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To be cited as: *Chem. Asian J.* 10.1002/asia.201801396

Link to VoR: http://dx.doi.org/10.1002/asia.201801396

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COMMUNICATION

Cell-selective delivery of microRNA with microRNA-peptide conjugate nanocomplex

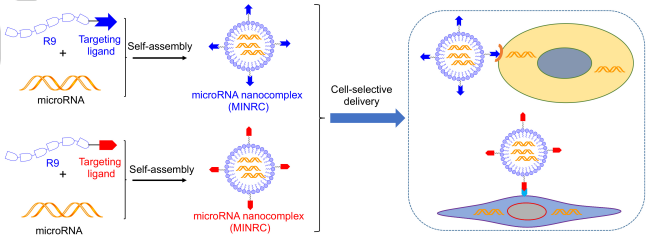
Xiao Xiao,[a] Xingxing Wang,[a] Haidong Gao,[b] Xi Chen,[b] Jinbo Li*[a,b] and Yan Zhang*[a,b]

Abstract: Targeted delivery of microRNA (miRNA) into specific cells has been regarded as an efficient strategy to enhance miRNA-targeted therapeutics. However, concurrent delivery of therapeutic miRNAs into different target cells that is conducive to multi-target therapy is still underdeveloped. Here, we report a novel strategy for cell-selective delivery of miRNA into different target cells through using miRNA nanocomplexes (MINRCs) formed by miRNA with peptide conjugates. The peptide conjugates comprised a cationic cell-penetrating peptide nona-arginine and a targeting ligand that is cyclic RGD or folic acid. Upon mixing in buffer, the peptide conjugates and miR-34a readily formed two MINRCs, respectively. These two MINRCs facilitated the targeted delivery of miR-34a into RGD receptor-positive U87MG cells or folate receptor-positive HeLa cells via ligand-receptor interaction. We also demonstrated that co-incubation of these two MINRCs with U87MG and HeLa cells led to cell-selective delivery of miR-34a.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that function by negatively regulating gene expression at the post-transcriptional level.[1] Since approximately 60% of human genes are their targets,[2] miRNAs extensively involve in not only normal physiologies but also pathologies.[3] Aberrantly expressed miRNAs have close links with the development and progress of various human diseases, including cancers.[4] Regulation of endogenous abnormal miRNAs has therefore been confirmed as an efficient therapeutic strategy.[5] Synthetic miRNA mimics that replenish tumor-suppressive miRNAs upon proper delivery into cells appear as promising drug candidates.[6] The demand of strategies for targeted delivery of miRNA that can enhance miRNA-targeted therapeutics is thus tremendously high.[7] However, cell-selective delivery of miRNA into different target cells in a multicellular context, which is conducive to multi-target therapy, still remains challenging.

In addition to conventional carriers such as lipids and nanovectors,[8] cationic cell-penetrating peptides have emerged as biocompatible and efficient carriers for nucleic acid delivery and gene therapy.[9] Targeted delivery of nucleic acids into specific cells is also achievable through using peptide conjugates by linking cationic peptides with cell-targeting ligands. For example, several peptide conjugates that are cationic nona-arginine (R9) modified with cell-binding ligands were used to transport small-interfering RNA (siRNA) into specific target cells through nanocomplexes formed by these peptide conjugates with siRNA.[10] Recently, we developed a multi-functional peptide conjugate that comprised R9, a targeting ligand and an activatable fluorescence probe for monitoring miRNA function.[11] The multi-functional peptide conjugate readily formed miRNA nanocomplex (MIRNC) upon mixing with miRNA for targeted miRNA delivery as well as real-time imaging of miRNA function. Therefore, it is possible to use different peptide conjugates to form MIRNCs with miRNAs and accomplish cell-selective delivery of miRNA into different target cells through using these MIRNCs.

Here, we report a novel strategy for cell-selective and concurrent delivery of miRNA into two different target cells by using MIRNCs (**Scheme 1**). In this method, R9 is respectively modified with two different targeting ligands, and the resulted peptide conjugates are used to form two MIRNCs with miRNA for selective delivery of miRNA into target cells expressing corresponding receptors.



Scheme 1. Schematic illustration of cell-selective delivery of miRNA with different miRNA nanocomplexes formed by targeting ligands-modified R9 and miRNA.

We chose cyclic (Arg-Gly-Asp-Phe-Lys) c(RGDfK) and folic acid (FA) as the targeting ligands to modify R9. c(RGDfK) and FA are the ligands of $\alpha_v\beta_3$ integrin that overexpresses on the membrane of U87MG cells[12] and folate receptor that is highly expressed on the membrane of HeLa cells,[13] respectively. To connect R9 with c(RGDfK) or FA, we used click chemistry[14] as the conjugation method. Briefly, alkyne-functionalized R9 was prepared via solid-phase peptide synthesis, and c(RGDfK) or FA were pre-installed with azide functionality. c(RGDfK)-R9 and FA-R9 peptide conjugates (**Scheme 2**) were then obtained through Cu(I)-catalyzed click reaction between alkyne-functionalized R9 and azide-functionalized c(RGDfK) or azide-functionalized FA. Following purification of c(RGDfK)-R9 and FA-R9 with high-performance liquid chromatography (HPLC), we verified these

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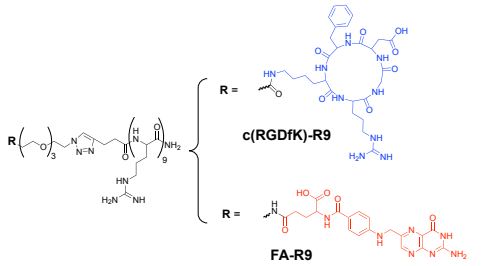
two peptide conjugates by mass spectrometry (MS) (supporting information).



Scheme 2. Chemical structures of c(RGDfK)-R9 and FA-R9 peptide conjugates.

Next, we first investigated whether c(RGDfK)-R9 and FA-R9 could self-assemble with miRNA to form nanocomplexes. Tumor-suppressive miR-34a[5a] was selected as the model miRNA for further studies. c(RGDfK)-R9 or FA-R9 were mixed with miR-34a in phosphate buffer saline (PBS) with molar ratios ranging from 0/1 to 70/1. The mixtures were allowed to stand at room temperature for 30 minutes and further subject to gel electrophoresis. **Figure 1a** shows the gel-shift analysis results. The amount of free miR-34a significantly decreased when the c(RGDfK)-R9/miR-34a and FA-R9/miR-34a ratios increased. Most of the free miR-34a was packaged in nanocomplexes when c(RGDfK)-R9/miRNA ratio was larger than 30/1. Free miR-34a completely disappeared when FA-R9/miR-34a ratio reached 30/1, indicating that miR-34a is totally encapsulated in the nanocomplexes. We then measured the sizes of formed nanocomplexes by dynamic light scattering (DLS). Continued decrease in the sizes, which ranged from 1200 to 100 nm or 200 to 100 nm, was observed for nanocomplexes formed by c(RGDfK)-R9/miR-34a or FA-R9/miR-34a (**Figures 1b** and **1c**). Transmission electron microscope images of the formed nanocomplexes (**Figure S1**) showed that the diameter sizes of nanocomplexes formed by c(RGDfK)-R9/miR-34a 50/1 or FA-R9/miR-34a 50/1 were ~80 nm or ~50 nm, respectively. These results confirm that c(RGDfK)-R9 or FA-R9 peptide conjugates are able to form nanocomplexes with miRNA and also suggest that targeting ligands can affect the self-assembly ability of R9 toward miRNA.

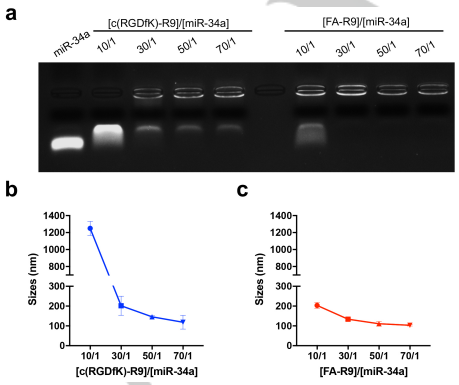


Figure 1. (**a**) Gel-shift analysis of free miR-34a in the mixtures of c(RGDfK)-R9 or FA-R9 and miR-34a with molar ratios at 10/1, 30/1, 50/1 and 70/1. (**b**, **c**) Hydrodynamic sizes of the nanocomplexes formed (**b**) c(RGDfK)-R9 or (**c**) FA-R9 with miR-34a. The molar ratios were 10/1, 30/1, 50/1 and 70/1. Data are shown as mean ± SEM (n=3).

Intracellular delivery efficiency of the formed nanocomplexes was further tested. Since only a few of free miR-34a was left after self-assembly with c(RGDfK)-R9 or FA-R9, we directly used the formed nanocomplexes without purification. Nanocomplexes formed by c(RGDfK)-R9/miR-34a at different molar ratios were then incubated with U87MG cells that express high level of $\alpha_v\beta_3$ integrin on the membrane.[12] After incubation for 24 h, the endogenous miR-34a levels in U87MG cells were measured by real-time quantitative PCR (RT-qPCR). As shown in **Figure 2a**, endogenous miR-34a levels significantly increased when the c(RGDfK)-R9/miR-34a ratio was larger than 10/1, and it reached maximum when the molar ratio was 50/1. To test the delivery efficiency of nanocomplexes formed by FA-R9/miR-34a, folate receptor-positive HeLa cells[13] were used. Endogenous miR-34a levels inside HeLa cells were also determined by RT-qPCR after the nanocomplexes were incubated with HeLa cells for 24 h. **Figure 2b** shows the RT-qPCR analysis results. The miR-34a levels remarkably increased along with the increase of FA-R9/miR-34a ratio and also reached plateau when the ratio was 50/1. We therefore used the nanocomplexes formed by c(RGDfK)-R9/miR-34a 50/1 and FA-R9/miR-34a 50/1 for further investigations, which were respectively dubbed as RGD-MIRNC-34a and FA-MIRNC-34a. It is worth noting that c(RGDfK)-R9 or FA-R9 peptide conjugates did not modulate endogenous miR-34a levels (**Figure S2**) and the delivery efficiency of these two nanocomplexes was comparable to that of the commercial transfection agent Lipofectamine 2000 (**Figure S3**).

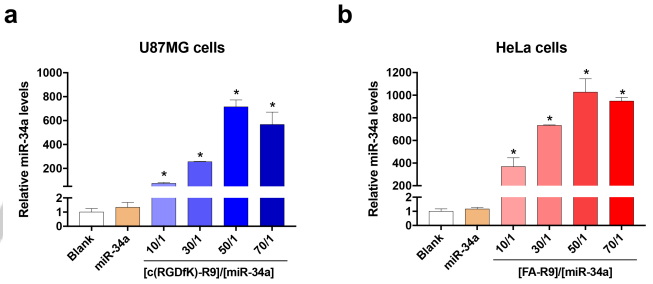


Figure 2. RT-qPCR analysis of endogenous miR-34a levels in (**a**) U87MG cells and (**b**) HeLa cells after incubation with free miR-34a or nanocomplexes formed by c(RGDfK)-R9/miR-34a or FA-R9/miR-34a with molar ratios at 10/1, 30/1, 50/1 and 70/1. The final concentration of miR-34a was 100 nM. Data are shown as mean ± SEM (n=3). $^*P < 0.05$, relative to blank control.

We have previously demonstrated that MIRNC could distinguish receptor-positive cells from receptor-negative cells and deliver miRNA into target cells through receptor-mediated endocytosis.[11] We speculate that RGD-MIRNC-34a and FA-MIRNC-34a may have the capability to find target cells in a mixture of co-cultured cells and deliver miRNA into their target cells in a cell-selective manner. To demonstrate this hypothesis, we first validated whether RGD-MIRNC-34a and FA-MIRNC-34a could recognize their target cells. We used $\alpha_v\beta_3$ integrin-positive U87MG and HUVEC cells, folate receptor-positive HeLa and KB cells, and RAW264.7 cells that are both $\alpha_v\beta_3$ integrin-negative and folate receptor-negative (**Figure S4**). These cells were then respectively incubated with RGD-MIRNC-34a or FA-MIRNC-34a for 24 h, followed by analysis of endogenous miR-34a levels via RT-qPCR. As shown in **Figures 3a** and **3b**, in comparison with

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that induced by RGD-MIRNC-34a, the endogenous miR-34a levels in U87MG and HUVEC cells after treatment with FA-MIRNC-34a were much lower. Similarly, the endogenous miR-34a levels in HeLa and KB cells remarkably up-regulated only after treatment with FA-MIRNC-34a but not RGD-MIRNC-34a (**Figures 3c and 3d**). It is noteworthy that no significant uptake of both FA-MIRNC-34a and RGD-MIRNC-34a in RAW264.7 cells was observed (**Figure 3e**). To further validate these results, we used R9 without targeting ligands to self-assemble with miR-34a to deliver miR-34a into these cells, resulting in only a slight increase in endogenous miR-34a levels (**Figure 3**). Moreover, we also used c(RDDfK) that cannot bind to $\alpha_v\beta_3$ integrin to modify R9 and prepared the nanocomplex RDD-MIRNC-34a through incubation of c(RDDfK)-R9 with miR-34a (50/1). When using RDD-MIRNC-34a to treat U87MG or HUVEC cells, we detected only a slight increase in endogenous miR-34a levels (**Figure S5**). These results demonstrate that MIRNCs are able to recognize target cells and deliver miRNA with high specificity and efficiency. It is also worth noting that miR-34a upon delivery into U87MG or HeLa cells by RGD-MIRNC-34a or FA-MIRNC-34a was able to repress target gene expression (**Figure S6**), which was demonstrated by using luciferase reporter systems as we reported before.[11, 15]

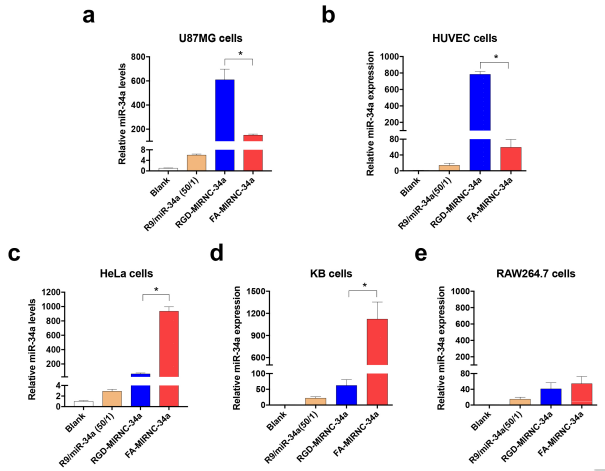

Figure 3. RT-qPCR analysis of endogenous miR-34a levels in (**a**) U87MG cells, (**b**) HUVEC cells, (**c**) HeLa cells, (**d**) KB cells and (**e**) RAW264.7 cells after incubation with nanocomplex formed by R9 and miR-34a (peptide/miRNA 50/1), RGD-MIRNC-34a, or FA-MIRNC-34a for 24 h. The final concentration of miR-34a was 100 nM. Data are shown as mean ± SEM (n=3). $^*P < 0.05$.

Finally, we demonstrated the hypothesis that cell-selective delivery of miRNA in a mixture of co-cultured cells could be achieved through using different MIRNCs. To directly view the selective delivery, we used Cy3/Cy5-labled miR-34a for fluorescence tracking. The nanocomplexes were prepared by respectively mixing c(RGDfK)-R9 with Cy3-miR-34a (50/1) or FA-R9 with Cy5-miR-34a (50/1). Time-dependent fluorescence imaging results confirmed that RGD-MIRNC-34a^{Cy3} or FA-MIRNC-34a^{Cy5} could respectively deliver miR-34a into their target cells through endocytosis (**Figures S7** and **S8**). We then co-cultured U87MG and HeLa cells, followed by co-incubation with RGD-MIRNC-34a^{Cy3} and FA-MIRNC-34a^{Cy5} for 24 h. To distinguish HeLa cells from U87MG cells, HeLa cells were pre-stained with Hoechst 33342 prior to co-culture with U87MG cells. **Figures 4a** and **4e** show the image for nucleus staining and bright field image of co-cultured cells, respectively. In the view, we could easily recognize HeLa and U87MG cells. Fluorescence signals from Cy3-miR-34a and Cy5-miR-34a inside co-cultured cells were then acquired to indicate the location of miR-34a delivered by RGD-MIRNC-34a or FA-MIRNC-34a. We found that Cy3-miR-34a mainly located in the cytoplasm of U87MG cells (**Figures 4b** and **4d**). In comparison, Cy5-miR-34a was mainly detected in the cytoplasm of HeLa cells (**Figures 4c** and **4d**). Moreover, RGD-MIRNC-34a and FA-MIRNC-34a could also selectively deliver miR-34a into U87MG cells and HeLa cells, respectively, when U87MG cells or HeLa cells were co-cultured with RAW264.7 cells (**Figures S9** and **S10**). These results confirm that RGD-MIRNC-34a and FA-MIRNC-34a are able to deliver miRNA into their target cells when incubation with co-cultured cells. Therefore, cell-selective delivery of miRNA in complex multicellular systems is achievable through using MIRNCs.

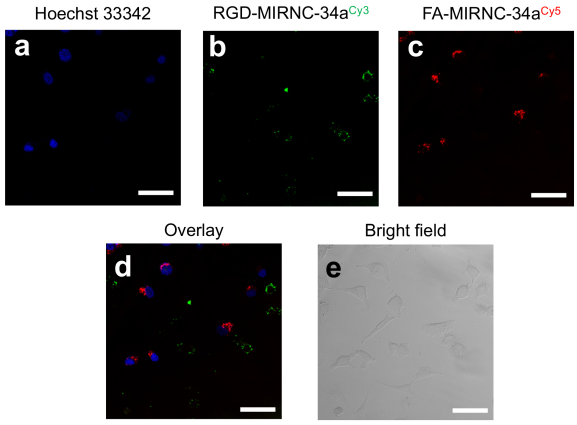

Figure 4. Bright field and confocal fluorescence images of co-cultured U87MG cells and HeLa cells (1/1) after incubation with RGD-MIRNC-34a^{Cy3} and FA-MIRNC-34a^{Cy5} for 24 h. The final concentration of Cy3/Cy5-labeled miR-34a was 100 nM. (**a**) Hoechst 33342 channel, nucleus staining of HeLa cells; (**b**) Cy3 channel; (**c**) Cy5 channel; (**d**) Merged image; (**e**) Bright field image. Scale bar: 75 μm.

In summary, we developed a novel strategy for cell-selective delivery of miRNA in a multicellular context. In this strategy, c(RGDfK)-R9 and FA-R9 peptide conjugates were the only necessary reagents, which were constructed by conjugating c(RGDfK) or FA with R9 through click chemistry. These two peptide conjugates readily formed two MIRNCs with miR-34a. RGD-MIRNC-34a and FA-MIRNC-34a were able to deliver miR-34a into corresponding target cells with high specificity and efficiency. Upon incubation with co-cultured target cells, RGD-MIRNC-34a and FA-MIRNC-34a delivered miR-34a into their target cells in a cell-selective manner. Therefore, this strategy offers a practical way to realize cell-selective delivery of miRNA in multicellular context. Further exploration of this strategy in multi-miRNA delivery and multi-target therapy in living animals is now underway in our group.

Acknowledgements

We would like to acknowledge the financial support from the National Natural Science Foundation of China (21672103, 21572102, 91753116, 21372115) and the Fundamental Research Funds for Central Universities (020514380133, 020514380137).

Keywords: microRNA • peptide conjugate • microRNA nanocomplex • targeted delivery • cell-selective delivery

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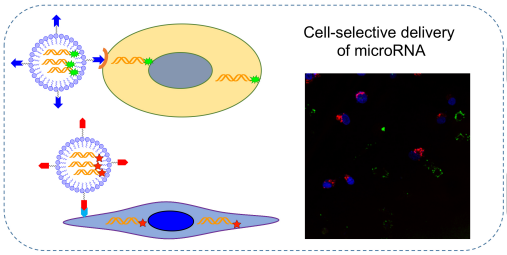

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