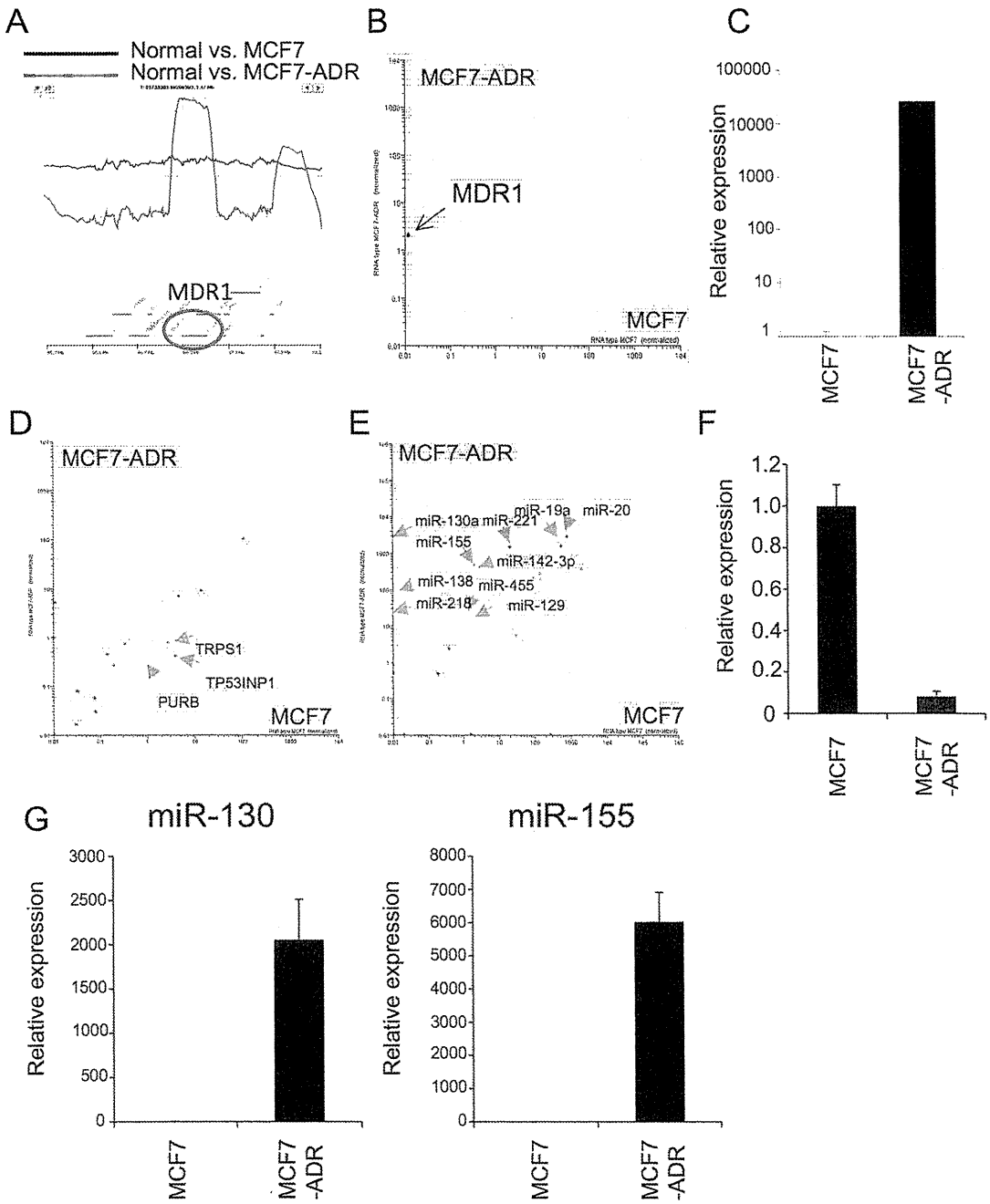

Figure 2 Genomic amplification and overexpression of MDR1, and a number of miRNA regulation of TP53INP1. (A) Twenty fold amplification of multi-drug resistance gene (MDR1) region in MCF7-ADR as compared with MCF7. (B and C) Overepression of MDR1 gene in MCF7-ADR by microarray and real-time PCR, respectively. (D) Scatter plot of miRNA target genes predicted by Targetscan. More than 15% of miRNAs up-regulated in MCF7-ADR (74 miRNAs) were predicted to potentially bind the 3'-UTR of these genes. Expression levels of three (TP53INP1, PURB and TRPS1) of them are clearly dwon-regulated. (E) Scatter plot of miRNAs that might bind to 3'UTR of TP53INP1 gene. (F and G) Confirmation of TP53INP1 gene, miR-130 and miR155 expression by real-time PCR. Standard deviation was calculated in triplicate determinants in the experiment.

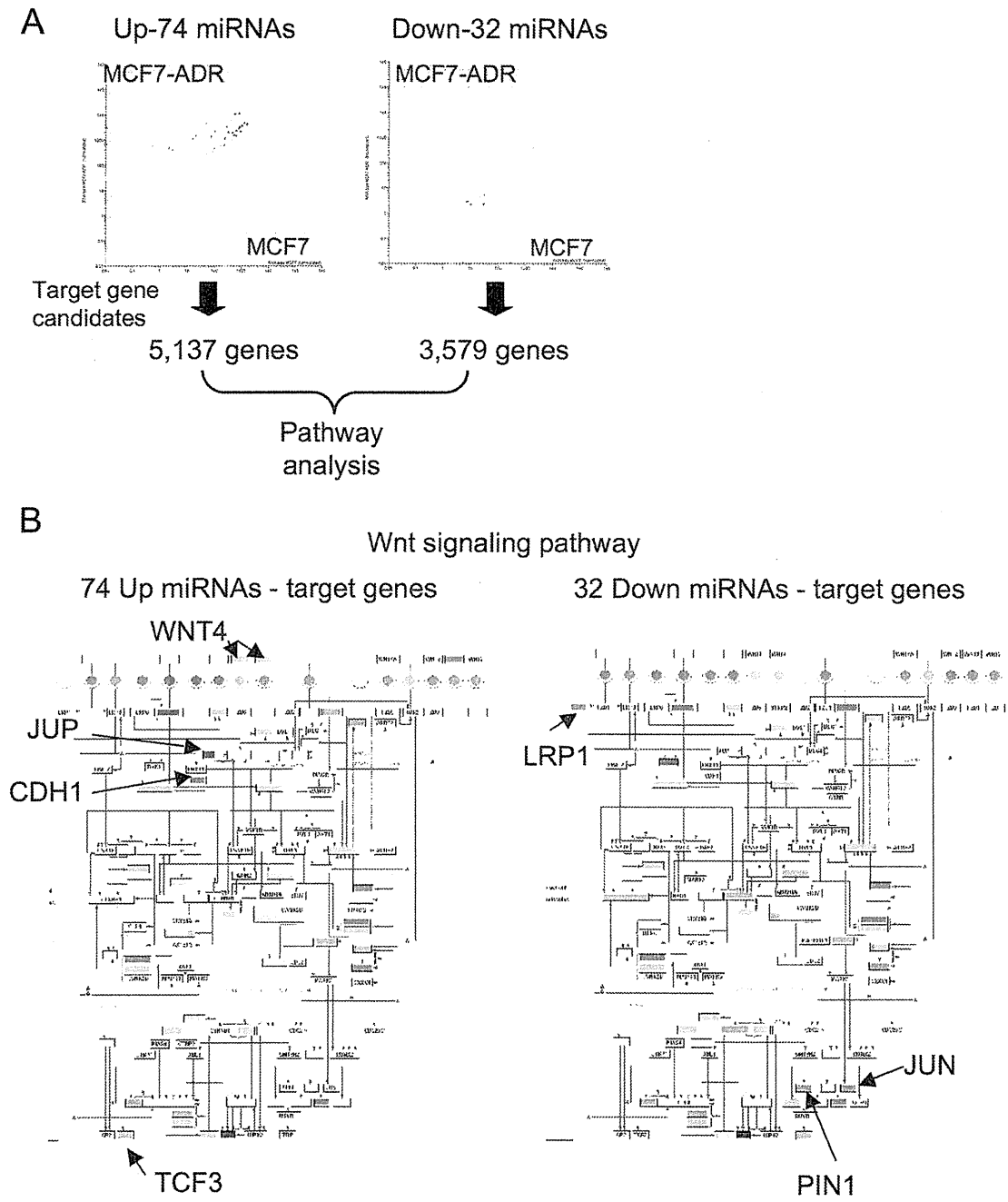

Figure 3 Pathway enrichment analysis of up- and down-regulated miRNAs in breast cancer cells. (A) Diagram of GO analysis of MCF7 and MCF7-ADR. In the comparison with MCF7 and MCF7-ADR, 5, 137 genes and 3, 579 genes are predicted as miRNA target candidates of 74 up-regulated miRNAs and 32 down-regulated miRNAs, respectively. With these predicted genes, enriched pathways are selected, as shown Table S1. (B) Wnt signaling pathway. 74 miRNA-targeted genes are plotted in Wnt signal pathway map (left), 32 miRNA-targeted genes are plotted (right). Red boxes show high expression genes, and green boxes show low expression genes.

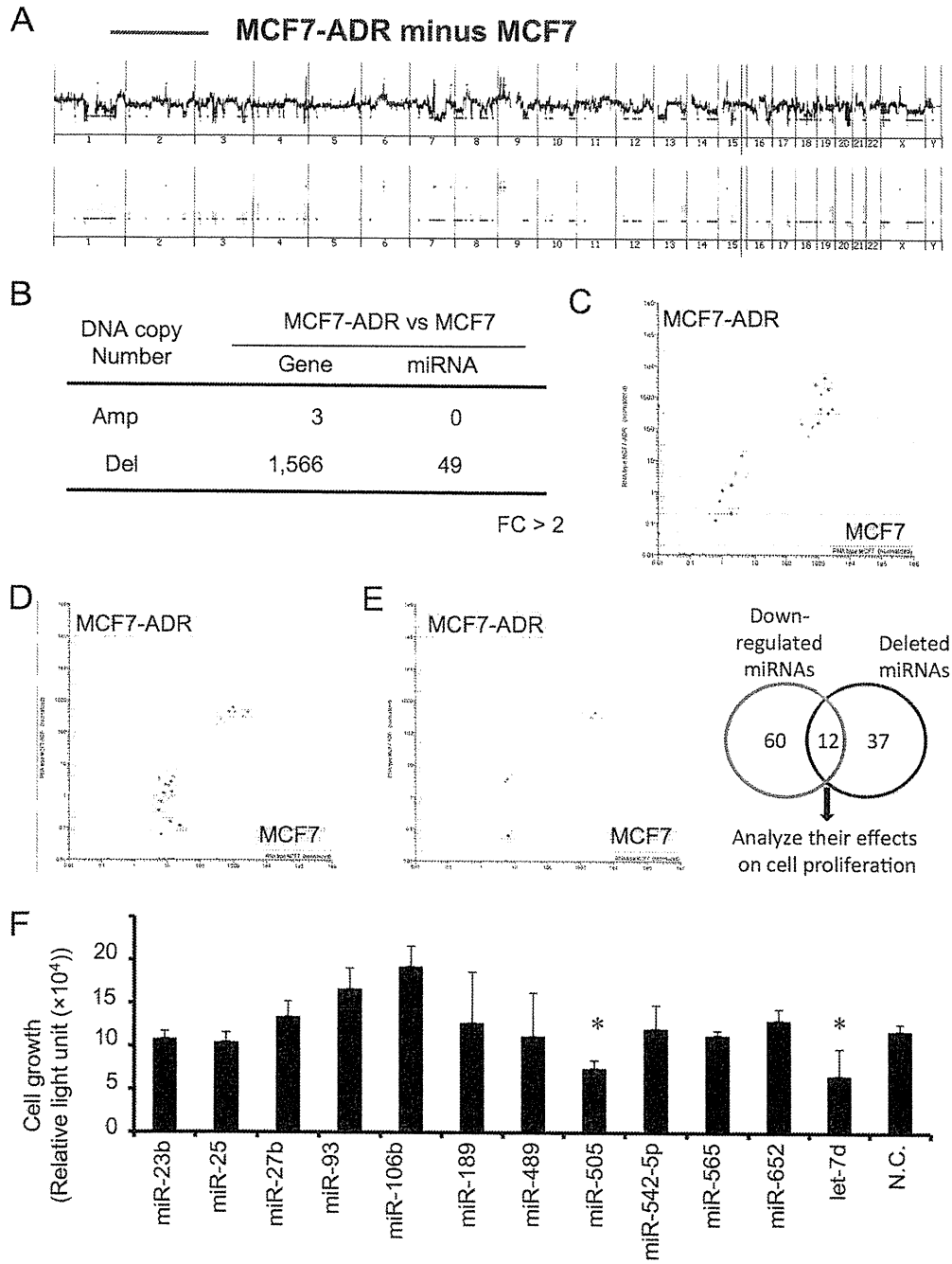

Figure 4 Screening of miRNAs responsible for drug resistance in MCF7-ADR. (A) Direct comparison of MCF7 and MCF7-ADR genomic status based aCGH. Modified Figure1B, differences in genomic status between MCF7 and MCF7-ADR are shown (FC > 2). (B) The number of genes and miRNAs located on the amplified or deleted genome regions in MCF7-ADR as compared with MCF7 (FC > 2). (C) Scatter plot of 49 miRNAs that locates in the deleted genomic regions in MCF7-ADR. (D) Scatter plot of 72 miRNAs whose expression was down-regulated in MCF7-ADR when compared with MCF7. (E) Twelve miRNAs (miR-23b, miR-25, miR-27b, miR-93, miR-106b, miR-189, miR-489, miR-505, miR-542-5p, miR-565, miR-652 and let-7d), which are overlapped between deleted and down-regulated in MCF7-ADR, are candidates responsible for drug resistance in MCF7-ADR. (F) Transfection analysis of selected 12 miRNAs in MCF7-ADR-Luc, which stably express luciferase. All miRNAs were transfected at 20 nM. Seventy-two hours after transfection, cell growth was estimated by luciferase activity in MCF7-ADR-Luc cells (n = 3-6 per group). $P < 0.05$.

status was deleted in MCF7-ADR cells as compared to MCF7. Expression levels of 72 miRNAs were significantly down-regulated ($p < 0.05$, Additional File 7 Table S3 and Figure 4D) and 49 miRNAs (Additional File 8 Table S4 and Figure 4C) were located in deleted regions (FC > 2) in MCF7-ADR cells. Twelve miRNAs were overlapped between the 72 down-regulated miRNAs and 49 deleted miRNAs (Table 1 and Figure 4E).

To examine the functions of these miRNAs, we tested the effects of 12 miRNAs on cell proliferation in MCF7-ADR cells. The 12 miRNAs (miR-23b, miR-25, miR-27b, miR-93, miR-106b, miR-189, miR-489, miR-505, miR-542-5p, miR-565, miR-652, and let-7d) were transfected into MCF7-ADR-Luc cells (Figure 4F). Interestingly, miR-25, miR-93, and miR-106b are known as polycistronic miRNAs [19] and their expression and genomic region were coincidently changed between MCF7-ADR and MCF7 (Additional File 9 Figure. S5). Consistent with previous report, transfection with miR-93 and miR-106b promoted cell proliferation as compared with negative control miRNA, suggesting that they actually act as oncogenic miRNAs [19,20]. Inversely, transfection of miR-505 and let-7d inhibited the cell proliferation of MCF7-ADR-Luc cells. Let-7 family is a well known tumor suppressive miRNA as described in many reports [21-23]. Inhibitory effects of cell growth by miR-505 and let-7d transfection are at similar level (Figure 4F). These data suggest that miR-505 is a novel tumor suppressive miRNA and plays a role in the regulation of cell proliferation similar to let-7d.

miR-505 inhibits cell growth by inducing apoptotic cell death in MCF7-ADR cells

Since transfection of miR-505 showed the inhibition of cell proliferation effectively and significantly (Figure 4F and 5A), we focused on miR-505 to evaluate its molecular function in this study. From the aCGH data, genomic region of miR-505 locus in MCF7-ADR was deleted (Figure 5B), in contrast it was intact in MCF7. The expression level of miR-505 was also decreased by real-time RT-PCR analysis in MCF7 and MCF7-ADR cells (Figure 5C). To further examine the mechanism of cell growth inhibition, we sought to check whether miR-505 is responsible for cell apoptosis in MCF7-ADR cells. A mature form of miR-505 was transfected into MCF7-ADR cells in the presence or absence of DOC (1 nM), as MCF7-ADR cells are resistant to DOC, and caspase-7 activity was measured to estimate apoptotic cell death in MCF7-ADR. The results of the caspase-7 assay indicated that transfection of miR-505 with DOC resulted in a marked induction of apoptosis ($p < 0.05$, Figure 5D), although no significant difference was seen in the samples without DOC. We validated this result by counting the Hoechst-stained cells showing apoptotic nuclear condensation and fragmentation and found that significantly higher apoptotic cell death was observed in cells with miR-505 than in control miRNA ($p < 0.05$, Figure 5E and 5F). Taken together, we concluded that transfection of miR-505 inhibit the growth of drug resistance cells, MCF7-ADR, through the inducing apoptosis.

Akt3, correlates inversely with miR-505 expression, modulates drug sensitivity

It has been already known that drug resistance in cancer cells was an acquired characteristic by activation of multiple drug resistance-responsible genes. To investigate what kind of the genes are responsible for the drug sensivity by miR-505 induce gene suppression, we combined gene expression data and gene ontology (Figure 6A). As for gene expression data, since the expression level of miRNA-regulating genes was up-regulated when miRNA expression was lower in MCF7-ADR than in MCF7 cells, up-regulated genes judged by the T-test (1, 758 genes, Additional File 10 Table S5) were selected as candidates of miR-505. As miR-505-targeted genes, apoptosis-related genes (153 genes, Additional File 11 Table S6) were chosen using a database (KEGG web site, http://www.genome.

Table 1 Selected 12 miRNAs

Name	MCF7-ADR/MCF7	Chromosome	Start	Stop
hsa-miR-565	0.48	3	45705468	45705564
hsa-miR-489	0.01	7	92951184	92951267
hsa-miR-25	0.16	7	99529119	99529202
hsa-miR-93	0.15	7	99529327	99529406
hsa-miR-106b	0.15	7	99529552	99529633
hsa-let-7d	0.37	9	95980937	95981023
hsa-miR-23b	0.18	9	96887311	96887407
hsa-miR-27b	0.12	9	96887548	96887644
hsa-miR-189 (miR-24)	0.00	9	96888124	96888191
hsa-miR-652	0.18	X	109185213	109185310
hsa-miR-542-5p	0.56	X	133501037	133505133
hsa-miR-505	0.53	X	138833973	138834056

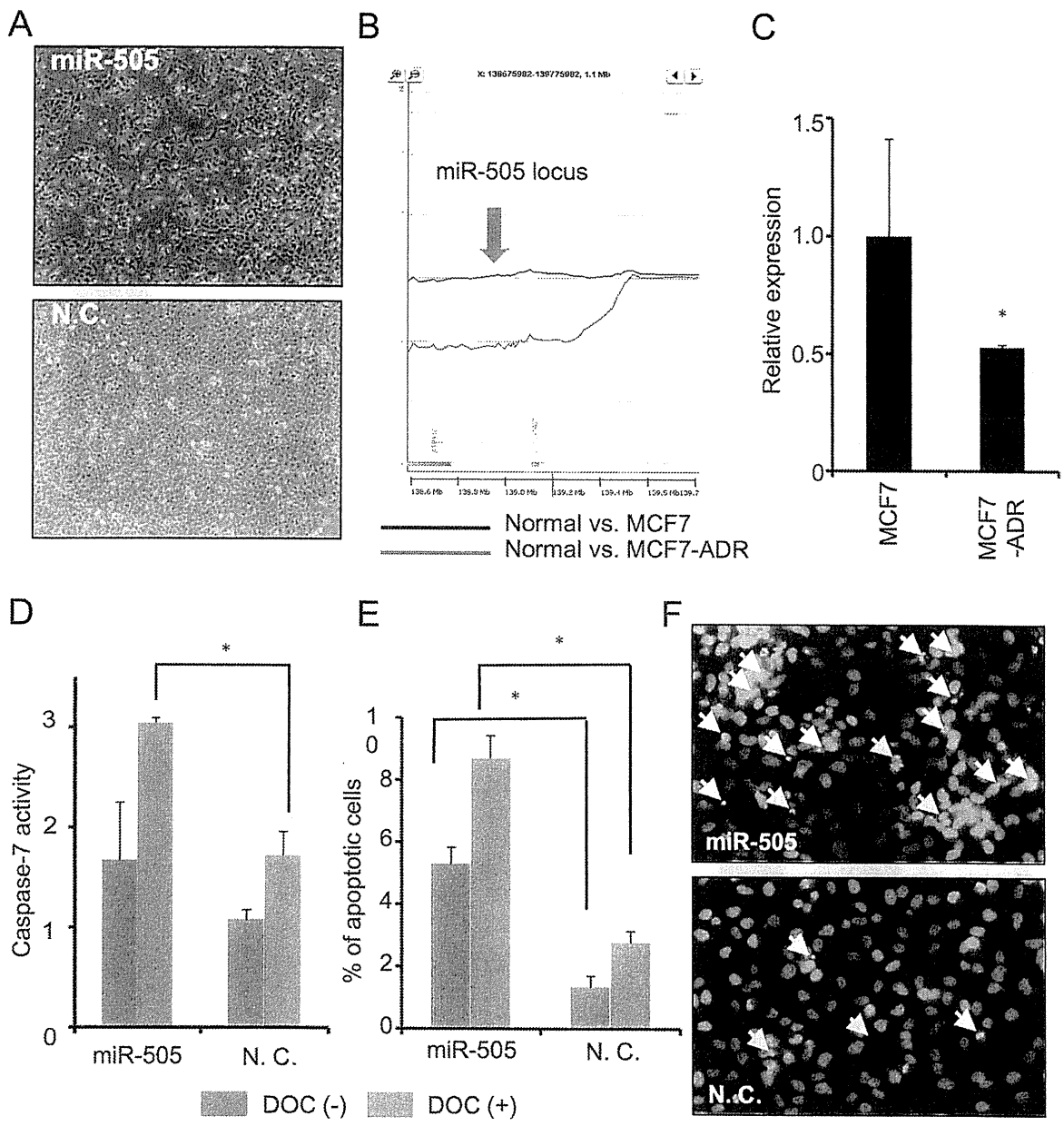

Figure 5 miR-505 inhibits cell growth by inducing apoptotic cell death in MCF7-ADR. (A) Images show typical MCF7-ADR cells transfected with miR-505 (top) and control miRNA (bottom) in 72 hours. (B) DNA copy number on the miR-505 locus of MCF7-ADR deleted as compared with that of MCF7. (C) Real-time RT-PCR analysis was performed to determine expression levels of miR-505 in parental cell line MCF7 and drug-resistant cell line MCF7-ADR. Error bar represents S.D. of triplicate determinations in a single experiment. $P < 0.05$. (D). Seventy-two hours after transfection, caspase-7 activity in the presence or absence of DOC (1 nM) was detected in miR-505 or control miRNA-transfected MCF7-ADR cells. $n = 6$, $P < 0.001$. (E) Condensed and fragmented apoptotic cells are counted in the presence or absence of DOC (1 nM) by staining with Hoechst dye. $n = 4$, $P < 0.05$. Mean ± S.D. is shown. (F) Images of apoptotic nuclear condensation and fragmentation. Seventy-two hours after transfection with miR-505 (top) or control miRNA (bottom), condensed and fragmented cells are observed. White arrows show apoptotic cells in culture.

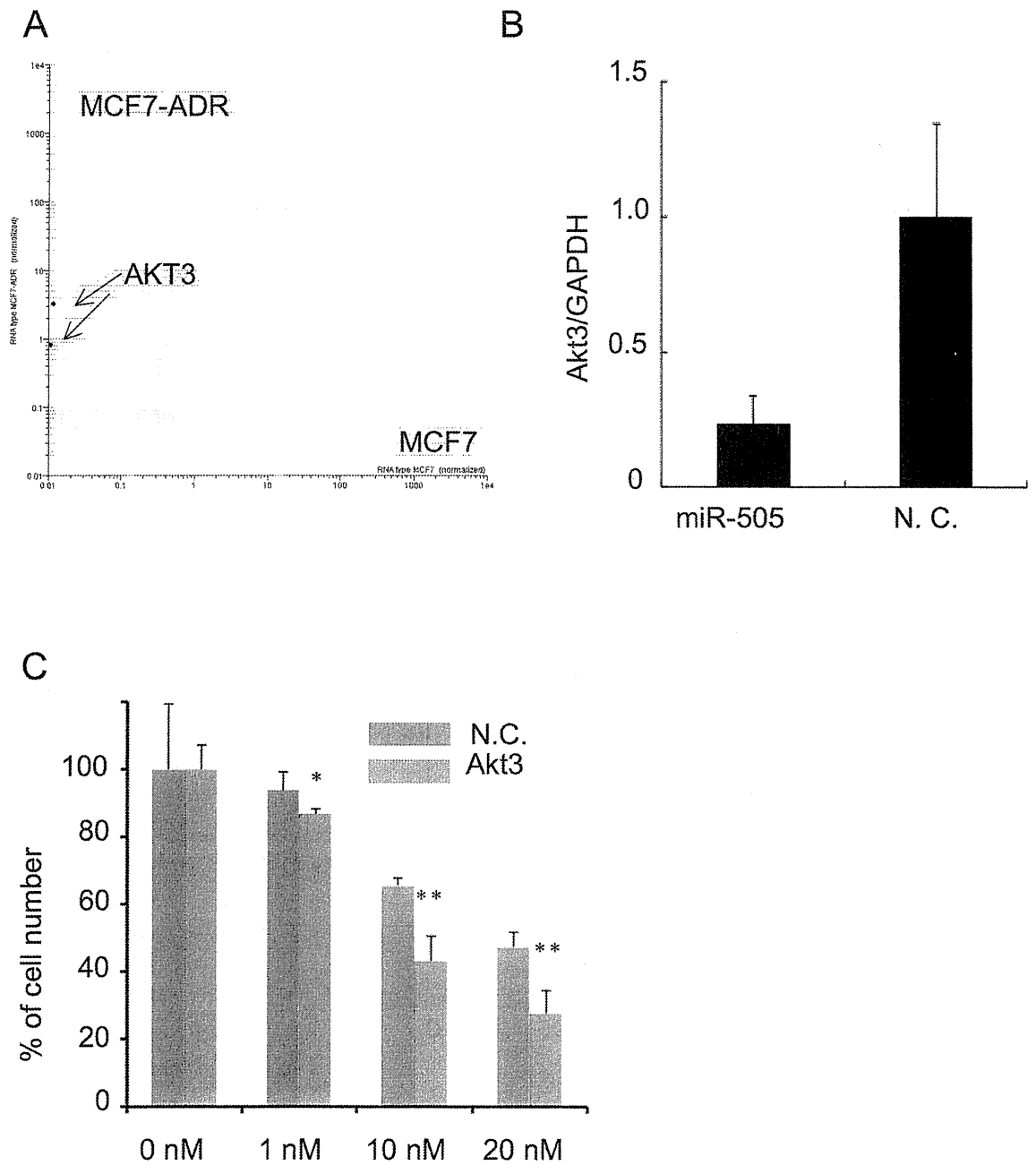

Figure 6 Akt3, whose expression was inversely correlated with miR-505, is associated with drug resistance in MCF7-ADR cells. (A) Scatter plot of 2 probes of Akt3 genes that up-regulated in MCF7-ADR cells. (B) Real-time RT-PCR analysis for Akt3 in miR-505 or control miRNA-transfected cells. Seventy-two hours after transfection with miR-505 or control miRNA, Akt3 expression levels were determined. Expression levels were normalized to GAPDH expression levels. N = 9, mean ± S.D. is shown. (C) Down-regulation of Akt3 induced cell growth arrest in MCF7-ADR cells at 1, 10 and 20 nM DOC conditions. Cell growth was calculated by luciferase activity. n = 3, *, $P < 0.05$. **, 0.01.

jp/kegg/) because transfection of miR-505 induced apoptotic cell death in MCF7-ADR-Luc cells. By combining these data, we postulated that Akt3 gene is candidate of miR-505-regulating gene, which is responsible for the drug resistance in breast cancer cell (Figure 6A). Notably, several studies have reported that Akt3 promotes melanoma development [24] and that the down-regulation of Akt3 distinctly inhibited the proliferation of ovarian cancer cell lines by slowing G2-M phase transition [25]. Given this evidence and the observed phenotype in miR-505-transfected MCF7-ADR-Luc cells, we sought to determine whether Akt3 was a target of miR-505 or not. As shown in Figure 6B, decrease in relative gene expression was observed with miR-505 in MCF7-ADR-Luc cells, suggesting that miR-505 suppresses the gene expression of Akt3. However, we found that miR-505 could not bind to the 3'-UTR of Akt3 gene (Additional File 12 Figure. S6), indicating that down-regulation of Akt3 after miR-505 overexpression was caused by indirect effect of miR-505-mediated gene suppression. Finally, transfection of Akt3 siRNA was conducted to examine whether down-regulation of the Akt3 gene induced cell growth arrest in the presence or absence of DOC. Three days after transfection, the expression of Akt3 was clearly suppressed (Additional File 13 Figure. S7), and cell growth rates were assayed with or without 1, 10, and 20 nM DOC conditions. A slight decrease in cell growth was observed with 1 nM DOC condition, and more remarkable decreases was detected with 10 and 20 nM DOC conditions in Akt3 siRNA-transfected MCF7-ADR cells than in control siRNA-transfected cells (Figure 6C). Therefore, our data show that Akt3, whose expression is correlated conversely with miR-505, regulates DOC sensitivity in MCF7-ADR cells.

Discussion

According to recent high-throughput analyses of both coding and non-coding genes, cancer progression is caused by genetic alteration involving structural and expression abnormalities of oncogenes and tumor suppressor genes [6,26]. In this study, we performed an integrated genomic analysis to link miRNA expression data to aCGH and gene expression microarray, using the parental cell line MCF7 and the drug-resistant cell line MCF7-ADR, to examine the molecular mechanism governing drug resistance in breast cancer. We found that the expression of miR-505 was down-regulated, and its genomic region was deleted in MCF7-ADR cells, which provided evidence that miR-505 was a tumor suppressive miRNA and had a pivotal role for inducing apoptosis in drug resistant cancer cells. In addition, by using the data of gene expression and bioinformatics analysis (gene function), our data identified Akt3 whose expression was conversely correlated with miR-505, which modulated drug sensitivity in MCF7-ADR.

Akt is a homolog of the retroviral oncogene v-Akt, which is ubiquitously expressed and has 3 members; Akt1, Akt2, and Akt3 [27]. Downstream genes of the Akt signal pathway modulate the cell cycle, DNA repair, and nitric oxide production. Moreover, Akt inhibits apoptotic cell death by inactivation of a key apoptotic molecule and is broadly activated in various kinds of cancer. Importantly, the Akt signal pathway is tightly related to drug resistance in cancer. Several studies have reported that inactivation of Akt promotes drug-induced apoptosis [28,29]. Therefore, inhibition of Akt3 is a therapeutic strategy for cancers by inducing apoptotic cell death and reversing drug resistance. However, our results showed that inhibition of Akt3 was less effective than transfection of miR-505 in cell growth arrest. A simple explanation of a low effect might be that miRNA could modulate the expression of a large number of downstream target genes in a highly orchestrated manner to control apoptosis and cell cycle processes

Concerning the variations in the DNA copy numbers and genomic aberrations, several reports have shown the deletions of miRNAs that act as tumor suppressors, namely miR-15, miR-16, and miR-34a. They are observed in cancer, and down-regulation of these miRNAs contributed to cancer progression, indicating that variations in DNA copy numbers are closely associated with miRNA expression and carcinogenesis [9,10]. Meanwhile, several data provided evidences that miRNA expressions were regulated by epigenetic modifications, such as DNA hypomethylation and hypermethylation. It has been demonstrated that miR-342 was methylated in colorectal cancer and the reconstitution of miR-342 induced apoptosis in a colorectal cancer cell line [30]. A recent study showed that miR-127, which was embedded in a CpG island, was expressed in normal fibroblast but silenced or down-regulated in cancer cells. The silencing of the miRNA promoter region of miR-127 was mediated by CpG island hypermethylation, which could be reversed by simultaneous treatment with the chromatin-modifying drugs 5-aza-2'-deoxycytidine and 4-phenylbutyric acid [31]. In addition, recent studies have shown that impaired miRNA processing contributes to a decrease in mature miRNA expression and accelerates tumorigenesis [32], and a number of groups have also revealed that miRNA expression is regulated by transcription factors and cytokines as well as coding genes [33]. In this study, we found 12 miRNAs whose expressions are down-regulated and their genomic regions are deleted in MCF7-ADR. Interestingly, some of them, such as miR25-93-106b and miR-23b-27b-189, are located in close proximity and their expressions are

expected to be regulated by the same transcriptional regulators. Curiously, miR-23b-27b-189 is localized on the Ch9q22.3, and LOH of this region is strongly correlated with cancer progression and lymph node metastasis [34-36]. In our assay we could not observe any significant differences, however, they could be related to malignancy in different aspects [37,38].

Pathway analysis, which was based on miRNA target prediction, proved that differentially-expressed miRNA cooperatively regulated a large number of signaling pathways, including Wnt, insulin, EGFR1, MAPK and TGF-β receptor, which are relevant to drug resistance as well as tumorigenesis. Concerning the Wnt signaling pathway, it has been reported that activation of the Wnt/β-catenin pathway plays critical roles in establishment of MLL leukemic stem cells and conferring drug-resistant properties [39]. Additionally, activation of Wnt/beta-catenin signaling in plasma cells induced chemoresistance [40], and RNAi-mediated gene silencing of β-catenin negatively regulated drug-induced apoptosis [41]. Therefore, Wnt/β-catenin signaling pathway would be a potential therapeutic target to sensitize drug-resistant cancer cell. Furthermore, other pathways were also reported to be associated with drug resistance and apoptosis. Sequential treatment of TGF-β induced MDR1 expression in rat hepatocytes [42]. In contrast, TGF-β also induces apoptotic cell death in hepatocytes and activation of the MAPK/ERK pathway confers resistance to TGF-β-induced cell death [43]. Our findings showed that a lot of pathways were commonly enriched in up- and down-regulated miRNA targets, however, it is hard to decide whether these pathways are positively or negatively regulated in MCF7-ADR, because a large number of target candidates exist in each signaling pathway. Further investigation such as systems biology would be needed to clarify this point.

We believe that the integrative genomic analyses as described here have a huge potential to fundamentally understand transcriptional regulatory networks and identify the novel molecular targets for therapy in the field of cancer biology. By integrating array data and bioinformatics, it could be possible to expeditiously explore the key molecule in the all aspects of pathophysiology. Our studies by means of an integrated genomic analysis not only identified miR-505 as a tumor suppressive miRNA that inhibited cell proliferation by inducing apoptotic cell death but also, more broadly highlighted that various genes and miRNAs orchestrate to temper the drug resistance by intricately controlling genomic status, gene and miRNA expression in cancer cells. Thus, it would be a useful approach to accelerate the understanding of cancer genetics and discover the key targets for diagnosis, prognosis and therapy.

Additional material

Additional file 1: Table S1. Primer list for real-time PCR.

Additional file 2: Figure. S1. Microarray analysis of gene expression and miRNA in MCF7-ADR and MCF7. (A) Scatter plot of gene expression. 41, 000 probes (n = 3). (B) Scatter plot of miRNA expression. 470 probes (n = 2).

Additional file 3: Figure. S2. Validation of accuracy of aCGH in MCF7 and MCF7-ADR. (A) aCGH analysis of MCF7. (B) aCGH analysis of MCF7-ADR. For each sample, the experiment was repeated once, wherein the dye was reversed between the experimental and the reference sample, in order to account for dye-incorporation bias. An aberration filter was set at 2 for the minimum number of probe region and 1 for minimum absolute average log2 ratio for regions in the CGH Analytics to reduce false positives.

Additional file 4: Figure. S3. aCGH analysis of MCF7 and MCF7-ADR as compared with normal human female genome. (A) Blue line shows normal vs. MCF7, and red line shows normal vs. MCF7-ADR (top). Amplified or deleted genome regions (fold change > 2) are highlighted (bottom). (B) The numbers of genes and miRNAs located on the amplified or deleted genome regions (FC > 2).

Additional file 5: Table S2. Up and Down miRNA target gene related pathways.

Additional file 6: Figure. S4. TGF-β signaling pathway. Seventy-four miRNA-targeted genes are plotted in TGF-β signaling pathway map (left), 32 miRNA-targeted genes are plotted (right).

Additional file 7: Table S3. List of down-regulated miRNAs in MCF7-ADR.

Additional file 8: Table S4. List of miRNAs located in genome deletion regions in MCF7-ADR.

Additional file 9: Figure. S5. Polycistronic miRNAs; miR-106-25 cluster. miR-106-25 cluster is located on the deleted genomic region, and the expression is coincidently downregulated.

Additional file 10: Table S5. List of up-regulated genes in MCF7-ADR judged by T-test in comparison with MCF7.

Additional file 11: Table S6. List of genes accosiated with apoptosis (KEGG web site).

Additional file 12: Figure. S6. The 3'-UTR assay of Akt3 by miR-505 in MCF7-ADR cells and HEK293 cells. (A) MCF7-ADR cells and (B) HEK293 cells were co-transfected with pre-miR-505 or pre-NC and the psi-Akt3_1 or with psi-Akt3_2. After 48 h, luciferase activities were measured. n.s. represents not significant.

Additional file 13: Figure. S7. Real-time RT-PCR analysis of Akt3 gene by transfection of siRNA in MCF7-ADR cells. Real-time RT-PCR analysis was performed to examine Akt3 from RNA extracted from MCF7-ADR cells transfected with either Akt3 siRNA or negative control siRNA. Akt3 expression levels were normalized to GAPDH expression levels. The mean ± S. D. of results from triplicate transfections is shown. Results represent the mean ± S. D. (n = 3). Since Akt3 siRNA-1 was most effectively inhibited the expression of Akt3 genes, it was used for the analysis of cell growth arrest.

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Authors' contributions
YYa, YYo, KM, RT, and FT carried out the experimental work, YYa, KM, TT, RH and YF provided data analysis, YYa, TK, NK and TO designed the study and YYa, NK and TO participated in writing the paper. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no conflict of interest. KM, TT, RH, and YF are Agilent employees.

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References

1. Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H, Haga N: **Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal.** *Cancer Sci* 2003, **94**:15-21.
2. Leslie EM, Deeley RG, Cole SP: **Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters.** *Toxicology* 2001, **167**:3-23.
3. Renes J, de Vries EG, Jansen PL, Muller M: **The (patho)physiological functions of the MRP family.** *Drug Resist Updat* 2000, **3**:289-302.
4. Leonessa F, Clarke R: **ATP binding cassette transporters and drug resistance in breast cancer.** *Endocr Relat Cancer* 2003, **10**:43-73.
5. Ambros V: **The functions of animal microRNAs.** *Nature* 2004, **431**:350-355.
6. Esquela-Kerscher A, Slack FJ: **Oncomirs - microRNAs with a role in cancer.** *Nat Rev Cancer* 2006, **6**:259-269.
7. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function.** *Cell* 2004, **116**:281-297.
8. Calin GA, Croce CM: **Chromosomal rearrangements and microRNAs: a new cancer link with clinical implications.** *J Clin Invest* 2007, **117**:2059-2066.
9. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM: **miR-15 and miR-16 induce apoptosis by targeting BCL2.** *Proc Natl Acad Sci USA* 2005, **102**:13944-13949.
10. Wei JS, Song YK, Durinck S, Chen QR, Cheuk AT, Tsang P, Zhang Q, Thiele CJ, Slack A, Shohet J, Khan J: **The MYCN oncogene is a direct target of miR-34a.** *Oncogene* 2008, **27**:5204-5213.
11. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM: **Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.** *Proc Natl Acad Sci USA* 2004, **101**:2999-3004.
12. Iwao-Koizumi K, Matoba R, Ueno N, Kim SJ, Ando A, Miyoshi Y, Maeda E, Noguchi S, Kato K: **Prediction of docetaxel response in human breast cancer by gene expression profiling.** *J Clin Oncol* 2005, **23**:422-431.
13. Honma K, Iwao-Koizumi K, Takeshita F, Yamamoto Y, Yoshida T, Nishio K, Nagahara S, Kato K, Ochiya T: **RPN2 gene confers docetaxel resistance in breast cancer.** *Nat Med* 2008, **14**:939-948.
14. Lipson D, Aumann Y, Ben-Dor A, Linial N, Yakhini Z: **Efficient calculation of interval scores for DNA copy number data analysis.** *J Comput Biol* 2006, **13**:215-228.
15. Wang H, Ach RA, Curry B: **Direct and sensitive miRNA profiling from low-input total RNA.** *Rna* 2007, **13**:151-159.
16. Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, Ng I, Man K, Wong N, To KF, Zheng BJ, Lai PB, Lo CM, Chan KW, Guan XY: **miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1.** *Cell Stem Cell* 2010, **7**:694-707.
17. Gironella M, Seux M, Xie MJ, Cano C, Tomasini R, Gommeaux J, Garcia S, Nowak J, Yeung ML, Jeang KT, Chaix A, Fazli L, Motoo Y, Wang Q, Rocchi P, Russo A, Gleave M, Dagorn JC, Iovanna JL, Carrier A, Pébusque MJ, Dusetti NJ: **Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development.** *Proc Natl Acad Sci USA* 2007, **104**:16170-16175.
18. Ito Y, Motoo Y, Yoshida H, Iovanna JL, Takamura Y, Miya A, Kuma K, Miyauchi A: **Decreased expression of tumor protein p53-induced nuclear protein 1 (TP53INP1) in breast carcinoma.** *Anticancer Res* 2006, **26**:4391-4395.
19. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM: **A microRNA polycistron as a potential human oncogene.** *Nature* 2005, **435**:828-833.
20. Sampath D, Calin GA, Puduvalli VK, Gopisetty G, Taccioli C, Liu CG, Ewald B, Liu C, Keating MJ, Plunkett W: **Specific activation of microRNA106b enables the p73 apoptotic response in chronic lymphocytic leukemia by targeting the ubiquitin ligase Itch for degradation.** *Blood* 2009, **113**:3744-3753.
21. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ: **RAS is regulated by the let-7 microRNA family.** *Cell* 2005, **120**:635-647.
22. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E: **let-7 regulates self renewal and tumorigenicity of breast cancer cells.** *Cell* 2007, **131**:1109-1123.
23. Mayr C, Hemann MT, Bartel DP: **Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation.** *Science* 2007, **315**:1576-1579.
24. Cheung M, Sharma A, Madhunapantula SV, Robertson GP: **Akt3 and mutant V600E B-Raf cooperate to promote early melanoma development.** *Cancer Res* 2008, **68**:3429-3439.
25. Cristiano BE, Chan JC, Hannan KM, Lundie NA, Marmy-Conus NJ, Campbell IG, Phillips WA, Robbie M, Hannan RD, Pearson RB: **A specific role for AKT3 in the genesis of ovarian cancer through modulation of G(2)-M phase transition.** *Cancer Res* 2006, **66**:11718-11725.
26. Calin GA, Croce CM: **MicroRNA signatures in human cancers.** *Nat Rev Cancer* 2006, **6**:857-866.
27. Vivanco I, Sawyers CL: **The phosphatidylinositol 3-Kinase AKT pathway in human cancer.** *Nat Rev Cancer* 2002, **2**:489-501.
28. Yuan XJ, Whang YE: **PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway.** *Oncogene* 2002, **21**:319-327.
29. Wan X, Yokoyama Y, Shinohara A, Takahashi Y, Tamaya T: **PTEN augments staurosporine-induced apoptosis in PTEN-null Ishikawa cells by downregulating PI3K/Akt signaling pathway.** *Cell Death Differ* 2002, **9**:414-420.
30. Grady WM, Parkin RK, Mitchell PS, Lee JH, Kim YH, Tsuchiya KD, Washington MK, Paraskeva C, Willson JK, Kaz AM, Kroh EM, Allen A, Fritz BR, Markowitz SD, Tewari M: **Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer.** *Oncogene* 2008, **27**:3880-3888.
31. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA: **Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells.** *Cancer Cell* 2006, **9**:435-443.
32. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T: **Impaired microRNA processing enhances cellular transformation and tumorigenesis.** *Nat Genet* 2007, **39**:673-677.
33. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M: **Interferon modulation of cellular microRNAs as an antiviral mechanism.** *Nature* 2007, **449**:919-922.
34. Mitelman F, Mertens F, Johansson B: **A breakpoint map of recurrent chromosomal rearrangements in human neoplasia.** *Nat Genet* 1997, **15(Spec No)**:417-474.
35. Simoneau M, Aboulkassim TO, LaRue H, Rousseau F, Fradet Y: **Four tumor suppressor loci on chromosome 9q in bladder cancer: evidence for two novel candidate regions at 9q22.3 and 9q31.** *Oncogene* 1999, **18**:157-163.
36. Sinha S, Singh RK, Alam N, Roy A, Roychoudhury S, Panda CK: **Alterations in candidate genes PHF2, FANCC, PTCH1 and XPA at chromosomal 9q22.3 region: pathological significance in early- and late-onset breast carcinoma.** *Mol Cancer* 2008, **7**:84.
37. Tsuchiya Y, Nakajima M, Takagi S, Taniya T, Yokoi T: **MicroRNA regulates the expression of human cytochrome P450 1B1.** *Cancer Res* 2006, **66**:9090-9098.

38. Wang Y, Rathinam R, Walch A, Alahari SK: ST14 (suppression of tumorigenicity 14) gene is a target for miR-27b, and the inhibitory effect of ST14 on cell growth is independent of miR-27b regulation. *J Biol Chem* 2009, 284:23094-23106.
39. Yeung J, Esposito MT, Gandillet A, Zeisig BB, Griessinger E, Bonnet D, So CW: beta-Catenin mediates the establishment and drug resistance of MLL leukemic stem cells. *Cancer Cell* 18:606-618.
40. Bjorklund CC, Ma W, Wang ZQ, Davis RE, Kuhn DJ, Kornblau SM, Wang M, Shah JJ, Orlowski RZ: Evidence of a Role for Activation of Wnt/{beta}-Catenin Signaling in the Resistance of Plasma Cells to Lenalidomide. *J Biol Chem* 286:11009-11020.
41. Senthivinayagam S, Mishra P, Paramasivam SK, Yallapragada S, Chatterjee M, Wong L, Rana A, Rana B: Caspase-mediated cleavage of beta-catenin precedes drug-induced apoptosis in resistant cancer cells. *J Biol Chem* 2009, 284:13577-13588.
42. Zhang X, Wang T, Batist G, Tsao MS: Transforming growth factor beta 1 promotes spontaneous transformation of cultured rat liver epithelial cells. *Cancer Res* 1994, 54:6122-6128.
43. Caja L, Sancho P, Bertran E, Iglesias-Serret D, Gil J, Fabregat I: Overactivation of the MEK/ERK pathway in liver tumor cells confers resistance to TGF-{beta}-induced cell death through impairing up-regulation of the NADPH oxidase NOX4. *Cancer Res* 2009, 69:7595-7602.

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Cellular and Molecular Life Sciences

REVIEW

Gene-manipulated embryonic stem cells for rat transgenesis

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Abstract Embryonic stem cells (ESCs) are derived from blastocysts and are capable of differentiating into whole tissues and organs. Transplantation of ESCs into recipient blastocysts leads to the generation of germline-competent chimeras in mice. Transgenic, knockin, and knockout gene manipulations are available in mouse ESCs, enabling the production of genetically modified animals. Rats have important advantages over mice as an experimental system for physiological and pharmacological investigations. However, in contrast to mouse ESCs, rat ESCs were not established until 2008 because of the difficulty of maintaining pluripotency. Although the use of signaling inhibitors has allowed the generation of rat ESCs, the production of genetically modified rats has been difficult due to problems in rat ESCs after gene introduction. In this review, we will focus on some well-documented examples of gene manipulation in rat ESCs.

Keywords ES cell · Rat · Transgenic · Pluripotency · Chimera

Introduction

Embryonic stem cells (ESCs) established from the inner cell mass (ICM) of preimplantation blastocysts [1] have been routinely derived from mice since 1981 [2, 3]. These cells have a stable developmental potential to form derivatives of all three embryonic germ layers, the endoderm, mesoderm, and ectoderm, even after prolonged culture [4] and have been used to study the mechanism of cell differentiation. Moreover, they are capable of generating germline chimeras following injection into the blastocyst [5]. Gene manipulation is available, and germline transmission of transgenic ESCs was achieved in 1986 [6]. Soon after this achievement was reported, gene-targeting mice were generated via homologous recombination in ESCs [7]. So far, a huge number of genetically modified mice have been produced via the manipulation of ESCs and used in a range of biomedical researches. However, this technique is unavailable in species other than mice because of a lack of stable ESCs.

The laboratory rat, the first mammalian species domesticated for scientific research, has been used as an animal model for research in physiology, toxicology, nutrition, behavior, immunology, and neoplasia for over 150 years [8–12]. Despite the utility to use rats in experiments, rat ESCs were not established until 2008. The reasons for the failure to develop ESCs in rats are related to the difficulty in maintaining pluripotency in culture despite trials using numerous strategies [13–17]. Our group generated rat ESCs harboring a potential to contribute to chimera but not to develop germ cells [18]. On the other hand, despite the lack of authentic ESCs, several technologies have been developed to alter rats genetically [19–26].

Rat transgenesis from ESCs with 2i+LIF medium

In 2008, germline-competent rat ESCs were first established from blastocysts by using a 2i+LIF medium composed of two signaling inhibitors (MEK inhibitor PD0325901; GSK3 inhibitor CHIR99021), a leukemia inhibitory factor (LIF), and a defined basal culture medium

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containing no fetal bovine serum (FBS) (Fig. 1) [27, 28]. The results of the two studies showed that FBS was a key factor in the induction of differentiation in rat ESCs [29]. This culture medium was also used for the generation of mouse ESCs [30]. Generally, mouse ESCs are cultured on feeder layers of mouse embryonic fibroblasts (MEFs). Further, it was found that the use of DIA-M cells [27] or a mixture of MEFs and L-cells as feeder layers [28] was optimal for isolating rat ESCs. In these conditions, although ESCs maintained pluripotency and contributed to chimeras, only two of nine cell lines achieved germline transmission. Rat-induced pluripotent stem cells (iPSCs) with the potential to contribute to chimeras were also generated by the addition of A-83-01 (Type 1 Tgf β receptor inhibitor) to 2i+LIF in a mouse ESC basal culture medium containing 20% knockout serum replacement (KSR). However, these iPSCs did not achieve germline transmission [31].

Gene introduction was available in the rat ESCs cultured in 2i+LIF medium. However, they were sensitive to electro-physical stimulation induced by the conventional electroporation method, which led to cell death. A nucleofection method was found to be more efficient and convenient for gene introduction in rat ESCs [28]. Furthermore, FBS was temporally added into an electroporation medium as well as a 2i+LIF cell-culture medium to aid viability [27]. Each group obtained stable transfectant clones in which the CAG-eGFP-IRES-pac plasmid was randomly integrated in their genome after selection with puromycin [27, 28]. Although five overt coat color chimeras were born after injection of the clone, they either died perinatally or were euthanized due to jaw abnormalities. The reasons for their abnormalities might have been chromosomal instability in the transfectant ESC line [27]. On the other hand, Hirabayashi et al. [32] succeeded in the germline transmission of a transfectant rat ESC line harboring a humanized Kusabira-Orange (huKO) gene using the 2i+LIF culture medium. A CAG/huKO-neo plasmid was introduced into ESCs by electroporation, and then stable clones were obtained by neomycin selection. In the 2i+LIF medium, 1,000 U/ml of rat LIF [33] was substituted for the human LIF used in the previous works (100 U/ml [27]; 10 U/ml [28]). It is possible that the rat LIF is better for the maintenance of rat ESCs [34]. Kobayashi et al. [35] overcame the difficulty to generate interspecific chimeras between rats and mice using rat ES or iPS cells cultured in a 2i+rat LIF medium. Thus, using rat LIF might be an option to keep rat ESCs stable.

Rat transgenesis from ESCs with YPAC medium

Our group developed a new culture medium (YPAC medium) including the additional signaling inhibitors of Rho-associated kinase (Y-27632) and A-83-01 to the 2i [36]. The four inhibitors, Y-27632, PD0325901, A-83-01, and CHIR99021, are collectively referred to as YPAC. A mouse ESC basal culture medium containing FBS (20% vol/vol) and MEFs was used, but LIF was not necessary in our study (Fig. 1). In the culture condition, the majority of cell lines (six out of six) demonstrated chimerism and germline transmission and could be stably transfected with a reporter transgene to produce genetically modified rats. These three cell lines were derived from each of the following strains: Wistar, LEA (Long Evans Agouti), and hybrid Wistar/LEA [36].

Since the medium contained 20% serum, the ESCs were tolerant to the damage induced by electric stimuli during gene introduction. In our procedure, a transgene in which the Venus gene was transcribed by the *Oct4*-promoter (*Oct4*-Venus) was introduced in the ESCs by the nucleofection method. When the manipulated cells were plated,

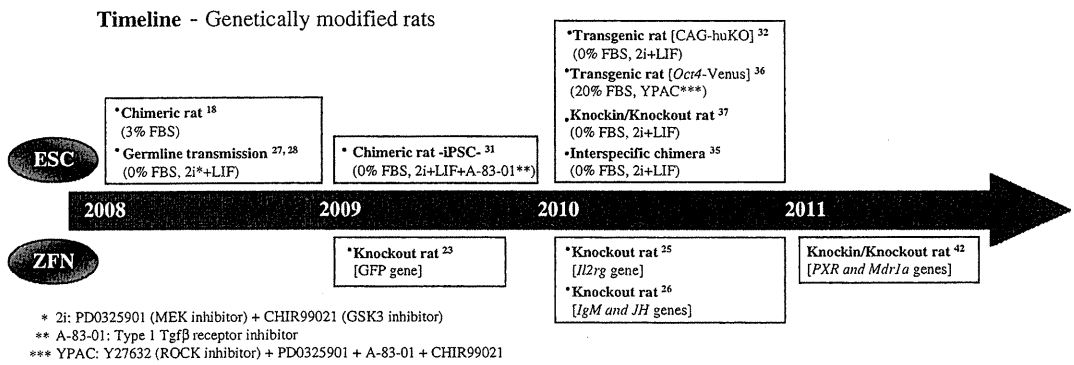

Fig. 1 Timeline of rat transgenesis using ESC or ZFN technology since 2008. The *parentheses* indicate the culture conditions. The *brackets* indicate transgenes or targeted genes

the use of matrigel (2% at final concentration) was effective for selecting stable clones because they are retained to adhere on MEFs [36]. It is generally known that rat ESC colonies tend to detach from MEFs [27, 28, 36]. This phenomenon enhances the ESCs' attachment to each other, leading to clone contamination. As the transgene did not include a selection cassette, Venus-positive clones were picked and expanded without drugs. In this cloning process, we found an advantage of using ESCs for the generation of transgenic rats because we were able to choose high-quality clones mimicking an endogenous *Oct4* expression pattern. While the majority of the clones exhibited a heterogeneous expression pattern in undifferentiated cells, only a few clones maintained homogenous expression after long-term culture (Fig. 2a). This homogeneity corresponds to the expression pattern of endogenous OCT4 protein. *Oct4*-Venus transgenic rats were generated through germline transmission of the selected clones without any adverse effects of gene introduction on chimera contribution (Fig. 2b). The Venus fluorescence was also detected in germ cells of the transgenic fetal gonads (Fig. 2c). Moreover, we could trace the fluorescence only in undifferentiated ESCs from their blastocysts during the establishment process and long-term culture (Fig. 2d) [36].

Gene targeting rats from ESCs

Tong et al. [37] achieved for the first time the production of knockout rats via homologous recombination in rat ESCs. A targeting vector was constructed to disrupt the tumor suppressor gene *p53* (also known as *Tp53*). Targeting efficiencies in two ESC lines derived from the DA (Dark Agouti) strain were 1.12–3.70%. Many properly targeted cell lines cultured in the 2i+LIF medium developed chromosome abnormalities. Over 65% of the cells were polyploid. This phenomenon is similar to that reported in previous works [27, 28]. However, after subcloning round and compact colonies, two out of 20 clones had euploid chromosome numbers leading to the production of a viable knockout [34]. This achievement is historic because the p53 knockout rat validates the culminated effort of many to enable targeted genetic engineering in rat ESCs. Another group also succeeded in gene targeting in a hypoxanthine phosphoribosyltransferase (*hprt*) locus by homologous recombination in rat ESCs [38]. Although these *hprt* heterozygous clones cultured in the 2i+LIF medium maintained pluripotency, aneuploid cells did emerge in the cultures. However, approximately 2% of geneticine-resistant colonies achieved recombination correctly. The efficiency was similar to that originally reported for mouse

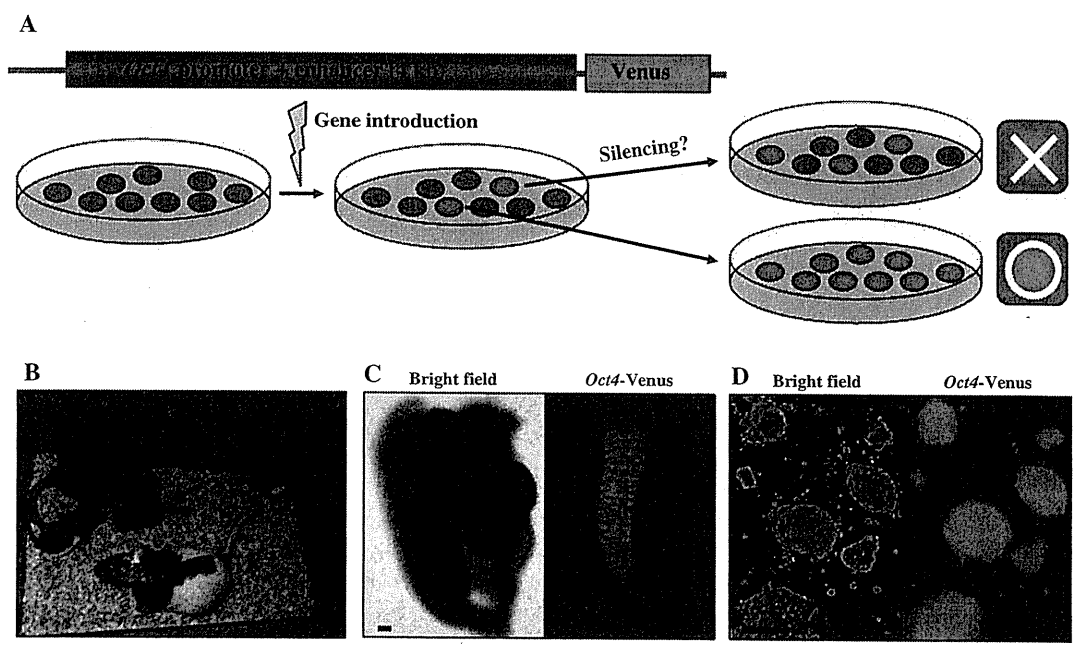

Fig. 2 Transgenesis in rat ESCs. **a** *Oct4*-Venus transgene is introduced in rat ESCs by a nucleofection method. Some clones receive random integration of the transgene, with subsequent green fluorescence. After subcloning and passaging, Venus fluorescence was decreased in a majority of the clones (*upper*), while a minority of the clones expressed the fluorescence homogeneously. **b** The *Oct4*-Venus transgenic rat (*arrow*) was produced through germline transmission of the recombinant ESCs from a chimeric rat. **c** *Oct4*-Venus positive-germ cells in E16.0 gonad of the transgenic rats. **d** An ESC line derived from the *Oct4*-Venus transgenic rat. Venus fluorescence was kept in undifferentiated cells after 18 passages. All *scale bars*, 100 μm

and human ESCs [39–41]. Thus, these reports suggest that rat ESCs are readily amenable to gene targeting by homologous recombination using the basic methodology that has proved so effective in mouse ESCs.

Discussion

Rat transgenesis via gene manipulation in ESCs was demonstrated in 2010, marking the beginning of a new era in rat genetics. Although some problems remain in the rat ESC handling, a combination of the methods described in this manuscript as well as newly devised techniques will lead to the discovery of a gold standard method to routinely generate genetically modified rats from ESCs. Recently, not only knockout but also knockin rats have been generated using ZFN-mediated homologous recombination [42]. This knockin strategy will make it possible to introduce temporal control and tissue-specific changes in genes in rat models by combining *Cre/loxP* and an inducible gene expression system. ZFN technology also possesses several advantages, such that the time frame to obtain mutant animals is short, ZFN-mediated homologous recombination in embryos does not require a selection marker, and time-consuming backcrossing is avoided [42]. However, this technology remains expensive to purchase, which is an obstacle for most researchers. In contrast, researchers can apply gene targeting by using ESCs, as is routinely done in mouse research. Therefore, ESC is also required to expand knockout rat lines. There is another advantage of using ESCs when generating transgenic rats. Useless transgenic animals are frequently generated with the conventional method. However, as described in this manuscript, we can choose ESC clones in which a transgene is correctly expressed, leading to the generation of high-quality transgenic rats [36]. Moreover, we can analyze gene function in chimeric animals by using ESCs. Recent reports have shown that this chimeric strategy is effective in identifying gene functions in vivo in terms of developing a more clinically relevant stochastic model [43]. Thus, we speculate that using both the ESC and ZFN strategies will be necessary for routine rat transgenesis.

We now have an opportunity to find new gene functions that have been concealed or questioned in mutant mice. We have accumulated genetic information and a vast amount of research data on physiology and pharmacology in rats. Thus, a combination of these studies will lead to the discovery of new and profound mechanisms of human diseases and the manufacture of medicines to cure patients. Furthermore, rats with their larger sizes make it possible to extract sufficient quantities of samples, such as blood, without killing the animals and to perform difficult surgeries, such as those in brain tissue; all of this emphasizes the advantages of gene-modified rats. We hope that researchers will create many genetically modified rats and open up a powerful new platform for the study of human diseases.

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References

1. Brook FA, Gardner RL (1997) The origin and efficient derivation of embryonic stem cells in the mouse. Proc Natl Acad Sci USA 94:5709–5712
2. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
3. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78:7634–7638
4. Thomson JA, Marshall VS (1998) Primate embryonic stem cells. Curr Top Dev Biol 38:133–165
5. Bradley A, Evans M, Kaufman MH, Robertson E (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309:255–256
6. Robertson E, Bradley A, Kuehn M, Evans M (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323:445–448
7. Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, First NL, Maeda N, Smithies O (1989) Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. Proc Natl Acad Sci USA 86:8927–8931
8. Gill TJ 3rd, Smith GJ, Wissler RW, Kunz HW (1989) The rat as an experimental animal. Science 245:269–276
9. Jacob HJ (1999) Functional genomics and rat models. Genome Res 9:1013–1016
10. Jacob HJ, Kwitek AE (2002) Rat genetics: attaching physiology and pharmacology to the genome. Nat Rev Genet 3:33–42
11. Aitman TJ, Critser JK, Cuppen E, Dominiczak A, Fernandez-Suarez XM, Flint J, Gauguier D, Geurts AM, Gould M, Harris PC, Holmdahl R, Hubner N, Izsvák Z, Jacob HJ, Kuramoto T, Kwitek AE, Marrone A, Mashimo T, Moreno C, Mullins J, Mullins L, Olsson T, Pravenec M, Riley L, Saar K, Serikawa T, Shull JD, Szpirer C, Twigger SN, Voigt B, Worley K (2008) Progress and prospects in rat genetics: a community view. Nat Genet 40:516–522
12. Jacob HJ, Lazar J, Dwinell MR, Moreno C, Geurts AM (2010) Gene targeting in the rat: advances and opportunities. Trends Genet 26:510–518
13. Brenin D, Look J, Bader M, Hübner N, Levan G, Iannaccone P (1997) Rat embryonic stem cells: a progress report. Transplant Proc 29:1761–1765
14. Buehr M, Nichols J, Stenhouse F, Mountford P, Greenhalgh CJ, Kantachuvesiri S, Brooker G, Mullins J, Smith AG (2003) Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. Biol Reprod 68:222–229
15. Demers SP, Yoo JG, Lian L, Therrien J, Smith LC (2007) Rat embryonic stem-like (ES-like) cells can contribute to extraembryonic tissues in vivo. Cloning Stem Cells 9:512–522
16. Fändrich F, Dresske B, Bader M, Schulze M (2002) Embryonic stem cells share immune-privileged features relevant for tolerance induction. J Mol Med 80:343–530
17. Vassilieva S, Guan K, Pich U, Wobus AM (2000) Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell

lines and effects of cytokines of the IL-6 family on clonal growth. Exp Cell Res 258:361–373
18. Ueda S, Kawamata M, Teratani T, Shimizu T, Tamai Y, Ogawa H, Hayashi K, Tsuda H, Ochiya T (2008) Establishment of rat embryonic stem cells and making of chimera rats. PLoS One 3:e2800
19. Zan Y, Haag JD, Chen KS, Shepel LA, Wigington D, Wang YR, Hu R, Lopez-Guajardo CC, Brose HL, Porter KI, Leonard RA, Hitt AA, Schommer SL, Elegbede AF, Gould MN (2003) Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. Nat Biotechnol 21:645–651
20. Kitada K, Ishishita S, Tosaka K, Takahashi R, Ueda M, Keng VW, Horie K, Takeda J (2007) Transposon-tagged mutagenesis in the rat. Nat Methods 4:131–133
21. Lu B, Geurts AM, Poirier C, Petit DC, Harrison W, Overbeek PA, Bishop CE (2007) Generation of rat mutants using a coat color-tagged Sleeping Beauty transposon system. Mamm Genome 18:338–346
22. Cozzi J, Anegon I, Braun V, Gross AC, Merrouche C, Cherifi Y (2009) Pronuclear DNA injection for the production of transgenic rats. Methods Mol Biol 561:73–88
23. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325:433
24. Izsvák Z, Fröhlich J, Grabundzija I, Shirley JR, Powell HM, Chapman KM, Ivics Z, Hamra FK (2010) Generating knockout rats by transposon mutagenesis in spermatogonial stem cells. Nat Methods 7:443–445
25. Mashimo T, Takizawa A, Voigt B, Yoshimi K, Hiai H, Kuramoto T, Serikawa T (2010) Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. PLoS One 5:e8870
26. Ménoret S, Iscache AL, Tesson L, Rémy S, Usal C, Osborn MJ, Cost GJ, Brüggemann M, Buelow R, Anegon I (2010) Characterization of immunoglobulin heavy chain knockout rats. Eur J Immunol 40:2932–2941
27. Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A (2008) Capture of authentic embryonic stem cells from rat blastocysts. Cell 135:1287–1298
28. Li P, Tong C, Mehrian-Shai R, Jia L, Wu N, Yan Y, Maxson RE, Schulze EN, Song H, Hsieh CL, Pera MF, Ying QL (2008) Germline competent embryonic stem cells derived from rat blastocysts. Cell 135:1299–1310
29. Kawamata M, Ochiya T (2010) Establishment of embryonic stem cells from rat blastocysts. Methods Mol Biol 597:169–177
30. Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A (2008) The ground state of embryonic stem cell self-renewal. Nature 453:519–523
31. Li W, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S (2009) Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell 4:16–19
32. Hirabayashi M, Kato M, Sanbo M, Kobayashi T, Hochi S, Nakauchi H (2010) Rat transgenesis via embryonic stem cells electroporated with the Kusabira-orange gene. Mol Reprod Dev 77:474
33. Takahama Y, Ochiya T, Sasaki H, Baba-Toriyama H, Konishi H, Nakano H, Terada M (1998) Molecular cloning and functional analysis of cDNA encoding a rat leukemia inhibitory factor: towards generation of pluripotent rat embryonic stem cells. Oncogene 16:3189–3196
34. Hirabayashi M, Kato M, Kobayashi T, Sanbo M, Yagi T, Hochi S, Nakauchi H (2010) Establishment of rat embryonic stem cell lines that can participate in germline chimerae at high efficiency. Mol Reprod Dev 77:94
35. Kobayashi T, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Ibata M, Sato H, Lee YS, Usui J, Knisely AS, Hirabayashi M, Nakauchi H (2010) Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. Cell 142:787–799
36. Kawamata M, Ochiya T (2010) Generation of genetically modified rats from embryonic stem cells. Proc Natl Acad Sci USA 107:14223–14228
37. Tong C, Li P, Wu NL, Yan Y, Ying QL (2010) Production of p53 gene knockout rats by homologous recombination in embryonic stem cells. Nature 467:211–213
38. Meek S, Buehr M, Sutherland L, Thomson A, Mullins JJ, Smith AJ, Burdon T (2010) Efficient gene targeting by homologous recombination in rat embryonic stem cells. PLoS One 5:e14225
39. Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51:503–512
40. Doetschman T, Maeda N, Smithies O (1988) Targeted mutation of the Hprt gene in mouse embryonic stem cells. Proc Natl Acad Sci USA 85:8583–8587
41. Zwaka TP, Thomson JA (2003) Homologous recombination in human embryonic stem cells. Nat Biotechnol 21:319–321
42. Cui X, Ji D, Fisher DA, Wu Y, Briner DM, Weinstein EJ (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 29:64–67
43. Zhou Y, Rideout WM 3rd, Zi T, Bressel A, Reddypalli S, Rancourt R, Woo JK, Horner JW, Chin L, Chiu MI, Bosenberg M, Jacks T, Clark SC, Depinho RA, Robinson MO, Heyer J (2010) Chimeric mouse tumor models reveal differences in pathway activation between ERBB family- and KRAS-dependent lung adenocarcinomas. Nat Biotechnol 28:71–78

MicroRNA-143 Regulates Human Osteosarcoma Metastasis by Regulating Matrix Metalloprotease-13 Expression

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Pulmonary metastases are the main cause of death in patients with osteosarcoma, however, the molecular mechanisms of metastasis are not well understood. To detect lung metastasis-related microRNA (miRNA) in human osteosarcoma, we compared parental (HOS) and its subclone (143B) human osteosarcoma cell lines showing lung metastasis in a mouse model. miR-143 was the most downregulated miRNA ($P < 0.01$), and transfection of miR-143 into 143B significantly decreased its invasiveness, but not cell proliferation. Noninvasive optical imaging technologies revealed that intravenous injection of miR-143, but not negative control miRNA, significantly suppressed lung metastasis of 143B ($P < 0.01$). To search for miR-143 target mRNA in 143B, microarray analyses were performed using an independent RNA pool extracted by two different comprehensive miR-143-target mRNA collecting systems. Western blot analyses revealed that MMP-13 was mostly protein downregulated by miR-143. Immunohistochemistry using clinical samples clearly revealed MMP-13-positive cells in lung metastasis-positive cases, but not in at least three cases showing higher miR-143 expression in the no metastasis group. Taken together, these data indicated that the downregulation of miR-143 correlates with the lung metastasis of human osteosarcoma cells by promoting cellular invasion, probably via MMP-13 upregulation, suggesting that miRNA could be used to develop new molecular targets for osteosarcoma metastasis.

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INTRODUCTION

Osteosarcoma is the most common primary bone malignancy and accounts for 60% of all malignant childhood bone tumors.[1] The age distribution is bimodal: the first major peak occurring during the second decade of life, and the second much smaller peak being observed in patients over 50 years of age. The distal femoral and proximal tibial metaphyses are the most common sites for osteosarcoma. Approximately 50% of cases are localized in the knee region.[2] With combined treatment (neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy), the 5-year survival of patients with no metastatic disease at diagnosis is 60–70%;[3–5] however, for patients who present with metastatic disease, the outcome is far worse at <30% survival.[6] Pulmonary metastasis is the predominant site of osteosarcoma recurrence and the most common cause of death. Unfortunately, survival has not improved for 20 years despite multiple clinical trials with increased intensity, and further gains with refinements of cytotoxic chemotherapy regimens alone are unlikely; therefore, for better prognosis, new therapeutic targets and approaches must be sought to suppress pulmonary metastasis of osteosarcoma.

MicroRNA (miRNA) belongs to a class of endogenously expressed, non-coding small RNA and contains about 22 nucleotides. Based on miRBase release 16.0, >1,000 human miRNA have been registered with a large number being evolutionarily conserved.[7] It has been shown that miRNA can regulate the expression of protein-coding genes at the post-transcriptional level through imperfect base pairing with the 3′-untranslated region (3′-UTR) of target mRNA.[8] miRNA is predicted to regulate the expression of at least 30% of all genes.[9] Growing evidence suggests that deregulation of miRNA may contribute to many types of human diseases, including cancer. Errors in the expression of miRNA have been observed in various types of cancers[10,11] and are also associated with the clinical outcome of cancer patients.[12,13] Consistently, miRNA has been implicated in the regulation of various cellular processes that are often deregulated during tumor development and progression,[8,14–17] suggesting that miRNA might be a target for cancer therapy.

The most direct way for molecules to correct altered miRNA expression is by treatment with RNA oligonucleotides. Therapeutic potentials using RNA oligonucleotides have been proposed, although our understanding of the role of miRNA in cancer is still very limited. There are two possible approaches: blocking oncogenic

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miRNA by anti-miRNA oligonucleotides or replacement of miRNA with tumor suppressor activity by miRNA mimetics. In fact, *in vitro* studies have revealed that anti-miR-17-5p treatment halts the growth of a human neuroblastoma cell line, LAN-5, overexpressing miR-17-5p.[18] Si *et al.* also reported that anti-miR-21 inhibited cell growth via increased apoptosis and decreased cell proliferation, which could partly be due to the downregulation of antiapoptotic Bcl-2 in a human breast cancer cell line, MCF-7.[19] Recently, Ma *et al.* reported that systemic administration of miR-10b antagomir inhibited lung metastasis of mouse breast cancer cells in a mouse model.[20] On the other hand, it has been reported that cell proliferation or invasion was suppressed by miRNA mimetics transfection into human cancer cells. For example, the introduction of synthesized miR-143 or miR-145 into a human B-cell lymphoma cell line, Raji, resulted in significant growth inhibition that occurred in a dose-dependent manner.[21] Crawford *et al.* reported that treatment with miR-126 decreased adhesion, migration, and the invasion of a human nonsmall-cell lung carcinoma cell line, H1703.[22] Valastyan *et al.* found that overexpression of miR-31 independently inhibited the invasive capacity of MDA-MB-231 breast cancer cells, extravasation into or survival in the lung parenchyma, and metastatic colonization.[23] Moreover, Tazawa *et al.* demonstrated in a mouse model that direct intratumoral injection of a miR-34a/atelocollagen complex successfully suppressed the growth of tumors derived from human colon cancer cells.[24] Furthermore, significant reduction of the tumor volume was observed until day 6 after miR-34a administration. Interestingly, the authors showed that the expression of miR-34a was downregulated in more than one-third of human colon cancers compared with counterpart normal colon mucosa. Therefore, these data suggested that restoring decreased miRNA in cancer cells was able to suppress the progression of cancer *in vivo*.

Our goal is to understand the mechanisms of metastases and, based on this knowledge, identify new targets that can be used for the development of new molecular markers and therapeutic approaches to inhibit metastasis from osteosarcoma. In this study, we explored miRNA and its target mRNA associated with cell invasion of osteosarcoma cells *in vitro* using two human osteosarcoma cell lines, HOS and 143B, and aimed to clarify whether spontaneous lung metastasis from osteosarcoma could be suppressed by restoring or blocking miRNA *in vivo* using a mouse model.

RESULTS

miRNA microarray analysis and validation of the array data by real-time RT-PCR

Two human osteosarcoma cell lines, HOS and 143B, were used to discover metastasis-related miRNA candidates. The 143B line was generated by transformation of HOS via v-Ki-ras and, unlike HOS, demonstrated high tumorigenecity and spontaneous metastatic potential after orthotopic intratibial inoculation.[25] Thus, by comparing the miRNA expression patterns of these cells, it is suggested that metastasis-related miRNA is extractable. miRNA microarray analysis was performed comparing HOS and 143B cells to evaluate the miRNA profiles of each cell. It was observed that the expression of many miRNAs in the two cell lines was different. Nineteen miRNAs were significantly upregulated, whereas nine miRNAs, including miR-143, were significantly downregulated in 143B compared to HOS (**Table 1**). It was suggested that the former were metastasis-promoting miRNA and the latter were metastasis-suppressing miRNA.

By miRNA microarray analysis, miR-143 was decreased about 1/10 as compared to HOS. Based on the microarray results, we examined the expression level of miR-143 with real-time reverse transcriptase (RT)-PCR. For that purpose, RNA pooled from the same RNA samples used for the microarray experiments was prepared. Additionally, we determined *RNU6B* as a reference gene for normalization of miRNA data. The PCR result was consistent with the microarray data because miR-143 was downregulated less than one-tenth the level in 143B (**Supplementary Figure S1**).

miRNA mimic or anti-miRNA oligonucleotide transfer allows efficient inhibition of 143B-luc cell invasion, but not proliferation, *in vitro*

To screen target genes showing inhibition of invasion in 143B cells transfected with firefly luciferase gene (*143B-luc*), the nine most strongly up- and downregulated miRNAs (miRNAs above dotted line in **Table 1**) were selected for *in vitro* screening (see Materials and Methods section). For monitoring cell invasion and proliferation, we analyzed luciferase activity. As shown in **Figure 1**, inhibition of cell invasion was significantly ($P < 0.05$) observed on 143B-luc cells transfected with miR-143, followed by miR-145 (not significant). No other miRNA mimics or anti-miRNA inhibited cell invasion in 143B-luc cells. On the other hand, no miRNA

Table 1 Significantly aberrant expression of miRNAs in 143B compared to HOS

Upregulated miRNA		Downregulated miRNA	
Name	Ratio (143B/HOS)	Name	Ratio (143B/HOS)
miR-584	N.D.[a]	miR-143	0.11
miR-146a	N.D.[a]	miR-145	0.12
miR-31	>10	miR-193b	0.15
miR-100	5.09	miR-28	0.28
miR-125b	5.07	miR-149	0.55
miR-222	4.85	miR-99b	0.58
miR-221	4.62	miR-133b	N.D.[b]
miR-29b	4.59	miR-140	N.D.[b]
miR-625	4.57	miR-335	N.D.[b]
miR-29a	4.30		
miR-21	3.62		
miR-148a	3.25		
miR-34a	2.99		
miR-652	2.70		
miR-361	2.48		
miR-210	2.47		
miR-374	2.46		
miR-455	2.20		
miR-23a	2.15		

Abbreviation: miRNA, microRNA.
[a]Not determined, because expression of the miRNA in HOS could not be detected by miRNA microarray analysis. [b]Not determined, because expression of the miRNA in 143B could not be detected by miRNA microarray analysis.

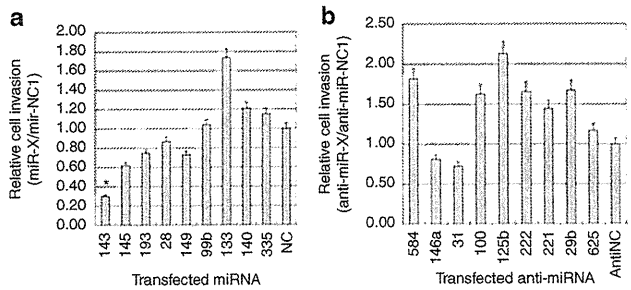

Figure 1 Matrigel invasion assay. The matrigel invasion assay was performed using a human osteosarcoma cell line (143B-luc) transfected with either synthetic (**a**) microRNA (miRNA) or (**b**) anti-miRNA, which were differentially expressed in 143B compared to HOS. Invaded cells were lysed and then analyzed for luciferase activity using the Bright-Glo Luciferase Assay System. Inhibition of luciferase production was normalized to the level of negative control pre-miR- (miR-NC1)- or anti-miR (anti-miR-NC)-transfected cells. The experiment was performed in triplicate and repeated three times. $^*P < 0.05$ versus control.

mimics or anti-miRNA used in this assay significantly affected cell proliferation (**Supplementary Figure S2**). These results revealed that miR-143 might be the miRNA with the most potential to suppress the metastasis of 143B-luc cells.

Suppression of spontaneous lung metastasis of osteosarcoma cells in mice with systemic miR-143 treatment

First, we determined the ability of 143B cells transfected with firefly luciferase gene (*143B-luc*) to develop a primary tumor and spontaneous lung metastasis in athymic mice ($n = 10$). Experimentally, 1.5×10^6 143B-luc cells were inoculated into the right knee, and we checked their location immediately after inoculation using an *in vivo* imaging system (IVIS) (**Supplementary Figure S3a**). Subsequently, we checked the inoculated animals weekly for luciferase bioluminescence by IVIS to monitor tumor growth and to visualize the presence of distant metastases in the animals. At 1 week after inoculation, primary tumors were macroscopically detectable in some mice, but no signals were detected in the pulmonary area. At 2 weeks, we observed the first sign of metastasis in some of the mice by IVIS (**Supplementary Figure S3b**). During the subsequent week, numerous metastases could be detected by IVIS. At 4 weeks after tumor cell inoculation, all animals showed signals in the pulmonary area by IVIS and they were sacrificed for histological examination. Many nodules were seen on the surface of the resected lungs (**Supplementary Figure S3c**), and they were confirmed microscopically as osteosarcoma metastatic lesions (**Supplementary Figure S3d**).

To assess the therapeutic potential of miR-143 against spontaneous lung metastasis of osteosarcoma, 50 μg miR-143 mimic or miR-negative control 1 (NC1) mixed with atelocollagen was administered intravenously into mice in each group ($n = 10$ each) at 1, 4, 7, 10, 13, 16, and 19 days after inoculation of 143B-luc cells. The development of a primary tumor and metastasis in the pulmonary area was monitored weekly by IVIS. At 1 week, the signal from firefly luciferase was detected at only the primary lesion in each group (**Figure 2a**). At 2 weeks, 4 of the 10 mice injected with miR-NC1 showed a signal from luciferase in the pulmonary area,

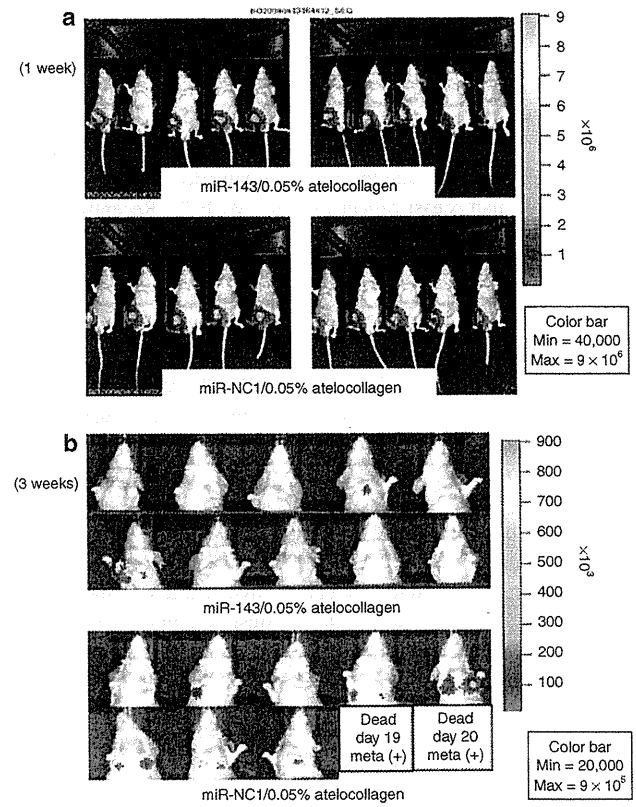

Figure 2 Inhibition of lung metastasis of osteosarcoma by systemic treatment of miR-143. All mice used in this experiment are shown. Luminescence was observed at only the right knee where 143B-luc cells were inoculated at (**a**) 1 week after inoculation. (**b**) At 3 weeks after inoculation, six of eight mice exhibited lung metastasis by *in vivo* imaging system (IVIS) and the other two mice died due to lung metastasis on day 19 and 20, respectively, in miR-NC1/atelocollagen-treated mice, whereas only 2 of the 10 mice in the miR-143/atelocollagen-treated group showed lung metastasis.

suggesting lung metastasis, but no signal was detected in the miR-143-injected mouse group. At days 19 and 20, 2 of the 10 mice in the miR-NC1 group died and lung metastasis was confirmed by autopsy. At 3 weeks, lung metastasis was detected by IVIS in 6 of the 8 live mice (miR-NC1 group). Interestingly, only 2 of the 10 mice injected with miR-143 showed lung metastasis (**Figure 2b**), a significant difference ($P < 0.01$).

To know whether the inhibitory effect of miR-143 on lung metastasis was not due to the direct inhibition of tumor growth in the primary tumor, all mice were sacrificed and the resected primary tumors were examined (**Supplementary Figure S4**). The weight (mean ± SD) of the primary tumor was 3.67 ± 0.59 g (miR-NC1-treated group) and 3.32 ± 0.65 g (miR-143-treated group), respectively, indicating that there were no differences between the two groups. Moreover, the expression of proliferative cell nuclear antigen in the primary tumor was examined by immunohistochemistry. Proliferative cell nuclear antigen-positive cells were observed in most of the tumor cells and no difference was shown between the two groups. These data suggested that miR-143 did not affect tumor growth in the primary lesion during the course of miRNA treatment.

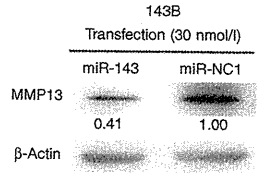

Figure 3 Downregulation of MMP-13 expression by miR-143. Western blot analyses of MMP-13 expression in 143B cells 48 hours after transient transfection of miR-143 or miR-NC1. Relative expression, quantified by Image J software is normalized to β-actin, and measured by the ratio of the indicated situation to miR-NC1.

Detection and identification of *miR-143* target genes

To elucidate metastasis-related *miR-143* target genes in 143B cells, candidate mRNA regulated by miR-143 was comprehensively collected by two different methods, anti-Ago2 antibody immunoprecipitation (Ago2 IP) and the labeled miRNA pull-down (LAMP) assay system (see Materials and Methods section). The collected RNA was validated by microarray analyses and 1,113 genes and 1,658 genes were detected, at least a 1.1-fold increase in miR-143 by Ago2 IP and LAMP, respectively. Of these, 78 genes were commonly detected by both methods (**Supplementary Table S1**). Furthermore, candidate target genes were selected using two criteria: (i) genes that were included in at least one of three publicly available databases, TargetScanHuman 5.1, PicTar, and miranda (September 2008 release) as miR-143 target genes, or (ii) genes that were involved in cell invasion or migration. Six genes met at least one of the two requirements (**Supplementary Table S2**). Western blot analyses revealed that the expression of MMP-13 was suppressed most in the six genes (**Figure 3** and **Supplementary Figure S5**).

Expression of miR-143 and MMP-13 in clinical samples

Finally, we evaluated the expression of miR-143 in human primary osteosarcoma in order to examine whether miR-143 expression there correlated with metastasis. Twenty-two biopsy samples of primary osteosarcoma without any metastases at first diagnosis were analyzed for the expression level of miR-143 by real-time RT-PCR. Seven of the 22 cases showed lung metastasis after resection of the primary tumor, and the other cases (n = 15) showed no metastasis for at least >1 year after the operation. The miR-143 expression data were normalized to the mean of miR-103, which was found to be among the most stably expressed miRNA in human tumor tissues.[26] Three of the fifteen lung metastasis-negative cases showed an extremely higher expression of miR-143, whereas this expression was low in the seven cases that had lung metastasis after the operation (**Figure 4**). The relative expressions of miR-143 were 0.61 ± 0.12 (lung metastasis-positive group) and 1.23 ± 0.43 (no metastasis group). These data suggested that a lower expression of miR-143 in osteosarcoma might tend to occur in lung metastasis, although the difference was not statistically significant between these groups (P = 0.19). MMP-13 expression was evaluated by immunohistochemistry. Five of the seven lung metastasis-positive cases and fourteen of the fifteen lung metastasis-negative cases were available for immunohistochemical examination. As shown in **Figure 4**, MMP-13-positive tumor cells were studded in all of

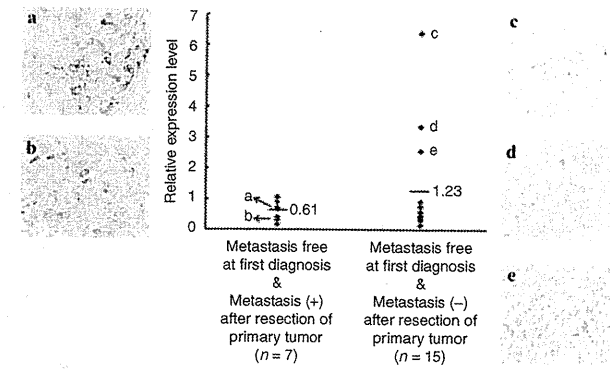

Figure 4 Expression of miR-143 in primary osteosarcoma tissue samples. Twenty-two primary osteosarcoma specimens were divided into two groups: metastasis-positive cases after resection of primary tumor (n = 7, left) and metastasis-free cases at least 1 year after resection of primary tumor (n = 15, right). miR-143 was measured by real-time reverse transcription (RT)-PCR. Individual data are the mean of triplicate measurements from single RNA samples. The expression level of miR-143 is normalized to miR-103. P-values were calculated using Welch's t-test. The mean value for each data set is shown as a horizontal line. MMP-13-positive tumor cells appeared in cases in the lung metastasis-positive group and showed (**a**,**b**) lower miR-143 expression. (**c**–**e**) On the other hand, no positive tumor cells were observed in three cases showing higher miR-143 expression in the lung metastasis-negative group. Each case (**a**–**e**) of dot data is consistent with the case indicated by immunohistochemistry data, respectively.

the five lung metastasis-positive cases (**Figure 4a**,**b**), whereas only five of the fourteen lung metastasis-negative cases showed MMP-13-positive cells. In other words, expression of MMP-13 in tumor cells was extremely low in 9 of the 14 lung metastasis-negative cases; in particular, no positive tumor cells were observed in three cases showing a higher expression of miR-143 (**Figure 4c–e**).

DISCUSSION

Altered expression of miRNA has recently been reported to impact human carcinogenesis and cancer progression.[10,11,27] In the present study, we found differentially expressed miRNA by comparing 143B and HOS cells, which resemble each other genetically, but showed different phenotypes of metastasis *in vivo*: inoculation of 143B cells, but not HOS cells, into a knee joint led to spontaneous lung metastasis in the athymic mice used in this study, as well as in a previous report.[25] It was considered that metastasis-promoting miRNA was upregulated and/or metastasis-suppressor miRNA was downregulated in 143B cells. Of these miRNAs, we found miR-143 to be the most downregulated miRNA in 143B cells by miRNA microarray analysis. Because cell invasion was inhibited by restoring miR-143 in 143B cells, we injected miR-143 with atelocollagen into spontaneous lung metastasis of an osteosarcoma mouse model to evaluate whether this miRNA could suppress lung metastasis from a primary tumor. Atelocollagen is a biomaterial with potential for use as a carrier for gene delivery.[28] We previously reported that a human enhancer of zeste homolog 2 (EZH2) and human phosphoinositide 3′-hydroxykinase p110α subunit (p110α) small interfering RNA–atelocollagen complexes administered intravenously into mice having a bone metastatic lesion of human prostate cancer markedly suppressed tumor growth in the lesion with no side effect caused by the atelocollagen.[29]

Recently, we also reported that systemic administration of miR-16 with atelocollagen successfully regressed prostate cancer in a bone metastatic lesion in a mouse model.[30] Thus, for prevention of lung metastasis from osteosarcoma, a new atelocollagen-mediated systemic delivery method could be a reliable and safe approach to achieve maximal function of miRNA *in vivo*, as well as small interfering RNA. In the present study, systemic administration of miR-143 with atelocollagen surprisingly suppressed lung metastasis in a spontaneous lung metastatic mouse model. On the other hand, treatment with miR-143 did not affect tumor growth in a primary lesion in an *in vivo* model. These data are consistent with *in vitro* data demonstrating that miR-143 transfection into 143B cells could suppress cell invasion but not cell proliferation, suggesting that miR-143 might specifically regulate the invasion and/or migration signal pathway(s) of osteosarcoma cells.

New approaches that can complement and improve on current strategies for the prediction of prognosis are urgently needed. Many independent studies on different tissues have demonstrated that miRNA expression patterns correlated with the prognosis of cancer patients,[17,31] which generally depends upon the occurrence of metastasis, because ~90% of deaths from solid tumors are caused by metastasis. Therefore, the expression of miRNA that regulate cell adhesion, migration and invasion could be a good diagnostic marker to predict cancer prognosis. Ma *et al.* reported that miR-10b is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion,[32] and they also demonstrated that systemic administration of miR-10b antagomir inhibited lung metastasis of mouse breast cancer cells in a mouse model.[20] Overexpression of miR-10b in otherwise nonmetastatic breast tumors initiates robust invasion and metastasis. Expression of miR-10b is induced by the transcription factor Twist, which is an epithelial-mesenchymal transition factor and is known to bind directly to the putative promoter of miR-10b. The miR-10b induced by twist inhibits translation of the mRNA encoding homeobox D10, resulting in increased expression of a well-characterized prometastatic gene, *RhoC*. Significantly, the level of miR-10b expression in primary breast carcinomas correlates with clinical progression. On the other hand, Tavazoie *et al.* showed that restoring the expression of miR-335 in a human breast cancer cell line MDA-MB-231 inhibited metastatic cell invasion.[33] miR-335 suppresses metastasis and migration through targeting of the progenitor cell transcription factor SOX4 and extracellular matrix component tenascin C. Moreover, the expression of miR-335 is downregulated in the majority of primary breast tumors from patients who relapse, and hence loss of the expression of miR-335 is associated with poor distal metastasis-free survival. These reports suggested that altered expression of metastasis-associated miRNA could be used for the prediction of prognosis. In the present study, expression analysis of miR-143 using clinical osteosarcoma samples showed that the mean of the expression level was two times higher in metastasis-free cases than in metastasis-positive cases. However, no statistical significance was shown, which might be because only three cases that showed a higher expression of miR-143 raised the mean in metastasis-free cases. In other words, however, it could be considered that osteosarcoma in which a relatively higher level of miR-143 is expressed might be considered a low risk for metastasis. It is suggested that the expression level of miR-143 at a primary osteosarcoma lesion might be a prognostic marker for lung metastasis.

It has been reported that reduced expression of tumor-suppressor miRNA was caused by chromosome deletions, epigenetical changes, abberant transcription, and disturbances in miRNA processing. Suzuki *et al.* reported that P53 enhances the post-transcriptional maturation of several miRNAs, including miR-143. P53 interacts with Drosha processing complex through association with DEAD-box RNA helicase p68. Thus, wild-type P53 could facilitate the processing of primary miR-143 to precursor miR-143, but mutated P53 interferes with functional assembly between Dorosha complex and P68, leading to attenuation of miR-143 processing activity in HCT116, a human colon cancer cell line.[34] Another study showed that upregulation of KRAS leads to downregulation of miR-143 in human pancreatic cancer cell lines;[35] however, the mechanism of this downregulation has not been investigated in osteosarcoma cells in detail. Thus, further studies are needed to reveal the precise mechanism of miR-143 downregulation in 143B cells. The downregulation of miR-143 expression was also reported in several human cancers, e.g., colorectal cancer,[36] prostate cancer,[37] cervical cancer,[38] ovarian cancer,[39] B-cell lymphoma;[21] thus, it is considered that miR-143 is a tumor-suppressor miRNA. In these cancers, downregulation of miR-143 resulted in the promotion of cell proliferation or inhibition of apoptosis, indicating that miR-143 acts as a suppressor on cell proliferation and viability. Akao *et al.* reported that the target gene of miR-143 was determined to be ERK5/MAPK7, the upregulation of which leads to cell growth via activation of c-Myc, in Raji cells, a human B-cell lymphoma cell line.[21] Recently, another paper showed that miR-143 suppressed cell proliferation by inhibiting KRAS translation in human colorectal cancer;[40] however, our data showed that restoring miR-143 in human osteosarcoma cell 143B could suppress cell invasion, but not cell proliferation in *in vitro* and *in vivo* studies. Also, western blotting showed that the expression levels of KRAS and ERK5 did not change by transfecting miR-143 into 143B cells (**Supplementary Figure S6**). These data suggested that miR-143 might have different targets in a cell-type-dependent manner. Additionally, Tome *et al.* reported that *in vivo* transfer of the *KRAS* gene from high-metastatic cancer cells to coimplanted low-metastatic cancer cells enhanced lung metastasis of human osteosarcoma cells,[41] indicating that KRAS is a key factor in the metastasis of osteosarcoma cells; however, our data showed that miR-143 could suppress lung metastasis of 143B cells without KRAS downregulation. Therefore, this might also indicate that miR-143-target genes play roles downstream of the KRAS-related metastasis-promoting pathway(s).

To find which targets are regulated by miR-143 in 143B cells, microarray analyses were performed after collecting target RNA by two independent comprehensive methods (Ago2 IP and LAMP), respectively. By extracting the common genes after the two different collection methods, 78 common genes were identified in >1,000 genes. Of those, six genes matched the requirements of (i) predicted genes by database (Target Scan, PicTar, or miRanda) or (ii) genes related to migration, invasion, or metastasis. Interestingly, protein expressions of at least four of the six genes were downregulated by miR-143 transfection in 143B cells, indicating that the miRNA-target gene detection system in the