

Investigation of LL-37-mediated siRNA transfection

Mustafa YALÇINKAYA, Şahru YÜKSEL*

Molecular Biology and Genetics Department, Center for Life Sciences and Technologies, Boğaziçi University, Bebek, İstanbul, Turkey

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Abstract: RNA interference is not only an important tool for studying protein function; it also holds many promises for the treatment of a variety of diseases in which the function of unwanted proteins needs to be suppressed. For small interfering RNA (siRNA) to be used as a therapeutic agent, the biggest hurdle is to find the right delivery agent that is both nontoxic to the cells and efficient in delivery. Synthetic and natural peptides from a variety of organisms have been investigated for this purpose. However, there is still no agent or formulation that has been approved for siRNA delivery. Antimicrobial peptide LL-37 has been reported to deliver negatively charged cargo molecules like DNA into the mammalian cells. Since it is nontoxic and nonimmunogenic, and it has the ability to penetrate through mammalian cells in complex with negatively charged molecules, LL-37 peptide appears to make an ideal delivery agent for siRNA therapy. In this report, LL-37/siRNA complexes have been characterized and LL-37-mediated transfection has been investigated in vitro. The results show that the efficiency of delivery was dependent on the cell type, and for LL-37 to be used as a siRNA delivery agent, conditions have to be optimized for each target tissue type.

Key words: Antimicrobial peptide, LL-37, siRNA, shRNA, transfection, cell-penetrating peptides

1. Introduction

RNA interference (RNAi) is a rapidly developing and evolving field holding many promises for laboratory and therapeutic applications. It was shown for the first time in 1998, in *Caenorhabditis elegans*, (1) and since then, this natural mechanism that takes part in the control of the gene expression has been identified in a wide range of organisms, from yeast to humans (2–4). RNAi is also crucial as a defense mechanism against infections and viruses, and it plays a key role during development (5). This groundbreaking discovery was not only rapidly adapted bench-side as a powerful and easy methodology to study gene function in vitro, but it also emerged as a revolutionary approach for the treatment of a variety of diseases including cancer, viral infections, and neurodegenerative diseases in clinical setting (4).

Most of the drugs on the market exert therapeutic effects through interacting with proteins and cellular receptors. However, RNAi-based approaches have an enormous advantage, as they are aimed at the inhibition of target protein production in the first place to provide a long-lasting and relatively durable effect. In addition, compared to conventional drugs that take years to develop, designing and choosing the siRNAs against the target gene(s) would require less time and will hopefully decrease

the cost of drug development. Theoretically, siRNAs can be designed to interfere with the translation of almost any mRNA, as long as the mRNA has a distinctive sequence and the chosen siRNAs are thoroughly investigated to not cause any nonspecific effects. This approach holds the potential to create a therapy against diseases that are not curable or are difficult to treat due to various reasons such as unavailability of inhibitors.

With all these advantages, one of the major hurdles that needs to be resolved for RNAi-based therapy is to find delivery agents that are both nontoxic to the cells and effectively transfer siRNA into the target cell upon systemic administration. This is because siRNAs can be rapidly degraded in circulation, or cleared from the system and not effectively internalized due to their high molecular weight and polyanionic nature when they are administered alone (6,7). The most frequently studied delivery systems for siRNA drugs for systemic administration are peptide-based systems, viral vectors, and polymer- or liposome-based systems. Mostly not being toxic at the doses that can be used for administration, cationic peptides with cell penetration properties offer an important advantage over the other types of delivery systems, such as viral vectors or polymer-based nanoparticles, that can cause toxicity or immunogenicity and insertional mutagenesis problems

* Correspondence: sahr.yuksel@boun.edu.tr

(8). Each group has its own potentials and limitations, which were extensively discussed in recent reviews (9–11).

Within the peptide group, there are synthetic peptides such as poly-lysine and natural peptides from bacteria, viruses, or others such as the HIV-1 trans-activator (Tat) gene product (12), penetratin, and transportin, with the ability to penetrate membranes and deliver cargo molecules into the host cells. In general, these are cationic structures, and the mechanism of membrane penetration is not fully clear for all of them. For instance, with Tat-based delivery systems, endosomal uptake is favored when the size of the peptide nucleic acid complex is between 500 and 700 nm, whereas particles smaller than 300 nm do not utilize this pathway (13). Unlike with viral systems, delivery of a variety of cargo molecules with cell-penetrating peptides include, but are not limited to, nucleic acids (6), proteins (14), and quantum dots (15). There is a growing list of such peptides that are extensively discussed in recent reviews (11).

The fact that LL-37 has been shown to deliver negatively charged cargo molecules such as genomic DNA or plasmid DNA into the host cells prompted us to investigate the possibility of whether this naturally present molecule could be used as a siRNA therapy tool. LL-37 is the only member of the cathelicidin family of antimicrobial peptides in humans. The propeptide, human cathelicidin-like antimicrobial peptide (hCAP18), is secreted from activated neutrophils and the conserved N terminal cathelin domain is cleaved off by protease 3, secreted from activated neutrophils, leaving the functional antimicrobial LL-37 (16). It is mainly expressed by bone marrow cells, such as monocytes and neutrophils, and epithelial cells of various organs in the body (17). The concentration of LL-37 in human plasma was reported to be 1.2–1.8 µg/mL for LL-37 and 1.18 µg/mL for hCAP18 (18). The ability of LL-37 to interact with DNA and LL-37-mediated delivery of plasmid DNA to the nuclear compartment was demonstrated for the first time in 2004 by Sandgreen et al. (19). In another groundbreaking study by Lande et al., LL-37 was identified as a factor involved in the internalization of genomic DNA by plasmacytoid dendritic cells (pDCs) in psoriasis skin lesions (20). The ability of LL-37 to make complexes with DNA was based on the net 6 positive charge on the peptide and negative charges on the backbone of DNA, and the complexed nucleic acids were protected from degradation by serum nucleases.

In this study, we report for the first time the characterization of LL-37 and siRNA complexes, as well as investigation of the LL-37-mediated delivery of these complexes into several mammalian cell lines.

2. Materials and methods

LL-37, LL 8-37, and TAMRA-LL-37 were purchased from Anaspec (Fremont, CA, USA) and resuspended in sterile distilled deionized water. siRNA against green fluorescent protein (GFP) was purchased from Dharmacon (Lafayette, CO, USA) and resuspended to the desired molarity in DEPC-treated phosphate buffered saline.

2.1. Gel shift assay

Peptide and siRNA were mixed in a final concentration of 10 mM HEPES (pH 7.4) at various peptide/siRNA charge ratios and incubated for 30 min at room temperature. Charge ratios were calculated as previously described (21). The mixture was run on a 15% nondenaturing polyacrylamide gel electrophoresis at 100 V for 2 h in 1X TBE and visualized by ethidium bromide staining.

2.2. Heparin release assay

Peptide/siRNA complexes were prepared at a 5:1 charge ratio as described above, then heparin (Wockhardt, France) was added at various concentrations (0.5 units to 0.02 units) and the mixture was incubated for 10 min at room temperature. The mixtures were run on a 15% nondenaturing PAGE at 100 V for 2 h in 1X TBE and visualized by ethidium bromide staining.

2.3. Particle size analysis

Peptide/siRNA complexes at a 5:1 charge ratio were visualized by scanning tunneling electron microscopy (STEM). A drop of sample mixture (approximately 20 µL) was loaded on carbon-coated copper 400-mesh grids. It was then incubated at room temperature for the formation of thin film and analyzed with an XL30 ESEM-FEG/EDAX in STEM mode (Philips Global). Liquid state analysis for the particle size was done with a Brookhaven Instruments 90 Plus Particle Size Analyzer at room temperature. An average of 10 measurements were taken for each data point.

2.4. Confocal microscopy

SKBR3 breast cancer cells were seeded on cover slips overnight. The next day, the cells were treated overnight in serum-free media with FITC-GFP siRNA (130 nM) (Invitrogen, USA) and TAMRA-LL-37 (17.5 µg/mL) complex. The cells were then washed extensively with PBS and 1 M NaCl to remove uninternalized peptide–siRNA complexes and then fixed in 4% paraformaldehyde. The FITC signal was amplified with TSA plus fluorescence systems (PerkinElmer, USA) according to instructions provided by the manufacturer and visualized with a Leica SP2 AOBS (Wetzlar, Germany). Nuclei were stained with DAPI.

2.5. Lentiviral transduction

Lentivirus production was carried out in packaging cell line HEK-293 FT for stable transduction of the HCC1143 cell line with lentiviruses expressing eGFP. The 4-plasmid

system (pHCMV-G, pRSV-Rev, pMDLg/pRRE, and fg12GFP) was a gift from MS Soengas and was used to transfect HEK-293 FT cells by the calcium phosphate method. Two days after the transfection, the supernatant containing the viral particles was filtered through a 0.45 μ M filter and incubated with HCC1143 cells for 8 h in the presence of polybrene. Fresh medium was then added and cells were passaged several times for the stabilization of GFP expression before being used in the experiments.

2.6. Cell culture and transfection with LL-37 complexes

HEK-293 FT (human embryonic kidney cells), Huh-7 (human hepatocellular carcinoma), MCF-7 (human breast adenocarcinoma cell line), MDA-MB 231 (human breast carcinoma cell line), SKBR3 (human breast adenocarcinoma cell line), HeLa (human cervical adenocarcinoma), HT-29 (human colon adenocarcinoma cell line), SKMEL (human skin melanoma cell line), and HCC1143 (human breast adenocarcinoma cell line) were propagated in media as suggested by the American Type Culture Collection. Cells were seeded overnight in 96-well plates with about 70% confluency. Complexes of pEGFPc3 and LL-37 peptide were prepared in different charge ratios in a serum-free medium and incubated with the cells. After 24 h, serum-free media containing the complexes were replaced with the regular growth medium. Fluorescence was monitored with fluorescence microscopy for up to 1 week.

3. Results and discussion

3.1. Characterization of LL-37 siRNA complexes

Gel shift analysis indicated that efficient complex formation between LL-37 and siRNA starts at a charge ratio of 3:1 and is mostly completed at a ratio of 5:1 (Figure 1a). The ability of complexes was analyzed in the presence of heparin, a highly anionic substance for competition with siRNA. The complexes were able to dissociate from each other at saturating levels of heparin, as shown in Figure 1b.

In order to determine the size and the stability of the formed complexes, electron microscopy and a nanoparticle size analyzer based on dynamic light scattering were used. According to the electron microscopy analysis, the particle size varied between 68 and 123 nm (mean: 90.8 ± 21.3) (Figure 1c), and with the nanosizer an average effective diameter of 186.4 ± 5 nm was obtained. Measurements of the complexes in solution showed that complexes retained approximately the same average effective diameter for around 42 h, suggesting that the complexes were very stable and do not degrade easily (Figures 1d and 1e). The slight differences in the size of the complexes measured by these 2 different instruments is most likely due to different states of the material at the time of analysis (in solution vs. dry).

3.2. Internalization of LL-37/siRNA complexes

The ability of LL-37 peptide-mediated internalization of siRNA was investigated by confocal microscopy. According to the data obtained, FITC-labeled siRNA was efficiently internalized and detected within the cytoplasm and perinuclear regions of the SKBR3 cells when delivered in complex with TAMRA-labeled LL-37 (Figure 2), but not when incubated alone with the cells. Most of the siRNA was detected as still complexed with LL-37, possibly in the endosomal compartment as conferred from the distinct punctual appearance. Another factor pointing toward the endosomal uptake mechanism for LL-37-mediated delivery was supported by the observation that the use of chloroquine, a weak base that inhibits lysosomal acidification, increased the efficiency of transfection (unpublished observation).

According to the study by Sandgreen et al. (19), LL-37 facilitates the delivery of plasmid DNA into the nucleus of mammalian cell lines. The fact that siRNA and LL-37 complexes were also detected in nuclear compartments of some of the cells in our study suggests that, independent of the nature of the cargo particle in complex with LL-37, some of the internalized material may end up in the nucleus.

3.3. LL-37-mediated silencing in vitro

Since the internalization with LL-37 was reported to be dependent on the lipid raft and proteoglycan composition of the target cell (19), we wanted to determine what kind of cells were more readily taking up LL-37 complexes. Nine cell lines, including HEK-293 FT, Huh-7, MCF-7, MDA-MB 231, HeLa, HT-29, SKMEL, SKBR3, and HCC1143, were used for this purpose. When LL-37/pEGFP complexes were transfected, not all of the cell types expressed the GFP with the same efficiency as expected. The most efficient transfection with pEGFP in complex with LL-37 was achieved with the HCC1143 breast cancer cell line.

The differences in the efficiency of internalization via these different cell types could be due to differences in the plasma membrane composition and lipid raft formation that might depend on parameters such as the amount of cholesterol, glycosphingolipids, types of phospholipids, and protein composition. The composition of the extracellular matrix of these cell types could also possibly influence the degree of uptake and the intracellular localization of these complexes.

Next, to determine the efficiency of gene silencing with LL-37, a cell line of HCC1143 stably expressing eGFP (HCC1143-GFP) was generated through lentiviral transduction. When LL-37/GFP siRNA complexes were incubated with HCC1143-GFP cells, partial silencing of GFP fluorescence was observed (Figure 3). The fragment of LL-37 extending from 8–37 amino acids was also tested

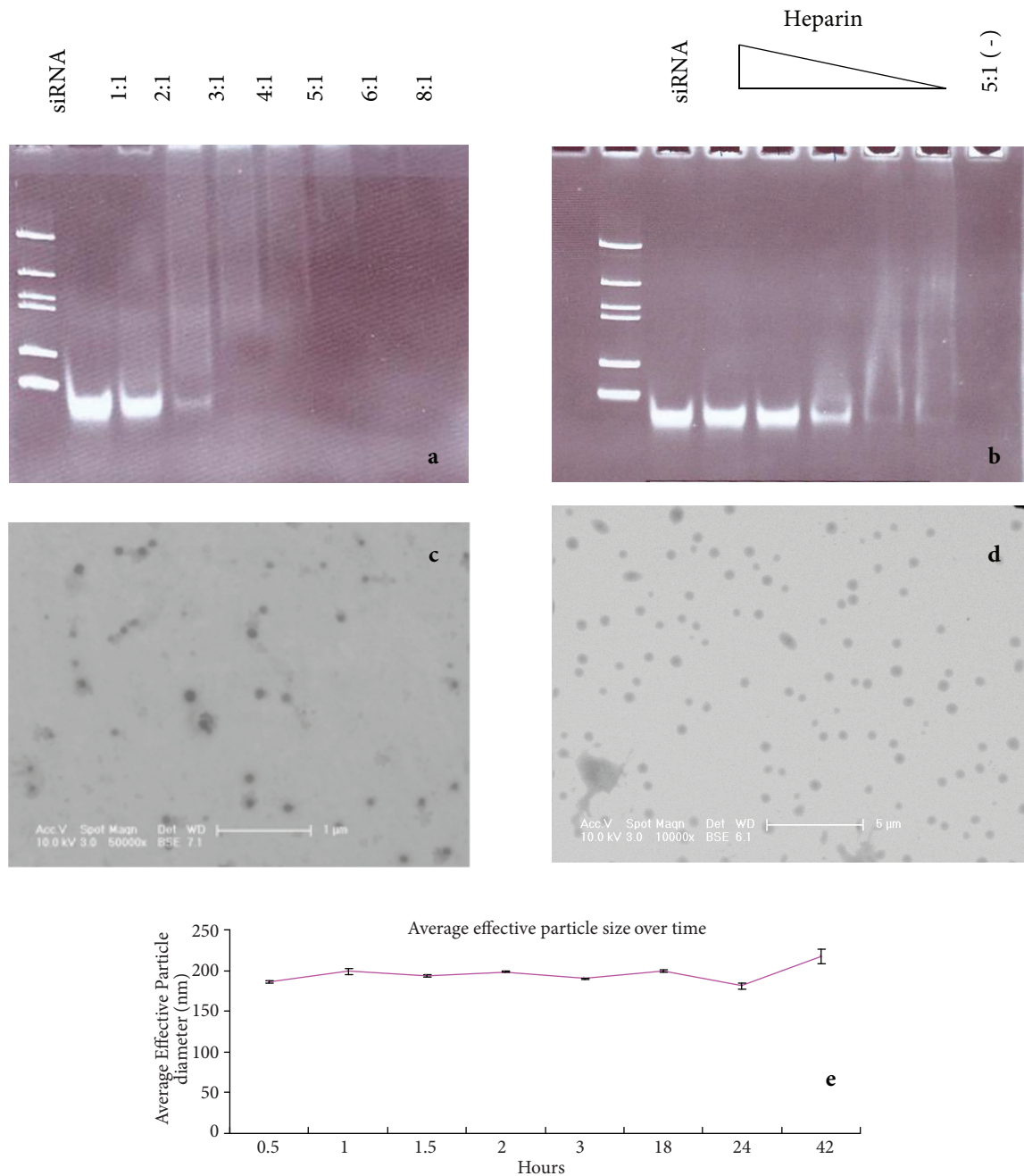


Figure 1. Characterization of LL-37/siRNA complexes: a) formation of complexes at increasing peptide charge ratio analyzed by gel shift assay; b) dissociation of the complexes prepared at 5:1 ratio by the addition of decreasing amounts of heparin; c) electron microscopy analysis of complexes prepared at 5:1 charge immediately and d) after 42 h of incubation at room temperature; e) liquid state analysis of complexes as by nanosizer at room temperature.

as an alternative to the full-length mature peptide. This fragment of LL-37, to a similar extent, was also able to induce GFP knockdown in HCC1143 GFP cells (Figure 3). It may be the increased amount of cytoplasmic siRNA, as opposed to endosomal siRNA, resulting in some silencing observed in HCC1143 cells. As opposed to the earlier literature reporting endosomal localization in human

pDCs treated with LL-37 genomic DNA complexes (20), in a very recent report, DNA was mostly (>70%) localized to cytoplasm after 24 h of incubation with LL-37-genomic DNA complexes in human monocytes (22). Among the primary human keratinocytes, fibroblasts, mature dendritic cells, and pDCs tested in this study, very high cytoplasmic localization of DNA was only seen in monocytes, and this

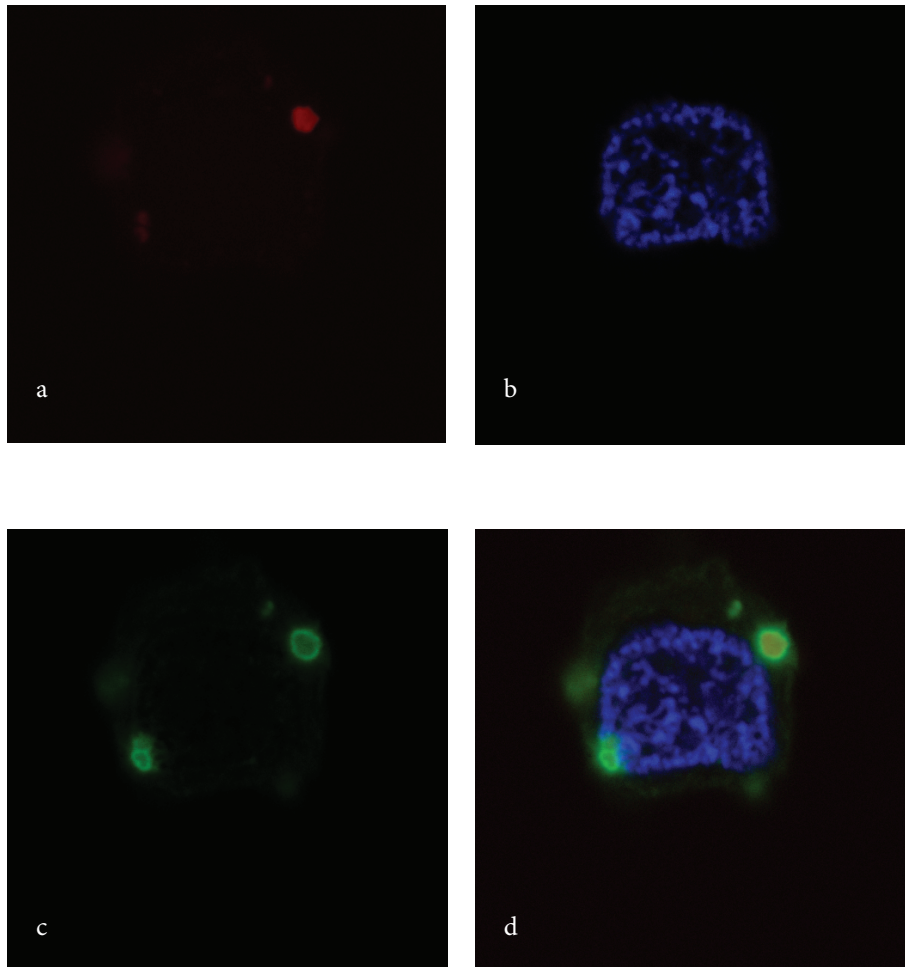


Figure 2. Localization of LL-37/siRNA complexes. Confocal images of SKBR3 cells treated with complexes prepared at 4:1 charge ratio of TAMRA-LL-37 (shown in red) and FITC GFP siRNA (shown in green) after overnight incubation in serum free media: a) peptide, b) nucleus, c) siRNA and d) overlay.

was independent of the sequence of the DNA. Based on this study, it is possible to speculate that LL-37-mediated delivery of siRNA and knockdown would be similarly very high in primary human monocytes. It still remains to be investigated how the LL-37-mediated delivery of the same cargo molecule results in such different subcellular localization in these 2 cell types. Understanding the mechanism might help us to find out which cells are more suitable for LL-37-mediated siRNA delivery and gene knockdown.

In conclusion, this work suggests that LL-37 might be useful for the delivery of siRNA to some cell lines. However, it is important to keep in mind that, based on the target tissues, sometimes slight modifications or manipulations might also be useful. For instance, it was reported that the incorporation of a polyhistidine tag to Tat peptide increased the efficiency of delivery by 7000-fold (23). The fact that some LL-37 complexes were shown to localize

to the nuclear compartment in this and earlier reports also suggests that LL-37 has the potential to be used for gene silencing when used for delivery in complex with small hairpin RNA (shRNA). The degree of nuclear localization might differ in different cell types and individual testing and optimization might be required. More excitingly, the recent literature on primary cells supports the idea that LL-37-mediated delivery might even be more promising in human monocytes, which remains to be tested.

It is highly likely that LL-37 might have some physiological role in the manipulation of the gene expression, such as making complexes with the microRNAs that can be found in the circulation and delivering them to another cell, since both the LL-37 peptides and microRNAs are shown to exist in the circulation. If this is shown to be true, such a mechanism would not only reveal a new way of intracellular communication mechanism, but also might have diagnostic and prognostic implications for some diseases.

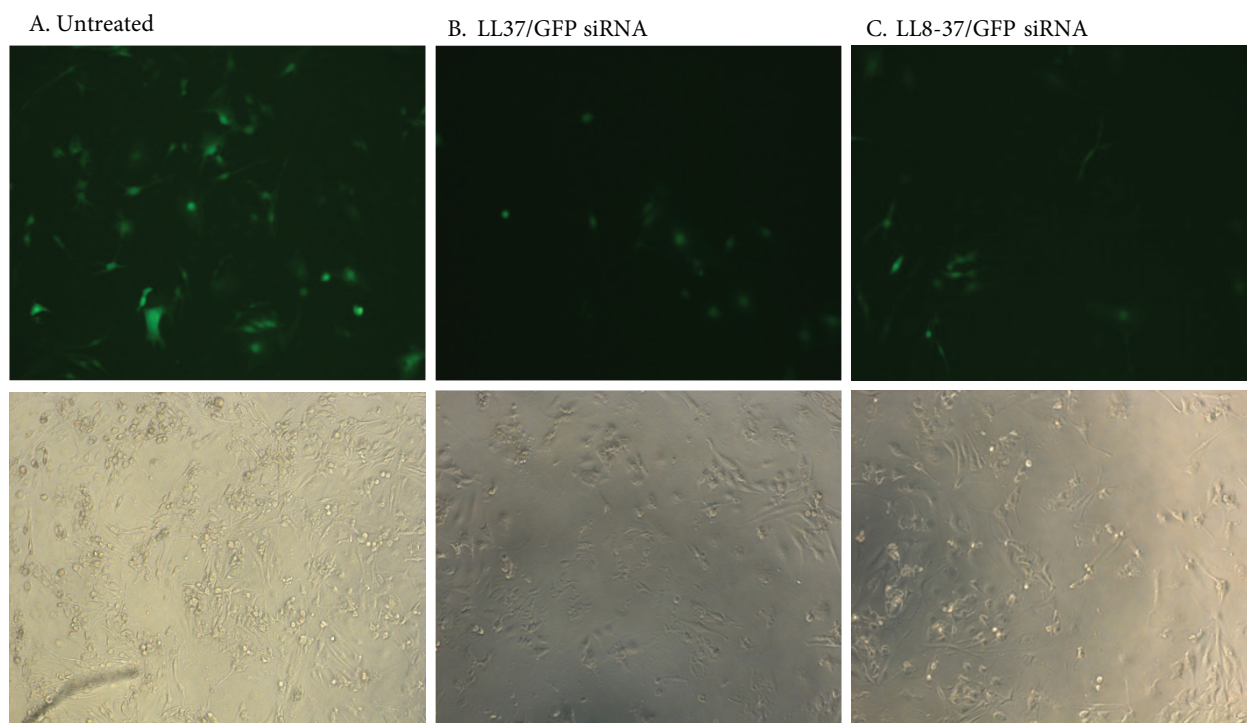


Figure 3. LL-37 mediated GFP knockdown in HCC1143-GFP cells. HCC1143-GFP cells were incubated with LL-37/GFP siRNA complexes for 24 h in serum-free media. Pictures were taken 48 h after the addition of fresh media. Fluorescence and bright field images of: a) untreated HCC1143-GFP cells, b) HCC1143-GFP treated with complexes prepared at 4:1 LL-37 to GFP siRNA charge ratio, and c) LL8-37-GFP siRNA complexes prepared at 3:1 ratio.

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