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Photo-regulation of Constitutive Gene Expression in Living Cells by Using of Ultrafast Photo-cross-linking Oligonucleotides

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3-Cyanovinylcarbazole nucleoside (^{CNV}K), which has ultrafast photo-cross-linking ability to complementary RNA was incorporated to the antisense oligonucleotide targeting green fluorescent protein (GFP) mRNA. We successfully demonstrated that the photoreactive antisense oligonucleotides effectively regulate GFP expression in a stable cell line expressing the GFP gene in a photo-responsive manner.

The development of methods for regulating gene expression is one of the most important issues in the field of drug discovery, biology and chemical biology. An antisense strategy[1] that can regulate specifically gene expression with the specific binding property of a short oligonucleotide, i.e. an antisense oligonucleotide (AS-ODN), and its complementary target mRNA is a promising technology in this field. In this strategy, chemically modified AS-ODNs, such as phosphorothioates,[2] peptide nucleic acids[3] and locked (bridged) nucleic acids,[4] have been used to avoid the degradation by nucleases in blood or cells, or to stabilize the hetero-duplex of AS-ODN and its target mRNA, and these AS-ODNs successfully regulate the gene expression in vivo.[5]

As another class of chemically modified AS-ODNs, photoresponsive AS-ODNs having a photo-cleavable linker,[6] an azobenzene[7] or a psolaren moiety[8] that can control the stability of the AS-ODN/mRNA hetero-duplex by photo-irradiation has been developed. Based on the photoresponsive manner of these AS-ODNs, the regulation of gene expression can be induced with the desired timing and position. Although these AS-ODNs are a promising tool for biochemistry and chemical biology, there are few reports on the photo-regulation of constitutive gene expression. In many cases, the photo-regulation of gene expression has been demonstrated using a transient gene expression system with co-transfection of a reporter gene and AS-ODNs.

In this study, we adopted the phosphorothioate AS-ODN having 3-cyanovinylcarbazole nucleoside (^{CNV}K),[9] which has ultrafast photo-cross-linking ability to the pyrimidine base in target DNA or RNA strand (Fig. 1), as a photo-cross-linker toward green fluorescent protein (GFP) mRNA. The photoresponsive gene silencing activity of these AS-ODNs for the regulation of constitutive GFP expression in a GFP stable cell line (GFP-HeLa) was evaluated (Fig. 2).

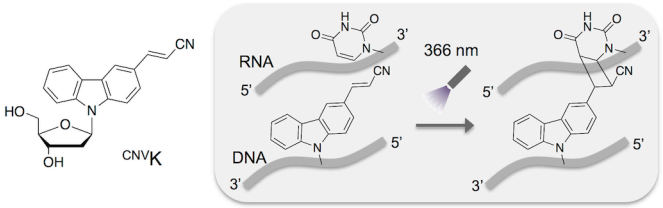

Figure 1. Structure of ^{CNV}K and the photo-cross-linking reaction of ^{CNV}K with U in DNA/RNA heteroduplex.

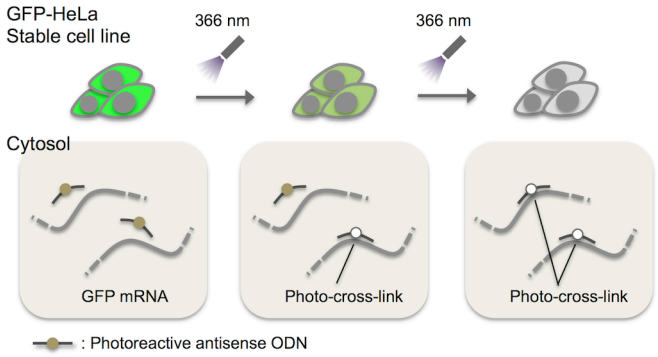

Figure 2. Schematic drawing of the photo-regulation of GFP gene expression in stable cell line with K-AS.

First, the photoreactivities of AS-ODNs (K-AS-a1~3; Sequence of all AS-ODNs are listed in Table 1) toward

Table 1. Sequences of AS-ODNs used in this study

Name	Target region	Sequence[a]
AS-a	14 – 38	ACCACCCCGGTGAACAGCTCCTCGC
K-AS-a1	14 – 38	A**X**CACCCCGGTGAACAGCTCCTCGC
K-AS-a2	14 – 38	ACCACCCCGGTGA**X**CAGCTCCTCGC
K-AS-a3	14 – 38	ACCAC**X**CCGGTGAACAGCTCCTCGC
K-AS inv	14 – 38	CGCTCCTCGACAAGTGGCCCCAC**X**A
AS-b	471 – 495	AGTTCACCTTGATGCCGTTCTTCTG
K-AS-b1	471 – 495	A**X**TTCACCTTGATGCCGTTCTTCTG
K-AS-b2	471 – 495	AGTTCACCTTGA**X**GCCGTTCTTCTG
AS-c	542 – 566	ATGGGGGTGTTCTGCTGGTAGTGGT
K-AS-c1	542 – 566	A**X**GGGGGTGTTCTGCTGGTAGTGGT
AS-d	592 – 616	ACTGGGTGCTCAGGTAGTGGTTGTC
K-AS-d1	592 – 616	A**X**TGGGTGCTCAGGTAGTGGTTGTC
K-AS-d2	592 – 616	ACTGGGTGCTCA**X**GTAGTGGTTGTC

[a] "**X**" indicates ^{CNV}K.

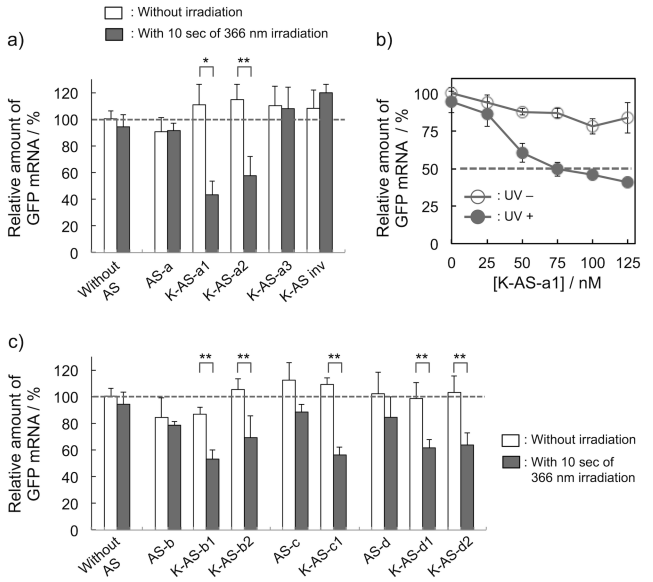

Figure 3. Down regulation of GFP expression in GFP-HeLa cells caused by photoirradiation with K-ASs treatment. (a) Relative amount of GFP mRNA in GFP-HeLa cells before and after the photoirradiation with the AS-a, K-AS-a1, K-AS-a2, K-AS-a3 and K-AS inv treatment ([antisense] = 100 nM). (b) Dose dependency of K-AS-a1 for the down regulation of GFP gene expression (photoirradiation: 366 nm, 10 sec). (c) Relative amount of GFP mRNA in GFP-HeLa cells before and after photoirradiation with the treatment of antisense targeting other site of AS-a ([AS-ODN] = 100 nM). The error bars indicate the standard error of the mean. *P < 0.01, ** P < 0.05.

complementary oligoRNA were estimated by denaturing PAGE analysis (Fig. S1). In the case of K-AS-a1 and a2, a band identical to the photo-cross-linked dimer appeared by 366 nm photoirradiation, whereas the band did not appear in the case of K-AS inv having an inverted sequence of K-AS-a1, suggesting that the photo-cross-linking reaction occurred in a sequence selective manner. The photo-cross-linked dimer also did not appear in the case of K-AS-a3 having a sequence designed to possess the guanine base at the photo-cross-linking site in target RNA strand, suggesting that the pyrimidine selectivity of the photo-cross-linking reaction, previously reported,[9a] was not affected by the phosphorothioate internucleoside modification.

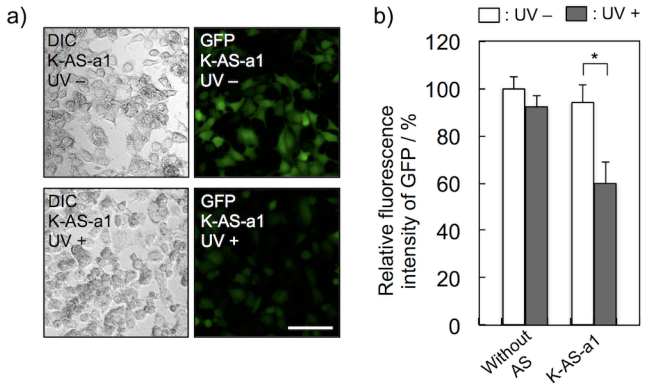

Figure 4. (a) Fluorescence microscopic image of GFP-HeLa cells after the treatment of K-AS-a1 (100 nM) and 10 sec of photoirradiation followed by 24 h incubation (Scale bar: 100 μm). (b) Fluorescence intensity of GFP in cells before and after the treatment of antisense (K-AS-a1) and 10 sec of 366 nm irradiation. The error bars indicate the standard error of the mean. * P = 0.02.

To evaluate the gene silencing activity of photoreactive AS-ODNs, AS-a, K-AS-a1~a3 and K-AS inv (100 nM) were transfected to GFP-HeLa cell, which expresses continuously GFP gene. After 10 sec of photoirradiation followed by 2 h incubation, total RNA was extracted and then the expression level of GFP mRNA was quantified by quantitative RT-PCR. As shown in Fig. 3a, only in the case of K-AS-a1 and 2,significant down regulation of GFP mRNA was induced by 366 nm photoirradiation, although the change in the expression level was not observed in the case of AS-a, K-AS-a3 and K-AS inv. These results were consistent with the photo-cross-linking ability of each K-AS ODNs discussed above, suggesting that the photo-cross-linking reaction is the key reaction of this antisense-based photoregulation of constitutive GFP expression. As shown in Fig. 3b, the photo-regulation efficiency has a dose dependent manner, and the 50% inhibition concentration (IC_{50}) was 75 nM. The IC_{50} was 2- to 3-fold smaller than that of standard phosphorothioate antisense ODNs for GFP,[10] suggesting that the irreversible covalent bond formation between K-AS ODNs and target mRNA enhances the antisense effect. The other K-ASs targeting other regions of GFP mRNA (K-AS-b, c, d) show the same levels of gene silencing effect as K-AS-a (Fig. 3c), although the secondary structure of these target regions would be different from each other (Fig. S2), suggesting the possibility that the K-AS invade the intramolecular duplex of the target region. Same strand invasion effect was observed in the case of a photoreactive molecular beacon having ^{CNV}K.[9i] As shown in Fig. 4a,b, the fluorescence intensity of GFP in cells was clearly decreased 40% by the photoirradiation with the treatment of K-AS-a1, suggesting that the K-AS-a1 can down regulate the cellular synthesis of GFP by 10 sec of photoirradiation. Since the expression level of β-actin, which is one of the representative endogenous house-keeping gene, and SIN3B, whose mRNA has highest homologous (83%) with AS-a among the human genome, were not affected by K-AS-a1 treatment and UV irradiation (Fig. S3), the K-AS can photo-regulate specifically

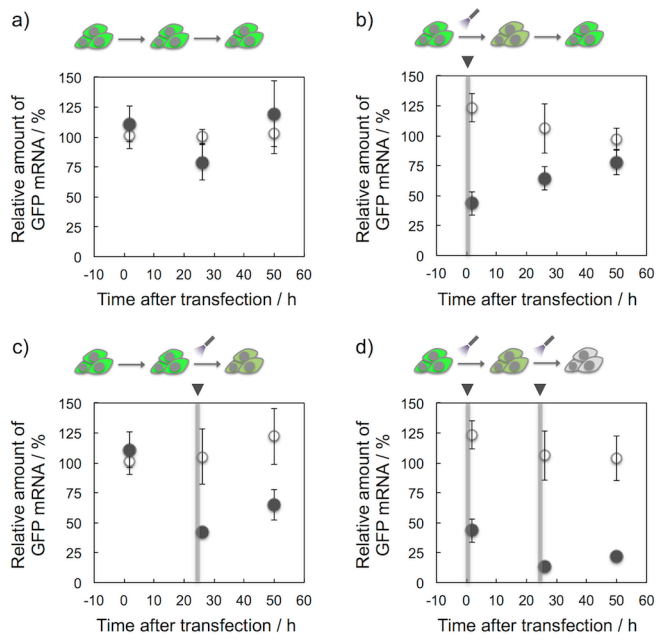

Figure 5. Temporal regulation of GFP gene expression by photoreactive AS-ODN. The open and solid circles indicate the absence and presence of K-AS-a1, respectively. After the transfection of K-AS-a1 (100 nM), photoirradiation (366 nm, 10 s) was performed at the timing indicated by the arrowheads (0, 24, 48 h). GFP mRNA was quantified by real-time RT-PCR at 2 h after the photoirradiation. The error bars indicate the standard error of the mean.

target gene expression. The 10 sec of photoirradiation scarcely affected the viability of GFP-HeLa cells (Fig. S4a) and the amount of pyrimidine (6-4) pyrimidone photoproducts (Fig. S4b), which is one of the representative pyrimidine photodimers caused by UV irradiation, suggesting that photodamage caused by the irradiation was insignificant.

The target selective and photoresponsive gene regulation effect of K-ASs may be a powerful tool in the fields of biotechnology and chemical biology. Finally, we tried to regulate temporally the GFP expression in cells by the photoirradiation with K-AS-a1. As shown in Fig. 5b, the GFP gene expression level was 60% decreased by the photoirradiation at 0 h after the transfection and then the expression level was monotonically increased, suggesting that the expression level was suppressed by the photo-triggered antisense effect and recovered by the spontaneous expression of GFP gene. On the other hand, in the case of the photoirradiation at 24 h after the transfection (Fig. 5c), the suppression was observed only at 26 h after the transfection, suggesting that the temporal regulation of gene expression was achieved by the photoirradiation with photoreactive AS-ODN. Ninety percent suppression of gene expression was observed in the case of photoirradiation twice (0 and 24 h) at 26 h after the transfection (Fig. 5d), suggesting that the expression level was temporally controllable by frequency of the photoirradiation. Surprisingly, the gene silencing effect of K-AS-a1 with the photoirradiation twice was comparable with the effect of siRNA treatment (Fig. S5), suggesting that our photoreactive AS-ODN has the potential to be a gene silencer which is better than siRNA with appropriate backbone modification. As phosphorothioate-modified ODNs are not degraded in cytoplasmic and nuclear extract of HeLa cells,[11] our photoreactive AS-ODNs would be remained in the cells through the incubation, although the dilution would be occurred through the cell growth. Since the doubling time of the HeLa cells is ca. 24 h, the concentration of photoreactive AS-ODN in the cells was diluted at least 4-fold after 48 h incubation. Together with the dose dependency of K-AS-a1 (Fig. 3b), the photoresponsive antisense effect might be decreased largely after 48 h incubation. Further modification of ODN that can enhance the binding affinity, such as 2'-MOE and LNA, can be expected for providing long-lasting photoresponsibility for the AS-ODN.

In conclusion, we clearly demonstrated that photoreactive AS-ODNs having ^{CNV}K act as an effective photo-regulator for constitutive GFP gene expression in living cells with only 10 sec of 366 nm irradiation, and that the antisense effect is temporally controllable by the photoirradiation. As the irradiation time required is shorter than that in the case of other photoresponsive AS-ODNs reported; it requires some minutes of UV irradiation;[12] we believe that our technology is superior for regulating endogenous gene expression in living cells without UV damage. As the timing and area of photoirradiation are completely controllable, this method largely contributes to spatiotemporal regulation of endogenous gene expression in cells or bioorgans. With the endoscopic drug delivery and localized photoirradiation, our photoreactive AS-ODNs may be a powerful drug for skin, cervical or gastrointestinal cancer treatment.

Notes and references

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Electronic Supplementary Information (ESI) available: experimental procedures, MALDI-TOF-MS data, melting temperature of AS-ODNs, PAGE analysis of the photo-cross-linking with complementary oligoRNA, expression level of other endogenous gene, and the gene silencing effect of siRNA for GFP. See DOI: 10.1039/c000000x/

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