The Intermediate Filament Vimentin Mediates MicroRNA miR-378 Function in Cellular Self-renewal by Regulating the Expression of the Sox2 Transcription Factor#§

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MicroRNAs are short noncoding RNAs that are implicated in cell self-renewal and cancer development. We show that miR-378 is up-regulated in human cancers and found that tumor cells transfected with miR-378 acquired properties of tumor stem cells, including cell self-renewal. Overexpression of miR-378 enhanced cell survival and colony formation. Isolated from a single-cell colony, the miR-378-expressing cells formed tumors in nude mice at low cell densities. These cells expressed higher levels of miR-378 and formed more and larger spheres and colonies. We found that the miR-378-expressing cells contained a large number of side population cells and could undergo differentiation. Cells transfected with miR-378 expressed increased levels of Sox2. Expression of miR-378 and Sox2 was found correlated significantly in cancer cell lines and in cancer patient specimens. We also observed opposite levels of vimentin in the cancer cell lines and human breast carcinoma specimens. We further demonstrated that vimentin is a target of miR-378, and ectopic transfection of vimentin inhibited Sox2 expression, resulting in decreased cell survival. Silencing vimentin promoted Sox2 expression and cell survival. Our study demonstrates that miR-378 is a regulator of stem cell marker Sox2 by targeting vimentin, which may serve as a new tool in studying the role of stem cells in tumorigenesis.

Conclusion: Our study demonstrates that miR-378 can trigger a signal cascade.

Significance: Our study reveals a novel signaling pathway in modulating cell stemness by miR-378 expression.

Recent studies indicated that expression of these genes is largely modulated by a subset of RNAs named microRNAs (miRNAs) (3–6). Indeed, the microRNA let-7 has been found to regulate tumor cell self-renewal, proliferation, and tumorigenesis (7, 8).

Over the past few years, miRNAs have emerged as a prominent class of gene regulators (9). miRNAs are single-stranded RNAs of 18–24 nucleotides in length and are generated by an RNase III-type enzyme from an endogenous transcript that contains a hairpin structure (10, 11). In animals, miRNAs initially are transcribed from genomic DNA to produce long primary transcripts (pri-miRNAs), which are processed by the RNase III-type enzyme Drosha to produce precursor miRNAs (pre-miRNAs) in the nucleus (12). Pre-miRNAs are then trans-located by exportin-5 to the cytoplasm (13). In the cytoplasm, pre-miRNAs are subjected to secondary processing by Dicer, a cytoplasmic RNase III-type enzyme (14, 15), where miRNAs function as guide molecules in post-transcriptional gene silencing by partially pairing with the 3’-untranslated region (UTR) of the target mRNAs, resulting in translational repression (16). By silencing various target mRNAs, miRNAs have key roles in diverse regulatory pathways, including control of development (17), cell differentiation (18, 19), survival (20, 21), cell proliferation (22), division (23), protein secretion (24), and viral infection (25). Most importantly, miRNAs have been known to play roles in cancer development (26–28).

Sox2 is a transcription factor with fundamental importance in the maintenance of the early, pluripotent stem cells of the epiblast (29). It is highly expressed in neural stem cells, where it maintains the neural stem cell properties (30, 31). Sox2 is also required to maintain neural stem cells in the eyes and brain and facilitates neuronal differentiation (32). By acting together with other transcription factors, Sox2 can re-establish pluripotency in terminally differentiated cells, resulting in reprogramming them to become pluripotent stem cells for further differen-

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Sox2 is also highly expressed in brain tumors (34). Silencing of Sox2 in human glioblastoma-derived cultures decreases their abilities in cell proliferation and tumor formation (35).

Previous studies have shown that miR-378 is expressed in a number of cancer cell lines (36), and it is involved in the expression of vascular endothelial growth factor (37). To understand the biological functions of miR-378, we have generated a miR-378 expression construct for functional studies. We demonstrated that tumor cell line U87, a human primary glioblastoma cell line, transected with miR-378, formed larger tumors and blood vessels (38). In this study, we investigated the role of miR-378 in tumor cell self-renewal and chemoresistance.

MATERIALS AND METHODS

Construct Generation—A miRNA construct expressing miR-378 was designed by our laboratory and generated as described previously (38). This plasmid has been used successfully in our laboratory. The control plasmid is the same except the pre-miR-378 sequence was replaced with a nonrelated sequence (atacagtcacgatactgaggtttttgaaaagctagttaaa), serving as a mock control.

Oil Red O Staining—Cultured cells were fixed in 10% formalin and washed with distilled water for 10 min. The fixed cells were rinsed with 60% isopropyl alcohol and stained with freshly prepared Oil Red O solution for 15 min. After being rinsed with 60% isopropyl alcohol, the cells were briefly stained with aluminum hematoxylin and mounted in glycerine jelly. After being dried, the stained cells were examined under a light microscope.

Treatment with Oleic Acid—U87 cells transfected with miR-378, anti-miR-378, or mock were cultured in 96-well tissue culture plates to subconfluence. The medium was changed to serum-free DMEM, followed by treating the cells with 200 μl of oleic acid solution overnight. The medium was removed, and the cells were treated with 100 μl of fixative solution (10% formalin) at room temperature for 10 min, followed by microscopic examination. The cells were stained with Oil Red O as described above. After washing and drying completely, 100 μl of 100% isopropyl alcohol was added to each well. The cells were incubated at room temperature for 10 min to release Oil Red O from the staining. The extract solution was then transferred to another 96-well plate. The plate was subjected to absorbance measurement at a wavelength of 405 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

Real-time PCR—The total RNA was extracted from ~1 × 10⁶ cells with the mirVana miRNA Isolation kit (Ambion) according to the manufacturer’s instructions, followed by reverse transcription to synthesize cDNA using 1 μg of RNA. Successive PCR was performed by a QuantiMir-RT kit using 1 μl of cDNA as a template (Qiagen, miScript Reverse Transcription kit, catalog 218060; miScript Primer Assay, catalog 218411; miScript SYBR Green PCR kit, catalog 218073). The primers specific for mature miR-378 were purchased from Qiagen. The primers used as real-time PCR controls were human-U6RNAf and human-U6RNAr.

Cell Survival Assay—Cells (1.5 × 10⁵ cells/well or 2 × 10⁵ cells/well) were seeded on 35-mm Petri dishes in DMEM containing 0–10% FBS and incubated for different time periods. The cell numbers were counted using trypan blue staining as described (39).

Side Population (SP) Cell Analysis—Cells were harvested from tissue culture dishes with trypsin and EDTA, washed, and suspended at a cell density of 1 × 10⁶ cells/ml in DMEM containing 2% FCS. The cells were preincubated at 37 °C for 10 min and then maintained at 37 °C for 90 min with 2.5 μg/ml Hoechst 33342 dye, either alone or in combination with 50 μM verapamil. The number of SP cells were measured with flow cytometry by using a dual-wavelength for analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350-nm UV light. Propidium iodide-positive (dead) cells, which were normally <15%, were excluded from the analysis.

Colony Formation in Soft Agarose Gel—Colony formation was assessed using a method described previously (40). In brief, 1 × 10³ cells were mixed in 0.3% low melting agarose in DMEM supplemented with 10% FBS and plated on 0.66% agarose-coated 6-well tissue culture plates. Colony formation was monitored weekly. Four weeks after cell inoculation, colonies were examined and photographed.

Tumorigenicity Assays in Nude Mice—Six-week-old CD1 strain nude mice were injected with the miR-378- and mock-transfected U87 cell lines (1 × 10⁵ cells/injection). Tumor growth was monitored weekly. Tumor volume (V) was estimated using a caliper by measuring the length (L) and width (W), where V = (L × W²)/2. Tumors were removed for further analysis 40 days after the injection as described (40).

Processing of Human Cancer Samples—Human cancer specimens (n = 14) and paired noncancerous normal specimens (n = 14) were obtained from patients who had been subjected to the surgery for tumor removal at The Affiliated People’s Hospital of Jiangsu University. All samples were immediately stored in liquid nitrogen until RNA extraction. We followed the guidelines of The Affiliated People’s Hospital protocol including patient consent and specimen collection.

Total RNA was isolated from the specimens with mirVana miRNA Isolation kit (Ambion) according to the manufacturer’s instructions. The RNAs were subjected to first strand cDNA synthesis by reverse transcription using 1 μg of RNA. PCRs were then performed with QuantiMir-RT Kit using 2 μl of cDNA as template. To perform these experiments, other kits were also needed, including the miScript Reverse Transcription kit and miScript SYBR Green PCR kit. For real-time PCR of miR-378, the primer was specific for mature miR-378 (5'-ctcctgactcgcagttctgtg). The primers for internal control U6 RNA products were assessed with Pearson’s correlation coefficient using GraphPad Prism software.

Statistical Analysis—The results (mean values ± S.D.) of all experiments were subjected to statistical analysis by t test. The level of significance was set at p < 0.01 and p < 0.05.
RESULTS

Cells Expressing miR-378 Exhibit Greater Effects on Cell Survival and Colony Formation—We have previously demonstrated that expression of miR-378 enhances tumor cell survival and promotes tumor growth and angiogenesis (38). In continuation of this study, we conducted analysis of miR-378 expression in 23 pairs of human cancer specimens and the adjacent noncancer tissues. The isolated RNAs were subjected to real-time PCR to analyze miR-378 expression. The levels of miR-378 in the tumor tissues were divided by that in the nontumor tissues. There were 16 cases of samples (x axis) displaying higher levels of miR-378 than the adjacent tissues. However, only 7 cases of samples showed lower levels of miR-378 than the adjacent tissues. Right, RNAs were isolated from wax blocks of human breast carcinoma specimens and the adjacent noncancer tissues followed by real-time PCR analysis of miR-378 levels. The breast carcinoma tissues expressed higher levels of miR-378 than the adjacent tissues. b, construct of miR-378 containing pre-miR-378, GFP, and neomycin is shown. c, U87 cells transfected with miR-378 or a control vector were maintained in serum-free conditions in Petri dishes to examine the effect of miR-378 on cell survival. d, the cells were also maintained in tissue culture plates in serum-containing medium and treated with C2-ceramide (20 mM) for 2 days for cell survival assay. e, U87 cells were stably transfected with anti-miR-378 or a control vector. The stably transfected cells were maintained in serum-free medium and treated with C2-ceramide for survival assay. f, in colony formation assays performed in soft agar, miR-378-expressing cells formed more colonies with larger sizes. g, the miR-378- and mock-transfected cells were cultured in 96-well plates to obtain one cell (one colony) per well. The miR-378-transfected cells formed larger colonies than the mock-transfected cells. The single colonies were then harvested and replaced in new wells (one colony/well) in stem cell culture medium. The miR-378-transfected cells could form colonies whereas the mock-transfected cells could not form colonies and tended to die readily. Error bars, S.D.

To understand how miR-378 affected cancer cell activities, we transfected miR-378 expression construct in U87 cells (Fig. 1b). The cells were cultured in Petri dishes and tissue culture plates without serum, and we observed that the miR-378-transfected cells could survive for a long period of time, characteristics of tumor stem cells (Fig. 1c). We have also tested whether the apoptosis-inducing agent C2-ceramide had any effect on miR-378-enhanced cell survival and observed that U87 cells transfected with miR-378 exhibited reduced cell death (Fig. 1d and supplemental Fig. S1a).

To test the effect of endogenous miR-378, an antisense construct was developed which bound and arrested the functions of miR-378 (anti-miR-378). Expression of anti-miR-378 reduced
cell survival significantly (Fig. 1e and supplemental Fig. S1b). In colony formation assays, we detected more colonies with larger sizes formed by the miR-378-expressing cells compared with the control (Fig. 1f). In single-cell culture, we found that the miR-378-transfected cells could form larger colonies than the mock-transfected cells (Fig. 1g, upper). The colonies obtained from the single-cell culture were passed to new wells, the miR-378-transfected cells could form large new colonies, but most of the mock-transfected cells died (Fig. 1g, lower).

**Higher Levels of miR-378 Expression Exhibit Greater Effects on Sphere Formation—**To examine whether or not the cells that formed colonies were those that expressed high levels of miR-378 and had tumor stem cell properties, we isolated cells from single colonies. Analysis of miRNA levels indicated that the cells isolated from the single colony expressed significantly higher levels of miR-378 compared with cells transfected with miR-378, but selected with flow cytometry thus containing a mix of cell population called miR-378 mix or miR-378M, or with a control vector (Fig. 2a).

We tested whether there is a correlation between miR-378 expression and stem cell-associated properties. Cells expressing low and high levels of miR-378 were cultured in serum-free medium in Petri dishes for 7 days to test the effect of miR-378 on survival. ***, p < 0.01.**

The colonies in the groups expressing miR-378 were much larger than the ones in the control group (lower). d, the cells were cultured in sphere formation medium F12 containing B27, to which bFGF (10 ng/ml), EGF (20 ng/ml), and L-glutamine were added. On day 4, the miR-378-expressing cells formed a greater number of spheres (upper) with larger sizes (lower) than the mock control. e, after 11 days of culture, most of the mock-transfected cells died, whereas the miR-378-transfected cells survived and continued to grow (upper). These cells were able to adhere to the tissue culture plates for survival and growth (lower). Error bars, S.D.
onies than the mock control (Fig. 2c). The cells expressing higher levels of miR-378 formed more colonies than the cells expressing lower levels of miR-378. The colonies in the groups expressing miR-378 were much larger than the ones in the control group.

To further confirm the stem-like properties, we cultured the cells in sphere formation medium and found that the miR-378-expressing cells formed greater number of spheres with larger sizes than the mock control (Fig. 2d). The cell expressing higher levels of miR-378 (miR-378C) formed larger spheres than the cell expressing lower levels of miR-378 (miR-378M). After long term culture, the spheres were able to survive and continue to grow (Fig. 2e). This is a normal process of stem cell differentiation. After long term culture as a monolayer, these cells obtained less stem-like phenotype. Our results suggest that miR-378 has dose-dependent effect of the stemness of tumor stem cells.

Tumorigenesis of the Tumor Stem-like Cells—It has been reported that tumor stem cells have great capacity in tumor formation (1, 2, 7, 41, 42). To test whether or not the colony derived from the single cell could form bigger tumors, we tested tumorigenic capacity of the three types of cells including miR-378M, miR-378C, and mock by injecting the cells into nude mice subcutaneously. Tumor incidence was examined 40 days after the injection. Due to injection of low cell number, the mock-transfected cells did not form visible tumors, whereas the miR-378M cells formed tumors with medium sizes and the miR-378C cells formed large tumors (Fig. 3a). Because the parental U87 cells form tumors slowly, it normally takes 4–5 weeks to form small tumors by injecting 1 × 10^5 cells/mouse. It is expected that 1 × 10^6 cells would not form visible sizes of tumors. This is in agreement with our previous report that miR-378 expression promotes tumor growth (38).

To further understand the effect of miR-378 on tumor stem cell behaviors, we isolated miR-378-transfected cells from the tumors formed in the nude mice by digesting the miR-378C-formed tumor tissues with collagenase and maintaining in medium containing G418. Sensitivities of these cells along with miR-378M, miR-378C, and mock cells to clinical chemodrugs docetaxel and epirubicin were tested. The cells isolated from the tumor tissues exhibited highest activity of resistance to both drugs (Fig. 3b). This is in agreement with the above results and previous report that miR-378 expression promotes tumor growth (38).

Expression of Stem Cell Markers and Differentiation of the miR-378-transfected Cells—We then analyzed the levels of CD133, a marker of stem cells, and found that expression of miR-378 dramatically up-regulated CD133 levels (Fig. 4a, left). The experiments were repeated three times for statistical analysis and we observed a significant up-regulation of CD133 in the miR-378-transfected cells (Fig. 4a, right). Analysis of SP fraction with Hoechst 33342 dye-based technique has been used to isolate stem cells. To investigate the prevalence of SP cells, we stained the miR-378- and mock-transfected cells with Hoechst 33342 dye and identified the SP fractions by its characteristic fluorescent profile in dual-wavelength analysis. The miR-378-transfected cells contained higher percentage of SP cells than the mock-transfected cells (Fig. 4b, left). The experiments were repeated three times for statistical analysis. A significant difference was seen (Fig. 4b, right). All of our results above suggest that cells transfected with miR-378 acquired stem cell-like properties. We next analyzed whether these cells acquired the properties of differentiation, a property of induced pluripotent stem cells. We cultured the cells in serum-free DMEM followed by treatment with oleic
The cells were then stained with Oil Red O, a dye routinely used to stain adipocyte. After Oil Red O staining, we examined the cells under a light microscope and detected red color in the cells stably transfected with miR-378 but not in the mock-transfected cells (Fig. 4c). Treatment with oleic acid, a chemical used to induce steatosis in a number of cells, promoted oleic acid uptake in miR-378-transfected cells more than in the mock-transfected cells (Fig. 4d), which was statistically significant (Fig. 4e).

**Up-regulation of Sox2 Expression**—To investigate how miR-378 expression conferred stem cell properties to U87 cells, we analyzed stem cell markers in the miR-378-transfected cells using Western blotting and observed that expression of stem cell marker Sox2 was up-regulated in the miR-378-transfected cells compared with the mock-transfected cells (Fig. 5a) but did not detect clear change of Oct4, Nanog, and c-myc (data not shown). We further examined the level of regulation by performing a real-time PCR using RNA isolated from the miR-378- and mock-transfected cells and found that expression of miR-378 increased Sox2 mRNA levels significantly (Fig. 5b). To confirm this result, we analyzed Sox2 mRNA using the anti-miR-378- and mock-transfected cells and detected a significant
down-regulation of Sox2 mRNA compared with the mock-transfected cells (Fig. 5c).

It has been reported that down-regulation of vimentin resulted in decrease in p21 mRNA and protein expression whereas overexpression of vimentin promoted p21 transcription by triggering p21 promoter activity (43), and down-regulation of p21 increases Sox2 expression (44). The p21-p53 pathway can serve as a barrier in generation of cell stemness (45). We examined whether the p53-p21 signal pathway was involved in the vimentin-mediated miR-378 function. We found that p53-p21 signal pathway was involved in the vimentin-mediated miR-378 function. We found that transfection of miR-378 decreased p21 level (Fig. 5d) and that silencing vimentin resulted in decreased p21 expression (Fig. 5e). On the other hand, expression of p16, p53, N-cadherin, GSK-3β, β-catenin was not affected by miR-378 expression. These results were consistent with the previous publications.

We then examined the correlation between miR-378 and Sox2 in a number of cell lines, including A549, A2058, C8186, OV2008, U87, and 4T1. We found that expression of miR-378 was correlated significantly with Sox2 levels. In the cell lines expressing higher levels of miR-378, Sox2 levels were also higher. Using the GraphPad Prism, we analyzed the correlation of miR-378 and Sox2 and found that expression of miR-378 was highly correlated with Sox2 (Fig. 5f).

Because Sox2 is a tumor stem cell marker and tumor stem cells are the key component in tumor formation and metastasis, we tested whether expression of miR-378 and Sox2 was correlated. Total RNAs were isolated from cancer patient specimens including tissues from the tumors and samples from the adjacent tissues. The RNA samples were subjected to real-time PCR
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FIGURE 6. Transfection of miR-378 enhances Sox2 expression. a–c, total RNAs were isolated from endothelial cells Ypen (a), NIH3T3 fibroblasts (b), and breast cancer cell line 4T1 (c) that had been transfected with miR-378 or mock control. The RNAs were subjected to real-time PCR analysis of miR-378 levels (left) and Sox2 expression (right). Error bars, S.D.

to analyze levels of miR-378 and Sox2. We found that expression of miR-378 was correlated significantly with Sox2 levels. The samples that expressed high levels of miR-378 also displayed high levels Sox2 (supplemental Fig. S2b). The samples that expressed low levels of miR-378 also showed low levels of Sox2. Their expression correlated significantly (Fig. 5g).

Finally, we tested whether or not transfection of miR-378 could alter Sox2 expression. We stably transfected miR-378 expression construct in cancer and noncancer cell lines including breast carcinoma cell line 4T1 (Fig. 6a), endothelial cell line Ypen (Fig. 6b), and fibroblast cell line NIH3T3 (Fig. 6c). After confirming that transfection of miR-378 increased the levels of mature miR-378 in the miR-378-transfected cells compared with the mock control, we found that increased expression of miR-378 resulted in increased levels of Sox2, indicating a direct role of miR-378 in Sox2 expression.

miR-378 Promotes Sox2 Expression by Targeting Vimentin—We analyzed expression of miR-378, Sox2, and vimentin, a marker of stem cell, in a number of cancer cell lines including MCF7, MDA-MB-468, MT-1, 4T1, DU145, HepG2, JHH-1, and U87. We observed that MT1 cells expressed extremely high levels of vimentin but with an undetectable level of Sox2 (Fig. 7a). However, JHH-1 and MDA-MB-468 expressed high levels of Sox2 but with an undetectable level of vimentin, whereas U87 expressed relatively high levels of Sox2 and vimentin. Interestingly, whereas we did not detect vimentin expression in JHH-1 and HepG2 cells, we found that both cell lines expressed high levels of miR-378 (Fig. 7a, bottom).

This reversed correlation suggests that transfection of miR-378 plays roles in up-regulation of Sox2 and down-regulation of vimentin. To understand how it worked, we examined the levels of Sox2 and vimentin in the miR-378- and mock-transfected cells and found that expression of miR-378 repressed vimentin levels while enhancing Sox2 expression (Fig. 7b). Real-time PCR analysis of vimentin level detected little difference (supplemental Fig. S3a), suggesting post-transcriptional regulation.

We then dissected the relationship between vimentin and Sox2 by transfecting U87 cells with a vimentin expression construct. Ectopic expression of vimentin was confirmed by Western blotting (supplemental Fig. S3b). We detected dramatic down-regulation of Sox2 in the vimentin-transfected cells compared with the mock control (Fig. 7c). The experiments were repeated three times, and the protein bands were scanned for densitometry analysis. Significant down-regulation of Sox2 was seen in the vimentin-transfected cells (Fig. 7c). We examined the correlation of Sox2 and vimentin in human breast carcinoma specimens. Tumor sections were immunostained with anti-Sox2 and anti-vimentin antibodies. We detected higher levels of Sox2 expression but lower levels of vimentin in all tumor areas than the nontumor areas (Fig. 7d and supplemental Fig. S4).

We next sought to test whether miR-378 could target vimentin expression. Computational analysis indicated that miR-378 potentially targets vimentin. The potential target sequence displays a seed region for miR-378 binding (Fig. 8a).

To obtain direct evidence that vimentin 3'-UTR is a target of miR-378, we generated a luciferase construct harboring a fragment of the vimentin 3'-UTR containing the miR-378 target sequence (Luc-VIM) and a mutant Luc-VIM-mut (Fig. 8a, left, nucleotides 1881–1919, GenBank excess number NM_003380; supplemental Fig. S3c). Luciferase activities were significantly repressed when Luc-VIM was co-transfected with miR-378 compared with the control vector harboring a nonrelated fragment (Ctrl) or the mutated sequence (Fig. 8a, right). Three individual experiments produced similar results. Mutation of the miR-378 target site partially abolished the miR-378 effects.

Examination of the target sequences indicated that the miR-378 target site in the vimentin 3'-UTR was conserved across different species (Fig. 8b). In the 12 species examined, all contained the identical seed region except Cavia porcellus which contains an “A” instead of “G” in the seed region. In general, the target sequences are highly conserved across different species.

To demonstrate that vimentin played an essential role in mediating miR-378 function, we delivered siRNA to silence vimentin expression (supplemental Fig. S3d). Silencing the vimentin level promoted Sox2 expression and tumor cell survival (Fig. 8c). Transfection of vimentin siRNA greatly reduced cell death, suggesting that vimentin-mediated Sox2 expression plays an essential role for miR-378-enhanced cell survival. To confirm this, we performed a rescue experiment. A vimentin expression construct was generated and transiently expressed in the miR-378-transfected cells. As shown in Fig. 8d, reintro-
duction of vimentin into the miR-378-cells reversed the effect of miR-378 on cell survival. Re-expression of vimentin was sufficient to cause cell death, suggesting that the effect of miR-378 on enhanced survival was at least partly taking place through repression of vimentin expression.

DISCUSSION
In this study, we demonstrated that cells transfected with miR-378 acquired stem cell properties. This was demonstrated by enhanced cell survival in serum-free conditions and increased colony formation. Because single-stem cells can form colonies in soft agar, cells transfected with miR-378 formed more and larger colonies. This suggests that expression of miR-378 facilitated transformation of nonstem cells to stem cells. To examine whether or not the miR-378-expressing cells had greater capacity in colony formation, we isolated single colonies from the mock- and miR-378-transfected cells and recultured them in stem cell culture medium. Larger spheres were obtained in the miR-378 colonies but not in the mock colonies. These results suggest that cells expressing miR-378 had a greater capacity of stem cell property. This may explain why cells transfected with miR-378 form much larger tumors than cells transfected with a control vector (38). A recent study also reported that miR-378 facilitates cell transformation and tumor formation by targeting the anti-proliferative BTG family member, TOB2, which appears to be a candidate tumor suppressor.

FIGURE 7. Relationship of miR-378, Sox2, and vimentin expression. a, upper, cell lysates prepared from a number of cancer cell lines were subjected to Western blot analysis probed with anti-Sox2, vimentin, and β-actin antibodies. An opposite correlation between Sox2 and vimentin expression was observed in several cell lines. Lower, expression of miR-378 was analyzed by real-time PCR in the cancer cell lines as indicated. b, cell lysate prepared from miR-378-transfected U87 cells was analyzed on Western blot probed with anti-vimentin, anti-Sox2, and anti-actin antibodies. Although the miR-378-transfected cells expressed higher levels of Sox2, the cells expressed lower level of vimentin than the mock-transfected cells. c, U87 cells were transiently transfected with vimentin expression construct, and cell lysates were subjected to Western blot analysis probed with anti-Sox2 antibody. Transfection with vimentin dramatically decreased Sox2 level. The experiments were repeated three times for densitometry scanning and statistically analysis. d, human breast carcinoma specimens were probed for expression of Sox2 and vimentin. Sox2 levels appeared to be higher whereas vimentin levels were lower in the tumor areas than the adjacent nontumor areas. Open arrows, duct structures; filled arrows, tumor cells. e, expression of Sox2 mRNA was measured by real-time PCR in U87 cells transfected with siRNA targeting vimentin, miR-378, and a control vector. Silencing vimentin increased Sox2 expression. Error bars, S.D.
FIGURE 8. Confirmation of miR-378 targeting vimentin. a, left, vimentin 3'-UTR was found to be the potential target of miR-378 and was thus inserted into the luciferase reporter vector pMir-Report. Mutations were generated on the potential target sequence (red). Right, U343 cells were co-transfected with the miR-378 construct and the luciferase reporter construct harboring vimentin 3'-UTR (Luc-VIM) or mutant vimentin 3'-UTR (Luc-VIM-mut). As a negative control, the luciferase reporter construct was engineered with a nonrelated fragment of cDNA (Ctrl). Luciferase activity assays indicated that the miR-378 construct repressed luciferase activity when it harbored the vimentin 3'-UTR, which was abolished when the potential miR-378 target site was mutated. Significant differences are indicated by asterisks. Error bars, S.D. (n = 3). **, p < 0.01. b, alignment of the miR-378 target site across Homo sapiens (NM_003380), Mus musculus (NM_011701), Rattus norvegicus (NM_031140), Bos taurus (NM_179969), Bos bovis (L13263), Bos taurus (NM_179969), Bos taurus (NM_179969), Cavia porcellus (EU827601), Canis familiaris (DQ190949), Cricetulus longicaudatus (X87227), Canis familiaris (XM_535175), Cavia porcellus (EU827601), Cercopithecus aethiops (DO190949), Equus caballus (XM_001916285), Macaca mulatta (XM_001093658), and Pan troglodytes (NM_001009148). The miR-378/vimentin target sites are in boldface. Conservation of the sequences is shown across all species. c, U87 cells transiently transfected with siRNA targeting vimentin or a control oligonucleotide were grown on 12-well plates in serum-free conditions. Upper, transfection with siRNA up-regulated Sox2 expression. Lower, surviving cells were harvested and counted after trypan blue staining for statistical analysis. **, p < 0.01. Error bars, S.D. (n = 3). d, U87 cells stably transfected with miR-378 were transiently transfected with vimentin expression construct. The cells were grown on 12-well tissue culture plates in serum-free conditions, followed by survival assays. Transfection of vimentin into the miR-378-expressing cells reversed the effect of miR-378 in enhancing cell survival. **, p < 0.01. Error bars, S.D. (n = 3). e, diagram showing how miR-378 functioned in the promotion of tumor growth.
in repressing proto-oncogene cyclin D1 (46). Further support was obtained by our observation that cells expressing miR-378 also produced higher levels of stem cell marker Sox2. Sox2 is a major regulator of stem cell activities (47–51). It is highly expressed in the brain and plays a critical role in the development of the neural system (32, 52). Because U87 cells were originally developed from astrocytes, we investigated how miR-378 modulated stem cell transformation by regulating Sox2 expression.

We further tested the ability of miR-378 in stem cell transformation. Breast tumor cell line 4T1 and nontumor cell lines, endothelial cell line Ypen and fibroblast cell line NIH3T3, were transiently transfected with miR-378. Expression of Sox2 was up-regulated significantly in all cell types. These results suggest that expression of miR-378 could induce transformation of normal cells to stem cells and could induce transformation of nontumor stem cells to tumor stem cells. Because in transient transfection, the cells were maintained for several days, it did not allow selectively maintenance of a particular cell type. Up-regulation of Sox2 levels suggests that miR-378 transfection induced Sox2 expression, perhaps by inducing stem cell transformation. Nevertheless, our results do not exclude the possibility that miR-378 induced stem cell transformation by regulating multiple factors associated with the transformation, because a miRNA has the capacity of regulating a great number of mRNA expression. This awaits further investigation.

The strong correlation of miR-378 and Sox2 in a number of cell lines and the human cancer specimens suggests that both molecules play important roles in the development of cancer. Up-regulation of miR-378 and Sox2 may be essential for tumor growth. Expression of miR-378 and Sox2 may be used as markers for cancer diagnosis. As well, they may be considered as targets for cancer treatments.

To confirm that the miR-378-transfected cells contained a larger population of stem-like cells, we analyzed the number of SP cells. Cells in the side population have distinguishing biological properties such as the stem cell-like characteristics. SP cells have been identified in cancer, and recent studies indicate that >40% of the SP cells are undifferentiated stem cells (53). The miR-378-transfected cells showed greater number of SP cells than the mock-transfected cells. This result confirmed that transfection of miR-378 conferred the cells with stem-like properties. The SP cells are expected to be able to differentiate to other downstream cellular lineages. A well known stem cell marker CD133 was also found up-regulated in the miR-378-transfected cells. These results support the conclusion that the miR-378-expressing cells have been transformed to stem-like cells.

Further support was obtained from the differentiation assay, because cells have the properties of undergoing differentiation. We tested whether the miR-378-transfected U87 astrocytoma cells could differentiate to adipocytes. In the conditions favoring formation and growth of adipocytes, the miR-378-transfected U87 cells showed red particles after Oil Red O staining, a method used to stain fatty acids. Treatment with oleic acid promoted the steatosis of the miR-378-transfected U87 cells. The results demonstrated that expression of miR-378 conferred the cells to differentiate to adipocytes. In normal conditions, the astrocytoma cells U87 do not produce fatty acids sufficiently to be detected by Oil Red O staining. The steatosis of the miR-378-transfected cells suggests differentiation of the cells to adipocytes.

The mechanism by which miR-378 up-regulates Sox2 expression appears to function through repression of vimentin expression. We demonstrated that miR-378 repressed vimentin expression and vimentin down-regulated Sox2 level. As a consequence, miR-378 can up-regulate Sox2 expression (Fig. 8e). There have been a few molecules reported to inhibit Sox2 expression, including Sox21 (54). Vimentin appears to be a new member to be included in the list as a Sox2 inhibitor. Because vimentin is a cytoskeleton molecule, how it exerts its role in down-regulation of Sox2 expression awaits further investigation. Nevertheless, the inverted correlation between vimentin and Sox2 in a number of cell lines and in the clinical cancer specimens supports our conclusion. Although vimentin has been used as a stem cell marker, it appears that ectopic expression of vimentin decreased the stemness capacity of the cells and induced cancer cell death. A human cancer specimen also displayed a reduction of vimentin expression in the tumors. These results suggest that vimentin may be developed as an approach for intervention of cancer progression.

Using stem cell markers, it is possible to isolate relatively large population of tumor stem cells. However, the isolated tumor stem cells tend to differentiate into regular tumor cells in normal culture conditions. This has made it difficult to study tumor stem cells. Our approach of expressing miR-378 not only allowed transformation or selection of tumor stem cells, but also allowed long term maintenance of tumor stem cells. Thus, it is possible to obtain a large population of tumor stem cells for research purpose and for screening of agents that can specifically induce tumor stem cell death. It is of significance to maintain a large population of tumor stem cells for clinical research in seeking agents for tumor treatments.

In summary, we have demonstrated that miR-378 can facilitate tumor cell self-renewal, allowing the cells to form more and larger colonies, spheres, and tumors, characteristics of tumor stem cells (Fig. 8e). Consistent with this is the detection of a large population of SP cells and CD133-positive cells present in the miR-378-transfected cells. Confirmation of the stemness was obtained by differentiation of the cells into adipocytes. The stem cell transformation appears to be the consequence of up-regulation of Sox2 expression, which occurred through repression of vimentin expression, because we have demonstrated that miR-378 targeted vimentin expression and vimentin was an inhibitor of Sox2. Our study provides a means to obtain large population of stem-like cells for basic research and clinical application.

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