Loss of SNAIL regulated miR-128-2 on chromosome 3p22.3 targets multiple stem cell factors to promote transformation of mammary epithelial cells

Peng-Xu Qian\textsuperscript{1}, Arindam Banerjee\textsuperscript{2}, Zheng-Sheng Wu\textsuperscript{1,3,4}, Xiao Zhang\textsuperscript{1}, Hong Wang\textsuperscript{1}, Vijay Pandey\textsuperscript{5}, Wei-Jie Zhang\textsuperscript{1}, Xue-Fei Lv\textsuperscript{1}, Sheng Tan\textsuperscript{1}, Peter E. Lobie\textsuperscript{5,*}, and Tao Zhu\textsuperscript{1,*}.

\textsuperscript{1}Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, P.R. China;
\textsuperscript{2}Liggins Institute, University of Auckland, Auckland, New Zealand;
\textsuperscript{3}Department of Pathology, Anhui Medical University, Hefei, Anhui, P.R China;
\textsuperscript{4}Department of Pathology, Shanghai Medical College, Fudan University, Shanghai, P.R China;
\textsuperscript{5}Cancer Science Institute of Singapore and Department of Pharmacology, National University of Singapore, Singapore;

*Correspondence to: Tao Zhu, MD, Ph.D., Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People’s Republic of China. Tel: +86 (551) 3602461, Fax: +86 (551) 3601505, E-mail: zhut@ustc.edu.cn. Or Peter E Lobie, MD, PhD, Cancer Science Institute of Singapore, National University of Singapore, Centre for Life Sciences, #03-06C, 28 Medical Drive, Singapore 117456. E-mail: csipel@nus.edu.sg

Running title: miR-128-2 as a tumor suppressor in breast cancer

Keywords: miR-128, chromosome 3p, mammary carcinoma, SNAIL, stem cell factor

Author contributions

Abbreviations
TSG, tumor suppressor gene; miRNAs, microRNAs; LOH, loss of heterozygosity;
3’UTR, 3’untranslated regions; ASO, antisense oligonucleotide; qRT-PCR, quantitative real-time polymerase chain reaction; DEAB, diethylaminobenzaldehyde; FFPE, formalin-fixed paraffin-embedded; OS, overall survival; RFS, relapse-free survival; ISH, in situ hybridization; H&E, Haematoxylin and eosin; IHC, immunohistochemistry; TUNEL, Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling; ChIP, chromatin immunoprecipitation; TGF-β, transforming growth factor-β; EMT, epithelial-to-mesenchymal transition; ALDH1, aldehyde dehydrogenase.

Disclosure statement: The authors declare no conflict of interest.

Word count: Abstract: 218; Manuscript (excluding abstract, references and figure legends): 4991
Abstract

A discontinuous pattern of LOH at chromosome 3p has been reported in 87% of primary breast cancers. Despite the identification of several tumor suppressor genes in this region, there has yet to be a detailed analysis of non-coding RNAs including microRNAs (miRNAs) in this region. In this study, we identified 16 aberrant miRNAs in this region and determined several that are frequently lost or amplified in breast cancer. miR-128-2 was the most commonly deleted miRNA. Embedded in the intron of the ARPP21 gene at chromosome 3p22.3, miR-128-2 was frequently downregulated along with ARPP21 in breast cancer where it was negatively associated with clinicopathologic characteristics and survival outcome. Enforced expression of miR-128 impeded several oncogenic traits of mammary carcinoma cells, whereas depleting miR-128-2 expression was sufficient for oncogenic transformation and stem cell-like behaviors in immortalized non-tumorigenic mammary epithelial cells, both in vitro and in vivo. miR-128-2 silencing enabled transforming capacity partly by derepressing a cohort of direct targets (BMI1, CSF1, KLF4, LIN28A, NANOG and SNAIL) which together acted to stimulate the PI3K/AKT and JAK2/STAT3 signaling pathways. We also found that miR-128-2 was directly downregulated by SNAIL and repressed by TGF-β signaling, adding two additional negative feedback loops to this network. In summary, we have identified a novel TGF-β/SNAIL/miR-128 axis that provides a new avenue to understand the basis for oncogenic transformation of mammary epithelial cells.
Introduction

Mammary epithelial oncogenic transformation and progression is a multistep process that purportedly involves varied genomic alterations and epigenetic modifications (1). These aberrations activate oncogenes, inactivate tumor suppressor genes, and fundamentally convert a normal cell into a symptomatic cancer cell clone, which is rendered with growth advantage and expands in an unregulated manner, leading to local invasion and distant metastasis (2). In the past four decades, significant progress has been made in the discovery of chromosomal aberrations in human breast cancer. In familial breast cancer, BRCA1 at 17q21 and BRCA2 at 13q12–13 have been isolated as breast cancer susceptibility genes, and their mutations account for the majority of hereditary breast cancer (3). LOH has been discovered on chromosomes 3p, 6q, 7p, 11q, 16q, and 17p in breast cancer, indicating TSGs might harbor in these regions (4). Of special interest, 87% of primary breast cancers exhibit a discontinuous pattern of LOH at chromosomes 3p and several genes in this region have been identified as TSGs, such as DUTT1 and FHIT (5). However, although large-scale genomic and molecular genetic technologies allow us to efficiently identify numerous breast cancer-related protein-coding genes in the abnormal chromosomal regions, detailed analyses of non-coding RNAs locating in these regions are largely not determined.

miRNAs are small, endogenous non-coding RNAs consisting of 21-24 nucleotides, which repress gene expression post-transcriptionally via recognizing complementary target sites in the 3’UTRs of cognate mRNAs (6). Accumulating evidence has revealed that miRNAs are involved in the regulation of varied biological processes and their dysregulation is associated with various human diseases, including cancer (7). It has been
estimated that more than half of known human miRNAs reside in, or close to, fragile chromosomal sites that are susceptible to deletion, amplification, or translocation in the establishment and progression of tumor (8). Moreover, systematic miRNA expression profilings have observed that characteristic miRNA signatures are associated with prognosis and progression of certain human cancers (7, 9).

Our recent study (10) observed significantly diminished expression of let-7g, embedded in the WDR82 gene on chromosome 3p21.1, in breast cancer compared with normal tissue. Previous studies have consistently reported that LOH is frequently discovered in primary breast cancers at the chromosome 3p allele, especially at 3p21.3, 3p22-24, 3p21.2-3p21.3, 3p25, 3p14.2, 3p14.3, and 3p12 (5, 11-12). For these reasons, we undertook to uncover specific miRNAs in these aberrant regions, with the hope that these breakpoint-associated miRNAs might provide insights into the causal mechanisms of breast cancer development.
Materials and Methods

Cell cultures. All the human breast cancer cell lines and non-tumorigenic human breast epithelial cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD) and cultured in conditions as ATCC recommended.

Cell function assays. All functional assays such as total cell number, soft agar colony formation assay, growth on two-dimensional or in three-dimensional matrigel, in vitro cell motility (migration, invasion and wound healing) assays were carried out as described (13). For monolayer cell proliferation, 3000 cells were seeded into 100 µl media (2% FBS) and viability was quantified every 24 hours. Colony scattering assays were performed as previously reported (14). All the images were taken under a microscope (Olympus, Tokyo, Japan).

Protein extraction and western blot analysis, RNA extraction and quantitative reverse transcription-PCR (qRT-PCR). These procedures were performed as described previously (10). The sequences of primers used in the real-time PCR experiments and the characteristics of the antibodies are listed in Supplementary Table S7, and S8, respectively.
Results

Aberrant miRNAs on chromosomal 3p in human mammary carcinoma.

In order to screen for miRNAs within the aberrant regions at the chromosome 3p allele, especially at 3p21.3, 3p22-24, 3p21.2-3p21.3, 3p25, 3p14.2, 3p14.3, and 3p12, we initially searched the miRbase 18.0 database (15) on the basis of their precise locations on the human chromosome and identified 34 presently known miRNAs (Supplementary Table S1). We then performed genomic real-time PCR to determine changes in DNA copy numbers in 20 archived breast cancer specimens compared with 4 breast tissue specimens from patients with benign breast diseases (normal) (Supplementary Fig. S1A-F). As summarized in Fig. 1A, we uncovered 11 novel miRNAs with genome copy losses (beyond 20% of the tested cases) in breast cancer. Intriguingly, we also detected 5 miRNAs with obvious genome copy gains. Among the 11 frequently lost miRNAs, we identified miR-128-2 as the most frequently lost miRNA (deleted in 75% cases; Fig. 1A), even higher than let-7g (60% deletion rate). This observation attracted our interest to determine the specific functional roles and regulatory mechanisms of miR-128-2.

miR-128-2 is frequently down-regulated together with ARPP21 in human breast cancer and is associated with poor survival outcome.

miR-128-2 is an intronic gene, which resides within the 17th intron of the ARPP21 gene on human chromosome 3p22.3 (Fig. 1B). The 21-kDa cAMP-regulated phosphoprotein (ARPP21), also known as “Regulator of Calmodulin Signaling (RCS)” which is enriched in brain, has been described as a candidate TSG and is frequently deleted in mammary carcinoma (16). We first detected the expression of ARPP21 using
qRT-PCR and observed a significant reduction in breast cancer (n=21) compared with normal specimens (n=15) (Fig. 1C). To assess whether deregulation of miR-128-2 expression is due to loss of DNA copy number, we next examined the expression levels of pri-miR-128-2 and mature miR-128. We found that both pri-miR-128-2 and mature miR-128 were remarkably down-regulated (Fig. 1D, E), and their expressions were significantly concordant with that of the host gene ARPP21 (Supplementary Fig. S1G, H), indicating that miR-128-2 is often co-expressed with ARPP21.

To further explore the clinicopathologic implications of miR-128 in the progression of breast cancer, we determined the specific abundance of mature miRNA-128 in breast cancer specimens (n=85) and normal breast tissue specimens (n=18) by in situ hybridization (ISH). We observed significantly decreased expression of miR-128 in mammary carcinoma compared to normal specimens ($P=0.039$, Fig. 1F, G). In normal mammary tissue miR-128 was predominantly localized to carcinoma cells with limited expression in stroma. Moreover, we further analyzed the relationships between miR-128 expression and clinicopathologic characteristics of breast cancer and the results are summarized in Supplementary Table S2. A statistically significant association was observed between lower expression of miR-128 and clinical stage ($P=0.036$), lymph node metastasis ($P=0.020$) and Ki-67 labeling index ($P=0.027$).

We next examined the relationship between the miR-128 expression and survival in the breast cancer patient cohort with 5-year follow-up (n=85) using Kaplan–Meier survival analyses. We found that patients with lower expression levels of miR-128 exhibited significantly worse survival outcomes compared to those with higher expression levels of miR-128 (Figure. 1H, I, Supplementary Table S3). Thus, these
results demonstrated that miR-128-2 is frequently down-regulated together with \textit{ARPP21} in human breast cancer and the expression level of miR-128 may be a potential prognostic indicator for patient survival.

\textbf{Forced miR-128 expression reduces oncogenicity, migration and invasion of mammary carcinoma cells in vitro.}

To determine the functionality of miR-128 in breast cancer cells, we first performed a preliminary screen for expression levels of \textit{ARPP21}, \textit{pri-miR-128-2} and mature miR-128 in two mammary epithelial cell lines and eight mammary carcinoma cell lines by qRT-PCR (Fig. 2A, and Supplementary Fig. S2A). We observed remarkably higher expression levels of \textit{ARPP21}, \textit{pri-miR-128-2} and mature miR-128 in two immortalized but otherwise normal HMECs, in contrast to lower or undetectable expression levels in mammary carcinoma cell lines. In addition, we observed a strong correlation between the expression level of mature miR-128 and \textit{ARPP21} (Pearson Coefficient=0.8749) and a further stronger correlation between the expression level of \textit{pri-miR-128-2} and \textit{ARPP21} (Pearson Coefficient=0.9689) (Supplementary Fig. S2B, C).

We next assessed the potential effect of miR-128 on the phenotype of the MDA-MB-231 cells by transfecting a synthetic miRNA mimic (Fig. 2B). Forced miR-128 expression decreased both the total cell number and colony formation number in soft agar by approximately 60% compared with cells transfected with scrambled control oligonucleotides (Fig. 2B). Smaller colonies and a 2-fold decrease in branching colonies were consistently observed with MDA-MB-231 miR-128 cells cultured in three-dimensional matrigel (Figure 2C). In addition, forced expression of miR-128 in MDA-
MB-231 cells produced a “cobble stone” like epithelial cellular morphology in monolayer adherent culture as compared to the control cells (Figure 2D). In colony-scattering assays, we observed a significantly larger proportion of compact cells (3-fold) and smaller proportion of scattered cells (3-fold) with forced expression of miR-128 in MDA-MB-231 cells (Figure 2E). Finally, forced expression of miR-128 in MDA-MB-231 cells led to a retarded wound closing (Fig. 2F) and reduction in cell migration by 3-fold and invasion by 2.5-fold compared with the control cells (Fig. 2G).

Conversely, miR-128 depletion in mammary epithelial MCF-10A cells by transfection of specific ASO (Supplementary Fig. S2D) significantly increased both the total cell number and colony formation number in soft agar compared with cells transfected with control oligonucleotides (Supplementary Fig. S2D). Moreover, miR-128 depletion in MCF-10A cells increased the number of branching colonies by three fold in matrigel (Supplementary Figure S2F). Furthermore, MCF-10A cells with reduced expression of miR-128 exhibited a spindle like and a more scattered, elongated and mesenchymal cellular morphology in monolayer adherent culture compared with the control cells (Supplementary Figure S2E). In colony-scattering assays, we observed a 4-fold larger proportion of scattered cells and 2-fold smaller proportion of compact cells in MCF-10A cells with diminished expression of miR-128 (Supplementary Figure S2G). Finally, miR-128 depletion in MCF-10A cells resulted in a more rapid wound closing (Supplementary Fig. S2H), and an obvious augmentation in cell migration by 2-fold and invasion by 2.5-fold compared with the control cells (Supplementary Fig. S2I).

To determine whether miR-128 impacts on EMT, we examined the expression of epithelial and mesenchymal markers by western blot. MDA-MB-231 cells transfected
with miR-128 expressed higher levels of the epithelial markers, E-cadherin and plakoglobin (γ-catenin) and lower levels of the mesenchymal markers, FN1 and vimentin. Conversely, MCF-10A cells transfected with miR-128 ASO expressed lower levels of epithelial markers and higher levels of mesenchymal markers (Supplementary Fig. S2J).

To exclude cell type specific effects, the parallel experimental approaches were deployed in MCF-7 with forced expression of miR-128 and conversely with HBL-100 with reduced expression of miR-128. We observed concordant effects in the different two cell lines (Supplementary Fig. S3A-L). Besides, we utilized TERT immortalized HMEC cells as another model of normal immortalized mammary epithelial cells (17). We infected TERT-HMEC cells with pBabe-miR-128 sponge or control retroviruses and performed colony formation assay. We observed larger colonies and a 3-fold increase in colony number of TERT-HMEC cells transfected with pBabe-miR-128 sponge compared with control cells (Supplementary Fig. S3M). Thus, these data indicate that forced expression of miR-128 dramatically alters the morphology and impairs oncogenicity and invasiveness of mammary carcinoma cells in vitro.

**Forced miR-128 expression impairs mammary carcinoma xenograft growth *in vivo.*

To further determine the function of miR-128 in mammary carcinoma cells *in vivo,* we constructed one retroviral vector expressing miR-128 by harboring pri-miR-128-2 which was transduced in MDA-MB-231 cells, and the another vector inhibiting miR-128 expression by inserting a cassette of bulged miR-128 sequences as described (18) which was transduced in non-tumorigenic MCF-10A. The two stable cell lines, denoted MDA-MB-231 pri-miR-128-2 exhibited elevated miR-128 expression by 2.8-fold and MCF-
10A miR-128 sponge reduced miR-128 expression by 4-fold, respectively (Fig. 3A).

We next injected $5 \times 10^6$ MDA-MB-231 pri-miR-128-2 cells and MDA-MB-231 control cells subcutaneously into the flanks of female immuno-deficient nude mice. Both groups formed palpable and measurable tumors, however, with a 5.2-fold increase of miR-128 expression, tumors formed by MDA-MB-231 pri-miR-128-2 were smaller than those formed by control cells (Figure 3B, C). Tumors formed by MDA-MB-231 pri-miR-128-2 cells were well confined and differentiated (upper right panel, Figure 3D), whereas those formed by MDA-MB-231 control cells were poorly encapsulated and highly invasive (upper left panel, Figure 3D). Furthermore, local infiltration into muscle, and tumor emboli in blood vessels, were observed in tumors formed by control cells (upper left panel, Figure 3D). Moreover, pulmonary metastases were readily detectable in the lungs of host mice 3.5 weeks after subcutaneous injection with MDA-MB-231 control cells. In contrast, no metastases were detected in mice injected with MDA-MB-231 pri-miR-128-2 cells (Figure 3D). In addition, Ki-67 or PCNA staining and TUNEL or Caspase-3 assays were performed on tumor sections, and the MDA-MB-231 pri-miR-128-2 tumors exhibited fewer Ki-67-labeled or PCNA-labeled cells (Figure 3E, Supplementary Figure S4A) and higher percentage of TUNEL-positive or Caspase-3-labeled cells (Figure 3F, Supplementary Figure S4B) compared with MDA-MB-231 control tumors.

Alternatively, $5 \times 10^6$ MCF-10A miR-128 sponge cells and MCF-10A control cells were subcutaneously inoculated into the flanks of host mice. Consistent with prior reports (19), the non-tumorigenic MCF-10A cell line is not capable of initiating tumor formation. However, MCF-10A miR-128 sponge cells readily formed visible and measurable tumors.
(Supplementary Figure S4C, D), suggesting that miR-128 depletion promoted tumor formation in vivo.

**Forced expression of miR-128 represses mammary carcinoma stem cell-like behavior.**

Dysregulated cancer stem cells have been postulated to be crucial for cancer initiation and progression (20). By extension, we wondered whether miR-128 could influence cancer stem cell-like behavior. We therefore transiently transfected miR-128 mimics into MDA-MB-231 cells and miR-128 ASO into MCF-10A cells. We then cultured these cells in conditioned mammosphere culture media in ultra-low attachment 6-well plates. MDA-MB-231 cells with forced expression of miR-128 exhibited both significantly decreased number (43%) and size of mammospheres compared to control cells (Figure 4A). Conversely, we observed a slightly yet significant increase (2.6-fold) in mammospheric growth of MCF-10A cells with miR-128 depletion (Figure 4A). To exclude the possibility that the increase in mammosphere number arose from the aggregation of quiescent cells, we performed self-renewal assays. We observed decreased numbers of secondary and tertiary mammospheres generated from MDA-MB-231 cells with increased miR-128 expression, whereas MCF-10A cells with decreased miR-128 expression exhibited increased numbers of secondary and tertiary mammospheres compared with their corresponding control cells (Figure 4B). Recent studies have identified ALDH1 enzymatic activity as a potential marker for breast cancer stem cells (21). To this end, we performed the ALDEFLUOR assay to determine whether miR-128 modulated the ALDH1 positive cell population. Forced expression of miR-128 in MDA-
MB-231 markedly reduced the percentage of ALDH1 positive cells by 3-fold whereas depletion of miR-128 expression increased the percentage of ALDH1 positive cells by approximately 3.5-fold (Figure 4C). Moreover, we have sorted MDA-MB-231-ALDH1+ and MDA-MB-231-ALDH1- populations, and observed decreased expression of miR-128, CD44 and CD133 and increased expression of CD24 in the MDA-MB-231-ALDH1+ population comparing with MDA-MB-231-ALDH1- population (Supplementary Figure S4E, F). We next determined whether miR-128 modulated the CD44+CD24neg/low population. Forced expression of miR-128 in MDA-MB-231 reduced the percentage of CD44+CD24neg/low population by 1.5-fold whereas depletion of miR-128 expression increased the percentage of CD44+CD24neg/low population by 1.3-fold (Supplementary Figure S4G).

To ascertain the influence of miR-128 on tumor-initiating capability in vivo, we subcutaneously injected MDA-MB-231-pri-miR-128-2, MCF-10A miR-128 sponge cells and their corresponding control cells at a series of limiting dilutions from $5 \times 10^5$ to $5 \times 10^3$ cells into nude mice and identified their ability to form visible tumors. After an incubation period of 6 weeks, as summarized in Fig. 4D, in mice injected with $5 \times 10^4$ cells, the tumor incidence derived from MDA-MB-231 pri-miR-128-2 cells (4/6) was lower than control cells (6/6). Strikingly, in mice injected with $5 \times 10^3$ cells, 4/6 mice inoculated with MDA-MB-231 control cells formed tumors, whereas no tumor was detected in mice inoculated with MDA-MB-231 pri-miR-128-2 cells. Conversely, no tumors were observed in mice injected with any numbers of MCF-10A control cells or $5 \times 10^3$ MCF-10A miR-128 sponge cells. However, tumors were formed in mice injected with $5 \times 10^5$ MCF-10A miR-128 sponge cells (2/6) and $5 \times 10^4$ MCF-10A miR-128 sponge
cells (1/6), suggesting that depletion of miR-128 expression confers normal mammary epithelial MCF-10A cell with tumor-initiating capacity. Collectively, these observations indicate that miR-128 dramatically interfere with mammary carcinoma stem cell-like behavior, both in vitro and in vivo.

To determine a potential association between miR-128 and ALDH1 expression in patients with breast cancer, we performed qRT-PCR on a cohort of 24 breast cancer specimens to determine the expression levels of miR-128 and ALDH1 mRNA. We observed that the expression of miR-128 was inversely associated with ALDH1 mRNA (Pearson coefficient=-0.4065) (Figure 4E). Additionally, we performed IHC staining on a cohort of 47 breast cancer specimens to determine the expression levels of ALDH1. Combined with the expression levels of miR-128 by ISH, we observed that the expression of miR-128 was inversely and significantly associated with ALDH1 expression (Pearson coefficient=-0.327, \( P=0.025 \)).

**miR-128 directly targets a cohort of stem cell factors.**

To determine the mechanism by which miR-128 exerts tumor suppressor properties, we initially employed three algorithms to predict the potential mRNA targets of miR-128—MiRanda (22), Pictar (23) and TargetScan (6). Based on the sequences in their 3’ UTRs, 98 mRNAs were predicted to be targets by all of the three programs (Fig. 5A, Supplementary Table S4). To further narrow the possible downstream effectors of miR-128, we next screened for the mRNA expression profile of an array of genes which have been widely known in cancer initiation and progression by qRT-PCR in two sets of paired cells (MDA-MB-231 cells transfected with either miR-128 mimics or control and MCF-
10A cells transfected with either miR-128 ASO or scrambled sequence control). Interestingly, as shown in the heat map (Fig. 5B), the data derived from the two complementary screening systems were highly concordant, and indicated that miR-128 modulates numerous key genes functionally involved in neoplastic progression (Supplementary Table S5). By combination of the two strategies, we identified a cohort of six genes (*BMI1, CSF1, KLF4, LIN28A, NANOG and SNAIL*), all of which are known transcriptional regulators implicated in stem cell development (24) and contain 3’UTR elements that are partially complementary to miR-128 (Supplementary Fig. S5A-G).

To confirm whether the six candidate genes are direct targets of miR-128, we cloned full-length fragments of their 3’UTRs into the luciferase reporter plasmid psi-CHECK2. We then transfected the miR-128 mimic with different 3’UTR constructs into MDA-MB-231 cells and observed that miR-128 markedly repressed the relative luciferase activities of the wild-type 3’UTR of *BMI1, CSF1, KLF4, LIN28A, NANOG and SNAIL* (Fig. 5C). Furthermore, mutation of the partially complementary miR-128 site(s) in these 3’UTRs abrogated responsiveness to miR-128 (Figure 5C). Additionally, we measured the effects of miR-128 on expression levels of the six targets. Forced expression of miR-128 sharply reduced the protein levels of all six targets by 60-90%, whereas inhibition of miR-128 resulted in an obvious increase by 4-fold to 10-fold (Figure 5D). To further ascertain whether miR-128 represses these six targets *in vivo*, we examined their expression by IHC staining on formalin-fixed paraffin-embedded sections derived from tumors formed in the xenograft studies. The six proteins were expressed at low, almost undetectable, levels in tumors derived from MDA-MB-231 pri-miR-128-2 cells compared with their control counterparts, respectively (Figure 5E). Thus, *BMI1,*
CSF1, KLF4, LIN28A, NANOG and SNAIL are all bona fide targets of miR-128.

miR-128 diminishes mammary carcinoma cell tumor-initiating capacity via AKT and STAT3.

Given our identification of these six stem cell factors as coordinate targets of miR-128, we reasoned there may be common signaling pathways involved in mediating the effects of miR-128. BMI1 has been reported to act as part of the polycomb repressor complex, which plays a role in stem cell renewal and malignant transformation through activation of AKT pathway (25-26). CSF1 is known as macrophage colony stimulating factor 1, which regulates progenitor cell differentiation by AKT phosphorylation (27). KLF4, LIN28A and NANOG are all well-known stem cell factors and reported to possess fundamental roles in iPS reprogramming, in which AKT and STAT3 pathways are activated to promote the induction of normal somatic cells to pluripotent stem cells (28-29). SNAIL (SNAI) is a zinc finger transcriptional repressor, which has been described to induce EMT via repression of E-Cadherin and activation of several signaling pathways, including STAT3 and AKT (30-31). Consequently, we examined the expressions levels of AKT and STAT3 by western blot. Diminished p-AKT and p-STAT3 activities were observed in MDA-MB-231-miR-128 cells, whereas elevated p-AKT and p-STAT3 activities were detected in MCF-10A cells with miR-128 depletion compared to their respective control cells (Fig. 6A). Moreover, we performed IHC staining to examine levels of p-AKT and p-STAT3 on sections of xenograft tumors. We observed decreased p-AKT and p-STAT3 in MDA-MB-231-pri-miR-128-2 cells compared with control cells (Fig. 6B), demonstrating that miR-128 deprives mammary carcinoma tumor-initiating
suppression via AKT and STAT3 phosphorylation both in vitro and in vivo.

To further demonstrate that p-AKT and p-STAT3 activities were required for the tumor-initiating capability of miR-128, MCF-10A miR-128 sponge cells were treated with either the specific AKT inhibitor IV and/or the STAT3 inhibitor □, WP1066, or specific siRNAs targeting AKT and/or STAT3. We observed markedly diminished p-AKT and p-STAT3 activities (Fig. 6C) and expression of AKT and STAT3 (Supplementary Fig. S6C) in MCF-10A miR-128 sponge cells, which subsequently abrogated miR-128 depletion–promoted tumor-initiating capacity (Fig. 6D, Fig. S6D). Thus, miR-128 modulates mammary carcinoma tumor-initiating capability via PI3K/AKT and JAK2/STAT3 pathways.

miR-128-2 is directly repressed by SNAIL and attenuated via TGF-β.

We next undertook to determine how miR-128 expression is specifically regulated. Given our observation that miR-128 was expressed at low levels in breast cancer cells and directly repressed multiple stem cell factors, we hypothesized whether any transcription factors might regulate miR-128 expression. To this end, we searched conserved transcription factor binding sites by use of rVista 2.0 (32), and identified two conserved E-box motifs, one at -991 bp (E-box 1, CACATG) and the other at -26 bp (E-box 2, CACATG) relative to the transcription start site (+1) of the human miR-128-2 stem-loop, respectively (Fig. 6E). SNAIL family (SNAIL, SLUG and SMUC), ZEB (ZEB1 and ZEB2), and the bHLH (E47 and TWIST1) families have been reported to bind to E-box sequences (CANNTG) present in the promoters of genes (30). To further determine which factors could impact on miR-128-2 expression, we constructed a 3-kb
fragment upstream of human miR-128-2 stem-loop and inserted the fragment into the luciferase reporter plasmid pGL3. We co-transfected this plasmid together with different vectors expressing SNAIL, SLUG or TWIST1. We observed reduced luciferase activity by SNAIL and a milder decrease by Slug, whereas no change was observed with TWIST1 (Supplementary Fig. S6A).

We next co-tranfected the SNAIL expressing vector with different constructs which contain 3-kb fragment upstream of human miR-128-2 stem-loop into MCF-10A cells and observed that SNAIL significantly repressed the relative luciferase activity of miR-128-2 (Fig. 6F). In addition, mutations of either of the two E-Box motifs abrogated responsiveness to SNAIL (Figure 6F), indicating that two E-Box sites are necessary and functional for SNAIL regulation of miR-128. Conversely, depletion of SNAIL by specific siRNA increased the activity of luciferase construct of miR-128-2, whereas mutations in either of the two E-Box sites abolished the increase (Supplementary Fig. S6B). We next performed ChIP assays to validate whether SNAIL controls miR-128-2 expression by binding to the two putative binding sites. With an amplicon at a distance of 2116 base pairs upstream of the human miR-128-2 stem-loop as negative control, ChIP revealed that SNAIL bound to both E-box 1 and E-box 2 (Fig. 6G). Concordantly, forced expression of SNAIL decreased the expression levels of mature miR-128, pri-miR-128-2 and ARPP21 (Fig. 6H).

We further examined the mammosphere-forming ability of the MCF-10A cells with forced expression of SNAIL and/or miR-128. MCF-10A cells with forced expression of SNAIL formed 8-fold more mammospheres than MCF-10A control cells, and the increased numbers of mammospheres was abrogated by transfection with miR-128.
Thus, these findings indicate that SNAIL enhances tumor-initiating capability of mammary epithelial cells by repressing miR-128-2 expression through directly binding to the two E-Box sites.

We further sought to determine whether certain growth factors secreted into the tumor microenvironment might contribute to promotion of mammary carcinoma tumor-initiating capacity through repression of miR-128 expression. Previous studies revealed that TGF-β is a potent inducer of SNAIL, which can endow mammary epithelial cells with stem cell-like characteristics (33-34). Treatment of MCF-10A cells with TGF-β resulted in reduced miR-128 expression (Fig. 6J), sustained over time (Fig. 6K). In addition, to verify the specificity of TGF-β treatment, we utilized SB431542, a specific TGF-β1 receptor kinase inhibitor (35). We observed that SB431542 abrogated the TGF-β induced reduction in miR-128 expression (Fig. 6L), suggesting that TGF-β decreased miR-128 expression by enhancing expression levels of SNAIL. Functionally, we observed that TGF-β-treated MCF-10A cells exhibited an 11-fold increase in mammosphere formation compared to untreated control cells (Fig. 6M). The increase in mammosphere numbers stimulated by TGF-β was abolished by forced expression of miR-128 (Figure 6M). Thus, TGF-β promotes mammary epithelial cell oncogenic transformation, to some extent, by miR-128-2 down-regulation.

Discussion
Genome instability has been proposed as one of the essential hallmarks of cancer, which generates random mutations and confers selective advantage on cancer cells, enabling their outgrowth and eventually increasing cancer risk (36). The fact that 50% of human miRNAs reside in or around fragile chromosomal sites prompted us to uncover these breakpoint-associated miRNAs and clarify their functions. In this study, we have identified 16 miRNAs which are localized at chromosome 3p and often aberrantly expressed in human breast cancer. Of the 11 miRNAs which are subject to chromosome loss, several of them have been well documented. Our group has observed that let-7g, located on chromosome 3p21.1, is remarkably reduced in invasive ductal carcinoma and may be considered as a prognostic biomarker for breast cancer (10). In addition, miR-26a-1 and miR-135a-1 have been reported to be frequently lost in a variety of human malignancies, including breast, prostate, liver, brain, pancreatic, colon and nasopharyngeal carcinoma. Moreover, miR-885-5p is down-regulated upon loss of the 3p25.3 region in neuroblastoma (37). Intriguingly, we also found that 5 miRNAs (Fig. 1A) were amplified in breast cancer compared with normal breast tissues. miR-191 has been validated to be amplified and is viewed as a candidate oncogenic miRNA in liver (38), stomach (39) and pancreas (40). LOH comprises not only monosomy, caused by loss of one allele, but also homozygous disomy and homozygous polysomies, caused by the concurrent loss of one allele and endomultiplication of the other allele (41). Thus, we have identified a series of aberrant miRNAs at chromosome 3p, which exhibits frequent LOH and a discontinuous pattern of allele loss in breast cancer. Further endeavors will unravel their precise roles in the pathogenesis of breast cancer.

Among the eleven down-regulated miRNAs, miR-128-2 was the most frequently...
deleted (75% deletion) in our cohort. Previous reports have identified miR-128 as a TSG. miR-128 was first identified to be enriched in brain and frequently reduced in glioma cells (42), and dysregulation of miR-128 conferred a variety of malignant hallmarks on neural cells, including sustained proliferative signaling (43), resisting apoptosis (44), activating invasion and metastasis (45) and promoting self-renewal (25). A recent study has proposed that reduced miR-128 in breast tumor-initiating cells produced chemotherapeutic resistance (46). Consistently, we observed that forced expression of miR-128 in invasive MDA-MB-231 cells inhibited self-renewal and tumor-initiating capacities whereas depletion of miR-128 endowed normal mammary epithelial MCF-10A cells with a malignant phenotype and initiated tumor formation in vivo. Moreover, we further provided proof that miR-128 could directly target a cohort of stem cell factors which have comprehensive roles in stem cell development. Additionally, we demonstrated that miR-128-2 is directly regulated by SNAIL, suggesting a potential mechanism of how miR-128-2 is precisely controlled in embryogenesis and tumorigenesis. Finally, we observed miR-128 is involved in the TGF-β signaling pathway, in which TGF-β promotes breast cancer initiation and progression, partially, via miR-128-2 repression. Thus, we have extended our current knowledge by delineating the role of miR-128 in tumor-initiation, implying miR-128 is a candidate molecular target for clinical prognosis or therapy.

It is of interest to note miR-128 could directly target a series of six genes: Bmi1, Csf1, Klf4, Lin28a, Nanog and Snail, all of which are critical determinants of normal stem cell maintenance and renewal (24), and contribute to tumor formation. One explanation as to why depletion of miR-128 triggers oncogenic transformation in normal
mammary MCF-10A cells is due to the liberation of these six fundamental factors, which subsequently modulate PI3K/AKT and JAK2/STAT3 pathways enabling cells to undergo oncogenic transformation. Our hypothesis is further supported by the observations that miR-128 modulates numerous key genes functionally involved in EMT and neoplastic progression (Supplementary Table S5).

The SNAIL family of zinc-finger transcription factors consist of SNAIL1 (SNAIL), SNAIL2 (SLUG) and SNAIL3 (SMUC), all potent inducers of EMT and important predictors of breast cancer metastatic potential and recurrence (47). Expression of SNAIL is precisely regulated by a complex signaling network at the transcriptional and post-transcriptional level. Here, our findings supplement this network with the novel description of two positive feedback loops (Fig. 7). First, SNAIL directly binds to E-Box sites upstream of miR-128-2 and represses miR-128-2 expression, which in turn further increases the expression of SNAIL via direct targeting by miR-128-2. Secondly, SNAIL indirectly enhances the expression of BMI1, CSF1, KLF4, LIN28A and NANOG by inhibiting miR-128-2, which cooperatively increases the activity of AKT and STAT3. It has been reported that AKT up-regulates the expression of SNAIL with (48) or without (49) the activation of NF-κB. The JAK2/STAT3 pathway was also recently been reported to increase the expression of SNAIL (50). Thus, we have identified another positive feedback loop between SNAIL and the AKT or STAT3 cascade. Furthermore, TGF-β pathway has been demonstrated to induce EMT and endow mammary epithelial cells with stem cell-like traits mainly via transcriptionally inducing SNAIL expression (33-34). Our results demonstrate that TGF-β promotes EMT and breast cancer initiation at least in part by miR-128-2 down-regulation, revealing a cascade (TGF-β→TGF-β1 Receptor→

Collectively, in this study, our data reveal that decreased miR-128-2 expression due to allele loss on chromosome 3p22.3 can endow non-tumorigenic mammary epithelial MCF-10A with malignant phenotypes and stem cell-like behaviors both in vitro and in vivo via de-repression of a cohort of stem cell factors and activation of the PI3K/AKT and JAK2/STAT3 pathways. In addition, miR-128-2 is down-regulated by SNAIL and repressed in the TGF-β signaling pathway. The SNAIL/miR-128 axis provides a novel understanding of the mechanism of mammary epithelial oncogenic transformation.

Acknowledgements: We thank Muh-Hwa Yang and Kou-Juey Wu (Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan) for kindly providing the SNAIL, SLUG and TWIST1-expression plasmids. TERT-HMEC cell line was kindly gifted from Hines WC (University of New Mexico School of Medicine, New Mexico, USA). This work was supported by the National Key Scientific Program of China (2012CB934002, 2010CB912804), Chinese Academy of Sciences (XDA01000000), National Natural Science Foundation of China (30971492, 81101597) and Cancer Science Institute of Singapore.

References

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**Figure Legends**

**Fig. 1** miR-128 and ARPP21 are frequently lost and inversely associated with poor
**prognosis.** *A*, List of aberrant microRNAs (deleted or amplified) on human chromosome 3p in breast carcinoma determined by genomic real-time PCR. *B*, Schematic diagram of the genomic locus of hsa-miR-128-2 (Homo sapiens-miR-128-2). *C*, Expression level of *ARPP21* in breast cancer specimens (n=21) and benign breast disease tissues (Normal) (n=15) were analyzed by qRT-PCR. GAPDH was used as an internal control. *D*, Expression level of *pri-miR-128-2* in breast cancer specimens (n=21) and benign breast disease tissues (n=15) were measured by qRT-PCR, β-ACTIN was used as an internal control. *E*, miR-128 expression was determined by qRT-PCR in breast carcinoma specimens (n=21) and benign breast disease tissues (n=15), U6 (U6 small nuclear RNA) as an internal control. *F*, Expression level of miR-128 was examined in mammary carcinoma specimens and normal mammary specimens. One representative example of tissue microarray sections analyzed by *in situ* hybridization is shown. *G*, Expression of miR-128 in human breast cancer (BC) specimens and normal breast tissues. *H* and *I*, Correlations between the expression level of mature miR-128 and 5-year patient overall survival (OS) analysis or Relapse-free survival (RFS) by Kaplan-Meier analysis.

**Fig. 2** Forced expression of miR-128 alters the morphology and reduces migration and invasion of MDA-MB-231 cells *in vitro. A*, Expression levels of miR-128 in ten
human mammary epithelial or carcinoma cell lines were analyzed by qRT-PCR. The result was normalized to U6 snRNA. **B,** Left: Expression levels of miR-128 in MDA-MB-231 cells transiently transfected with miR-128 mimic. Middle: Total cell number assays. 5×10³ MDA-MB-231 cells transfected with miR-128 or control were seeded with 10% FBS and counted after 6 days. Right: Soft agar assay. 2×10³ cells were seeded in 0.35% agarose and colonies formed were counted after incubation for 14 days. **C,** Branching morphogenesis after cell growth in 3D Matrigel for 10 days was observed under a phase-contrast microscope. The number of branching colonies per 200 cells was counted. **D,** Monolayer adherent morphology of MDA-MB-231 cells with forced expression of miR-128. Cultures were imaged at approximately 40% confluence and at 100X, 200X, and 400 X magnifications. **E,** 1×10³ MDA-MB-231-miR-128 cells or control cells were seeded in 10cm petri dish. Colonies formed by each cell type, were categorized and counted as per the extent of colony scattering. Percentages of each colony category in the total counts were plotted as indicated. **F,** wound-healing assay of MDA-MB-231 cells transfected with miR-128 mimic or scrambled sequence oligonucleotides. Magnification: X100. **G,** transwell migration assay and matrigel invasion assay of MDA-MB-231 cells transfected with miR-128 mimic or scrambled sequences. A representative experiment is shown in triplicate along as mean ± s.e.m. in **A, B, C, E and G.** **P<0.01; ***P<0.001** (Student’s t-test).

**Fig. 3 Forced expression of miR-128 impairs MDA-MB-231 cells xenograft growth and induces apoptosis in vivo.** A, qRT-PCR analysis of the expression level of miR-128
in MDA-MB-231 cells infected with pBabe-pri-miR-128-2 or control vector and MCF-10A cells infected with pBabe-miR-128 Sponge or scrambled sequence oligonucleotide. The result was normalized to U6 snRNA. B, Tumor growth curve of MDA-MB-231 control or MDA-MB-231-pri-miR-128-2 cells implanted s.c. into the flanks of female nude mice. C, Representative images of tumors formed in nude mice that received subcutaneous injections of MDA-MB-231 cells transfected with the indicated vectors. D, H&E stained sections of tumors and lungs isolated from mice that received subcutaneous injection of MDA-MB-231-pri-miR-128-2 or control cells at week 4. Abundant local infiltration into the muscle, and tumor emboli in blood vessels, were visualized in the margins of the primary tumor nodule (upper, red arrow), whereas pulmonary metastases were readily detectable in the lungs (bottom, red arrow). Images were taken at 200X, and 400 X magnifications. E, Cell proliferation was assessed by Ki-67 staining on the sections of tumors. F, Apoptosis was measured by TUNEL labeling. A representative experiment is shown in triplicate along as mean ± s.e.m. in A, E, and F. **P<0.01; ***P<0.001(Student’s t-test).

Fig. 4 miR-128 represses the CSC-like population in mammary carcinoma cells. A, MDA-MB-231 and MCF-10A cells with forced or depleted expression of miR-128
were seeded in ultra low attachment plates in mammospheric growth media. After 14 days, growth was measured by AlamarBlue. Representative images of mammosphere generated by either forced expression or depleted expression of miR-128 cells of MDA-MB-231 and MCF-10A. Bar, 50µm. **B**, miR-128 inhibited the self renewal potential of MDA-MB-231 cells (upper), whereas depletion of miR-128 enhanced the self renewal potential of MCF-10A cells (bottom). MDA-MB-231 and MCF-10A cells with forced or depleted expression of miR-128 were grown under mammospheric condition and were sequentially cultured from first generation (G1) till third generation (G3) and growth compared with respective control cells. **C**, miR-128 modulates the ALDH1+ cell population in MDA-MB-231 and MCF-10A cells with forced or depleted expression of miR-128. Cells were incubated with ALDEFLUOR substrate (BAAA) and fluorescence was measured by flow cytometry. Cells with DEAB (Diethylaminobenzaldehyde), a specific inhibitor of ALDH1, were used as control. **D**, Tumor Incidence of MDA-MB-231 stably transfected with pri-miR-128-2 or MCF-10A stably transfected with miR-128 Sponge and then injected into host mice in limiting dilutions. **E**, miR-128 is inversely associated with ALDH1 in breast cancer samples. Pearson’s correlation coefficient was determined between the expression levels of ALDH1 and miR-128 by qRT-PCR in a cohort of 24 breast cancer specimens. A representative experiment is shown in triplicate along as mean ± s.e.m. in **A, B, and C**. *P<0.05; **P<0.01(Student’s t-test).

**Fig. 5** miR-128 directly targets a cohort of stem cell factors. **A**, Schematic displaying *in silico* analyses that led to identification of 98 putative miR-128 target genes by three
computational algorithms. **B**, Heat map depicting the mRNA expression profile of an array of genes involved in cancer initiation and progression by qRT-PCR in two sets of paired cells (MDA-MB-231 cells transfected with either miR-128 mimics or control and MCF-10A cells transfected with either miR-128 ASO or scrambled sequence control. Red squares correspond to up-regulation of mRNA levels, while green squares correspond to down-regulation of mRNA levels. **C**, Luciferase activity of luciferase reporters with wild-type or mutant 3’UTRs were performed after co-transfection with miR-128 mimic or negative control in MDA-MB-231 cells. The luciferase activity of each sample was normalized to Firefly luciferase activity. **D**, The protein levels were measured by western blot after transfection with miR-128 mimics or control in MDA-MB-231 cells (left) or after transfection with miR-128 ASO or control in MCF-10A cells (right). β-ACTIN was used as an internal control. **E**, The protein levels of targets were detected by immunohistochemical staining on sections of xenograft tumors formed by MDA-MB-231-pri-miR-128-2 or control cells, at week 4 after subcutaneous transplantation. Images were taken at 400 X magnifications. Bar, 20 μm. A representative experiment is shown in triplicate along as mean ± s.e.m. in **C**. *P<0.05; **P<0.01; NS, not significant (Student’s t-test).

Fig. 6 miR-128 affects cellular pathways via AKT and STAT3 phosphorylation, and
is directly repressed by SNAIL and responsive to TGF-β. A, Western blot showing down-regulation of AKT and STAT3 phosphorylation in MDA-MB-231 cells with forced expression of miR-128, and increased AKT and STAT3 phosphorylation in MCF-10A cells with depletion of miR-128 compared with their control cells, respectively. B, Immunohistochemical staining revealed reduced AKT and STAT3 phosphorylation on sections of xenograft tumors formed by MDA-MB-231-pri-miR-128-2 (bottom) compared with control cells (upper). Images were taken at 400 X magnifications. Bar, 20 μm. C, Western blot showing decreased AKT and STAT3 phosphorylation in miR-128-depleted MCF-10A cells treated with the specific AKT inhibitor IV and/or STAT3 inhibitor III. D, In vitro quantification of mammosphere formation in miR-128-depleted MCF-10A cells (2×10^3) treated with the specific AKT inhibitor IV and/or STAT3 inhibitor III. E, Bioinformatics analyses of predicted binding sites for SNAIL at promoter of miR-128. Schematic representation of the 2.2-kb regulatory region upstream of the human miR-128-2 stem-loop. Two E-box motifs were predicted, one at -991 bp (E-box 1) and the other at -26 bp (E-box 2) relative to the transcription start site (+1) of human miR-128-2 stem-loop. F, Luciferase assays for promoter activity. MCF-10A cells were cotransfected with different promoter constructs (wide-type or mutant) and vector expressing SNAIL. All cells were co-transfected with a plasmid expressing Renilla luciferase as an internal control. G, ChIP assay in MCF-10A cells transfected with a vector expressing SNAIL. PCR was performed with primers specific for human miR-128 E-box 1 and E-box 2, respectively. Amplicon at a distance of 2116 base pairs upstream of
the human miR-128-2 stem-loop was used as negative control. H, qRT-PCR analysis for expression levels of mature miR-128, pri-miR-128-2 and ARPP21 after transfection with a vector expressing SNAIL in MCF-10A cells. U6 snRNA and GAPDH served as internal controls, respectively. I, In vitro quantification of mammosphere formation by MCF-10A cells (2×10³) co-transfected with pc-SNAIL or control and miR-128 mimic. J, Relative expression of miR-128 in MCF-10A cells treated with recombinant TGF-β (2.5 ng/ml) for 12 days and transfected with or without miR-128 mimic. K, Relative expression of miR-128 in MCF-10A cells treated with recombinant TGF-β (2.5 ng/ml) for 3, 6, 9, 12 days, respectively. L, TGF-β depletion of miR-128 expression was mediated through TGF-β1 receptor. MCF-10A cells were treated with TGF-β in the presence or absence of 5 μM SB431542, a specific TGF-β1 receptor kinase inhibitor. M. In vitro quantification of mammosphere formation by MCF-10A cells (2×10³) described in J. U6 snRNA was used as an internal control in J, K and L. A representative experiment is shown in triplicate along as mean ± s.e.m. in D and F-M. *P<0.05; **P<0.01; ***P<0.001; NS, not significant (Student’s t-test).

Fig. 7 Proposed model for the critical roles of miR-128 in the mediation of
mammary epithelial oncogenic transformation and progression. TGF-β specifically represses miR-128 expression via phosphorylation of TGF-β1 receptor to increase SNAIL expression. SNAIL further directly binds to E-Box sites and down-regulates miR-128-2, which in turn represses a cohort of stem cell factors, including BMI1, CSF1, KLF4, LIN28A and NANOG as direct targets and subsequently promotes oncogenic transformation of mammary epithelial cells via activation of p-STAT3 and p-AKT pathways. Two positive feedback loops are proposed as indicated, one between SNAIL and miR-128, and the other between SNAIL and p-STAT3 and p-AKT activities via affecting SNAIL transcription or posttranscriptional processing.
**Figure 1**

A. Table showing changes in genome copy frequency for various microRNAs.

<table>
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<tr>
<th>ID</th>
<th>Chromosome</th>
<th>Change of genome copy</th>
<th>Frequency, %</th>
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</thead>
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<tr>
<td>hsa-mir-128-2</td>
<td>3p22.3</td>
<td>Deletion</td>
<td>75%</td>
</tr>
<tr>
<td>hsa-let-7g</td>
<td>3p21.1</td>
<td>Deletion</td>
<td>60%</td>
</tr>
<tr>
<td>hsa-mir-4792</td>
<td>3p24.2</td>
<td>Deletion</td>
<td>35%</td>
</tr>
<tr>
<td>hsa-mir-26a-1</td>
<td>3p22.2</td>
<td>Deletion</td>
<td>35%</td>
</tr>
<tr>
<td>hsa-mir-5193</td>
<td>3p21.31</td>
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<td>35%</td>
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<td>3p21.32</td>
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</tr>
<tr>
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<td>3p21.31</td>
<td>Deletion</td>
<td>25%</td>
</tr>
<tr>
<td>hsa-mir-135a-1</td>
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<td>Amplification</td>
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</tr>
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</table>

B. Diagram showing changes in genome copy frequency for ARPP21.

C. Graph showing relative expression of ARPP21.

D. Graph showing relative expression of pri-miR-128-2/pre-β-actin.

E. Graph showing relative expression of miR-128/U6.

F. Image showing ISH with LNA-miR-128 probe.

G. Table showing miR-128 expression in Normal and Breast Cancer groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Negative, n (%)</th>
<th>Positive, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>6 (33.3)</td>
<td>12 (66.7)</td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>85</td>
<td>51 (60.0)</td>
<td>34 (40.0)*</td>
</tr>
</tbody>
</table>

H. Graph showing OS probability over months after surgery.

I. Graph showing RFS probability over months after surgery.
Figure 2

A

![Graph showing relative expression of miR-128 in HMECs compared to breast cancer cell lines.](image)

B

**MDA-MB-231**

![Bar graph showing fold-change in miR-128 expression, total cell number, and colony formation.](image)

C

**3D Matrigel Assay**

![Images of control and miR-128 treated MDA-MB-231 cells.](image)

D

**Monolayer adherent morphology**

![Images of control and miR-128 treated breast cancer cells.](image)

E

**Colony-Scattering Assay**

![Graph showing number of branching colonies per 200 cells and percentage of colony types.](image)

F

**Wound healing Assay**

![Images showing wound healing for control and miR-128 treated MDA-MB-231 cells.](image)

G

**Transwell Assay**

![Graph showing fold-change in migration and invasion.](image)
Figure 3

A

Relative Expression of miR-128/68

<table>
<thead>
<tr>
<th></th>
<th>pBabe</th>
<th>pBabe-pri-miR-128-2</th>
<th>Control</th>
<th>miR-128 Sponge</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>***</td>
<td>**</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>MCF-10A</td>
<td></td>
<td></td>
<td></td>
<td>**</td>
</tr>
</tbody>
</table>

B

Tumor Volume (mm³)

C

MDA-MB-231 pBabe

MDA-MB-231 pBabe-pri-miR-128-2

D

Fat Pad Injection Tumor Growth Curve

E

Ki-67 Staining

F

TUNEL
**Figure 4**

### A

- **MDA-MB-231**
  - Fold-Mammosphere Numbers
  - Control vs. miR-128
  - Graph showing fold change in mammosphere numbers.

- **MCF10A**
  - Fold-Mammosphere Numbers
  - Control vs. miR-128 ASO
  - Graph showing fold change in mammosphere numbers.

### B

- **MDA-MB-231**
  - Fold-Mammosphere Numbers
  - Control vs. miR-128
  - Graph showing fold change in mammosphere numbers.

- **MCF-10A**
  - Fold-Mammosphere Numbers
  - Control vs. miR-128 ASO
  - Graph showing fold change in mammosphere numbers.

### C

- **MDA-MB-231**
  - ALDH1+ve cells (%)
  - Control vs. miR-128
  - Graph showing ALDH1+ve cell percentages.

- **MCF10A**
  - ALDH1+ve cells (%)
  - Control vs. miR-128 ASO
  - Graph showing ALDH1+ve cell percentages.

### D

<table>
<thead>
<tr>
<th>Cells</th>
<th>Tumors Incidence/Number of Injections</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>MDA-MB-231 pBabe</td>
<td>8/8</td>
</tr>
<tr>
<td>MDA-MB-231 pri-miR-128-2</td>
<td>8/8</td>
</tr>
<tr>
<td>MCF-10A Control</td>
<td>0/8</td>
</tr>
<tr>
<td>MCF-10A miR-128 Sponge</td>
<td>4/8</td>
</tr>
</tbody>
</table>

### E

- **Pearson Coefficient**
  - Relative Expression of ALDH1/GAPDH
  - Graph showing linear relationship.
  - $R^2 = 0.1652$
  - $P = 0.02$
  - Pearson Coefficient = -0.4065
Figure 5

A. TargetScan Stem Cell Factors

BMI1, CSF1, KLF4, LIN28A, NANOG, SNAIL

miRanda Pictar

B. MDA-MB-231 with miR-128 mimics

MCF-10A with miR-128 ASO

MDA-MB-231 with miR-128 mimics

C. MCF-10A with miR-128 ASO

C. Relative Renilla/Firefly luciferase activity

psi-Check2

BMI1 3'UTR

BMI1 3'UTR Mut

CSF1 3'UTR

CSF1 3'UTR Mut

KLF4 3'UTR

KLF4 3'UTR Mut

NANOG 3'UTR

NANOG 3'UTR Mut

psi-Check2

LIN28A 3'UTR

LIN28A Mut 1

LIN28A Mut 2

LIN28A Mut 3'UTR Mut

LIN28A Mut 1+2

SNAIL 3'UTR

SNAIL 3'UTR Mut

Control

miR-128

NS NS NS NS NS NS NS NS

D. MDA-MB-231

Control miR-128

37kD

72kD

58kD

26kD

39kD

34kD

43kD

BMI1

CSF1

KLF4

LIN28A

NANOG

SNAIL

β-ACTIN

E. MCF-10A

Control miR-128 ASO

pri-miR-128-2

pri-miR-128-2
Figure 6

A. MDA-MB-231 and MCF-10A cells were treated with Control miR-128 or miR-128 ASO. Western blot analysis was performed to determine the levels of p-AKT, AKT, p-STAT3, STAT3, and ACTIN.

B. MDA-MB-231 and MCF-10A cells were treated with Control miR-128 or miR-128 ASO. The expression of AKT and STAT3 was analyzed by Western blot.

C. MCF-10A cells were treated with miR-128 ASO, AKT Inhibitor IV, or STAT3 Inhibitor III. Western blot analysis was performed to determine the levels of p-AKT, AKT, p-STAT3, STAT3, and ACTIN.

D. MCF-10A cells were treated with miR-128 ASO, AKT Inhibitor IV, or STAT3 Inhibitor III. The fold-mammosphere numbers were analyzed.

E. MCF-10A cells were treated with miR-128 ASO, AKT Inhibitor IV, or STAT3 Inhibitor III. The expression of miR-128 was analyzed by RT-qPCR.

F. MCF-10A cells were treated with pcDNA3.1 or pc-Snail. The relative Firefly/Renilla luciferase activity was determined.

G. MCF-10A cells were treated with vector or pc-Snail. The expression of EBox1, EBox2, miR-128, ARPP21, and pri-miR-128 was analyzed by RT-qPCR.

H. MCF-10A cells were treated with Control or pc-Snail. The expression of miR-128 was analyzed by RT-qPCR.

I. MCF-10A cells were treated with pc-Snail or miR-128. The fold-mammosphere numbers were analyzed.

J. MCF-10A cells were treated with TGF-β or miR-128. The expression of miR-128 was analyzed by RT-qPCR.

K. MCF-10A cells were treated with TGF-β or miR-128. The expression of miR-128 was analyzed by RT-qPCR.

L. MCF-10A cells were treated with SB431542 or TGF-β. The expression of miR-128 was analyzed by RT-qPCR.

M. MCF-10A cells were treated with SB431542 or TGF-β. The fold-mammosphere numbers were analyzed.
Figure 7

```
  TGF-β
    ↓
  TGF-β1 Receptor
    ↓
  SNAIL
    ↓
  hsa-miR-128-2
    ↓
  BMI1  CSF1  LIN28A  NANOG  KLF4
    ↓
  p-STAT3  p-AKT
    ↓
  Oncogenic Transformation

  NF-κB
```

Genes and proteins involved in TGF-β signaling and related pathways are illustrated, including TGF-β, TGF-β1 Receptor, SNAIL, hsa-miR-128-2, BMI1, CSF1, LIN28A, NANOG, KLF4, p-STAT3, p-AKT, and NF-κB.
Loss of SNAIL regulated miR-128-2 on chromosome 3p22.3 targets multiple stem cell factors to promote transformation of mammary epithelial cells


Cancer Res  Published OnlineFirst September 26, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-1507

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