Light-Induced Angiogenesis Hot Paper

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# Regulating Angiogenesis with Light-Inducible AntimiRs**

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MicroRNAs (miRNAs, miRs) are small noncoding RNAs that posttranscriptionally regulate gene expression by mRNA degradation or translational repression.[1,2] While the effect of a given miR on one mRNA is often modest, owing to the large number of different targets miRs can be used to regulate gene expression patterns.[3] miRs play crucial roles in tissue homeostasis and altered expression levels of miRs are related to many diseases.[4,5] Angiogenesis, the process of the formation of new blood vessels from existing ones, is required to maintain oxygen supply to tissues after ischemia and has been shown to be regulated by several miRs.[6] The pro-angiogenic miR-126, for instance, regulates angiogenesis by targeting negative regulators of vascular endothelial growth factor signaling, whereas members of the miR-17-92 cluster exhibit anti-angiogenic properties.[7,8] Specifically, the inhibition of miR-92a by antisense oligonucleotides improved neovascularization and augmented the recovery of heart function after critical ischemia in mice and large animal models.[8,9]

Antagomirs and locked nucleic acid (LNA)-based antimiRs were shown to inhibit miR activity in vitro and in vivo after injection. They penetrate almost all tissues and have a long-lasting effect.[10] LNA-based antimiRs were shown to be safe and efficient in first clinical trials in humans.[11,12] Both antagomirs and LNA-based antimiRs directed against miR-92a showed similar therapeutic benefits in experimental studies.[8,9]

However, the broad and often ubiquitous expression of miRNAs and their multiple functions in different tissuesmay preclude complicate the systemic application of antimiRs. An external trigger signal to spatially and temporally restrict antimiR activity in a defined cellular context and avoid the systemic inhibition of a given miRNA would overcome the problem that miRNAs may have opposing functions in tissues. Light is a suitable external trigger signal as it can be applied in a defined and precise way and is an orthogonal signal that does not interfere with other cellular processes if a suitable wavelength is used. Recently, novel techniques have become available that allow light-controlled activation as deep as 10 mm in a tissue sample.[13,14]

Several studies concerning the light-induced activation of nucleic acids with "caged" oligonucleotides have been published.[15] Two studies investigated the use of light-induced regulation of miRNA expression and showed that light-activated antagomirs directed against miR-122 and miR-21, in the first study, and an antisense oligonucleotide against a *C. elegans* miRNA, in the second study, can be used to block miRNA expression in model systems.[16,17] The two studies made use of artificial reporter proteins or manipulated organisms. In an additional study caged constructs were used to turn let-7 miRNA on and off in developing zebrafish embryos.[18] Two of these studies made use of non-nucleosidic photocleavable linkers; the other one used caged 2′-*O*Me-uridine. The latter strategy restricts the applicability to suitable sequences containing multiple uridines. To our knowledge, the use of light-induced antimiRs to affect endogenous target gene expression and physiological functions in primary human cells with a therapeutic motivation has not been explored so far. Herein we report on light-induced antimiRs directed against the anti-angiogenic miR-92a to improve angiogenesis.

To develop a light-inducible antimiR against miR-92a, we designed a 21-mer oligonucleotide bearing 2′-*O*Me-RNA nucleotides that were modified with 1-(2-nitrophenyl)ethyl (NPE) photolabile protecting groups at the nucleobases (Figure 1b) and five phosphorothioate linkages at both ends (Figure 1a and Table 1 in the Supporting Information). After uncaging (Figure 1 in the Supporting Information), the antimiRs can hybridize with perfect complementarity to miR-92a (Table 1 in the Supporting Information). We synthesized three different caged antimiRs with three, five, and six caged residues spread over the whole sequence. Caged 2′-*O*Me-phosphoramidites, for site-specific incorporation of caged moieties into the antimiRs, were synthesized starting from suitable commercially available precursors (Schemes 1–3 in the Supporting Information). The modified phosphoramidites were incorporated into caged oligonucleotides $\mathbf{A}^{\mathbf{3c}}$, $\mathbf{A}^{\mathbf{5c}}$, and $\mathbf{A}^{\mathbf{6c}}$. As controls a noncaged antimiR ($\mathbf{A}^{\mathbf{pos}}$) against miR-92a and an antimiR ($\mathbf{A}^{\mathbf{neg}}$) that does not have any endogenous target and does not influence miR-92a level compared to untransfected control cells (Figure 3 in the Supporting Information) and a caged version thereof ($\mathbf{A}^{\mathbf{neg\ c}}$) were synthesized. After purification (Figure 1 in the Supporting Information) and characterization (Table 2 in the Supporting Information) antimiRs were transfected into human umbilical vein endothelial cells (HUVECs). Four hours after transfection, cells were irradiated with a transilluminator ($\lambda =$

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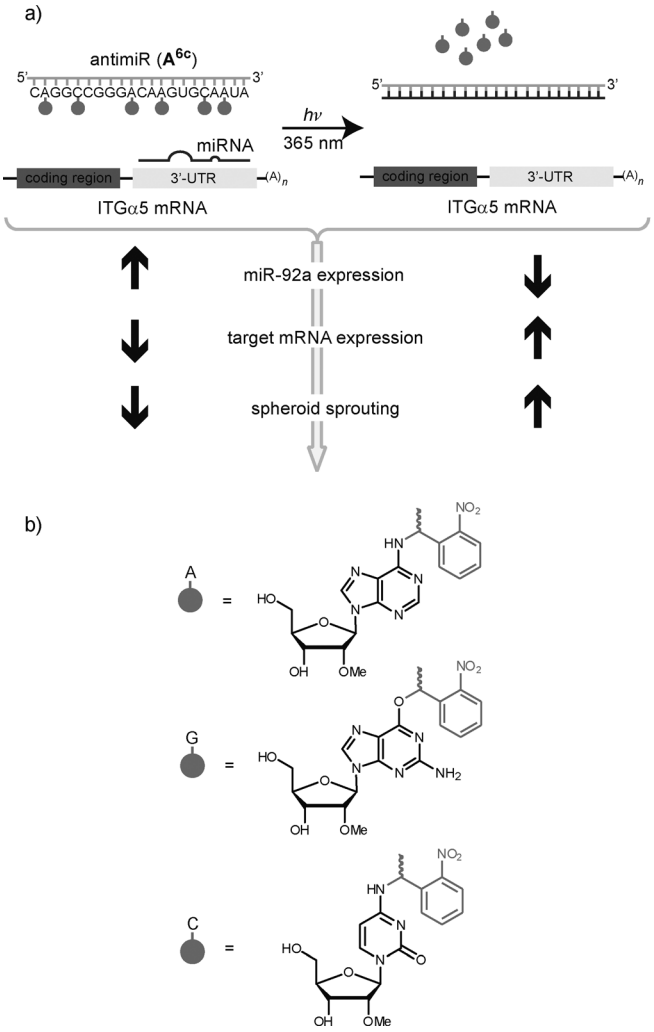


***Figure 1.*** a) AntimiRs with caged nucleobases cannot base-pair to miRNAs. Uncaging by light irradiation results in reduced miRNA levels, derepression of target mRNA, and, in the miR-92a system, to enhanced angiogenesis. b) Overview of nucleobase-caged residues used in this study. For synthesis see the Supporting Information.

365 nm, 5–6 mW) or kept in the dark for 25 min. Two days (48 h) after irradiation, the cells were lysed and miR-92a expression was determined by a Taqman microRNA assay.

Whereas the antimiR containing three caged moieties ($\mathbf{A^{3c}}$) still showed some inhibitory activity and partially inhibited miR-92a expression in the absence of irradiation, increasing the number of caged nucleotides to six ($\mathbf{A^{6c}}$) fully inactivated the antimiR and prevented the reduction of miR-92a expression in the absence of irradiation (Figure 2a). This is in accordance with the fact that melting point of the $\mathbf{A^{6c}}$/miR-92a duplex is lower than that of $\mathbf{A^{3c}}$/miR-92a (Table 3 in the Supporting Information). Exposure of $\mathbf{A^{3c}}$, $\mathbf{A^{5c}}$, and $\mathbf{A^{6c}}$ antimiR-transfected HUVECs to light induced an activation of the inhibitory activity of all caged antimiRs and resulted in a significant reduction of miR-92a to levels achieved with the active control antimiR $\mathbf{A^{pos}}$ (Figure 2a). Light-induced activation of antimiR $\mathbf{A^{6c}}$ was already detectable after 5 min of light exposure but was more efficient after prolonged exposure of up to 25 min (Figure 2b).

To exclude the effects of the irradiation itself, cells transfected with the noncaged control $\mathbf{A^{pos}}$, the nontargeting control $\mathbf{A^{neg}}$, and its caged derivative $\mathbf{A^{negc}}$ were also irradiated. No significant difference to the nonirradiated samples could be observed, indicating that irradiation itself does not have any effect on miR-92a expression (Figure 2a). Furthermore no differences in cell morphology between irradiated and nonirradiated samples were detectable. The observed effect is sequence specific since neither $\mathbf{A^{pos}}$ nor $\mathbf{A^{6c}}$ with or without irradiation had any effect on miR-126 expression (Figure 2 in the Supporting Information).

Next we tested whether endogenous mRNA levels of miR-92a target genes are also regulated by light-inducible antimiRs. The expression of ITGα5, a known target of miR-92a in endothelial cells, is 1.6-fold higher when miR-92a levels are reduced by the active antimiR control $\mathbf{A^{pos}}$; this is in contrast to the effect of the inactive antimiR control $\mathbf{A^{neg}}$ which does not influence miR-92a and ITGα5 mRNA expression (Figure 2c). In the absence of irradiation, only $\mathbf{A^{3c}}$ significantly augmented ITGα5 mRNA expression (Figure 2c), which is consistent with the half maximal inhibition of miR-92a expression by this antimiR (Figure 2a). In contrast, $\mathbf{A^{5c}}$ only slightly increased ITGα5 expression and $\mathbf{A^{6c}}$ exhibited no effect on ITGα5 expression in the absence of light. However, after light exposure, ITGα5 expression was significantly augmented in $\mathbf{A^{5c}}$- and $\mathbf{A^{6c}}$-transfected endothelial cells to levels that were observed in positive-control antimiR $\mathbf{A^{pos}}$-transfected cells (Figure 2c). The effect on target gene derepression by light-induced antimiRs was dose-dependent and correlated with miR-92a down-regulation. A maximal effect was achieved by transfecting 50 nM $\mathbf{A^{6c}}$ (Figure 2d; Figures 4 and 5 in the Supporting Information). Again irradiation itself does not have any effect on target mRNA levels supporting the fact that irradiation at this wavelength does not harm cells and that the observed effects are sequence specific (Figure 2c).

MiR-92a is a known regulator of angiogenesis in endothelial cells.[8] To determine whether light-induced antimiRs have a biological activity, we used the spheroid sprouting assay to measure the angiogenic activity of HUVECs after transfection with antimiRs.[19,20] Consistent with the anti-angiogenic activity of miR-92a, inhibition of miR-92a by $\mathbf{A^{pos}}$ leads to an enhancement of angiogenic sprouting (Figure 3a,b). The introduction of three caged moieties in the antimiR increased the sprouting activity in the absence of light, which is consistent with the lack of inactivation of the biological activity of $\mathbf{A^{3c}}$ shown above. In contrast, antimiR $\mathbf{A^{6c}}$, in which six moieties are modified, did not affect sprouting in the absence of irradiation in comparison to $\mathbf{A^{neg}}$ (Figure 3a). However, irradiation of $\mathbf{A^{6c}}$-transfected cells augmented the sprouting activity of endothelial cells to levels that were achieved with $\mathbf{A^{pos}}$. In summary these data show that in vitro angiogenesis can be regulated by light-inducible antimiRs against miR-92a.

In conclusion, we showed that miR activity in primary human cells can be controlled by light by using caged antimiRs. To completely abolish the activity of antimiRs, five to six caged residues were necessary. Notably, the caged oligonucleotides are stable in cell culture for at least three

  

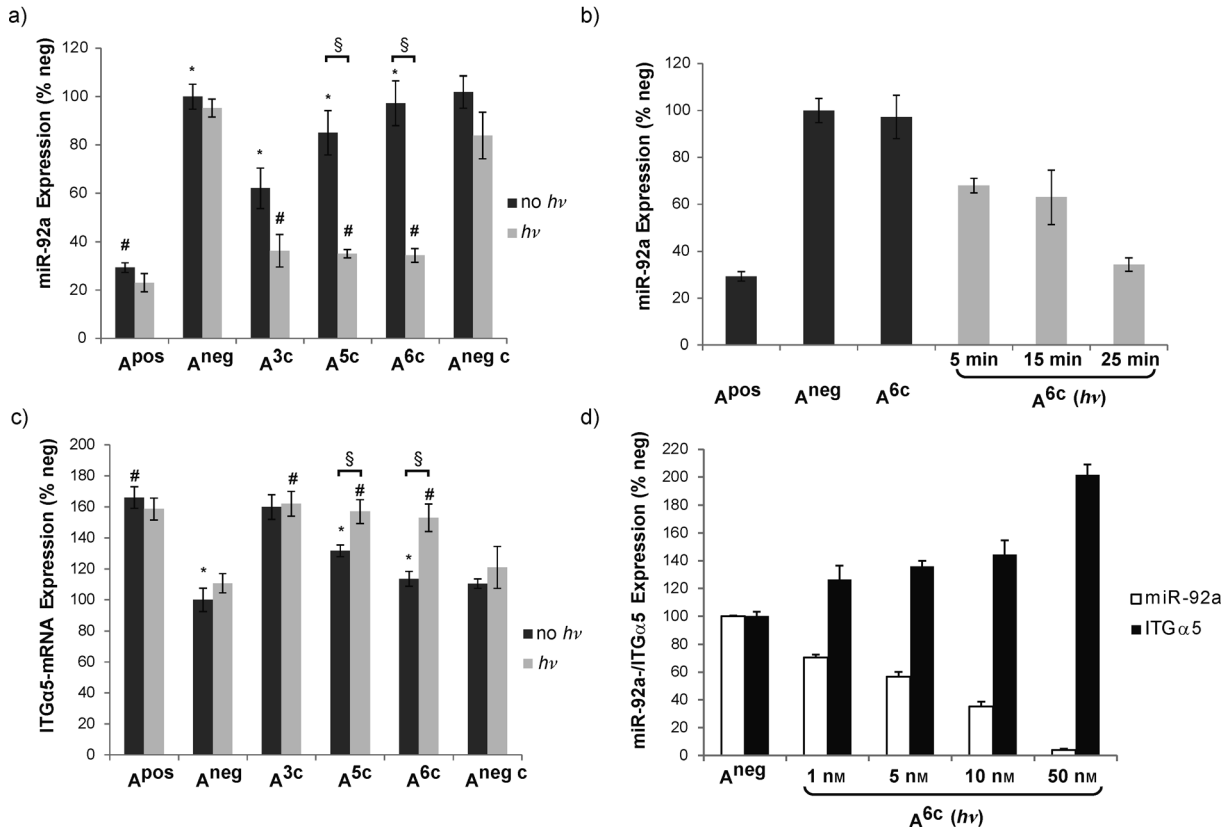


***Figure 2.*** Photoactivation of antimiRs against miR-92a. a) miR-92a expression after transfection of HUVECs with the different antimiRs (10 nM) and irradiation for 25 min (light gray bars) or in the absence of irradiation (dark gray bars). b) miR-92a expression after transfection of HUVECs with antimiR $\mathbf{A^{6c}}$ or control antimiRs and irradiation for different times (5, 15, 25 min; light gray bars) or in the absence of irradiation (dark gray bars. c) ITGα5 mRNA expression after transfection of HUVECs with the different antimiRs (10 nM) and irradiation for 25 min (light gray bars) or in the absence of irradiation (dark gray bars). d) miR-92a (white bars) and ITGα5 mRNA (black bars) expression after transfection of HUVECs with different concentrations (1, 5, 10, 50 nM) of antimiR $\mathbf{A^{6c}}$ or control antimiR $\mathbf{A^{neg}}$ and irradiation for 25 min. Data are expressed relative to the nontargeting control $\mathbf{A^{neg}}$. Error bars represent the standard error of the mean (SEM), $n=4$. $^{\#}p<0.01$ compared to $\mathbf{A^{neg}}$, $^{*}p<0.05$ compared to $\mathbf{A^{pos}}$, $^{\S}p<0.05$ (t-test).

days, indicating that the photolabile protecting groups are not removed by repair enzymes. Light-induced activation of the caged antimiRs fully activated the biological properties leading to efficient miR-92a inhibition and derepression of its target gene ITGα5. Most importantly, we showed for the first time that angiogenesis of endothelial cells can be augmented by light-induced activation of caged antimiRs implicating that light-inducible antimiRs may have a potential as therapeutic agents. AntimiRs have been used as therapeutics in various mice models and several larger preclinical animal studies.[9,21,22] Furthermore, a recent clinical trial reported the safe and efficient use of antimiRs directed against miR-122 in humans.[12] Given that systemic inhibition of miRNAs may result in unwanted side effects due to the different functions of miRNAs in the tissues and/or oncogenic effects as reported for some miRNAs, our present study may offer a strategy for local activation, which may allow spatial targeting of the antimiR effects. This might be particularly useful for the treatment of surface tissue such as the skin, where, for example, wound healing could be enhanced by augmenting angiogenesis. In addition, local delivery and activation during surgery or by catheters may be possible. Previous experiments have shown that a wide range of photolabile protecting groups can be used with different light-absorption characteristics.[15] In particular by using red or IR light and applying two-photon irradiation technologies or using upconversion techniques, penetration depths of up to 10 mm can be achieved.[13,14,23,24]

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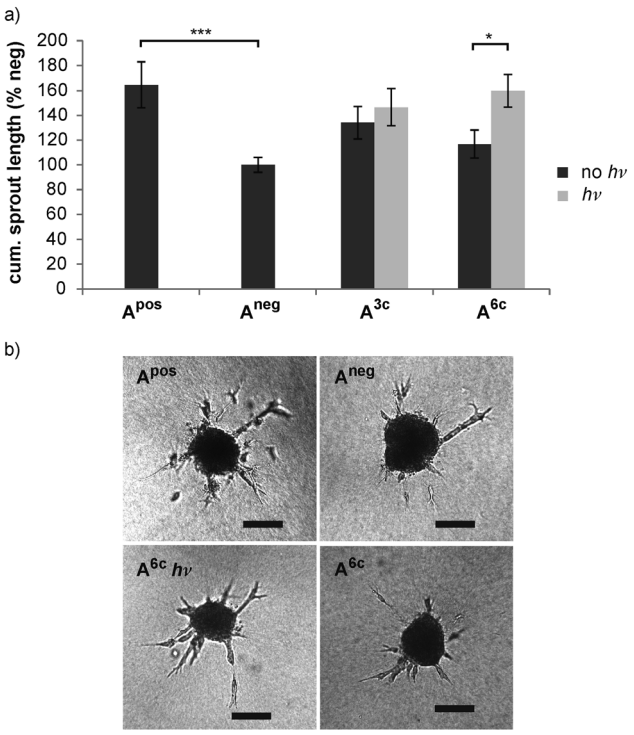


***Figure 3.*** Effect of light-inducible antimiRs on angiogenesis in endothelial cells. a) Cumulative sprout length of spheroids (mean of 10 spheroids per condition) generated from HUVECs after transfection with different antimiRs (10 nM) in the presence (light gray bars) or absence (dark gray bars) of irradiation. On day (24 h) after irradiation transfected cells were used for spheroid preparation. Sprouting of spheroids was measured 72 h posttransfection. Error bars represent the standard error of the mean (SEM), $n=5$. $*p<0.05$, $***p<0.001$ (Anova Bonferroni's multiple comparison test). b) Representative spheroid pictures for the conditions $\mathbf{A}^{pos}$ (upper left), $\mathbf{A}^{neg}$ (upper right), $\mathbf{A}^{6c}$ after irradiation (lower left), and $\mathbf{A}^{6c}$ without irradiation (lower right). The scale bar corresponds to 100 μm length.

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