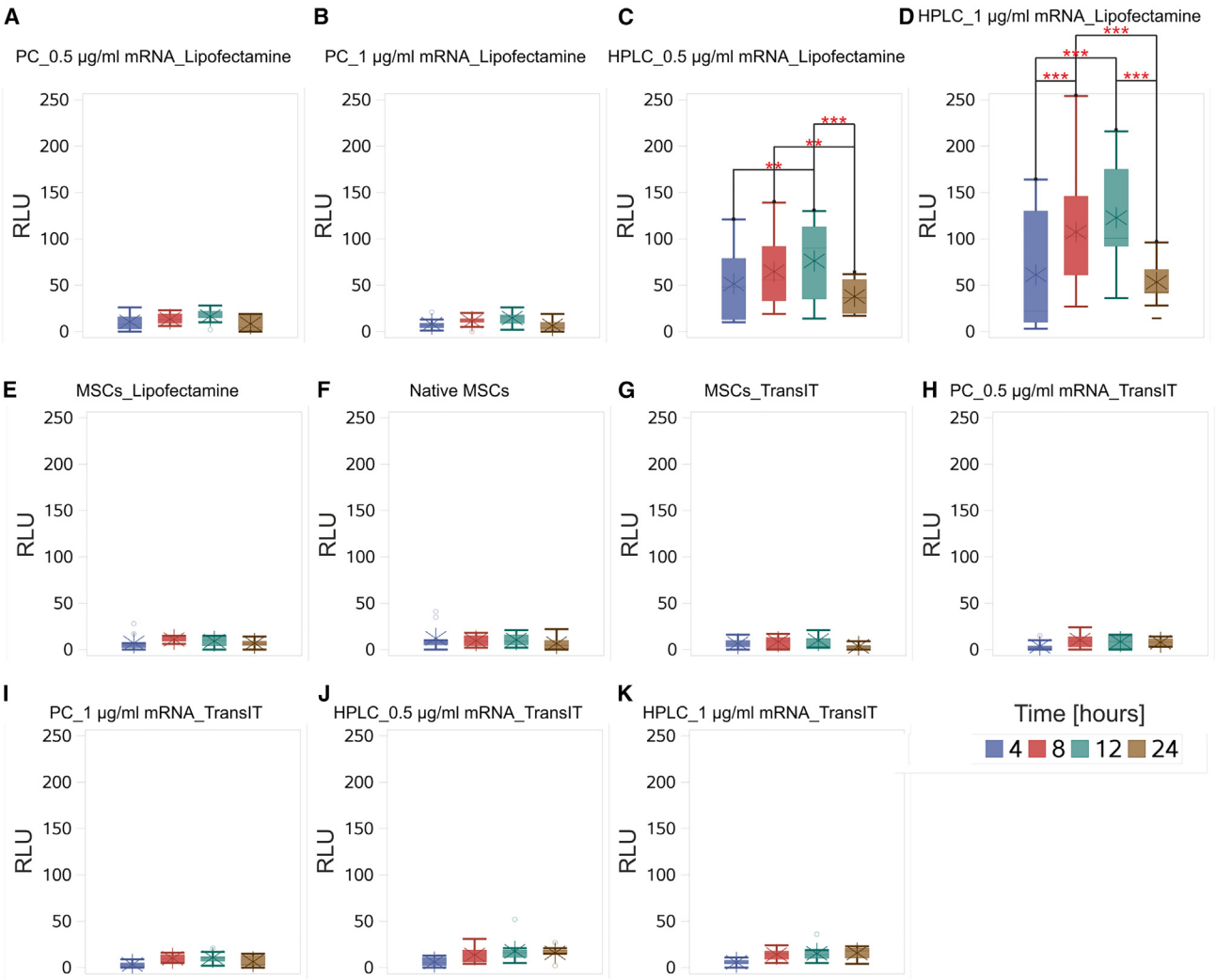

Figure 2. The impact of mRNA preparation, concentration, and transfection method on protein expression efficiency in transfected MSCs, time points within groups

MSCs were transfected with firefly luciferase coding mRNA. Luminescence readouts were performed across four time points: 4, 8, 12, and 24 h after mRNA transfection. Purification of mRNA was performed using either PC or HPLC. The mRNA was tested in two different concentrations: 0.5 or 1 µg/mL. Transfection was performed based on either TransIT-mRNA or Lipofectamine 2000 reagent. (A–K) Groups. The following symbols of the level of statistical significance were adopted: 0.01**; <0.001***; absence of symbol indicates no statistical significance ($p > 0.05$). RLU, relative light units.

luminescence enhancement across the experiment, both between the 4- and 8-h time points (4h_β-S-ARCA_D1_HPLC vs. 8h_β-S-ARCA_D1_HPLC: $p < 0.001$), as well as between the 8- and 12-h time points (8h_β-S-ARCA_D1_HPLC vs. 12h_β-S-ARCA_D1_HPLC: $p = 0.0081$). Of note, no significant differences in luminescence readouts were observed between any of the six groups beyond the 12-h time point (Figures 3D–3F and 4). Native MSCs and MSCs_Lipofectamine generated comparable, weak luminescence signals at each time point (Figures 3, 4E, and 4F).

In conclusion, we observed strong bioluminescence readouts in the luciferase mRNA-based assay with MSCs transfected with mRNAs capped with ARCA caps containing pseudouridine moiety and sulfur atom in β position in D1 and D2 diastereoisomer, with the D1 diastereoisomer generating a more consistent signal enhancement across the experiment.

Metabolic activity of MSCs transfected with mRNA capped with different 5′ cap analogs

Finally, we assessed the influence of the transfection procedure itself on MSCs' condition by measuring their metabolic activity. To this end, we performed a sensitive cholecystokinin (CCK)-8 colorimetric assay based on reducing water-soluble tetrazolium 8 by dehydrogenase activities in living cells. Upon reduction, tetrazolium

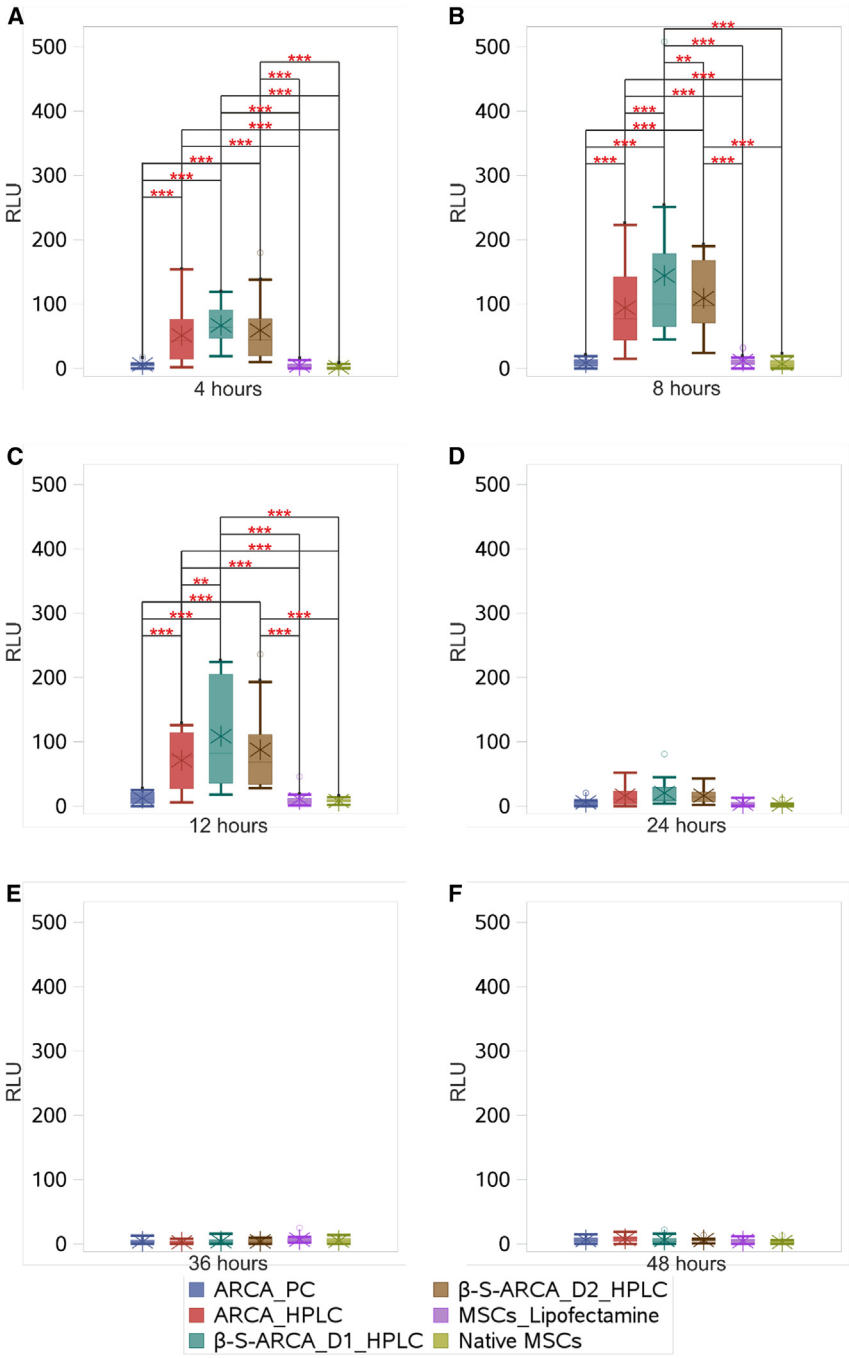

Figure 3. Different 5′ cap analogs influence protein expression pattern and efficiency in transfected MSCs, groups within time points

MSCs were transfected with firefly luciferase coding mRNA. Luminescence readouts were performed across six time points: 4, 8, 12, 24, 36, and 48 h after mRNA transfection (A, B, C, D, E, and F, respectively). In addition, MSCs were transfected with HPLC-purified mRNAs capped with ARCA caps containing pseudouridine moiety and sulfur atom in β position in two versions: diastereoisomers D1 and D2. The two mRNAs were also compared with mRNAs capped with unmodified ARCA purified by either HPLC or PC. Native, non-transfected MSCs and MSCs with sole Lipofectamine 2000 Transfection Reagent were treated as controls. The following symbols of the level of statistical significance were adopted: 0.01**; <0.001***; absence of symbol indicates no statistical significance ($p > 0.05$). RLU, relative light units.

salt generates a yellow formazan dye, directly proportional to cells' metabolic activity.[21] As done previously, we compared HPLC-purified mRNAs capped with ARCA caps containing pseudouridine moiety and sulfur atom in β position in two versions: diastereoisomers D1 ("β-S-ARCA_D1_HPLC") and D2 ("β-S-ARCA_D2_HPLC"), and mRNAs capped with unmodified ARCA purified by either HPLC ("ARCA_HPLC") or PC ("ARCA_PC"). Native, non-transfected MSCs ("native MSCs") and MSCs with sole Lipofectamine 2000 Transfection Reagent ("MSCs_Lipofectamine") were treated as controls for the above experiments. Transfected and control MSCs were subjected to absorbance measurements across 7 consecutive days, with a 24-h interval between readouts. To facilitate cross-comparison between groups, the name of each group was preceded by the name of a relevant readout time point (d1, d2, d3, d4, d5, d6, or d7) and an underscore.

We observed the first significant differences on day 2 when, expectedly, ARCA_HPLC started demonstrating significant superiority over ARCA_PC (d2_ARCA_HPLC vs. d2_ARCA_PC: $p = 0.0206$), which was consistent throughout the experiment (Figures 5 and 6A). No differences were noticed on day 2 between ARCA_HPLC, β-S-ARCA_D1_HPLC and β-S-ARCA_D2_HPLC (d2_ARCA_HPLC vs. d2_β-S-ARCA_D1_HPLC: $p = 0.6315$; d2_ARCA_HPLC vs. d2_β-S-ARCA_D2_HPLC: $p = 0.2272$; d2_β-S-ARCA_D1_HPLC vs. d2_β-S-ARCA_D2_HPLC: $p = 0.4657$). Surprisingly, however, no differences were observed on day 2 between D1 and D2 diastereoisomers and ARCA_PC (d2_ARCA_PC vs. d2_β-S-ARCA_D1_HPLC: $p = 0.0653$; d2_ARCA_PC vs. d2_β-S-ARCA_D2_HPLC: $p = 0.2631$). On day 3, HPLC-based mRNA purification again translated to a stronger absorbance than PC (d3_ARCA_HPLC vs. d3_ARCA_PC: $p < 0.001$). Interestingly, the difference specifically between ARCA and β-S-ARCA D1, and not β-S-ARCA D2, diminished (d3_ARCA_HPLC vs. d3_β-S-ARCA_D1_HPLC: $p = 0.1759$; d3_ARCA_HPLC vs. d3_β-S-ARCA_D2_HPLC: $p = 0.0093$), although no difference was observed between D1 and D2 diastereoisomers (d3_β-S-ARCA_D1_HPLC vs.

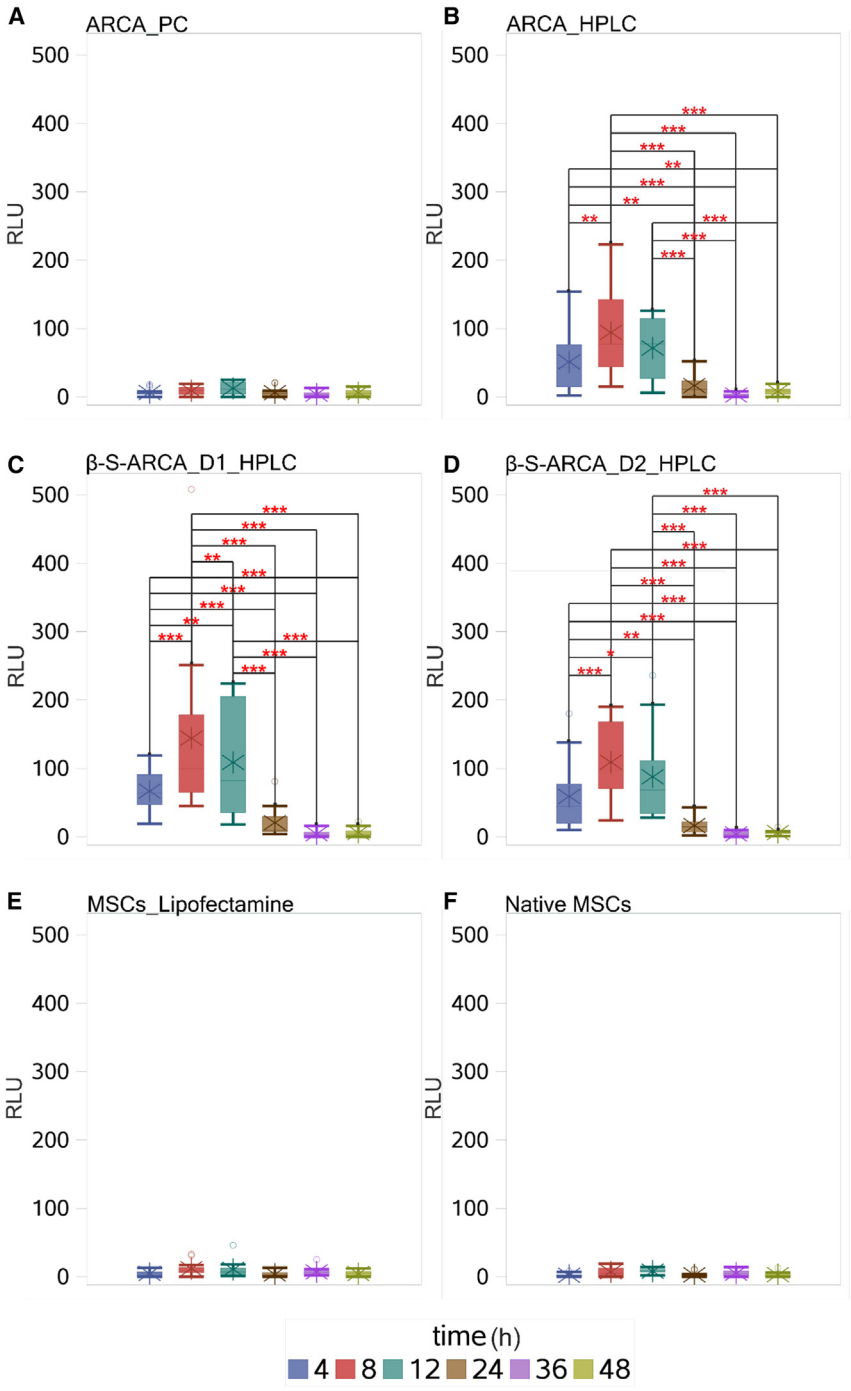

Figure 4. Different 5′ cap analogs influence protein expression pattern and efficiency in transfected MSCs, time points within groups

MSCs were transfected with firefly luciferase coding mRNA. Luminescence readouts were performed across six time points: 4, 8, 12, 24, 36, and 48 h after mRNA transfection. MSCs were transfected with HPLC-purified mRNAs capped with ARCA caps containing pseudouridine moiety and sulfur atom in β position in two versions: diastereoisomers D1 and D2. The two mRNAs were also compared with mRNAs capped with unmodified ARCA purified by either HPLC or PC. Native, non-transfected MSCs and MSCs with sole Lipofectamine 2000 Transfection Reagent were treated as controls. (A–F) Groups. The following symbols of the level of statistical significance were adopted: $p < 0.05$*; 0.01**; <0.001***; absence of symbol indicates no statistical significance ($p > 0.05$). RLU, relative light units.

d3_β-S-ARCA_D2_HPLC: p = 0.2064). However, on day 4, ARCA_HPLC again started demonstrating a more prominent absorbance than both D1 and D2 diastereoisomers, and no difference was observed between D1 and D2 diastereoisomers (d4_ARCA_HPLC vs. d4_β-S-ARCA_D1_HPLC: p = 0.0095; d4_ARCA_HPLC vs. d4_β-S-ARCA_D2_HPLC: p < 0.001; d4_β-S-ARCA_D1_HPLC vs. d4_β-S-ARCA_D2_HPLC: p = 0.4423). This result was mostly repeated on the next day (d5), only this time, the D1 diastereoisomer demonstrated a stronger absorbance than D2 (d5_ARCA_HPLC vs. d5_β-S-ARCA_D1_HPLC: p = 0.0065; d5_ARCA_HPLC vs. d5_β-S-ARCA_D2_HPLC: p < 0.001; d5_β-S-ARCA_D1_HPLC vs. d5_β-S-ARCA_D2_HPLC: p = 0.0058). On day 6, however, D1 and D2 diastereoisomers were, again, undistinguishable, unlike ARCA versus both D1 and D2 diastereoisomers (d6_β-S-ARCA_D1_HPLC vs. d6_β-S-ARCA_D2_HPLC: p = 0.1313; d6_ARCA_HPLC vs. d6_β-S-ARCA_D1_HPLC: p = 0.0037; d6_ARCA_HPLC vs. d6_β-S-ARCA_D2_HPLC: p < 0.001). Finally, on day 7, the D1 diastereoisomer demonstrated a slightly stronger absorbance than D2 (d7_β-S-ARCA_D1_HPLC vs. d7_β-S-ARCA_D2_HPLC: p = 0.0441). Both stereoisomers had weaker absorbance than ARCA_HPLC, only this time, the difference between ARCA and D2 was much more profound than between ARCA and D1 (d7_ARCA_HPLC vs. d7_β-S-ARCA_D1_HPLC: p = 0.0172; d7_ARCA_HPLC vs. d7_β-S-ARCA_D2_HPLC: p < 0.001). Interestingly, a comparison between the adjacent time points revealed that both ARCA_HPLC and β-S-ARCA_D1_HPLC had shown significant absorbance enhancement between days 4 and 5 and days 5 and 6, unlike β-S-ARCA_D2_HPLC with enhancement only between days 5 and 6 (Figures 6B–6D). Of note, ARCA had enhanced absorbance also between days 3 and 4; meanwhile, D1 had enhanced absorbance between days 6 and 7 (Figures 6B and 6C). Native MSCs and MSCs_Lipofectamine demonstrated comparable, strong absorbance between the second and seventh days of the experiment (Figures 5B–5G, 6E, and 6F). Compared with the control, non-

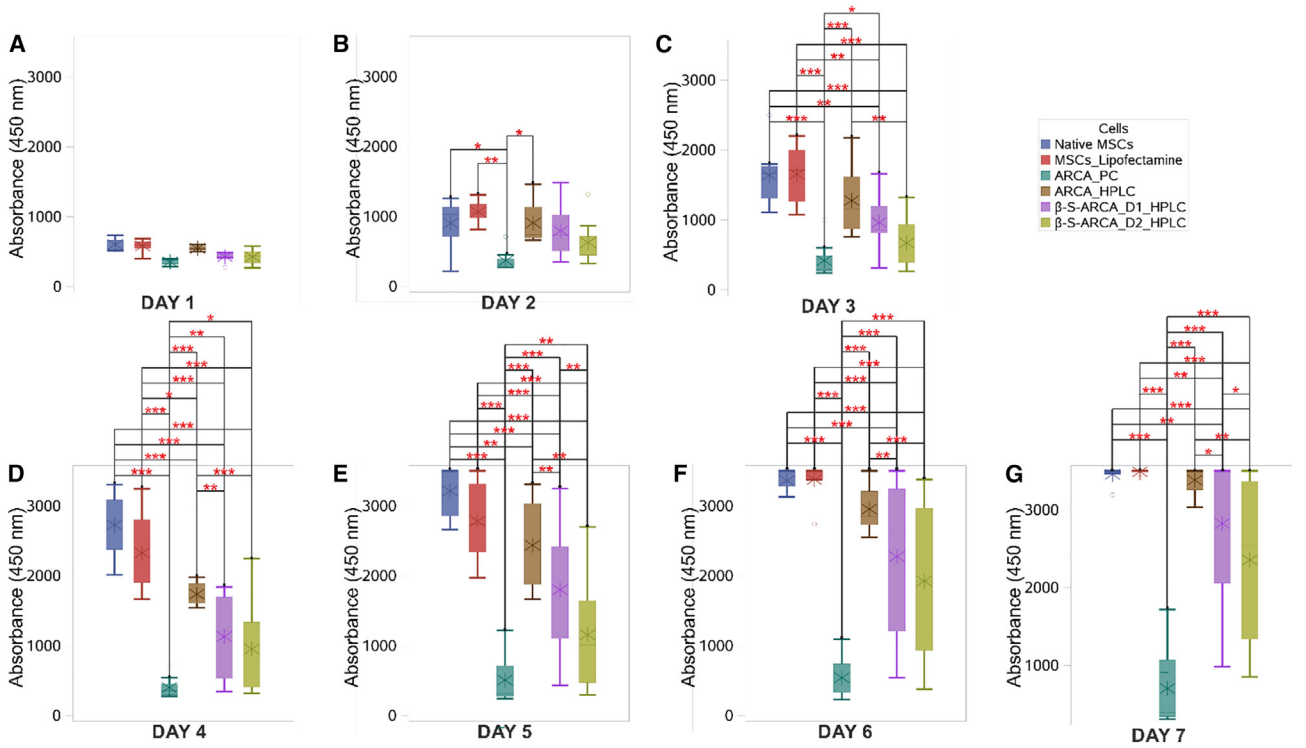

Figure 5. Metabolic activity of MSCs transfected with mRNA capped with different 5′ cap analogs, groups within time points

Absorbance readouts were performed daily across 7 days (A, B, C, D, E, F, and G). MSCs were transfected with HPLC-purified mRNAs capped with ARCA caps with pseudouridine moiety and sulfur atom in β position in two versions: diastereoisomers D1 and D2. The two mRNAs were also compared with mRNAs capped with unmodified ARCA purified by either HPLC or PC. Native, non-transfected MSCs and MSCs with sole Lipofectamine 2000 Transfection Reagent were treated as controls. The following symbols of the level of statistical significance were adopted: $p < 0.05$*; 0.01**; <0.001***; absence of symbol indicates no statistical significance ($p > 0.05$).

transfected groups, ARCA, diastereoisomer D1, and diastereoisomer D2 had weaker absorbances across the experiment between days 2 and 7 (Figures 5B–5G and 6B–6D).

To sum up, MSC transfection with all used cap analogs resulted in a decreased metabolic activity of cells at some point. However, the technique used to purify the mRNA significantly impacted the severity of the observed impairment. Cells transfected with HPLC-purified mRNA showed substantially higher metabolic activity than those transfected with column-purified material. MSCs transfected with ARCA-capped mRNA purified by HPLC reached the values of metabolic activity closest to the native cells. At the end of the experiment, ARCA-capped mRNA purified by HPLC demonstrated higher metabolic activity than D1 and D2 diastereoisomers. Moreover, MSCs transfected with D1 diastereoisomer had stronger absorbance than MSCs transfected with D2 diastereoisomer.

Human bone-marrow derived MSCs cellular response after transfection of differently capped mRNAs

To assess cellular responses, we transfected human bone-marrow derived (hBM)-MSCs with ARCA_PC luciferase mRNA and ARCA_HPLC/β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC luciferase mRNAs. In addition, using RT-qPCR, we quantified the mRNA levels for innate immune responses related genes such as *IFIT1*, *IFIT5*, *OAS1*, *NOD2*, *RNASEL*, *MX1*, *EIF2AK2* (PKR), *RIGI*, *IFIH1* (MDA5), and decapping processes related genes *DCP2* and *DCPS* (Figures 7A–7H). mRNA levels were quantified at two time points: 8 h after transfection and 48 h after transfection.

Eight hours after transfection of hBM-MSCs with ARCA_PC luciferase mRNA, the expression of immune response-related genes, including *IFIT1*, *OAS1*, *MX1*, *RIGI*, and *MDA5* were strongly upregulated (Figures 7A, 7C, 7F, 7H, and 7I), whereas *IFIT5*, *NOD2*, *RNASEL*, and *PKR* were moderately upregulated (Figures 7B, 7D, 7E, and 7G) when compared with native MSCs/MSCs_Lipofectamine as well as cells transfected with ARCA_HPLC/β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC capped luciferase mRNAs.

In the case of expression of immune response-related genes in ARCA_PC luciferase mRNA transfected MSCs at 48 h, only *MX1* was strongly upregulated (Figure 7F), whereas IFIT5, NOD2, RNASEL were moderately upregulated (Figures 7B, 7D, and 7I) and *IFIT1*, *OAS1*, *RNASEL*, *PKR*, *RIG1*, and *MDA5* were altered not

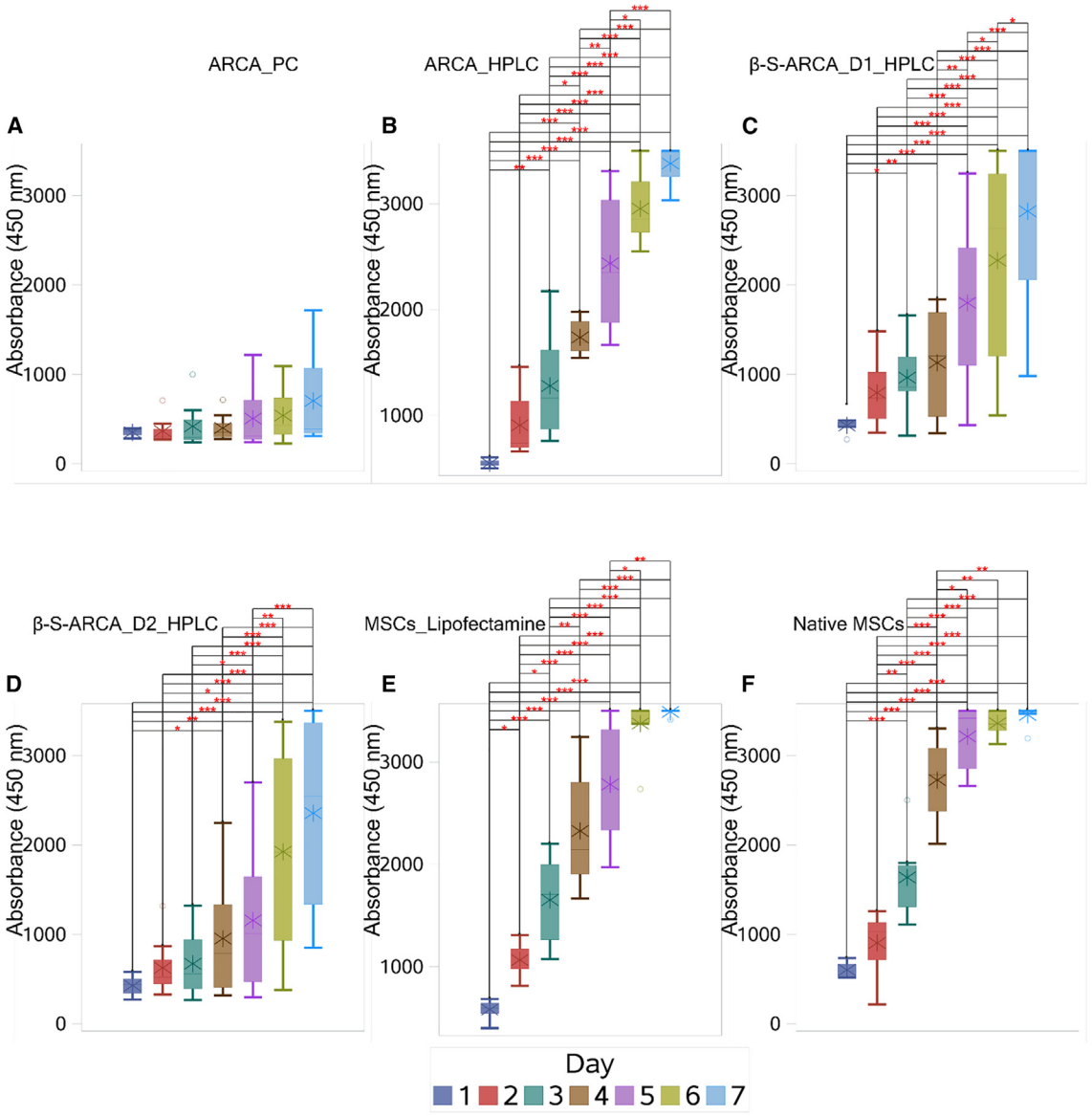

Figure 6. Metabolic activity of MSCs transfected with mRNA capped with different 5′ cap analogs, time points within groups

Absorbance readouts were performed daily across 7 days. MSCs were transfected with HPLC-purified mRNAs capped with ARCA caps with pseudouridine moiety and sulfur atom in β position in two versions: diastereoisomers D1 and D2. The two mRNAs were also compared with mRNAs capped with unmodified ARCA purified by either HPLC or PC. Native, non-transfected MSCs and MSCs with sole Lipofectamine 2000 Transfection Reagent were treated as controls. (A–F) Groups. The following symbols of the level of statistical significance were adopted: $p < 0.05$*; 0.01**; <0.001***; absence of symbol indicates no statistical significance ($p > 0.05$).

significantly (Figures 7A, 7C, 7E, 7I, 7G, and 7H) when compared with native MSCs/MSCs_Lipofectamine or cells transfected with ARCA_HPLC/β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC capped luciferase mRNAs.

IFIT1, *IFIT5*, *OAS1*, *RNASEL*, *MX1*, *RIG1*, and *MDA5* expression decreased significantly in MSCs transfected with ARCA_PC luciferase mRNA between 8 and 48 h post-transfection (Figures 7A, 7B, 7E, 7F, 7H, and 7I).

For decapping processes related genes expression at 8 and 48 h *DCP2* mRNA level was moderately upregulated in ARCA_PC luciferase mRNA transfected hBM-MSCs compared with ARCA_HPLC/β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC IVT luciferase mRNAs transfected cells as well as Native MSCs and MSCs Lipofectamine (Figure 7J). While *DCPS* mRNA level detected at 48 h in hBM-MSCs transfected with ARCA_HPLC/β-S-ARCA_D1_HPLC was slightly lower than level in Native MSCs (Figure 7K).

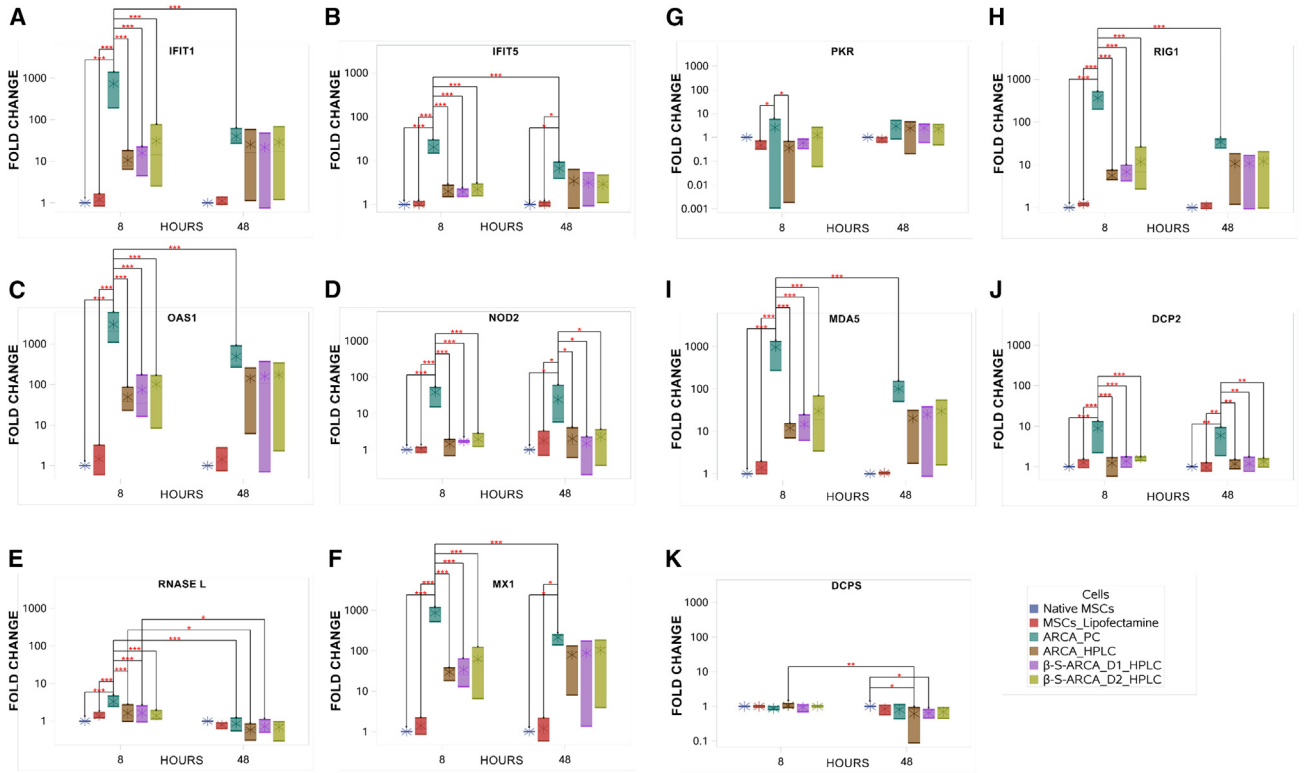

Figure 7. Gene expression changes in hBM-MSCs upon transfection with crude IVT luciferase mRNA/HPLC purified IVT luciferase mRNAs

hBM-MSCs cells were transfected with 1 ng/mL of crude ARCA capped IVT luciferase mRNA (ARCA_PC) and ARCA capped HPLC-purified modified IVT luciferase mRNAs (ARCA_HPLC, β-S-ARCA_D1_HPLC, β-S-ARCA_D2_HPLC) capped IVT luciferase mRNAs. At 8 h and 48 h after transfection, RT-qPCR analysis for genes involved in immune responses (A–I) and decapping process (J–K) was carried out by measuring mRNA levels. The data were obtained with crude IVT mRNA/HPLC purified IVT mRNAs generated in three independent IVT reactions. Data are presented as mean value of mRNA level change from three independent experiments. β-Actin was used as a reference for fold change calculation. The type III test of fixed effects was used for statistical analysis at each time point (8 h and 48 h). In all cases, annotations were represented on plots only where significant differences ($p < 0.05$) were found. Statistical significance annotation: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

We also evaluated stability of the IVT luciferase mRNAs at 48 h in comparison with mRNA level detected in 8 h time point in hBM-MSCs transfected with ARCA_PC/ARCA_HPLC/β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC. At 48 h after post-transfection, luciferase mRNA was detected in all variants of transfected cells (ARCA_PC/ARCA_HPLC/β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC). Our results show that a lower percentage of mRNA was maintained in cells transfected with ARCA_PC in comparison with β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC (Figure 8). This indicates a lower stability of ARCA_PC mRNA compared with β-S-ARCA_D1_HPLC/β-S-ARCA_D2_HPLC.

Overall, we noticed that MSCs also elicit an innate immune response like other characterized cells.[22] We observed that, at 48 h, the levels for most of the mRNAs were reduced from their levels at 8 h, indicating that immune responses are time dependent. Only *DCP2* has slightly upregulated in the group of decapping-related genes.

DISCUSSION

We have demonstrated that adequately purified and enveloped mRNA is highly effective for the short-term engineering of MSCs, such as applicable for MSC trafficking.[9] Notably, depending on the therapeutic application, various characteristics of mRNA transfection may be desirable. In particular, the engineering of stem cells may have other needs than immunotherapeutic purposes or induction of immune response by vaccines. Overall, stem cells are susceptible to induction of innate response, while such response can even be desirable to potentiate immune response induction in case of vaccine applications. Therefore, our study emphasizes metabolic activity and induction of innate intracellular immune response in our stem cells of choice, namely, MSCs, which may have multiple therapeutic applications as extensively reviewed by us.[4]

Protein expression is a primary reason for mRNA application; therefore, it is of utmost importance. Accordingly, our previous study showed that the ARCA cap outperforms a conventional cap analog (7-methylguanosine)[23]; consequently, we have not considered repeating this comparison. In addition, our previous studies employed a lipid-based transfection agent: Lipofectamine 2000, routinely used for various *in vitro* transfection purposes across research enterprises for years. However, we are observing a proliferation of different transfection agents; therefore, it was warranted to

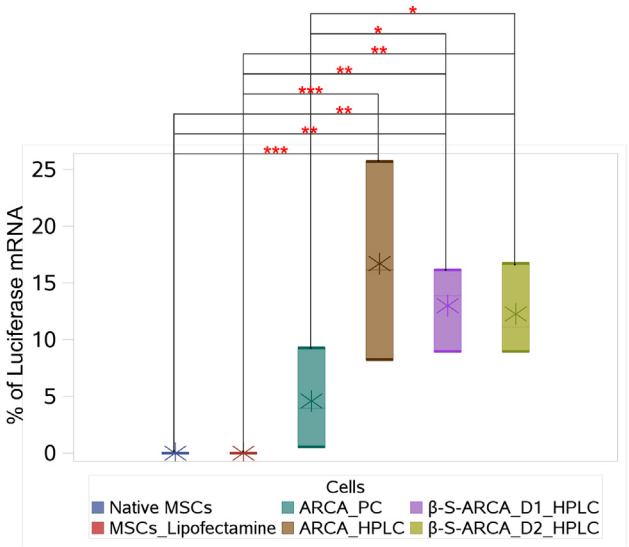

Figure 8. Stability of the IVT luciferase mRNAs at 48 h

We set the luciferase mRNA levels at 8 h as 100 percent in each treatments (ARCA_PC, ARCA_HPLC, β-S-ARCA_D1_HPLC, β-S-ARCA_D2_HPLC) and evaluated the percentage of remaining luciferase mRNAs at 48 h. The type III test of fixed effects was used for statistical analysis. In all cases, annotations were made on plots only where significant differences ($p < 0.05$) were found. Statistical significance annotation: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

compare it to others. Here, we compared lipid-based Lipofectamine 2000 with polymer-based TransIT. We have shown a clear advantage of Lipofectamine 2000 over TransIT. However, TransIT may be specially dedicated to plasmid DNA transfection[22]; thus, it underperformed for mRNA transfection. Accordingly, we continued our study with Lipofectamine 2000. Finally, it is worth mentioning that there has been considerable progress with *in vivo* mRNA application by lipid nanoparticles (LNPs), especially in the field of vaccines.[24] Therefore, LNPs could potentially also be beneficial for *in vitro* transfections. On the other hand, it was shown that LNPs are highly inflammatory *in vivo*,[25] which may be very helpful for vaccines, including COVID-19 prevention.[20] However, if LNPs also induce an innate intracellular response *in vitro*, they would not match the current Lipofectamine 2000-based performance for stem cell engineering. Overall, it seems that Lipofectamine 2000 fulfills the criteria for successful *in vitro* mRNA transfection.

The purification of biological agents is critical. Stem cells may be susceptible to any impurities, which can negatively impact such sensitive cell populations; therefore, it was essential to gain insight into this process. It is furthermore imperative since an IVT reaction results in many unwanted impurities and byproducts.[18,26] There are two main factors that cause an unfavorable cellular response to IVT mRNA transfection, the incorporation of canonical unmodified uridine into the transcript body and the presence of dsRNA in the preparation. dsRNA is formed as a result of elongation of the 3′ end of RNA that hybridizes to complementary sequences, or the formation of antisense RNA.[25,27,28]

Activation of the immune system results in the inhibition of protein synthesis and degradation of foreign RNA. This response normally targets pathogens that attack the cell, but if the IVT mRNA is contaminated with immunogenic molecules, its translation will also be impaired. This response is further enhanced with repeated administration of the new RNA-based preparation, which affects the effectiveness of the treatment. Thus, this is an important factor requiring attention, especially for therapies using more than one administration.

One of the most effective ways to separate dsRNA from the actual transcript product is to use reversed-phase HPLC purification.[29] Thus, we have compared NucleoSpin RNA Clean-Up PC dedicated to mRNA isolation for analytical purposes with mRNA subjected to additional purification by HPLC.[30,31] While HPLC was suggested as a purification method long ago, its impact has never been investigated in the context of stem cells, particularly MSCs.[29] We have demonstrated that additional HPLC-based purification is absolutely pivotal for mRNA transfection. The mRNA subjected to PC purification is poorly translated into proteins and affects metabolic activity and innate intracellular immune response; thus, this purification method is unacceptable for therapeutic mRNA transfection. On the contrary, HPLC-purified mRNA capped by ARCA produces a decent amount of proteins, does not produce any innate intracellular immune response at any investigated time point, and does not affect metabolic activity most of the time (except days 4 and 5 post-transfection in comparison with naive MSCs, but not Lipofectamine 2000-treated MSCs). Therefore, we can conclude that ARCA-capped, HPLC-purified mRNA is neutral to recipient MSCs and, thus, promising for mRNA therapeutics.

Next, we compared the original ARCA cap with two β-S-ARCA modifications. The β-S-ARCA D1 and D2 diastereoisomers are produced by substituting non-bridging oxygen on the β-phosphate.[32] Previous work has demonstrated higher expression and a better therapeutic function of immature dendritic cells subjected to transfection of mRNA capped by D1 β-S-ARCA diastereoisomer,[33] which warranted analogical studies in the context of MSCs. Indeed, we have shown that capping of mRNA by D1 β-S-ARCA improved reporter protein production at the 4-, 8-, and 12-h time points (protein production at 24 h was already negligible). On the other hand, capping mRNA by D2 β-S-ARCA delivered protein production at the lower level similar to mRNA capped by original ARCA. In this context, it is imperative to include metabolic activity in the overall assessment of all caps. While on day 1 there were no differences in metabolic activity among groups, ARCA resulted in only a transient decrease of metabolic activity on days 4 and 5 and caught up with non-transfected MSCs over the following days. In contrast, β-S-ARCA D1 and D2 led to a more profound deterioration of metabolic activity, which also started on day 4, but lasted until the end of the experiment on day 7. While we did not explicitly test MSC function, the decreased metabolic activity of β-S-ARCAs D1 and D2 until the end of the experiment raises concerns over the long-term impact of using both diastereoisomers on the stem cells. Interestingly, this impact starts to be observed on

day 4, long after exogenous mRNA-based protein production expires and at the moment of MSCs entering into a logarithmic growth phase. Ultimately, we did not find differences between the three HPLC-purified caps in the induction of expression of genes involved in innate intracellular immune response and mRNA decapping. To summarize, while β-S-ARCA D1 improves protein expression over the first 4–12 h, it also negatively impacts MSC metabolic activity starting on day 4. Overall, the positive impact of D1 β-S-ARCA on protein production seems to be outweighed by the delayed negative effect on the MSC metabolic activity.

Conclusions, further outlook, and study limitations

We have demonstrated Lipofectamine 2000-based *in vitro* transfection of HPLC-purified, ARCA-capped mRNA is relatively neutral to MSCs while securing decent protein production. However, while D1 β-S-ARCA improves protein production, it negatively impacts MSCs' metabolic activity without increased induction of expression of investigated genes involved in innate intracellular immune response and mRNA decapping. Thus, it would be worth performing RNA-seq of MSCs transfected with mRNA bearing various caps at different time points in the future to better understand the drop of metabolic activity by D1 β-S-ARCA and D2 β-S-ARCA diastereoisomers. Moreover, single-cell RNA-seq combined with induced protein production at the single-cell level could give us an even more profound insight but a higher financial cost. Since competition between ARCA and β-S-ARCA remains unresolved within the framework of the current study, it would be worth performing an *in vitro* functional assessment of MSCs, in terms of their differentiation potential and trophic factor release, as we previously performed for MSC labeling.[34] Moreover, a study similar to our previous *in vivo* assessment of MSC engineering by potentially therapeutic mRNA would be warranted to further compare both caps.[35] While ARCA and its modifications are still widely used, progress has been recently observed in a method of cap introduction to mRNA by co-transcriptional, CleanCap analog, which simplifies and, in this way, lowers the costs of mRNA production.[35] Thus, CleanCap Reagent AG (3′OMe) analog, as a one-step solution, was instrumental in the rapid development of COVID-19 vaccines and the widespread distribution of their high quantities worldwide.[20] While vaccine research benefits from immune activation, it is radically opposite for stem cell engineering; therefore, a head-to-head comparison of ARCA and CleanCap analogs on the efficacy and safety of MSC engineering would also be vital. While vaccine research has been an enormous boost to mRNA technology, an even bigger revolution may be ahead. There is an explosion in genome editing research,[36] which has been initially clinically translated through mRNA-based *in vivo* delivery.[37] Notably, genome editing, like our stem cell engineering, also requires a lack of immune activation; therefore, potentially, our findings could be widely applicable to the exciting new world of genome editing. However, we have not touched here cap-independent protein production based on the internal ribosome entry sites.[38] The growing mRNA applications also call for the first Nobel prize in this field.

MATERIALS AND METHODS

hBM-MSC culture

hBM-MSCs isolated under xeno-free conditions (RoosterVial-hBM-1M-XF) from healthy adult donors of both sexes aged 18–30 were purchased from RoosterBio, Inc., USA (Cat. No. MSC-031). hBM-MSCs were supplied with a certificate confirming their phenotype, three lineage differentiation capacity, release rate of immunomodulatory and angiogenic cytokines. The last two of these parameters (immunomodulatory and angiogenic cytokine release rate) were within the normal range, but they differed between cells from individual donors. Cells from different donors were randomly used in replicates of the experiments. Cells were thawed and maintained in, hereinafter referred to as "standard medium"—RoosterNourish-MSC-XF, composed of RoosterBasal-MSC (Cat. No. SU-005) supplemented with RoosterBooster-MSC-XF (Cat. No. SU-016) from RoosterBio, Inc., in a humidified atmosphere at 37°C and 5% CO_2 using 75-cm^2 cell culture flasks (Cat. No. 156499; ThermoFisher) for two passages, with culture medium exchanged every second day. For further experiments, hBM-MSCs in the second passage were treated with Trypsin-EDTA (0.05%), phenol red (Cat. No. 25300054; ThermoFisher), and transferred to 96-well plates (Cat. No. 167008; ThermoFisher) at 2.5×10^4 cells/well or 6-well plates (Cat. No. 140675; ThermoFisher) at 7×10^5 cells/well.

Synthesis of luciferase encoding mRNAs

For IVT reaction, a PCR product containing the firefly luciferase coding sequence and the SP6 promoter sequence purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) was used as the dsDNA template. RNA capping was carried out co-transcriptionally using cap analogs: $m_2^{7,3'-O}$GpppG (ARCA),[15] β-S-ARCA D1, and β-S-ARCA D2[20] (molar ratio of cap: GTP was 5:1). A standard transcription reaction contained: transcription buffer, 25 ng/μL dsDNA template, 0.5 mM ATP/CTP/UTP, 0.1 mM GTP, 0.5 mM dinucleotide cap analog, 0.5 U/μL Ribolock Ribonuclease Inhibitor, and 1 U/μL of SP6 RNA polymerase (Thermo Fisher Scientific). The reaction mixture was incubated overnight at 37°C, after which 0.025 U/μL of DNaseI (Thermo Scientific) was added and further incubated for 20 min at 37°C to remove template DNA. The whole reaction mixture was then subjected to 3′ end polyadenylation in 30 min reaction at 37°C in a poly(A) buffer containing 1 mM ATP, 0.125 U/μL of poly(A) polymerase (NEB), and 0.4 U/μL Ribolock (Thermo Fisher Scientific). To remove free phosphate groups from the 5′ ends, transcripts were treated with alkaline phosphatase (FastAP, Thermo Fisher Scientific) for 15′ at 37°C in a reaction mixture containing FastAP buffer, 0.025 U/μL FastAP, and 0.33 U/μL Ribolock. The transcripts were purified using the NucleoSpin RNA Clean-Up (Macherey-Nagel) according to the manufacturer's instructions. Quality of transcripts was checked on 1% 1 × TAE agarose gels and concentration was measured spectrophotometrically. Part of the sample at this stage of purification was saved for testing. The rest of the sample was further purified. To remove dsRNA by-products of IVT reaction, mRNAs were purified on

RNASepPrep—RNA Purification Column (ADS Biotec) using an HPLC technique. A linear gradient of buffer B (25% acetonitrile in 0.1 M triethylammonium acetate pH 7.0) in buffer A (0.1 M triethylammonium acetate pH 7.0) from 35% to 55% over 20 min at 4 mL/min was applied. Fractions containing mRNA were concentrated on an Amicon Ultra centrifugal filters (regenerated cellulose 10NMLW, Merck Millipore), precipitated with NaAc and isopropanol mixture overnight at −20°C and dissolved in water. The integrity of transcripts was checked on a 1% 1 × TAE agarose gel and concentration was determined spectrophotometrically.

hBM-MSC transfection with mRNAs

The hBM-MSCs in the third passage were engineered with mRNAs coding firefly luciferase at a final concentration of 0.5 or 1 μg/mL, using polymer-based TransIT-mRNA Transfection Kit (Cat. No. MIR 2225; Mirus Bio LLC) or lipid-based Lipofectamine 2000 Transfection Reagent (Cat. No. 11668027; ThermoFisher). Between four and six independent transfections were performed with each method.

Transfection using TransIT-mRNA Transfection Kit was performed according to the manufacturer's protocol. Briefly, TransIT-mRNA and mRNA Boost reagents were pre-warmed to room temperature (RT). Then, to prepare mRNA complexes, Opti-MEM Reduced Serum Medium (Cat. No. 11058021; ThermoFisher) was mixed with appropriate mRNA in 10% of the final transfection mixture volume to obtain concentrations of 5 or 10 μg/mL. Next, TransIT-mRNA and mRNA Boost reagents were added at a volume of 2 μL each per 1 μg mRNA and incubated for 5 min at RT for the complexes to form. Finally, obtained complexes were added dropwise to hBM-MSCs to obtain the final mRNA concentration of 0.5 μg/mL or 1 μg/mL, followed by a cell incubation in a humidified atmosphere at 37°C and 5% CO_2 for 24 h.

With modifications, the transfection using Lipofectamine 2000 Transfection Reagent was also performed according to the Manufacturer's protocol. Briefly, hBM-MSCs were washed three times with PBS and placed in Opti-MEM Reduced Serum Medium in a volume representing 50% of the final volume of the transfection mixture. Next, appropriate mRNA was mixed with Opti-MEM Reduced Serum Medium to a final concentration of 4 or 8 μg/mL. At the same time, Lipofectamine 2000 Transfection Reagent was mixed with Opti-MEM Reduced Serum Medium at a 1:28 ratio, reflecting 12.5% of the final volume of the transfection mixture. Next, Opti-MEM Reduced Serum Medium/mRNA and Opti-MEM Reduced Serum Medium/Lipofectamine 2000 Transfection Reagent mixtures were incubated at RT for 5 min, mixed at 1:1 ratio, and re-incubated at RT for an additional 20 min. The obtained mixture was then made up to 50% of the final volume of the transfection mixture with Opti-MEM Reduced Serum Medium and finally added to hBM-MSCs kept in Opti-MEM Reduced Serum Medium to obtain 100% of the final volume of the transfection mixture, with the final mRNA concentration of 0.5 μg/mL or 1 μg/mL. Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 4 h, followed by washing three times with PBS and placed in a standard medium.

hBM-MSCs subjected to appropriate transfection procedures excluding mRNA and native hBM-MSCs served as two independent controls.

Luminescence measurement

The luminescence of cultured hBM-MSCs was measured on 96-well plates. At 4, 8, 12, 24, 36, and 48 h after transfection with firefly luciferase coding mRNAs, cells underwent luminescence readouts using FLUOstar Omega microplate reader (BMG Labtech). At each time point, standard medium was replaced by a standard medium supplemented with 25 μM DMNPE-caged Luciferin (Cat. No. LC10000; Oz Biosciences), and luminescence measurements were immediately acquired. The experiments were performed in duplicate and subsequently repeated four to six times.

hBM-MSC metabolic activity measurement

The metabolic activity of cultured hBM-MSCs was also measured on 96-well plates. Twenty-four hours after transfection with mRNAs coding firefly luciferase, the Cell Counting Kit-8 Proliferation/Cytotoxicity Assay (CCK-8; Cat. No. CK04; Dojindo Laboratories) was used according to the manufacturer's protocol. Briefly, 10 μL CCK-8 reagent was added to each well, which contained hBM-MSCs in 100 μL of the standard medium. Plates were then gently shaken and incubated in a humidified atmosphere at 37°C and 5% CO_2 for 2 h. After 2 h, readouts were performed using a FLUOstar Omega microplate reader at 450 nm absorbance. Each well was subjected to a single measurement, then discarded. The measurements were performed across 7 consecutive days with a 24-h interval between readouts. The experiments were performed in duplicate and repeated four times.

Quantitative real-time PCR analysis

The hBM-MSCs cultured on 6-well plates were transfected with 1 μg/mL mRNA using Lipofectamine 2000 Transfection Reagent. After 8 and 48 h, cells were detached from wells by trypsinization and centrifuged at 1,000 rpm for 5 min. Then total RNA was isolated from cell pellets using the Total RNA Mini isolation kit (A&A Biotechnology) according to the manufacturer's instructions. First, RNA was quantified spectrophotometrically, and its quality was analyzed using A260/A280 ratio. Next, 500 ng RNA was used to obtain cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Finally, quantitative PCR was performed on LightCycler@ 480 System (Roche). Briefly, cDNA (obtained from 500 ng RNA), mRNA-specific primers (Table S1), Maxima SYBR Green qPCR Master Mix (2×) (Thermo Fisher Scientific) were mixed and a reaction run with a thermal profile of an initial 10 min melting step at 95°C, followed by 45 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The relative fold change of mRNAs was normalized to β-actin mRNA by the $2^{-\Delta\Delta Ct}$ method.[39]

Statistical analysis

Statistical calculations for protein expression and cell metabolism were made using PROC MIXED (SAS 9.4). The type III test of fixed effects was used to determine statistical significance. In addition, the

least mean square difference test was performed to compare means. Boxplots show the data distribution for the assay value in the compared populations. The length of the bars represents the quadrant (Q1–Q3) data; the whiskers indicate the minimum and maximum values within the fences (±1.5 Q), the line within the bar determines the median, while the arithmetic means are shown as asterisk inside the box and outliers are presented as circles outside the whiskers. In all experiments, the level of statistical significance has been set at a p value of <0.05. In addition, the following symbols of the level of statistical significance were also adopted: *p < 0.05; **0.01; ***<0.001.

DATA AND CODE AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author based on reasonable requests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.07.006.

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AUTHOR CONTRIBUTIONS

M.J., B.L., and E.D. conceived the project, analyzed the data, and wrote the manuscript. A.A. and R.G. designed and performed most of the experiments and analyzed the data. A.S. synthesized mRNA for experiments. N.S. performed RT-PCR analysis. P.R. performed the measurement of metabolic activity and luminescence. All authors participated in writing and critically reading the manuscript.

DECLARATION OF INTERESTS

M.J. is a co-founder of IntraART, Ti-com, and ART-EX, but they are not related to the current manuscript.

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