microRNA-124 is down regulated in nerve injured motor neurons and it potentially targets mRNAs for KLF6 and STAT3

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Abbreviations

CT, cycle threshold; ISH, *in situ* hybridization histochemistry; KLF6, Krüppel-like factor 6; miRNA, microRNA; STAT3, signal transducer and activator of transcription 3; 3'-UTR, 3' untranslated region

Abstract

MicroRNA (miRNA) is a small non-coding RNA that regulates gene expression by degrading target mRNAs or inhibiting translation. Although many miRNAs play important roles in various conditions, it is unclear whether miRNAs are involved in motor nerve regeneration. In this study, we identified the possible implication of miR-124 in nerve regeneration using a mouse hypoglossal nerve injury model. The significant down-regulation of miR-124 was observed in injured hypoglossal motor neurons after nerve injury, and this transient down-regulation showed a clear inverse correlation with the up-regulation of KLF6 and STAT3, known as axon elongation factor and regeneration associated molecules, respectively. Furthermore, the luciferase assay and *in vitro* gain of function methods supported that both genes could be potent targets of miR-124. These results suggest that injury-induced repression of miR-124 may be implicated in the regulation of expression of several injury-associated transcription factors, which are crucial for appropriate nerve regeneration.

Keywords: neuronal regeneration, microRNA, nerve injury, hypoglossal nerve, expression regulation, transcription factor

1.1 INTRODUCTION

MicroRNAs (miRNAs) are 20-23 nucleotide regulatory non-coding RNAs that repress gene expression post-transcriptionally. Previous reports have revealed that miRNAs control the production of hundreds of proteins, and function in the fine-tuning of gene expression (Baek et al., 2008; Selbach et al., 2008). In addition, many miRNAs have been shown to be involved in normal development of neural tissues. Indeed, conditional Dicer KO mice, which lack functional miRNAs in specific regions, displayed severe abnormalities in neuronal development and function (Davis et al., 2008; Haramati et al., 2010). Although these reports suggest that miRNAs are implicated in fine-tuning and regulation of gene expression for appropriate neural functions, the functional consequences of miRNAs under pathological conditions such as nerve injury remains unclear.

miR-124 is a well-known neuronal miRNA, and is highly conserved throughout evolution. From the early embryonic period, miR-124 is expressed specifically in the nervous system, and is known to play essential role in neuronal development. Overexpressed miR-124 shifts the gene expression profile from a non-neuronal cell type into a neuronal cell type (Conaco et al., 2006). Furthermore, miR-124 has been shown to repress the expression of genes with anti-neuronal activities, including repressor-element-1-silencing transcription factor (REST) (Conaco et al., 2006), SCP1 (Visvanathan et al., 2007), and Sox9 (Cheng et al., 2009). Other reports have shown that miR-124 indirectly controls alternative splicing (Makeyev et al., 2007) and appropriate pathfinding (Baudet et al., 2011) in developing neuronal cells. Recently, Sanuki et al. showed that miR-124 is required for hippocampal axogenesis and retinal cone survival using gene disruption studies (Sanuki et al.,

2011). Although these studies have suggested that miR-124 is one of the most important molecules in neuronal development, it remains unclear whether miR-124 also plays a role in neuronal regeneration.

Here, we identified the changes in expression of miR-124 following mouse motor nerve injury. After nerve transection, miR-124 was substantially down-regulated in injured motor neurons. We also searched for candidate target genes of miR-124 in injured motor neurons, and showed functional binding of miR-124 to the 3'untranslated region (3'-UTR) of Krüppel-like factor 6 (KLF6) and signal transducer and activator of transcription 3 (STAT3), which display clear inverse correlation with the expression of miR-124 after nerve transection. Because both target genes have been shown to be nerve regeneration associated molecules, the present results suggest that injury-induced repression of miR-124 can regulate the expression of several injury-related molecules for appropriate nerve regeneration.

1.2 EXPERIMENTAL PROCEDURES

1.2.1 Animal procedure

Eight-week-old male C57BL/6 mice (n=76) were anesthetized with pentobarbital (45 mg/kg) via intraperitoneal injection and were placed in a supine position. The right hypoglossal nerve was cut just proximal to the bifurcation of the nerve. Animal experiments were performed in accordance with the Guidelines of the Osaka City University Medical School regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number of animals used and their suffering.

1.2.2 Quantitative RT-PCR (miRNA).

Forty mice were used for the extraction of RNA. The control and injured side of the hypoglossal nuclei were dissected at 1, 3, 7, and 14 days after nerve injury under a microscope and quickly dipped into liquid nitrogen. Total RNA was extracted from the control and injured hypoglossal nuclei using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturers' protocol. The TaqMan microRNA reverse transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), specific TaqMan microRNA Assays for miR-124 (Applied Biosystems) and TaqMan Universal PCR master mix (Applied Biosystems) were used for quantitative analyses. The reverse-transcription reaction was initiated with 10 ng of tissue-derived total RNA. Negative controls were included with every real-time RT-PCR assay, and no amplification of the signal was observed when water was added instead of the cDNA sample. The ABI 7900HT system (Applied

Biosystems) was used for amplification and detection. Relative expression was calculated by using the comparative cycle threshold (CT) method. The relative microRNA levels were normalized to endogenous U6 small nuclear RNA expression for each sample. The CT value was obtained from the amplification plot with the aid of SDS software.

1.2.3 *In situ* hybridization (miRNA)

Sixteen mice were used for *in situ* hybridization. Brains were removed quickly at 1, 3, 7, 14 days after axotomy, and frozen in powdered dry ice. Sections were cut at 16 μ m using a cryostat. The sections were thaw-mounted on 3-aminopropyltriethoxysilane-coated slides, and stored at -80°C until ready for use. RNA oligonucleotides with a 5' end digoxigenin modification were custom synthesized commercially (Japan Bio Services Co., LTD., Saitama, Japan). The sequence of RNA oligonucleotides for detection of miR-124 were 5'-AAGGCAUUCACCGCGUGCCUUA-3' (Deo et al., 2006). All procedures were performed as previously described (Thompson et al., 2007).

1.2.4 Target prediction

Several freeware programs available on the internet are commonly used to search for potential miRNA–mRNA binding. We used three such items of software, TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de>), and microT (<http://www.microrna.gr/microT>). All three identified KLF6 and

STAT3 as having a relatively likely putative binding site for miR-124.

1.2.5 Semi-quantitative RT-PCR (mRNA).

Total RNA (500 ng aliquots) extracted from hypoglossal nuclei was converted to cDNA with Superscript reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA) and oligo (dT) nucleotides. Specific primers for detection of mRNA for KLF6 and STAT3 were used. The primers were as follows: for KLF6, primers targeted bases 2093–2110 and 2613–2630 of GenBank Accession No. NM_011803.2; for STAT3, primers targeted bases 3036–3055 and 3563–3583 of GenBank Accession No. NM_011486.4. Reaction products were separated electrophoretically on a 1.5% (w/v) agarose gel and visualized by staining with EZ-Vision One DNA Dye (Amresco Inc., Solon, OH, USA). Data were expressed as the mean densities for each band relative to the density of the GAPDH band in the same sample. The density was measured at two points for each band.

1.2.6 *In situ* hybridization (mRNA)

Twenty mouse brains were removed quickly at 1, 3, 5, 7, and 14 days after axotomy, and frozen in powdered dry ice. The sections were prepared as described above. The anti-sense KLF6 and STAT3 RNA probe were prepared by *in vitro* transcription with T7 RNA polymerase using the pGEM-T Easy vector (Promega, Fitchburg, WI, USA), which inserted partial cDNAs for KLF6 (Accession No. NM_011803.2, nucleotides

2093–2630) or STAT3 (Accession No. NM_011486.4, nucleotides 3036–3583). ³⁵S-labeled cRNA probes were prepared by *in vitro* transcription using T7 RNA polymerase (Promega) and 35S-UTP (PerkinElmer, Waltham, MA, USA). All procedures including hybridization, film autoradiography and emulsion autoradiography were performed as described previously (Nagata et al., 2006).

1.2.7 Luciferase assay

The 3'-UTR regions of the mouse KLF6 (NM_011803.2, nucleotides 2093–2630) and STAT3 (NM_011486.4 nucleotides 3036–3583) were PCR amplified using the specific primers (same as used in RT-PCR). Purified PCR products were cloned downstream of the firefly luciferase gene in the pGL3 plasmid. We also generated a mutant 3'-UTR of the KLF6 and STAT3 genes with substitutions of 6 bp, respectively, using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The sequence of mutant KLF6 or STAT3 3'-UTR segments contained 5'- ttgaccttcataatgg**ACGGAA**a -3' or cagcctgccctgtggg**ACGGAA**a -5', respectively (italic bold nucleotides are mutated). HeLa cells were plated at 50,000 cells per well in 24-well plates. The following day, cells were transfected with 200 ng pGL3 vector, including the 3'-UTR of KLF6 or STAT3 (with either wild-type or mutant miR-124 binding sites) and 20 ng *Renilla reniformis* luciferase encoding plasmid (pRL-TK, Promega, USA) using Fugene HD Transfection Reagent (Promega). After 12h, miR-124 mimic (Dharmacon, Thermo Fisher, Lafayette, CO, USA) or negative control mimic (Dharmacon) at a final concentration of 20 nM was transfected using Lipofectamine RNAiMAX Reagent (Invitrogen). Luciferase

assays were performed using a dual-luciferase assay system (Promega) 36 hours after the first transfection.

1.2.8 Treatment of HeLa cells with miR-124 mimic

HeLa cells were plated at 25,000 cells in 24-well plates and were transfected with a miR-124 mimic (Dharmacon) or negative mimic control (Dharmacon) at a final concentration of 20 nM. After 24 hours, the cells were harvested for real time PCR assays. RNA extraction was performed using the RNeasy mini kit (Qiagen, Valencia, CA, USA).

1.2.9 Quantitative RT-PCR (mRNA).

Total RNA (400 ng aliquots) was converted to cDNA with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The resultant cDNA was diluted 1:50. The quantitative PCR procedures were carried out according to the manufacturer's protocol, and real time PCR master mix (Toyobo) and specific TaqMan Gene Expression Assays for Homo sapiens GAPDH, KLF6, STAT3, and KLF9 (Applied Biosystems) were used. The ABI 7900HT system (Applied Biosystems) was used for amplification and detection. The relative expression was calculated using the comparative CT method. Relative mRNA levels were normalized to endogenous GAPDH mRNA expression for each sample. The CT value was obtained from the amplification plot with the aid of SDS software.

1.2.10 Quantitative and statistical analysis

Quantitative data are represented as the mean $\pm$ SD based on at least three separate experiments. Differences among groups were statistically analyzed by one-way analysis of variance followed by Tukey–Kramer post hoc tests. Comparison between two experimental groups was based on a two-tailed unpaired Student's t-tests. A P-value lower than 0.05 was considered significant.

1.3 RESULTS

1.3.1 *miR-124 decreases after hypoglossal nerve injury*

Among the several miRNAs, we focused on miR-124 for an analysis of nerve injured motor neurons. The reasons were as follows: first, miR-124 is one of the most abundant miRNAs in neural tissue. Second, miR-124 plays an essential role in neuronal development. Third, previous studies have shown that miR-124 is downregulated after central nervous system injury (Nakanishi et al., 2010; Liu et al., 2011; Strickland et al., 2011) and sensory nerve injury (Wu et al., 2011). First we examined the alteration of miR-124 expression using a mouse hypoglossal nerve injury model. After transection of the unilateral hypoglossal nerve, both injured and uninjured hypoglossal nuclei were dissected at 1, 3, 7, 14 days post injury, and then RNA was extracted. Real time PCR assay showed that the expression of miR-124 decreased on the injured side when compared with the uninjured control side (Fig. 1A). miR-124 expression was gradually down-regulated from 3 day after injury, and significantly decreased at 14 day (Fig. 1A). To reveal the detailed expression profiles and to further identify the cell types expressing miR-124, *in situ* hybridization histochemistry (ISH) was performed using DIG-labeled probes. miR-124 was ubiquitously expressed by most of neurons, including cranial motor neurons, under normal conditions. After nerve injury, compared to the uninjured control, miR-124 expression was markedly decreased at 3 days (Fig. 1B); however, expression was not altered on the control side and surrounding cells. The repression of miR-124 expression was maintained until at least 14 days (Fig. 1B). Most miR-124-positive cells in the hypoglossal nucleus were large and round in shape, and were assumed to be motor neurons.

1.3.2 *KLF6 and STAT3 mRNAs increase after nerve injury*

Next, we explored the target genes of miR-124 using three different search engines: TargetScan (Ruby et al., 2006), PicTar (Lall et al., 2006), and microT (Maragkakis et al., 2009). Although many candidate genes were identified *in silico*, we selected some candidate genes and focused on their expression after hypoglossal nerve injury. Interestingly, most of the genes examined were unchanged in expression after nerve injury (data not shown). However, PCR analyses showed prominent increases in KLF6 and STAT3 mRNAs (by approximately 2-fold) after nerve injury (Fig. 2A, B). We then confirmed the elevated expression using ISH. Emulsion autoradiography demonstrated a prominent increase of both mRNAs in injured motor neurons (Fig. 2C). Furthermore, the hybridization signals on the hypoglossal nuclei of the film autoradiogram were measured to determine the mRNA expression profiles during recovery. We detected that the increase of KLF6 mRNA expression started from 1 day post injury, peaked at 3 days, and then returned to basal levels (Fig. 2D). The expression profile of KLF6 mRNA was inversely correlated with that of miR-124 (Fig. 1A, B and 2D). Consistent with a previous report (Schwaiger et al., 2000), STAT3 mRNA was up-regulated after nerve transection (Fig. 2). STAT3 mRNA expression displayed almost the same expression pattern as that of KLF6 mRNA, with the exception that the fold induction was much smaller than that of KLF6.

1.3.3 *KLF6 and STAT3 mRNAs are possible targets of miR-124*

We conducted the luciferase assay to check whether both KLF6 and STAT3 mRNAs were the targets of miR-124. To this end, the reporter plasmid vectors containing the luciferase gene with the partial sequence 3'-UTR of KLF6 or STAT3 were used (see section 1.1.8). The other constructs with a mutated 3'-UTR of KLF6 or STAT3 and pGL3 vector were also used as a negative control (Fig. 3A, B). These vectors were transfected into HeLa cells with either miR-124 mimic, designed to increase the levels of miR-124, or negative control mimic. Incorporation of KLF6 3'-UTR in the reporter resulted in a significant decline in luciferase activity to $63.9 \pm 10.4\%$ (Fig. 3C). The effects could only be seen when the miR-124 mimic were co-transfected, but not when the control mimic was co-transfected (Fig. 3C). In contrast, incorporation of the mutated KLF6 plasmid did not show a change of the luciferase activity. Incorporation of the STAT3 3'-UTR also induced a somewhat decline in luciferase activity to $78.4 \pm 9.3\%$ (Fig. 3C), but did not reach a statistically significant difference. There were no effects on luciferase activity when the mutated STAT3 construct or pGL3 vector were transfected.

Finally, we examined whether miR-124 was able to suppress the levels of endogenous KLF6 and STAT3 mRNA. *In vitro* gain of miR-124 function methods were applied by transfecting non-neuronal HeLa cells with miR-124 mimic reagent. The expression levels were quantitatively measured by real time PCR. After treatment with miR-124 mimic reagent, expressions of KLF6 and STAT3 mRNAs were decreased by approximately 40% and 55%, respectively, compared with the no transfection control (Fig. 4). In contrast, the control mimic did not show such effects. Furthermore, miR-124 mimic did not induce any expression change of non-target

gene KLF9 mRNA. These results indicated that the endogenous KLF6 and STAT3 mRNAs could be regulated by miR-124.

1.4 DISCUSSION

In this study we demonstrated that miR-124 was markedly down-regulated after nerve injury, and this down-regulation would enhance the expression levels of the target mRNAs for KLF6 and STAT3, which are crucial transcription factors for nerve regeneration (Veldman et al., 2007; Moore et al., 2009; Bareyre et al., 2011).

In response to nerve injury, a substantial number of genes are expressed and suppressed to protect neurons and elongate axons for functional recovery, and failure of gene expression regulation can lead to degeneration of injured neurons. Gene expression is assumed to be precisely regulated by several mechanisms such as transcription factors and epigenetic regulators. To date, accumulating evidence demonstrates that transcription factors such as ATF3, STAT3, and KLF6 as well as their complexes serve as switches for the expression of groups of molecules, which were involved in proper nerve regeneration, (Nakagomi et al., 2003; Raivich et al., 2004; Kiryu-Seo et al., 2008; Moore et al., 2009; Kiryu-Seo and Kiyama, 2011). However, recent identification of miRNAs strongly suggests additional gene expression regulation mechanisms for nerve regeneration.

miRNAs inhibit target genes via post-transcriptional mechanisms, and this is considered to be one of the best candidates to regulate the expression of several genes in parallel by a single species of miRNA. In this study, we have shown that miR-124 is down-regulated in the injured hypoglossal nucleus in response to nerve injury, which would be a potent mechanism for expression of nerve regeneration associated molecules. The down-regulation of miR-124 in other neural disorders has been previously demonstrated (Nakanishi et al.,

2010; Liu et al., 2011; Strickland et al., 2011; Wu et al., 2011); however, the present study further addressed the possible targets of the miRNA during nerve regeneration.

The present study revealed that KLF6 is one of the target mRNAs of miR-124 (Fig. 3C and 4). KLF6 is a transcription factor belonging to the family of Kruppel-like zinc finger transcription factor. Similar to other KLFs, KLF6 has a DNA binding domain at the C-terminus region, and recognizes GC-rich DNA sequences in its target gene promoter. Previous reports have shown that KLF6 is distributed throughout the nervous system and several non-neural sites from the embryonic period (Slavin et al., 1999; Laub et al., 2001). In the adult mouse brain, KLF6 is widely distributed, and predominantly expressed in neuronal cells and endothelial cells (Jeong et al., 2009). In the present study, we have shown that KLF6 mRNA is significantly up-regulated in injured motor neurons, suggesting a crucial role of KLF6 during nerve regeneration. Consistent with this result, previous studies have implicated KLF6 in axon regeneration using retinal ganglion cells from mammals and zebrafish (Veldman et al., 2007; Moore et al., 2009). These and additional studies suggest that KLF6 and another family member of KLF7, which has 84% homology to KLF6, cooperatively function in axon regeneration (Blackmore et al., 2012). In fact, we also observed an increase in KLF7 mRNA in injured motor neurons in our model (data not shown). However, it should be noted that at least under the search *in silico*, KLF7 was not identified as a target for miR-124. This may suggest other gene regulation mechanisms in which KLF7 expression is promoted, or that KLF7 ensues in parallel with miR-124 down regulation, which enhances

KLF6 mRNA. In this study we identified a relatively quicker increase of KLF6 mRNA after nerve injury, whereas the decrease of miR-124 expression was slower and maintained longer. This discrepancy in expression time course may be explained by an existence of additional regulatory system for KLF6 expression. Previous reports have shown that myocyte enhancer factor 2 induces KLF6 expression (Salma and McDermott, 2012) and conversely miR-181a decreases KLF6 expression (Zhang et al., 2012). Although it is not clear whether these regulators regulate KLF6 expression in injured motor neurons, it is likely that not only miR-124, but also other factors, may coordinately modulate KLF6 expression after hypoglossal nerve injury.

We also identified STAT3 as another possible target of miR-124 in this study. A recent study has also provided evidence that miR-124 inhibits STAT3 signaling by binding to STAT3 3' UTR directly (Wei et al., 2013), which is consistent with our finding. STAT3 is a well-known transcription factor for neuron protection and nerve regeneration. STAT3 resides in the cytosol in an inactivated state, and peripheral nerve injury promotes its transcription, tyrosine phosphorylation, and nuclear translocation (Haas et al., 1999; Schwaiger et al., 2000; Qiu et al., 2005). We have previously demonstrated the crucial implication of STAT3 signaling in nerve regeneration using the same nerve injury model (Hirota et al., 1996; Kato et al., 2002; Nakagomi et al., 2003; Kiryu-Seo et al., 2008). In addition, Schweizer et al. demonstrated that STAT3 played an essential role in facial motor neuron survival by expressing Reg-2 and Bcl-xl after nerve injury (Schweizer et al., 2002). Recently, Bareyre et al. revealed that STAT3 was primarily needed to induce a neuronal growth program, but not required

for perpetuation of axonal outgrowth (Bareyre et al., 2011). As such, miR-124 down-regulation-mediated induction of STAT3 expression could be a crucial mechanism for proper nerve regeneration. Interestingly, Hatziapostolou et al. (Hatziapostolou et al., 2011) demonstrated that miR-124 targeted the IL6 receptor (IL6R), and consequently suppressed the STAT3 signaling pathway. In contrast, suppression of miR-124 expression induced IL6R and IL6 expression, suggesting that down regulation of miR-124 may also be implicated in IL6R induction in nerve injured motor neurons (Hirota et al., 1996). Taken together, miR-124 could be an overall suppressor of IL6-IL6R and its pathway, and the down regulation of miR-124 may be a crucial event for IL6 signaling-mediated neuron protection and axon regeneration.

In conclusion, we observed the down-regulation of miR-124 expression in injured motor neurons, and this change in expression was inversely correlated to the up-regulation of KLF6 and STAT3 mRNA, which are crucial for nerve regeneration. Furthermore, we demonstrated that miR-124 suppressed KLF6 and STAT3 expression using the luciferase assay and the *in vitro* gain of function method. Although we have addressed two target genes in this study, additional targets could exist, and may play crucial roles in nerve regeneration.

Additionally, it remains to be determined whether both KLF6 and STAT3 are actually *in vivo* targets of miR-124 in injured motoneurons by using a conditional knockout mouse in which miR-124 is specifically deleted in nerve injured motoneurons. In any event, the implication of the microRNA system in gene expression regulation for motor neuron regeneration may be an additional mechanism to fully understand the

mechanisms underlying nerve regeneration.

Figure Legends

Fig. 1. miR-124 expression decreased after hypoglossal nerve transection. (A) Quantitative RT-PCR analysis of miR-124 in control and injured hypoglossal nuclei of mice. (B) *In situ* hybridization demonstrating miR-124 expression after hypoglossal nerve injury. cc, central canal; cont, control side; inj, injured side. Scale bar: 100 μ m. Bars represent the mean $\pm$ SD. ** $p < 0.01$ vs. control side.

Fig. 2. Induction of KLF6 and STAT3 mRNA expression in injured hypoglossal motor neurons. (A) RT-PCR analysis of KLF6 and STAT3 in control and injured hypoglossal nuclei of mice. cont, control side; inj, injured side. (B) Quantification of panel A. (C) *In situ* hybridization demonstrating KLF6 and STAT3 mRNA expression. cc, central canal; cont, control side; inj, injured side. Scale bar: 40 μ m. (D) Alteration of KLF6 and STAT3 mRNA expression after nerve injury. Relative mRNA signal intensity in the injured side compared with that in the control side was calculated. Bars represent the mean $\pm$ SD. ** $p < 0.01$ vs. control side.

Fig. 3. KLF6 and STAT3 are miR-124 targets. (A, B) Design of miR-124 reporter vector containing SV40 promoter, luciferase gene, and KLF6 3'-UTR or STAT3 3'-UTR. Sequences of miR-124, predicted miR-124 binding sites in the KLF6 or STAT3 UTR, and mutated binding sites. (C) Luciferase activity was measured 36 h after transfection of the reporter plasmids into HeLa cells. Reporter plasmids were transfected either together with 20 nM miR-124 or together with 20 nM control mimic. Activity is plotted relative to the control mimic.

Data are the mean $\pm$ SD of at least three independent experiments. * $p < 0.05$ vs. control mimic.

Fig. 4. miR-124 induced the down-regulation of endogenous KLF6 and STAT3. Relative expression of KLF6 mRNA, STAT3 mRNA and non-target KLF9 mRNA. RNA was extracted 24 h after transfection of 20 nM miR-124 mimic (gray bar) or 20 nM control mimic (black bar). A no transfection control was also set up (white bar). Expression levels were normalized to the expression of GAPDH. Data are the mean $\pm$ SD. At least three independent experiments were performed. ** $p < 0.01$ vs. control mimic or no transfection control.

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A **B**

Fig.1 Nagata et al.

A **B** **C** **D**

Fig.2 Nagata et al.

Fig.3 Nagata et al.

Fig 4. Nagata et al.