## 4. Discussion

MAPO1 was identified as one of the protein elements functioning at a certain step following the induction of apoptosis [16]. In *Mapo1*-defective cells, mitochondrial membrane depolarization and caspase-3 activation were not observed even after exposure to MNU, although the cells retain the ability for mismatch repair protein-dependent DNA damage detection and signaling. Subsequent studies have revealed that MAPO1 is identical to FNIP2 and FNIPL, reported by Hasumi et al. [23] and Takagi et al. [24], respectively. This protein is bound to folliculin, encoded by the *FLCN* tumor suppressor gene, and AMP-activated protein kinase (AMPK). To analyze the possible roles of folliculin and AMPK in the induction of apoptosis, we introduced siRNAs specific for the *Flcn* or *Ampkα* gene and then treated the cells with MNU. The flow cytometric analyses performed to measure the sub-$G_1$ population of cells revealed that folliculin and AMPK, as well as MAPO1, were involved in MNU-induced apoptosis. Taken together, these data suggest that MAPO1 forms a protein complex(es) with folliculin and AMPK, and plays a role in a signal transduction pathway of apoptosis.

It is known that AMPK is one of the signaling kinases that negatively regulates cell growth and proliferation and is phosphorylated itself under conditions of energetic stress [26–29]. Several recent papers have observed the pro-apoptotic potential of activated AMPK [30–33]. In this report, we found a gradual increase in the levels of AMPK phosphorylation in *Mapo1*-proficient cells after MNU treatment, implying a possible involvement of the activation of AMPK in the MNU-induced apoptosis pathway. In *Mapo1*-deficient cells, AMPK activation in this manner was hardly detectable, even after the treatment with MNU. Furthermore, the treatment of cells with AICAR, a specific activator of AMPK, resulted in AMPKα phosphorylation and mitochondrial membrane depolarization in a *Mapo1*-dependent manner. These findings extended onto the case of *Flcn*-knockdown cells. Taken together, it is likely that MAPO1 and FLCN positively regulate the activation of AMPK through their mutual interaction in the apoptotic signaling pathway, triggered by an alkylating agent. MAPO1 and FLCN proteins have been reported to undergo some modifications in cells [17,24]. The treatment with an alkylating agent might affect the modified states of these proteins, and might cause the activation of the protein complex, thus leading to AMPK activation. Another folliculin-interacting protein, FNIP1, which is homologous to MAPO1, is also capable of binding to AMPK [17]. The activation of AMPK might therefore be regulated in more complex ways under the balance of MAPO1 and FNIP1 activities.

Another important problem which remains to be solved is how the AMPK–MAPO1–FLCN complex is activated by the signal delivered from the mismatch repair protein complex, which itself is activated through the interaction with DNA carrying base mismatches. The signal may be delivered by direct physical contact between the two complexes or through the involvement of other protein factors. The protein linking analyses, aided by mass spectrometry, have been performed, but no evidence to show the physical association of the two complexes was obtained (unpublished results). It seems likely, therefore, that some other protein factor(s) might be involved in the signal transduction process. To identify such factors, it would be relevant to extend this approach using retrovirus-mediated gene-trap mutagenesis studies.

Germline mutations in the *FLCN* gene have been identified in patients with Birt-Hogg-Dubé (BHD) syndrome, which is an autosomal dominant disorder characterized by hamartomas of skin follicles, spontaneous pneumothorax, and renal tumors [20–22]. Furthermore, *BHD* heterozygous knockout mice were revealed to develop kidney cysts and tumors as they aged, while *BHD* homozygous null mice displayed early embryonic lethality [34,35]. The recent findings, including this report, strongly suggest that folliculin has physical and/or functional interactions with the AMPK-mTOR signaling pathway [17,34,36]. Mutations in several other tumor suppressor genes, such as *LKB1*, *TSC1* and *TSC2* [29,37], have also been shown to lead to dysregulation of AMPK-mTOR signaling and to the development of other hamartomatous syndromes. Our present findings that folliculin is involved in the induction of apoptosis might shed some light on the physiological roles of *BHD/FLCN* and other related tumor suppressor genes. We are currently establishing *Mapo1* knockout mice to analyze the possible roles of the gene in the suppression of tumor predisposition resulting from environmental stresses.

## Conflict of interest statement

The authors declare that there are no conflicts of interests.

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# Therapeutic Effects of MicroRNA-582-5p and -3p on the Inhibition of Bladder Cancer Progression

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Many reports have indicated that the abnormal expression of microRNAs (miRNAs) is associated with the progression of disease and have identified miRNAs as attractive targets for therapeutic intervention. However, the bifunctional mechanisms of miRNA guide and passenger strands in RNA interference (RNAi) therapy have not yet been clarified. Here, we show that miRNA (miR)-582-5p and -3p, which are strongly decreased in high-grade bladder cancer clinical samples, regulate tumor progression *in vitro* and *in vivo*. Significantly, the overexpression of miR-582-5p or -3p reduced the proliferation and invasion of UM-UC-3 human bladder cancer cells. Furthermore, transurethral injections of synthetic miR-582 molecule suppressed tumor growth and metastasis in an animal model of bladder cancer. Most interestingly, our study revealed that both strands of miR-582-5p and -3p suppressed the expression of the same set of target genes such as *protein geranylgeranyltransferase type I beta subunit* (*PGGT1B*), *leucine-rich repeat kinase 2* (*LRRK2*) and *DIX domain containing 1* (*DIXDC1*). Knockdown of these genes using small interfering RNA (siRNA) resulted in the inhibition of cell growth and invasiveness of UM-UC-3. These findings uncover the unique regulatory pathway involving tumor suppression by both strands of a single miRNA that is a potential therapeutic target in the treatment of invasive bladder cancer.

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## INTRODUCTION

RNA interference (RNAi), a natural cellular process that regulates gene expression, is the most significant recent contribution to the field of cell biology.[1] Recently, RNAi-based therapies, which are harnessed to control the expression of pathogenic proteins, have been demonstrated in humans and have provided alternative powerful approaches to the traditional small molecule therapies. There has been an increase in the development of RNAi therapies for accessible tissues, such as the skin, retina, liver, and airways, due to their ability to be efficiently and safely delivered without unwanted side effects.[2–4]

Urinary bladder cancer remains one of the most costly cancers with regard to treatment and the monitoring of cytological changes, such as surveillance cystoscopy and periodic imaging.[5] However, despite the existence of appropriate therapies, patients are continually under the threat of ongoing recurrence and progression to muscle.[5,6] Therefore, the development of new treatment strategies to reduce the risk of recurrence and progression based on novel molecular networks is strongly desired. The facts described above provide an insight into an innovative approach that harnesses the power of the RNAi pathway; *i.e.*, the bladder maximizes the effect of RNAi therapy because of its accessibility and closed environment. Although some previous studies showed that the intravesical injection of small interfering RNA (siRNA) has potential as a treatment,[7,8] whether an intravesical strategy can overcome the progression to muscle and metastasis in invasive bladder cancer remains unclear.

In cancer research, the copy number variation of DNA was a focus during the 1990s and the beginning of the 2000s as a result of the development of technologies such as array-based comparative genomic hybridization and microsatellite analysis,[9–11] because copy number gains or losses were believed to be specific markers for functional protein-coding genes. However, if protein-coding genes were not located in these aberrant regions, disease candidate regions were excluded from the functional analysis. On the other hand, recent studies found that microRNAs (miRNAs), which are key post-transcriptional regulators, are the main candidates for cancer-predisposing genes and that approximately half of the miRNAs are located at chromosomal regions that are genetically altered in cancers.[12–14] Indeed, since their discovery, a widespread dysregulation of miRNAs caused by copy number variation is commonly observed in human cancers and has been shown to be involved in diverse physiological/pathological processes.[15–19] In this study, we hypothesized that a novel tumor-suppressive miRNA or onco-miR can be identified more efficiently and more expeditiously by utilizing a vast amount of previously generated information about aberrant chromosomal regions related to bladder cancer.

Cytogenetic studies of bladder cancer have revealed a number of genetic aberrations.[10] For discrimination of miRNAs which denote the abnormal expression in poorly prognostic patients

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on bladder cancer, we focused on the copy number variation in UM-UC-3 cells which is one of the most invasive bladder cancer cell lines. It was reported that a number of genomic regions were deficient or amplified in that cell line.[20] Furthermore, in those genomic regions, 3p11-12, 4q33-34, and 5q12-13 were selected as analysis sets because the correlations between the copy number loss and bladder cancer progression in clinical samples had been reported by at least two groups.[11,21–24] Although these regions include some miRNA genes, such as miR-1305, miR-1324, miR-578, miR-582-5p, and miR-582-3p, the modulation of the miRNA expression profiles and the exact functional mechanism are not thoroughly understood. In the present study, we report that miR-582-5p and miR-582-3p are concurrently downregulated in invasive bladder cancer, which is correlated with a copy number loss of chromosome 5q12. These significant reductions of miR-582-5p and -3p were also observed in clinical samples and strongly correlated with tumor grade. Furthermore, the restorations of miR-582-5p or -3p strongly inhibit cell proliferation and invasion. In the lung metastasis mouse model of human bladder cancer, involving orthotopic transplantation, the injection of the miR-582/cationic liposome complex prevented tumor growth and lung metastasis. This demonstration is the first, to our knowledge, to show inhibition of tumor metastasis in an orthotopic model by intravesical miRNA injection.

Two mature miRNAs can be generated from the same stem-loop pre-miRNA.[25] These 5p and 3p miRNAs, although excised from a single primary transcript, have different sequences and therefore target different mRNAs. Despite nearly a decade of studies on miRNA, the effect of strand-specific mature miRNAs has not yet been fully understood. In the present study, we also provide evidence that the expression of *protein geranylgeranyltransferase type I beta subunit* (*PGGT1B*), *leucine-rich repeat kinase 2* (*LRRK2*), and *DIX domain containing 1* (*DIXDC1*), which are involved in the cell growth and the invasiveness, is bifunctionally regulated by the effects of both strands of miR-582-5p and -3p. These findings suggest that a novel pathway involving miR-582 and cancer-related genes has the potential to be a critical target for the therapeutic treatment of invasive bladder cancer.

## RESULTS

### Downregulation of miR-582-5p and -3p in bladder cancer cell lines

To identify miRNAs downregulated in bladder cancer cell lines, we performed quantitative reverse transcription-PCR (qRT-PCR) for miR-1305, miR-1324, miR-578, and miR-582-5p and -3p, which are located in aberrant genomic regions correlated with tumor progression (**Supplementary Tables S1 and S2**). The results showed no downregulation of miR-1305 in bladder cancer cells (**Supplementary Figure S1a**), and the expression of miR-1324 and miR-578 was not detected in either bladder cancer cell lines or normal bladder tissue RNA, but we found that miR-582-5p and -3p are strongly downregulated in invasive bladder cancer cell lines (UM-UC-3, 5637, MBT2, J82, TCCSUP) (**Figure 1a**). Furthermore, the copy number of chromosome 5q12, in which the miR-582 gene is located, was lower in UM-UC-3 cells than in HT1376 cells (**Figure 1b**). HT1376 cells were reported to not have a loss in 5q12.[24] However, the degree of downregulation of miR-582 expression in

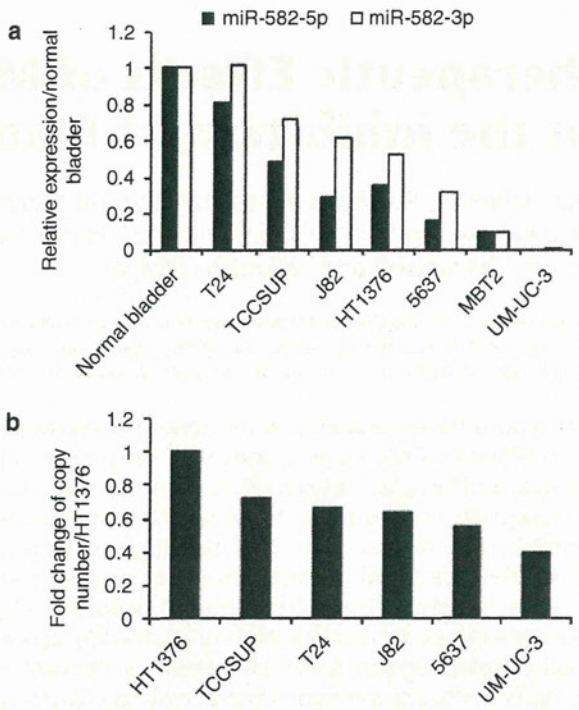


Figure 1 Expression level of miR-582-5p and -3p in human bladder cancer cell lines. (**a**) Expression level of miR-582-5p and -3p in human bladder cancer cell lines and a mouse bladder cancer cell line (MBT2). The relative expression of miR-582 for each of the cell lines was calculated compared with the level in normal bladder tissue RNA. (**b**) Copy number change of the miR-582 loci on chromosome 5q12. The HT1376 cell line was used as the control for this experiment.

UM-UC-3 cells seems too strong compared with the reduction of the copy number. This means that a remarkable reduction of miR-582-5p and -3p expression might be invoked by a combination of copy number loss and other factors. To identify whether the expression of these mature miRNAs are reduced in a transcriptional or biogenic process, we assessed the expression levels of pri-miR-582 and *phosphodiesterase 4D* (*PDE4D*) because miR-582 is located in the intronic region of *PDE4D*. The expression levels of pri-miR-582 and *PDE4D* are strongly reduced in UM-UC-3 and 5637 cells (**Supplementary Figure S1b,c**). These results indicate that the expression of miR-582-5p and -3p is reduced by the combination of copy number loss and transcriptional attenuation.

### Downregulation of miR-582-5p and -3p in clinical samples

We examined the expression levels of miR-582-5p and -3p in laser capture-microdissected bladder cancer tissue regions ($n$ = 53) and matched adjacent normal regions ($n$ = 31) derived from 28 patients (**Supplementary Table S3**). Relative to normal regions, the tumor regions showed markedly lower miR-582-5p and -3p expression levels (**Figure 2a**). In particular, we found that these levels tend to decrease more remarkably in invasive tumors (tumor stage ≥pT1) than in superficial tumors (tumor stage pTis and pTa) (**Figure 2b**) (miR-582-5p: superficial tumor $P$ = 0.006, invasive tumor $P$ = 0.003; miR-582-3p: superficial tumor $P$ = 0.035, invasive tumor $P$ < 0.001). Furthermore, a strong correlation was

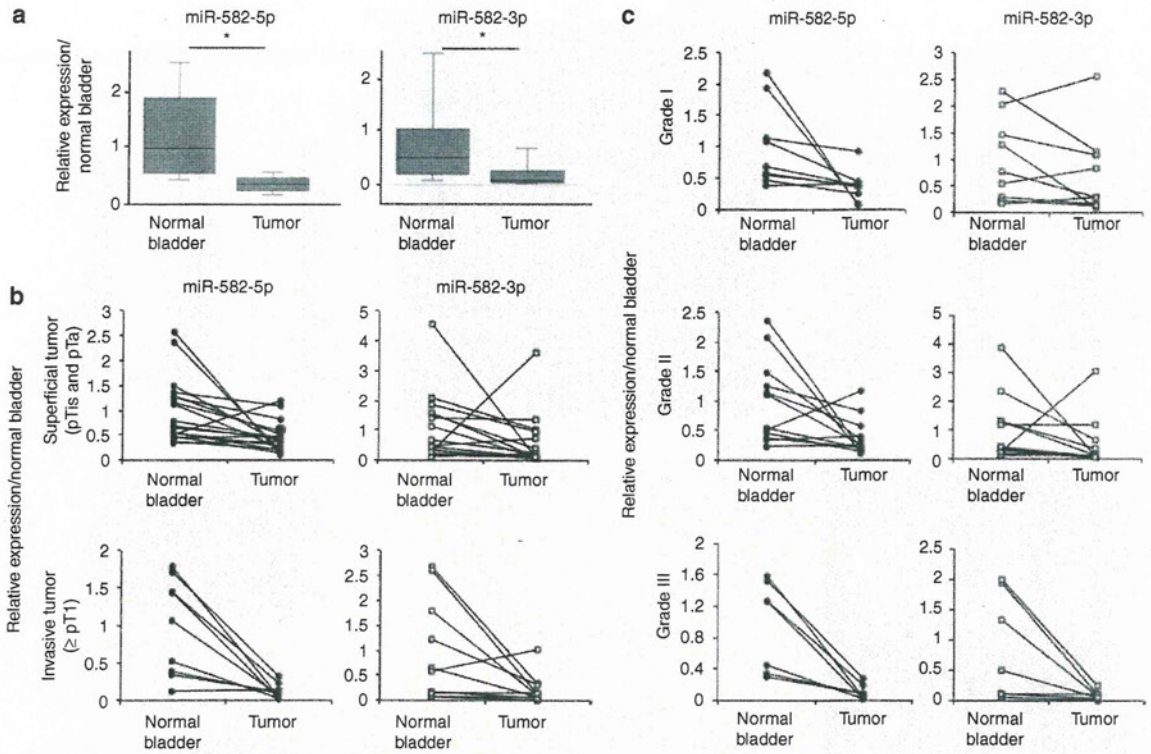


Figure 2 Expression level of miR-582-5p and -3p in bladder cancer tissue samples. (**a**) Expression of miR-582-5p and -3p in clinical samples, which show the downregulation in the tumor region compared with the adjacent normal tissues (tumor: $n = 53$, normal bladder: $n = 31$). The boxes and whiskers range from 25% to 75% and from 10% to 90%, respectively. *$P < 0.01$ versus normal bladder. (**b**) Comparison with the tumor stages in carcinoma cells paired with normal bladder tissue (superficial tumor: $n = 18$, invasive tumor: $n = 10$). (**c**) Comparison with the tumor grades in carcinoma cells paired with normal bladder tissue. All values for the miRNA expression levels were normalized to hsa-miR-103 as an internal control (Grade I: $n = 9$, Grade II: $n = 12$, Grade III: $n = 7$).

**Table 1 Downregulation in clinical samples (fold change >2.5)**

| | Superficial (pTis, pTa) | Invasive (≥pT1) | Grade I | Grade II | Grade III |
|---|---|---|---|---|---|
| miR-582-5p (%) | 7/18 (38.9) | 9/10 (90) | 3/9 (33.3) | 6/12 (50) | 7/7 (100) |
| miR-582-3p (%) | 9/18 (50) | 7/10 (70) | 2/9 (22.2) | 8/12 (66.7) | 6/7 (85.7) |

also observed between tumor grade and the downregulation of miR-582-5p and -3p (**Figure 2c**) (miR-582-5p: Grade I $P = 0.042$, Grade II $P = 0.028$, Grade III $P = 0.006$; miR-582-3p: Grade I $P = 0.206$, Grade II $P = 0.270$, Grade III $P = 0.046$). In addition to this, the ratio of samples showing strong downregulation greater than a 2.5-fold change increased as the tumor stage and grade advanced (**Table 1**). These data suggested that miR-582-5p and -3p levels vary with the malignancy potential and are potential therapeutic markers in the treatment of invasive bladder cancer.

## Effect of miR-582-5p and -3p on cell proliferation and invasive ability

To investigate the functional role of miR-582-5p and -3p in bladder cancer, we performed a cell proliferation assay and a cell invasion assay. UM-UC-3, J82, and TCCSUP cells were transfected transiently with miR-582, miR-582-5p, miR-582-3p, and negative control (NC) siRNA. miR-582 is synthesized to mimic endogenous mature miR-582, whereas miR-582-5p and -3p are designed to match perfectly and complementarily to each strand, similar to siRNA. Three days after transfection, the cell viability was decreased from 30% to 80% in the miR-582-, miR-582-5p-, or miR-582-3p-transfected cells compared with the NC cells (**Figure 3a**). The matrigel invasion assay showed that the cell invasion ability was significantly (>70%) decreased in miR-582-, miR-582-5p-, or miR-582-3p-transfected UM-UC-3, J82, and TCCSUP cells (**Figure 3b,c**). These results suggest that the cell proliferation and invasive abilities of bladder cancer cells were severely affected only by either strand of miR-582-5p or miR-582-3p. In addition, a reduction of invasiveness was observed in 5637 cells, although T24 cells did not show a significant response to miR-582 in the cell proliferation and cell invasion assays (**Supplementary Figure S2a,b**). The expression and functional data suggest that the tumor-suppressive role of miR-582 is correlated with the reduction of its expression level in bladder cancer cells.

To ensure that the functions of miR-582-5p and -3p are not at a supraphysiological level, we repeated these experiments using a stable miRNA vector that synthesizes mature miRNAs by biological processing. UM-UC-3 cells were transduced with a lentiviral construct stably expressing precursor miR-582, and the expression level was examined (**Supplementary Figure S3a**). Furthermore, the functional analysis revealed that UM-UC-3 cells stably expressing miR-582 (UM-UC-3-miR-582) show a significant repression of cell proliferation and invasion (**Supplementary Figure S3b,c**).

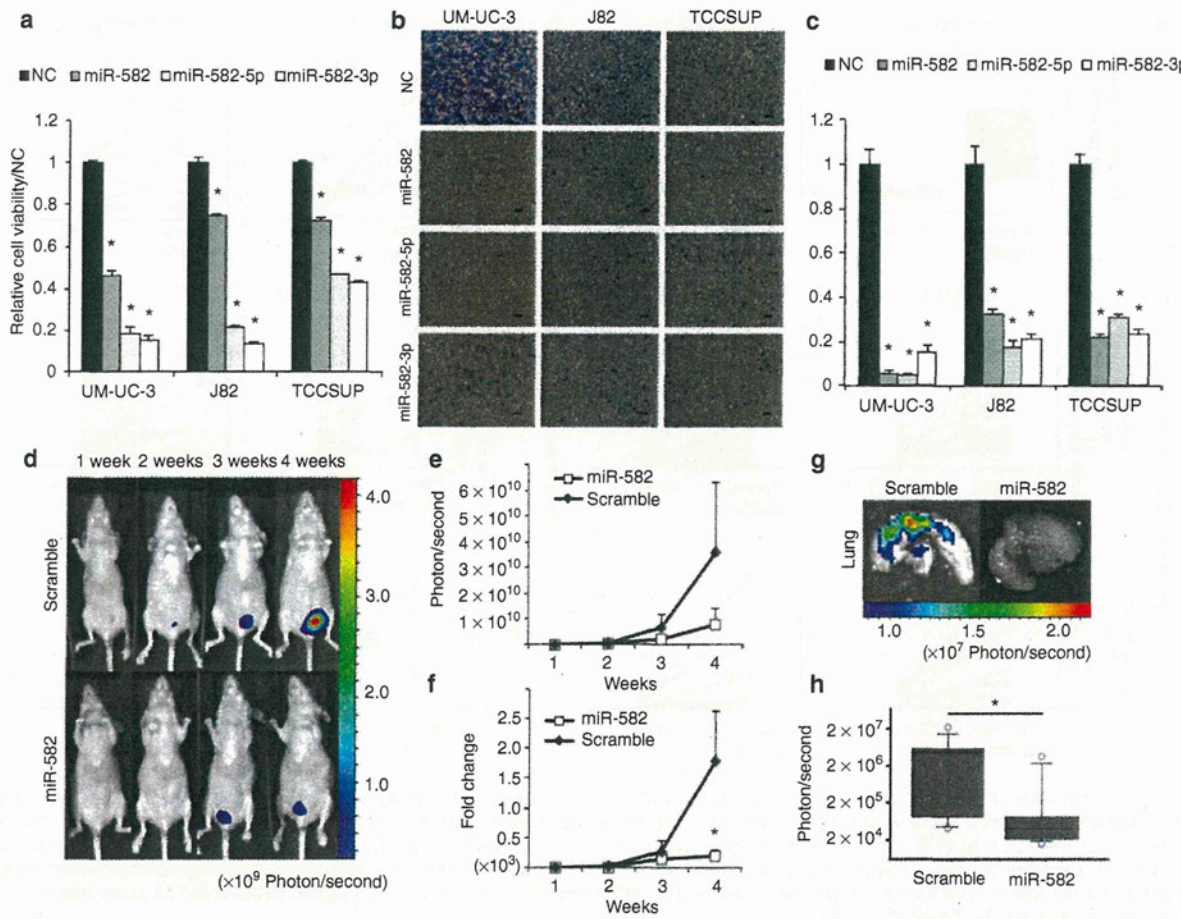


Figure 3 Function of miR-582 in human bladder cancer *in vitro* and *in vivo*. (**a**) Effect of miR-582 on cell proliferation of UM-UC-3, J82 and TCCSUP. The proliferation values were normalized to the values from cells treated with NC ($n = 6$). *$P < 0.01$ versus NC. (**b**,**c**) Effect on cell invasion. The invasive values were normalized to the values from cells treated with NC ($n = 4$). *$P < 0.01$ versus NC (scale bar: 100 μm). (**d**) Inhibition of tumor growth by miR-582 treatment in a murine orthotopic xenograft model of human bladder cancer. The mice were injected with $5 \times 10^6$ UM-UC-3-luc cells into the bladder on day 0. miR-582 and miR-582-scramble (10 μg) with cationic liposomes in a 70 μl volume were injected into the bladder on days 4, 6, 8, 11, 13, and 15 after tumor injection. (**e**,**f**) The growth curves of the orthotopically transplanted UM-UC-3-luc cells were measured by IVIS. Filled diamonds: treatment with miR-582-scramble; Open squares: treatment with miR-582 (scramble: $n = 8$, miR-582: $n = 10$). (**g**) Inhibition of lung metastasis by miR-582 treatment in an orthotopic bladder cancer mouse model. The images were obtained by IVIS on day 26 after transplantation. (**h**) Quantitation of bioluminescence emitted from the whole lungs of mice on day 26. The boxes and whiskers range from 25% to 75% and from 10% to 90%, respectively. *$P < 0.05$ versus scramble. NC, negative control.

By contrast, as shown in a loss-of-function assay using the locked nucleic acid–modified anti-miRNA oligonuleotides against each strand of miR-582-5p and -3p, cell proliferation and invasion are promoted in UM-UC-3-miR-582 cells (**Supplementary Figure S3d,e**). These results indicate that the presence of either miR-582-5p or -3p was sufficient to suppress tumor growth and invasiveness.

## Inhibition of tumor growth and metastasis *in vivo* with miR-582 treatment

To assess the therapeutic potential of miR-582, we used an orthotopic bladder cancer mouse model featuring UM-UC-3-luc cells, which have the capacity to form tumors in the bladder and spread to the lungs of mice. The cationic liposome, LIC-101 (Nippon Shinyaku, Tsukuba, Japan), was used to deliver miR-582 into the tumor cells in the mouse bladder. The miR-582/LIC-101 or miR-582-scramble/LIC-101 complexes were injected transurethrally into the bladder at 5, 7, 9, 11, 13, and 15 days after tumor transplantation (**Figure 3d**). These results demonstrated that miR-582 successfully suppressed tumor growth *in vivo*; in particular, we found that miR-582 provided a greater than fourfold reduction of the tumor growth in the fourth week (**Figure 3e**). Furthermore, there was a significant difference in fold changes relative to the photon counts in the first week (miR-582 = 2,253.5 ± 851.7, scramble = 187.1 ± 108.9, $P = 0.042$) (**Figure 3f**). At the end of the experiment, 4 weeks after transplantation, the mice treated with miR-582-scramble showed a more frequent presence of tumors in the lung (5 of 8; 63%) than in the miR-582 group (1 of 10; 10%) (**Figure 3g**). Moreover, there was a significant difference between the two groups regarding the luminescence of the lung (**Figure 3h**). These results indicated that the intravesical administration of the miR-582/LIC101 complex could be a novel therapeutic strategy for the inhibition of tumor progression and metastasis in bladder cancer.

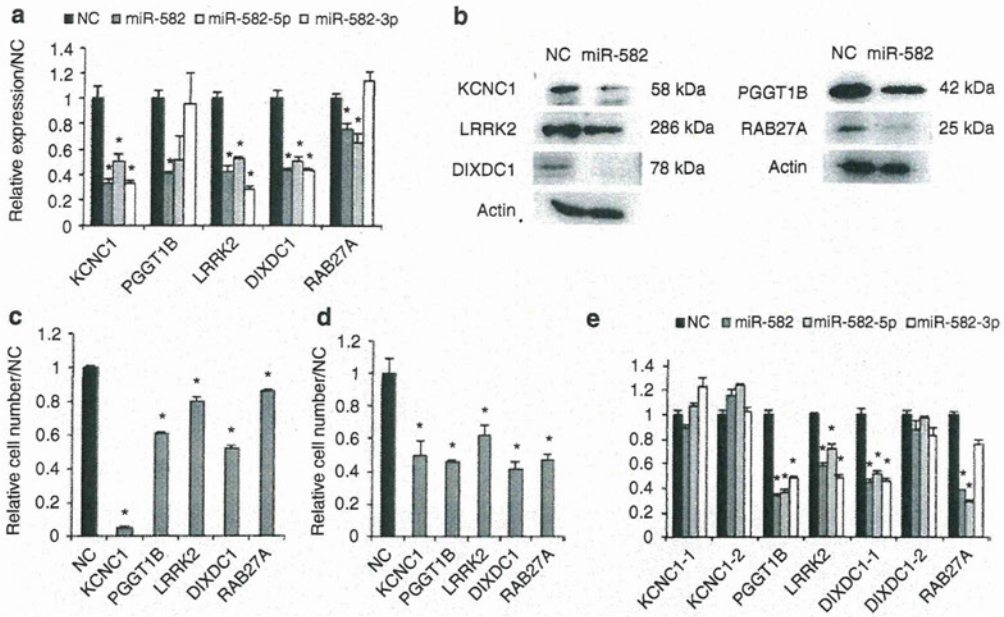


*Figure 4 Identification of PGGT1B, LRRK2, DIXDC1, RAB27A, and KCNC1 as miR-582-5p and -3p target genes.* Target validations of *KCNC1*, *PGGT1B*, *LRRK2*, *RAB27A*, and *DIXDC1* were confirmed by qRT-PCR, western blot analysis, and cell proliferation, cell invasion, and luciferase reporter assays. (**a**) qRT-PCR was performed in UM-UC-3 cells at 24 hours after transfection with miR-582. The relative expressions of the target mRNAs were calculated compared with the level in NC ($n = 3$). (**b**) Western blot analysis was performed in UM-UC-3 cells at 48 hours after transfection. Actin was used as the loading control. (**c**) Effect of target siRNAs on the proliferation of UM-UC-3. The proliferation values were normalized to values from cells treated with NC ($n = 6$). (**d**) Effect of target siRNAs on the invasion of UM-UC-3. The invasive values were normalized to the values from cells treated with NC ($n = 3$). (**e**) Each luciferase reporter construct containing a target 3′UTR was cotransfected with miR-582, miR-582-5p or -3p, or NC into UM-UC-3 cells ($n = 6$). *$P < 0.05$ versus NC. NC, negative control; qRT-PCR, quantitative reverse transcription-PCR.

**Table 2 Summary of the number of target sites for miR-582-5p and -3p**

| Target 3′UTR | | Number of predicted binding site | |
|---|---|---|---|
| | | miR-582-5p | miR-582-3p |
| KCNC1 | Site-1 | 1 | 0 |
| | Site-2 | 1 | 1 |
| PGGT1B | | 5 | 1 |
| LRRK2 | | 1 | 2 |
| RAB27A | | 4 | 0 |
| DIXDC1 | Site-1 | 2 | 2 |
| | Site-2 | 0 | 1 |

## Identification of miR-582-5p and -3p target genes

To identify miR-582-5p and -3p target genes, hemagglutinin (HA)-tagged Ago2-immunoprecipitation (Ago2-IP) and mRNA array analysis were performed. The UM-UC-3-Ago2 cells stably expressing HA-tagged-Ago2 were transfected with miR-582, and the Ago2-RNA complexes were precipitated with the anti-HA antibody, which was followed by the microarray analysis. The total RNA from UM-UC-3-Ago2 cells transiently transfected with miR-582 and from UM-UC-3-miR-582 cells was also analyzed (**Supplementary Figure S4a**). As a result, 259 genes were identified as candidates for directly targeted genes, and 1,559 genes were identified as candidates for indirectly targeted genes that are downstream of the direct targets (**Supplementary Tables S4 and S5**). In addition to these experimental approaches, we used two target prediction algorithms (TargetScan: http://www.targetscan.org, miRanda: http://www.microrna.org) as *in silico* approaches. After calculating the fold change and conducting an overlapping prediction analysis, we validated the identified 42 genes by qRT-PCR (**Supplementary Figure S4b**), western blotting analysis, and cell proliferation, cell invasion, and 3′UTR reporter assays. The results of the qRT-PCR and western blotting analyses demonstrated that the expression levels of the mRNA and protein of five genes—*potassium voltage-gated channel subfamily C member 1* (*KCNC1*), *PGGT1B*, *LRRK2*, *DIXDC1*, and *ras-related GTP-binding protein* (*RAB27A*)—were more downregulated by transfection with miR-582, or miR-582-5p, or -3p than the NC siRNA-treated cells (**Figure 4a,b**). Furthermore, silencing of these target genes by siRNAs showed a remarkable inhibition of cell proliferation and cell invasion in UM-UC-3 cells (**Figure 4c,d**). These five genes have several putative binding sites for miR-582-5p or -3p in their 3′UTRs (**Table 2** and **Supplementary Table S6**). Indeed, the expression of each luciferase reporter gene fused with the 3′UTR of *PGGT1B*, *LRRK2* and *DIXDC1* was suppressed by transfection with miR-582, miR-582-5p, or -3p (**Figure 4e**). Conversely, the expression of that of *RAB27A* was suppressed by miR-582 and miR-582-5p. There was no significant difference in the expression of that of *KCNC1*. These results indicated that miR-582-5p and -3p regulate the expression of *PGGT1B*, *LRRK2*, and *DIXDC1* by binding their 3′UTRs. One strand, miR-582-5p, can directly bind 3′UTR of *RAB27A* and regulate the expression. In contrast, the downregulation of *KCNC1* by transfection with miR-582-5p and -3p is not a direct consequence of miR-582.

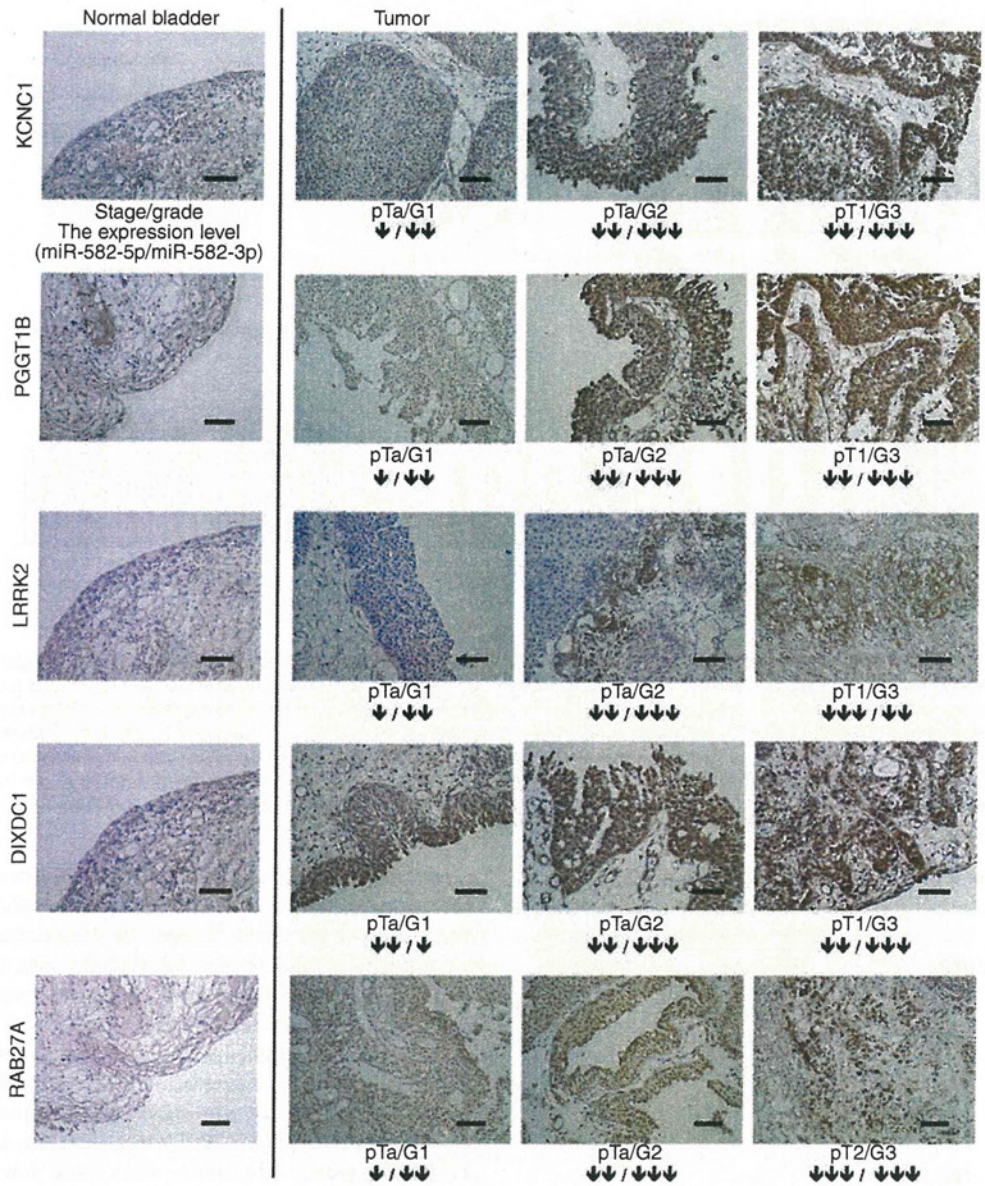


Figure 5 Representative examples of immunohistochemical expression of different gene products in human bladder cancer. Target gene localization in clinical samples by IHC. Left panel: representative normal bladder biopsy (scale bar: 100 μm); Right panel: representative bladder cancer with strong staining observed. A black arrow shows the fold change of the downregulation of miR-582-5p or -3p in tumor tissue compared with the adjacent normal tissues (single black arrow: fold change <2.0, double black arrows: fold change = 2.0 ~ 5.0, triple black arrows: fold change >5.0). Magnification: ×20. IHC, immunohistochemistry.

In addition, we performed an immunohistochemical analysis to observe the expression of these target genes in clinical samples of human bladder cancer. Strong nuclear or cytoplasmic staining was observed in the tumors but not in the adjacent normal bladder tissues (**Figure 5**). Furthermore, there was a positive correlation between the expression levels of target genes and tumor stage or grade of bladder cancer, and miR-582 was downregulated, whereas, in the low-stage or -grade bladder cancer, a high expression of target genes was not notably observed. These results suggest that the expressions of these cancer-related genes are a clinically relevant biological process, and the levels of the expressions are correlated with malignancy potential in bladder cancer.

## DISCUSSION

Genetic changes potentially affect all medical conditions and are associated with thousands of diseases.[26] The amplification of oncogenes or deletion of tumor-suppressor genes can broadly influence tumor initiation and progression.[21,24] Although many previous reports have indicated the correlation between region-specific gain or loss of DNA and tumor progression in bladder cancer, it has not yet been fully elucidated as to which genes, including

noncoding RNAs, are responsible for these different observations. In particular, the losses of chromosomes 3p11-12, 4q32-34, and 5q12-13 loci were reported to be associated with clinicopathological factors in bladder cancer.[11,21–24] Here, we provided evidence on the relationship between the copy number loss of 5q12 and tumor progression via post-transcriptional regulation by miRNA. We demonstrated a novel pathway of paired tumor-suppressive miRNAs, miR-582-5p and -3p located on 5q12, which are specifically attenuated in invasive bladder cancer cells by copy number loss. Furthermore, the expression levels of miR-582-5p and -3p were remarkably lower in bladder cancer tissues and correlated with the tumor grade. These clear correlations suggest an important role for miR-582 on bladder cancer progression.

Despite these considerable dysregulations, there are no reports concerning the expression profiling and functional role of miR-582 in bladder cancer. Thus, focusing on miR-582 as a new candidate tumor-suppressive miRNA in bladder cancer is natural. As expected, the restoration of miR-582-5p or -3p expression inhibited the cell proliferation and invasion in UM-UC-3 cells. Generally, most miRNA duplexes can be functionally asymmetric due to unequal election for assembly into RISC and distinct sequence features. Although previous reports showed that miR199a-5p and -3p or miR-297b-5p and -3p target same mRNAs,[27,28] for this reason, it is extremely rare that each strand of mature miRNA, 5p and 3p, has an identical property in the same physiological/pathological processes. The present experiment indicated that both strands of miR-582-5p and -3p have tumor-suppressive activities based on their sequences.

The number of RNAi-based preclinical and clinical trials has grown over the past several years, and these trials provide opportunities for success.[2,4] These studies were conducted mainly in specific organs, such as eyes, dermis, and lung, which were relatively accessible by topical or local administration. Thus, accessibility is a key requirement for successful RNAi *in vivo* to be delivered tissue specifically or cell specifically. Here, in a closed environment of the bladder, the intravesical injection of miRNA is expected to offer high specificity to a tissue and noninvasive administration. Indeed, a previous study showed that the low-toxic cationic liposome, LIC-101, and the dsRNA complex could be successfully delivered into the bladder tissue and were able to affect tumor growth.[7] However, whether transurethral injection is effective against a severe problem, such as invasion into the muscle and metastasis, remains unknown. In this study, we were successful at providing evidence for this procedure by showing that the instillation of the synthetic miR-582/LIC-101 complex inhibited both lung metastasis and tumor growth. For the first time, our findings have provided a new insight into the availability of a bladder cancer lung metastasis model mouse in RNAi therapeutic trials.

miRNAs are known to have the potential to target thousands of different mRNAs, and each mRNA is also targeted by multiple miRNAs.[29,30] Although target prediction algorithms (TargetScan, miRanda) such as *in silico* approaches based on prior experimental studies can help to predict miRNA-binding targets, computational algorithms are imperfect and insufficient to identify some essential or novel target genes. Hence, in addition to *in silico* analysis, as an optimized and high-throughput biochemical assay, we evaluated the use of Ago2-IP.[31–33] The validation studies revealed that miR-582-5p and -3p have a tumor-suppressive function by both the inhibition of translation and the degradation of *KCNC1*, *PGGT1B*, *LRRK2*, *DIXDC1*, and *RAB27A*. Although miR-582 did not regulate *KCNC1* expression directly, it is very interesting that *KCNC1* has a novel remarkable function in cell proliferation (**Figure 4c**), because it usually works as a potassium ion transporter. *PGGT1B* is involved in the geranylgeranylation of RhoA and the activation of *MMP-9*, resulting in the disruption of F-actin organization and the progression of cell motility, invasion, and metastasis.[34,35] The kinase domain of *LRRK2* is homologous to B-RAF kinase, which is well known to drive malignant melanoma. Furthermore, *LRRK2* is related to the direct phosphorylation of *Akt1*, resulting in cell survival and the prevention of apoptosis.[36,37] *DIXDC1*, which works as a receptor of the Wnt signaling pathway, is related to the upregulation of *CyclinD1* and the downregulation of p21. The siRNA knockdown of *DIXDC1* was reported to cause G1/S phase arrest in colon cancer.[38,39] *RAB27A*, a member of the RAS oncogene family, is well known to be a driver of melanoma and breast cancer, promoting cell proliferation, invasion, and metastasis potential.[40,41] It is also involved in the regulation of membrane trafficking and exosome formation.[42] This study carries considerable potential to provide novel insights into the pathogenic mechanisms of exosomes regulated by miR-582-5p in invasive bladder cancer.

In conclusion, this study provides two novel findings in bladder cancer. First, we presented evidence of a unique regulatory pathway involving a couple of tumor-suppressive miRNAs, miR-582-5p and -3p, that are downregulated in invasive bladder cancer. The significant dysregulation of the miR-582 network may contribute to the progression of other invasive cancers, and we are actively investigating this hypothesis. Second, an *in vivo* experiment showed the possibility of the application to a therapeutic target against the continued threat of progression to muscle-invasion and metastasis. Importantly, our findings provide an insight into the unique regulatory pathway, which has a coordinated role between miRNA-5p and -3p. These novel findings also provide a potential value of an extreme contribution to the development of the cancer therapy as a prominent class of RNAi therapy.

## MATERIALS AND METHODS

***Cell culture.*** The human bladder cancer cell lines UM-UC-3, 5637, J82, TCCSUP, T24, HT1376, and RT4 and the mouse bladder cancer cell line MBT2 were obtained from the American Type Culture Collection (Manassas, VA). The UM-UC-3, J82, TCCSUP, T24, and MBT2 cells were cultured in Modified Eagle's Medium (Sigma-Aldrich, St Louis, MO) containing 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and an antibiotic-antimycotic (Invitrogen) at 37 °C in 5% $CO_2$. The 5637 cells were cultured in RPMI (Invitrogen) containing 10% heat-inactivated fetal bovine serum and an antibiotic-antimycotic at 37 °C in 5% $CO_2$. The T24 and RT4 cells were cultured in McCoy'5A (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum and an antibiotic-antimycotic at 37 °C in 5% $CO_2$.

***Patients and human samples.*** All human bladder tissue samples were derived from the resected bladders of 29 patients who underwent transurethral resection or radical cystectomy at the Department of Urology, St. Marianna University between 2009 and 2011. The samples of tumor and normal epithelium were fixed in formalin, embedded in paraffin, and sectioned for use in microscopic analysis and laser capture microdissection. All materials were obtained with written informed consent, and the study protocols were approved by the Institutional Review Board at the National Cancer Center Research Institute and St. Marianna University.

***RNA extraction.*** Total RNA was extracted from cultured cells using the QIAzol and miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

***Real-time PCR (qRT-PCR).*** The expression of miRNA was quantified by TaqMan miRNA assays (Applied Biosystems, Foster City, CA). The PCR was performed in 96-well plates using the 7300 Real-Time PCR System (Applied Biosystems). All reactions were performed in duplicate. Human-RNU6B or hsa-miR-103 was used as an invariant control.[43]

For the mRNA expression analysis, total RNAs were reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using a random hexamer primer. The synthesized cDNAs were quantified by TaqMan Gene expression analysis or SYBR Green I qRT-PCR. The β-actin housekeeping gene was used to normalize the variation in the cDNA levels. The primer pairs used for gene amplification are listed in **Supplementary Table S7**.

***Quantitative PCR of miR-582 loci on chromosome 5q12.*** Genomic DNA was extracted from cultured cells using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The qPCR for the miR-582 locus on chromosome 5q12 was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and the primer sequences were 5′-ccacaacaagtcaatctgtgc-3′ and 5′-tattgaaggggttctggtg-3′. The housekeeping gene RNase P was also quantified as a control reference gene using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and the TaqMan RNase P Detection Reagents Kit (Applied Biosystems).

***Transient miRNA/siRNA transfection.*** Synthetic hsa-miR-582, hsa-miR-582-5p, hsa-miR-582-3p, and hsa-miR-582-scramble duplexes were obtained from the Bonac (Kurume, Japan). The Allstars Negative Control siRNA was purchased from Qiagen. The siRNAs targeting *KCNC1*, *PGGT1B*, *DIXDC1*, *LRRK2*, and *RAB27A* were purchased from the Bonac. The cells were transfected with 25 nmol/l of either the miRNA or siRNA using DharmaFECT 1 (Thermo Fisher Scientific, San Jose, CA) according to the manufacturer's protocol. The miRNA and siRNA sequences are given in **Supplementary Table S8**.

***Cell proliferation assay (MTS assay).*** For the cell proliferation assay, 24 hours after transfection, $3 \times 10^3$ cells were seeded in a 96-well plate. After 3 days of culture, the cell viability was measured using the Tetra Color One assay kit (Seikagaku Kohgyo, Tokyo, Japan) according to the manufacturer's instructions. The absorbance at 450 nm was measured using Envision (PerkinElmer, Norwalk, CT).

***Cell invasion assay.*** The invasive ability of the bladder cancer cells was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm pore size; Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, the cells were transfected with miRNA or siRNA and, on the following day, $1 \times 10^5$ cells were plated in the upper chamber. The lower chamber was supplemented with a medium containing 10% fetal bovine serum. After 24 hours incubation, the cells on the upper surface were scraped off, and the invasive cells attached to the lower surface of the membrane inserts were fixed and stained with Diff-Quik (Sysmex, Kobe, Japan). The invading cells were observed and counted under a microscope in four random fields. All assays were performed in triplicate.

***Generation of stable cell lines expressing miR-582 or HA-tagged Ago2 or luciferase.*** To construct a lentiviral vector for miR-582, pre-miRNA encompassing the stem-loop was amplified from genomic DNA isolated from HT1376 cells by PCR. The PCR product was digested and cloned into the pCDH cDNA cloning lentivector (Cat#CD513B-1; SBI, Mountain View, CA). To construct a lentiviral vector for luciferase, the luc construct was amplified from the pGL4 luciferase reporter vector by PCR. The PCR product was digested and cloned into the pLenti6/V5 Directional TOPO vector (Promega, Madison, WI). The lentiviral vector Lenti-miR-582, Lenti-scramble shRNA (Cat#MZIP000PA-1, SBI), Lenti-HA-Ago2 (RA703B-1, SBI), or Lenti-Luc and the lentiviral packaging plasmids (Invitrogen) were cotransfected into L293T cells. After 48 hours, the lentiviruses in the supernatant were collected and used to infect the UM-UC-3 cells. After antibiotic selection for 2 weeks, stable clones were obtained.

***Analysis of miR-582 treatment in a mouse model of bladder cancer.*** The animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. For the generation of the lung metastasis mouse model, we referred to some previous studies.[7,44–46] Six- to seven-week-old female Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane on day zero and subsequent days. The murine bladder was injected with UM-UC-3-luc cells intravesically at $5 \times 10^6$ cells/50 μl/bladder after 15 minutes of trypsin treatment. The development of subsequent tumor growth and metastasis was monitored once a week by *in vivo* imaging. In brief, the mice were injected with 150 mg/kg D-luciferin (Promega) intraperitoneally and imaged 10 minutes later to count the photons from the whole bladder or lung using the IVIS imaging system (Xenogen, Alameda, CA) according to the manufacturer's instructions. The data were analyzed using the LivingImage software (version 2.5; Xenogen). On day 4, the bioluminescence from the implanted cancer cells was measured, and the mice were divided into two treatment groups with equivalent levels of bioluminescence. The transurethral treatment with the miR-582 and LIC101 (Nippon Shinyaku, Kyoto, Japan) complexes at a ratio of 1:16 (w/w) in a volume of 70 μl (10 μg/site) was performed on days 5, 7, 9 11, 13, and 15.

***Ago2-IP.*** The RNA-binding protein immunoprecipitation was performed using an immunoprecipitation kit (RNA-binding protein immunoprecipitation–assay kit for microRNA; MBL, Nagoya, Japan) following the manufacturer's instructions. In brief, UM-UC-3 cells stably expressing HA-Ago2 were transfected with either miR-582 or NC for 48 hours and immunoprecipitated using anti-HA agarose beads (Wako, Osaka, Japan). The Ago2-bound RNA was eluted from beads with the HA peptide (Wako), and the QIAzol reagent was added to extract the total RNA. Ago2-bound total RNA was cleaned further using miRNeasy columns and then subjected to microarray analysis.

***Microarray analysis.*** Total RNA was harvested from UM-UC-3-miR-582, UM-UC-3-shNC, and UM-UC-3-HA-Ago2 cells that were transfected transiently with miR-582 or NC for 48 hours. The Ago2-bound RNA was prepared from the Ago2-IP experiments. The total RNA was labeled with Cy3 using a Low Input Quick Amp Labeling Kit (Agilent, Palo Alto, CA) and hybridized to a SurePrint G3 Human GE 8×60 K array (Agilent) according to the manufacturer's instructions. The data analysis was performed using GeneSpring GX11.5.

***Luciferase reporter assay.*** The 3′-UTRs of human *KCNC1*, *DIXDC1*, *LRRK2*, *PGGT1B*, and *RAB27A* were amplified by PCR from genomic DNA and cloned at the NotI and XhoI sites into pGMT Easy vector (Promega). The PCR primers and oligonucleotide sequences for the constructs are listed in **Supplementary Table S9**. All the constructs were further confirmed by sequencing.

For the luciferase activity analysis, each construct was cotransfected with the miRNA duplex in a 96-well plate using the DharmFECT Duo transfection reagent (Thermo Fisher Scientific) for 24 hours, and the luciferase assays were performed with the Dual-Glo Luciferase assay system (Promega) according to the manufacturer's instructions.

***Western blotting.*** Forty-eight hours after transfection, the cells were homogenized in an M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). The proteins in the total cell lysate or bound to the anti-HA agarose beads were separated by SDS-PAGE gels, which were calibrated using Precision Plus protein standards (Bio-Rad, Richmond, CA). The primary antibodies against KCNC1 (1: 250, ab84823), DIXDC1 (1: 250,

ab67763), LRRK2 (1: 250, ab57329), PGGT1B (1: 250, ab55615), and RAB27A (1: 200, ab55667) were purchased from Abcam (Cambridge, MA), and the primary antibody against ACTIN (1:10,000, MAB1501) was purchased from Millipore (Billerica, MA). The dilution ratio of each antibody is indicated in parentheses. The HRP-linked anti-mouse secondary antibody (GE Healthcare, Buckinghamshire, UK) was used at a dilution of 1:5,000. The bound antibodies were visualized by chemiluminescence using the ECL Plus Western blotting detection system (GE HealthCare), and luminescent images were analyzed using a LuminoImager (LAS-3000; Fuji Film, Inc., Tokyo, Japan).

***Immunohistochemistry.*** All tumors resected from human bladders were fixed with 10% buffered formalin and embedded in paraffin. Sections with a 5-μm thickness were examined using immunohistochemistry. The sections were deparaffinized, the antigens were retrieved by autoclaving in a 10 mmol/l citrate buffer (pH 6.0), and the endogenous peroxidase activity was blocked with the Immuno Pure Peroxidase Suppressor (Pierce, Chester, UK). The primary antibodies used in this study were the same as those used in the western blotting analysis (1:50), followed by incubation with peroxidase-coupled anti-mouse IgG (ImmPRESS Reagent; Vector labs, Burlingame, CA). The immunoreactions were visualized with diaminobenzidine, and the sections were counterstained with hematoxylin.

***Statistical analysis.*** Results are expressed as the mean ± SE. The statistical analyses were conducted using the Bonferroni multiple-comparison test, and the analyses of the luminescence of the lung were conducted using the nonparametric Mann–Whitney–Wilcoxon test. These analyses were performed with the Expert StatView analysis software (version 4; SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

## SUPPLEMENTARY MATERIAL

**Figure S1.** Expression of miR-1305, pri-miR-582 and PDE4D in human bladder cancer cell lines.
**Figure S2.** Functions of miR-582 in T24 and 5637 cells.
**Figure S3.** Functional analysis of UM-UC-3-miR-582 cells.
**Figure S4.** Identification of miR-582-5p and -3p target genes using HA-Ago2 IP and microarray analysis.
**Table S1.** Copy number losses detected in UM-UC-3 cell line.
**Table S2.** Copy number losses correlated with pathological stage in human bladder cancer.
**Table S3.** Stage and Grade distribution of the patients.
**Table S4.** A total of 259 genes as candidates for miR-582 direct targets.
**Table S5.** A total of 1,559 genes as candidates for miR-582 indirect targets.
**Table S6.** Summary of miR-582-5p and -3p target site.
**Table S7.** PCR primer sequences and oligonucleotide sequences.
**Table S8.** miRNA and siRNA oligonucleotide sequences.
**Table S9.** PCR primer sequences for Luciferase assay.

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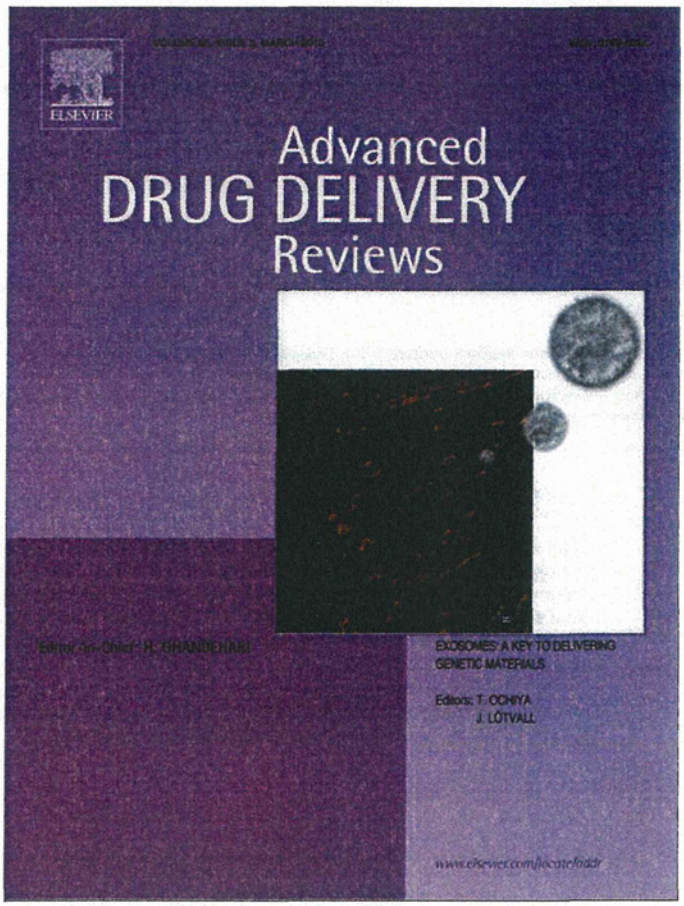

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# Exosomal tumor-suppressive microRNAs as novel cancer therapy☆ "Exocure" is another choice for cancer treatment

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ABSTRACT

MicroRNAs (miRNAs) act to fine-tune cellular responses in a variety of biological circumstances such as development, organogenesis, and homeostasis. The dysregulation of miRNA expression accelerates disease progression, including metabolic disease, immunological disease and cancer, through the gene network disorder. Therefore, understanding the miRNA maturation process may unravel the mechanisms of cancer malignancy; however, the life of miRNA has not been clarified. In this article, we summarize the recent findings regarding the novel forms of miRNA, especially secretory miRNAs, focusing on exosomal miRNAs. Recent research has revealed that exosomal miRNAs affect many aspects of physiological and pathological conditions, and may be useful as novel therapy. Here, we propose a method for the delivery of tumor-suppressive miRNAs to desired sites using exosomes, and we named this method "exocure".



**Contents**



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## 1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that are approximately 22 nt in length [1]. Although more than 1500 mature human miRNA sequences are currently listed in the miRNA database [2], the function of many has not been clarified. The maturation process of miRNAs includes several post-transcriptional processing steps [1]. First, the primary miRNA transcripts (primary miRNAs; pri-miRNA) are generated from the genomic sequence. These long transcripts are mainly transcribed by RNA polymerase II. Second, these transcripts are processed to precursor miRNA (pre-miRNA) in 60–110 nt fragments in the nucleus by Drosha, an RNase III enzyme. Then the pre-miRNAs are transported to the cytoplasm by exportin-5. Dicer, another RNase III enzyme, processes the pre-miRNA into double-stranded RNA, which is a mature miRNA of approximately 22 bp. The mature miRNA is composed of an RNA-induced silencing complex (RISC) and binds to the complementary sequence in the 3′ untranslated region (3′ UTR) of target mRNAs, resulting in the degradation of the mRNA and/or inhibition of protein translation [3]. This multistep processing is regulated by many factors such as epigenetic modifications of the genomic sequence and by transcription factors, RNase and RNA-binding proteins. If there is a misregulation of one of these processing components, the miRNA expression will be dysregulated, leading to disease progression such as cancer malignancy [4]. Recently, it was reported that miRNAs do not exist only in the cell but are also secreted outside of cells [5,6]. In this review, we will discuss recent reports that indicate that exosomes carrying "secretory miRNAs" mediate various biological phenomena. In addition, we propose the usage of an exosome-delivered tumor-suppressive miRNA in cancer therapy.

## 2. Tumor-suppressive miRNAs are novel anti-cancer agents

It is known that the downregulation of miRNA expression leads to cancer development through various mechanisms such as genomic deletions, amplifications, mutations, epigenetic silencing, the dysregulation of transcription factors that target specific miRNAs, or the inappropriate processing of miRNA precursors [4]. These downregulated miRNAs are thought to act as tumor suppressors. There are various types of tumor-suppressive miRNAs; however, little is known regarding their precise mechanism of action (Table 1).

### 2.1. let-7

The expression of let-7 miRNA is significantly downregulated in lung cancer, and the overexpression of let-7 in a lung adenocarcinoma cell line suppressed lung cancer cell growth in vitro through the downregulation of KRAS and HMGA2 [7–10]. Interestingly, let-7 miRNA expression was markedly reduced in breast cancer tumor-initiating cells [11], which have self-renewal ability and resistance to chemotherapy and radiotherapy, and the expression of let-7 miRNA increased after differentiation [12]. The re-expression of let-7 in breast cancer tumor-initiating cells reduced proliferation, mammosphere formation and metastasis in a xenograft model through the reduction of KRAS and/or HMGA2. Many downregulation mechanisms of let-7 have been reported [13–19]. Importantly, disruption of the let-7 precursor processing by LIN28 and LIN28B, which are reported as overexpressed in primary human tumors, human cancer cell lines and pluripotent stem cells, is essential for controlling proper miRNA expression [14].

### 2.2. miR-16

MiR-15a and miR-16-1 are deleted or downregulated in the majority of chronic lymphocytic leukemia (CLL) cells [20–22], and re-expression of these miRNAs induced apoptosis through the downregulation of BCL2. In addition, the miR-15a and miR-16 levels significantly decrease in advanced prostate tumor cells [23]. Takeshita et al. reported that the injection of miR-16 with atelocollagen (a highly purified type I collagen that possesses low immunogenicity and is produced by treating calf dermis with pepsin) via the tail vein of mice significantly inhibited the growth of prostate bone metastases in a therapeutic bone-metastasis model [24].

### 2.3. miR-143

The suppression of miR-143 expression has been reported in several human cancers, including colorectal, prostate, cervical and ovarian [25–30]. Induction of miR-143 expression in those cancer cells resulted in the inhibition of cell proliferation or the induction of apoptosis through the suppression of its target genes such as KRAS and ERK5. Furthermore, Osaki et al. showed that miR-143 was the most downregulated miRNA in metastatic human osteosarcoma cell lines relative to the parental cell lines, and transfection of miR-143 into metastatic human osteosarcoma cell lines significantly decreased cell invasiveness but not proliferation [31]. In addition, intravenous injection into mice of miR-143 significantly suppressed the formation of lung metastases from metastatic human osteosarcoma cell lines. Moreover, cells positive for MMP13, a target of miR-143 in osteosarcoma cells, was found in lung metastasis-positive cases but not in at least three cases with higher miR-143 expression levels and without metastases.

### 2.4. miR-22

Xu et al. demonstrated that miR-22 expression is upregulated in senescent human fibroblasts and epithelial cells. In contrast, its expression is downregulated in various cancer cell lines, and the overexpression of miR-22 in those cases induces growth suppression

**Table 1**
The list of typical tumor suppressive miRNAs.

| microRNA | Type of cancer | Target gene | Phenotype | References |
|---|---|---|---|---|
| let-7 | Lung cancer | KRAS | Inhibition of cell proliferation | [7–19] |
| | Breast cancer | HMGA2 | | |
| miR-16 | Chronic lymphocytic leukemia | BCL2 | Induction of apoptosis | [20–24] |
| | Prostate cancer | CCND1 | Inhibition of cell proliferation | |
| | | WNT3A | | |
| miR-143 | Ovarian cancer | | | [25–31] |
| | Prostate cancer | ERK5 | Induction of apoptosis | |
| | Cervical cancer | KRAS | Inhibition of metastasis | |
| | Osteosarcoma | | Inhibition of cell proliferation | |
| miR-22 | Colorectal cancer | Sp1 | | [32] |
| | Breast cancer | CDK6 | Induction of growth suppression | |
| | | SIRT1 | Induction of senescent phenotype | |

Representative cases are shown in the "type of cancer" and "target gene".

and a senescent phenotype in human normal and cancer cells through the downregulation of CDK6, SIRT1, and Sp1 genes [32]. In addition, in vivo injection of miR-22 inhibits tumor growth and metastasis through the induction of senescence in inoculated breast cancer cell lines, suggesting that miR-22 can be used as a senescence inducer, and this approach may be a novel cancer treatment method.

Taken together, these findings prompted the idea that delivery of tumor-suppressive miRNAs that are downregulated in cancer cells may provide a therapeutic option in combination with other cancer treatments such as chemotherapy or antibody therapy.

## 3. Exosomal miRNAs are novel humoral factors for cell–cell communication

As described above, the expression levels of tumor-suppressive miRNAs are known to be downregulated in cancer cells. Therefore, restoring the expression of these miRNAs might lead to the suppression of cancer progression. However, the nucleic acid delivery method is the most significant problem for nucleic acid therapy [33,34]. In 2007, Valadi et al. found that miRNAs are contained inside exosomes [35]. This study showed the possibility that miRNAs are not only intracellular gene regulators but are also humoral factors, suggesting that miRNAs could act as tools for cell–cell communication. Exosomes are lipoprotein complexes including small-membrane vesicles of endocytic origin (30–100 nm) [35]. Exosomes are formed through the inward budding of endosomal membranes that give rise to intracellular multivesicular bodies (MVBs) that later fuse with the plasma membrane, releasing the exosomes to the extracellular space [36–39].

### 3.1. The exosomal miRNAs are functional in recipient cells

Following the report from Valadi et al., three reports showed the functionality of exosomal miRNAs (Table 2). Pegtel et al. showed that miRNAs encoded by the EB virus are secreted from EBV-infected B cells through exosomes. These miRNAs repress the EBV target immunoregulatory genes in primary EBV-associated lymphomas [40]. Zhang et al. reported that exosomes from human monocyte/macrophage cell lines deliver miR-150 into human microvascular endothelial cells, and the expression levels of c-Myb were downregulated in microvascular endothelial cells and enhance their cell migration [41]. We demonstrated that secreted miR-146a, whose expression is known to be downregulated in prostate cancer, was transferred from miR-146a-overproducing HEK293 cells to cancer cells, where it suppressed its target gene and led to cell growth inhibition [42].

### 3.2. Immune cells use exosomal miRNAs for cell–cell communication

After the publication of these studies, many researchers reported the function of exosomal miRNAs in a variety of physiological and pathological phenomena (Table 2). The antigen-driven unidirectional transfer of exosomal miRNAs from the T cell to antigen-presenting cells has been observed, and this transfer results in the downregulation of target gene expression in recipient cells [43]. Furthermore, dendritic cells (DCs) release exosomal miRNAs, which then transfer to acceptor DCs [44]. Interestingly, DCs release exosomes with different miRNAs depending on the maturation of the DCs. Taken together, these two papers regarding immune cells describe a novel mechanism of complex immune cell communication mediated by exosomal miRNAs. However, the transfer of miRNAs between cells is not limited to immune cells.

### 3.3. The function of exosomal miRNAs in cancer development

The exosomal miR-223 from tumor-associated macrophages is transported to breast cancer cells, supporting the idea that macrophages regulate the invasiveness of breast cancer cells through exosome-mediated delivery of oncogenic miRNAs [45]. In addition to breast cancer cells, exosomal miRNAs derived from hepatocellular carcinoma cells can be taken up by other cells and target transforming growth factor β activated kinase-1, resulting in the enhancement of transformed cell growth in recipient cells [46].

Recently, we showed that proliferation of a prostate carcinoma cell line was inhibited by the addition of the exosome fraction isolated from a non-cancerous prostate epithelial cell line [47]. These observations suggest that exosomal miRNAs derived from non-cancerous cells were transferred to cancerous cells and inhibit proliferation. Indeed, some sets of tumor-suppressive miRNAs such as miR-16, miR-205, and miR-143 were downregulated in prostate cancer cell lines at the cellular and extracellular levels. This observation supports the idea that secretory tumor-suppressive miRNAs are transferred from non-cancerous to cancerous cells in accordance with the miRNA concentration gradient. To examine in depth the contribution of secretory tumor-suppressive miRNAs in cancer initiation, we generated miR-143 overproducing HEK293 cells. We found that a prostate cancer cell line showed an approximately 50% decrease in proliferation through the suppression of the miR-143 target gene KRAS after the addition of an exosome derived from the miR-143-overproducing HEK293 cells. Importantly, the decrease was reversed by the transfection of anti-miR-143 in the prostate cancer cell line. These data indicate that the cell growth inhibition is attributable to the secretory miR-143 contained in the exosome of miR-143-overexpressing HEK293 cells [47].

**Table 2**
The list of exosomal miRNA mediating cell–cell communication both in basic research and research of therapeutic purpose.

| Type of small RNA | Donor cells | Recipient cells | Phenotype | Target gene | References |
|---|---|---|---|---|---|
| EBV-miRNAs | LCL (EBV-transformed lymphoblastoid B cells) | MoDC (monocyte-derived dendritic cells) | | CXCL11<br>LMP1 | [40] |
| miR-150 | THP-1 (human monocyte/macrophage cell line) | HMEC-1 (human microvascular endothelial cell) | Promote cell migration | c-Myb | [41] |
| miR-146a | HEK293 | PC-3M | Growth inhibition | ROCK1 | [42] |
| miR-335 | J77 (T-cell line) | Raji (B-cell line) | | SOX-4 | [43] |
| miR-451<br>miR-148a | Bone marrow derived DCs | DC2.4 (mouse dentritic cell line) | | [a] | [44] |
| miR-223 | SKBR3 (human breast cancer cell line) | IL-4-activated macrophages (human monocyte-derived macrophages) | Promote invasion | Mef2c | [45] |
| Hep3B enriched miRNAs[b] | Hep3B (hepatocellular carcinoma) | Hep3B | Reduction in cell viability | TAK1 | [46] |
| miR-143 | PNT-2 HEK293 | PC-3M | Growth inhibition | KRAS ERK5 | [47] |
| miR-133a | H9c2 (rat cardiomyoblasts) | 293FT | | [a] | [60] |
| siRNA | Bone marrow derived DCs | Mouse brain | | GAPDH | [48] |
| siRNA | Huh-7 (human hepatoma cell line) | Mouse hepatocyte | | CD81 | [49] |

[a] The sensor vector, which is complementary sequence of miRNA, was used in this study.
[b] The target gene TAK1 was predicted by various types of miRNAs that were highly expressed in the exosome isolated from Hep3B.

## 4. Exosomes can be used as a small RNA delivery system

As described previously, resolving the issue of miRNA delivery is essential for cancer treatment by tumor-suppressive miRNAs. Therefore, it is natural to examine exosomal tumor-suppressive miRNAs for cancer treatment.

### *4.1. Exosomes can be used for siRNA delivery*

Recently, the exosomal delivery of siRNAs to the mouse brain was reported [48]. In this report, self-derived DCs, which express the exosomal membrane protein Lamp2b fused with the neuron-specific RVG peptide 3, was used for the reduction of immunogenicity. Exosomal siRNA against GAPDH, which was loaded by electroporation, was intravenously injected, and it was delivered specifically to neurons, microglia, and oligodendrocytes in the brain, resulting in specific gene knockdown. In addition, using human hepatoma cells producing the viral entry receptor CD81 siRNA, siRNA delivery was confirmed, causing suppression of CD81 expression in mouse hepatocytes in vivo [49].

### *4.2. Exosomes can be used for miRNA delivery*

As shown above, siRNA can be delivered by exosomes. It is plausible that tumor-suppressive miRNAs can be similarly delivered to cancer cells in vivo. As we have previously shown [47], the loading mechanism of miRNA and siRNA into exosomes is the same; therefore, this technique might be used for miRNA-mediated therapy. To address this possibility, we injected conditioned medium obtained from miR-143-overproducing or parental HEK293 cells into nude mice implanted with prostate cancer cells. The tumor expansion was restrained for 8 days with intratumor administration of miR-143-enriched conditioned medium. Consequently, the tumor masses shrank by approximately 0.5 fold on day 8. In addition, the expression of miR-143 target genes such as KRAS and ERK5 decreased following miR-143-transduced conditioned medium injections.

In our report, exogenously-transduced miR-143 did not suppress the proliferation of non-cancerous cells, suggesting that excessive amounts of tumor-suppressive miRNAs did not provide an additional growth inhibitory effect on normal cells, in which the expression of tumor-suppressive miRNAs is maintained at physiological levels [48].

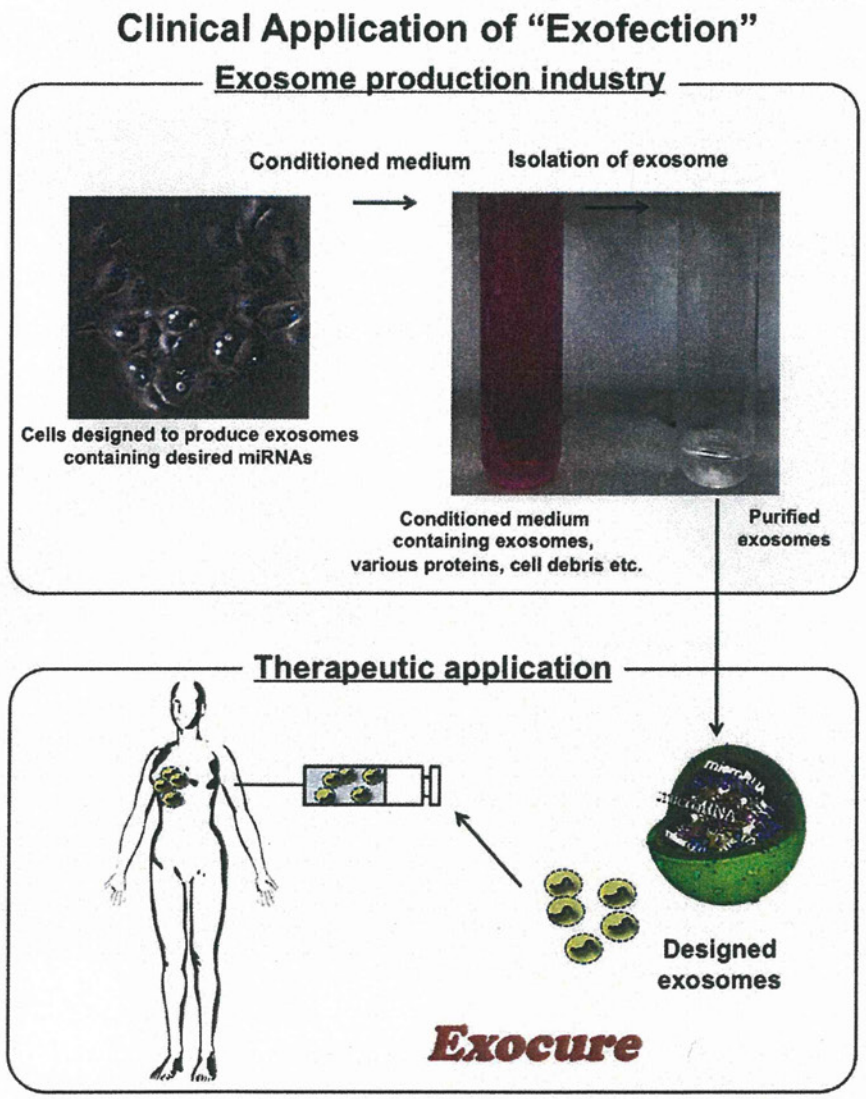


**Fig. 1.** A schematic explanation of "exocure". Exosome containing desired miRNAs, such as miR-16, miR-143 and so on, is produced by the "exosome production industry", and then these designed exosomes are delivered to cure the patient's disease. For this purpose, some of the issues which are showed in Fig. 2, need to be solved.

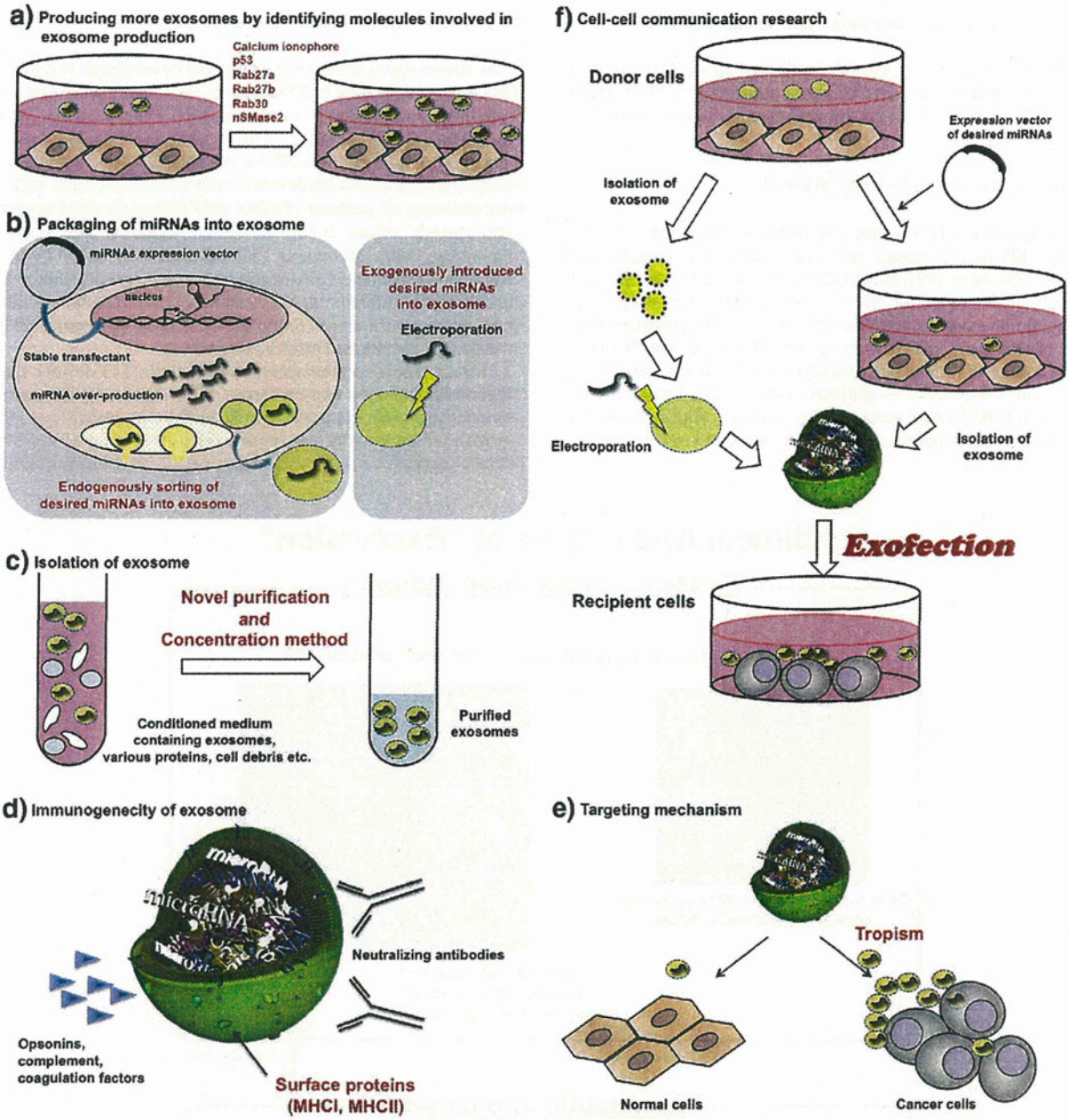


**Fig. 2.** Issues for the clinical use of "exocure". A schematic explanation of how the novel treatment "exofection" was established and the issues to be solved. To prepare exosomes containing tumor-suppressive miRNAs suitable for clinical usage, clarifying the mechanisms of exosome secretion is important. It is known that there are several molecules that regulate exosome secretion such as Rab27, Rab28, Rab30, nSMase2, p53 and calcium ionophore. Although the precise mechanism of exosome secretion has not yet been clarified, it is important to find the molecules that regulate exosome secretion (a). Currently, there are two kinds of methods to introduce desired miRNA into exosomes. One is to establish the cell line that stably overexpress desired miRNAs [47,49]. This enables us to obtain increased amount of desired miRNA in exosomes. The other one is exogenously introducing desired miRNAs using electroporation [48]. There are no knowledge about advantages and disadvantages in these two methods, moreover, the mechanism in which the tumor-suppressive miRNAs are sorted into exosomes is also unknown (b). These studies will enable us to obtain enough of the required exosomes for treatment. In addition, the establishment of exosome isolation methods is needed. The current, most popular isolation method, ultracentrifugation, is time-consuming and complicated, and its recovery rate is poor. Establishing more effective isolation methods is essential (c). Immunogenicity is an important factor to consider for the delivery of exosomes containing tumor-suppressive miRNAs. Exosomes are known to have reduced immunogenicity compared to other carriers such as viruses (d) [62]. Furthermore, the targeting mechanism of exosomes needs to be resolved (e). It has been known that exosomes have a tropism for target cells; however, the precise mechanism has not been found. Clarifying these five issues would enable us to establish the novel cancer treatment "exocure". The in vitro study of exosomal miRNAs might reveal many aspect of cross-kingdom research field, we suggest that this methods used for studying cell–cell communication be named "exofection" (f).

In addition, no overt side effects were observed in exosome-mediated gene delivery in vivo by dendritic cell-derived exosome [48]. Taken together, these reports suggest that exosomal tumor-suppressive miRNA therapy does not have serious side-effects [47,48].

## 5. Perspectives

In this review, we have summarized the knowledge regarding exosomal miRNAs for cancer therapy. We want to emphasize that

exosomal tumor-suppressive miRNAs are promising molecules for cancer therapy, (Fig. 1) although their use may have several difficulties (Fig. 2). First, the exosome must be abundant or highly enriched in order to utilize in therapy. It is known that exosome production was regulated by several molecules such as nSMase2, Rab27a, Rab27b, Rab35, p53 and calcium ionophore; however, the precise production mechanisms have not been clarified [50–59]. If the exosome production mechanism was clarified, we could develop "exosome-producing cells" by cell engineering. Second, the methods introducing desired tumor suppressive miRNAs into exosomes should be considered. There are two possible methods to introduce desired miRNAs into exosomes. One is the overexpression of desired miRNAs in the cells, resulting in the increased amount of miRNAs inside exosomes [42,47]. The other one is exogenously introducing miRNA into exosomes by electroporation [48]. Only few reports employed these methods and more studies need to be carried out for these methods to recognize more effective methods. Thirdly, methods for isolation of exosomes from conditioned medium need to be developed. The current, most popular isolation method, ultracentrifugation, is time-consuming and complicated, and its recovery rate is poor. Establishing more effective isolation methods is essential. Moreover, the exosome-producing cells need to be carefully chosen. Because of the tropism against the target cancer cells, we need to understand the mechanism of exosome uptake. In addition, the immunogenicity of exosomes is poorly understood. Resolving these issues may result in a safe and cost-effective exosome delivery method. Last, the functions and mechanisms of tumor-suppressive miRNAs need to be clarified. The choice of target miRNAs may aid in the decisions regarding cancer therapy such as radiotherapy and/or chemotherapy. Because the exosome is an ideal and promising delivery material for small RNA therapy, we suggest that this method be named "exocure" (Fig. 1).

In addition, this method can also be utilized for research of cell–cell communication. As shown in Table 2, these reports regarding the exosomal miRNAs open up a novel research field for the cell–cell communication. Surprisingly, recent reports showed that exogenous plant miRNAs can be found in the blood sera of animals and this plant miRNAs were considered to exist inside exosomes, suggesting that plant miRNAs can regulate the expression of target genes in mammals [61]. Although more studies need to be done about plant miRNAs in human body fluids, studying the exosomal miRNAs might unveil the mystery of this cross-kingdom gene regulation. To study the precise function of exosomal miRNAs, the methods that we proposed in Fig. 2 can be used. The in vitro study of exosomal miRNAs might reveal many aspect of cross-kingdom research field, we suggest that this methods used for studying cell–cell communication be named "exofection".

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