MicroRNA-212 displays tumor-promoting properties in non-small cell lung cancer cells and targets the hedgehog pathway receptor *PTCH1*

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ABSTRACT **Dysexpression of microRNAs has been found in many tumors, including lung cancer. The hedgehog (Hh) signaling pathway plays an important role during normal development, and the abnormal regulation of its members has also been related to many tumors. However, little is known about the relationship between microRNA and the Hh pathway. In this paper, we report microRNA-212 (miR-212) playing a role in non-small cell lung cancer (NSCLC) and targeting *PTCH1*, a receptor of the Hh pathway. We found that miR-212 was up-regulated when cells were treated with 4ß-12-*O*-tetradecanoylphorbol-13-acetate (TPA). We ectopically expressed miR-212 in NSCLC cell lines to examine the influence of miR-212 overexpression. The results showed that overexpression of miR-212 in NSCLC cells promoted cell cycle progression and cell proliferation, migration, and invasion. The promoting effects of miR-212 on cell proliferation, migration, and invasion were partially reversed by the miR-212 inhibitor anti-miR-212. These results suggested that miR-212 might have tumor-promoting properties. Potential targets of miR-212 were predicted, and we showed tumor suppressor *PTCH1* was a functional target of miR-212. *PTCH1* may be responsible for the effect of miR-212 on cell proliferation. Altogether, our results indicated that miR-212 was involved in tumorigenesis, and the oncogenic activity of miR-212 in NSCLC cells was due, in part, to suppression of *PTCH1*.**

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths and has the most rapidly increasing incidence rate in developed countries, as well as in China (Jemal *et al.*, 2011). It is well known that genetic alterations could occur at the chromosomal level (e.g., large gains and deletions), at the nucleotide level (e.g., nucleotide mutation), or at the epigenetic level (e.g., DNA methylation). Such a change could result in the activation of oncogenes (e.g., *Ras, Myc*) and other growth-promoting genes (e.g., *ERBB1, IGF-IR*) and the inactivation of tumor suppressor genes (e.g., *p53, p16INK4A, Rb, FHIT*). Moreover, emerging evidence suggests the potential involvement of altered regulation of microRNAs (miRNAs) in the pathogenesis of human cancers (Calin *et al.*, 2002, 2004; Calin and Croce, 2006; Michael *et al.*, 2003; Eis *et al.*, 2005; Lu *et al.*, 2005; Esquela-Kerscher and Slack, 2006).

MicroRNAs are a class of 22-nucleotide, noncoding RNAs that are evolutionarily conserved and function as negative regulators of gene expression. The levels of individual miRNAs vary dramatically in different cell types and different developmental stages, suggesting that miRNAs play a role in cell growth, differentiation, and programmed cell death (Lagos-Quintana *et al.*, 2001; Bartel, 2004). MiRNAs are aberrantly expressed or mutated in human cancer, indicating that they may function as a novel class of oncogenes or tumor suppressor genes (Calin *et al.*, 2002; Takamizawa *et al.*, 2004; Chan *et al.*, 2005; He L *et al.*, 2005; Iorio *et al.*, 2005; Lu *et al.*, 2005; Zhang *et al.*, 2006). Deregulation of miRNAs has been detected in many

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Abbreviations used: anti-miR-212, miR-212 inhibitor; anti-miR-NC, negative control inhibitor; Ct, comparative threshold cycle; GFP, green fluorescent protein; FBS, fetal bovine serum; Hh, hedgehog; miR-212, microRNA-212; miR-212m, synthetic miR-212 mimic; miRNA, microRNA; miR-NC, negative control mimic; mt-3′UTR, *PTCH1*-3′UTR-mutant; NSCLC, non–small cell lung cancer; PBS, phosphate-buffered saline; PKC, protein kinase C; RL, *Renilla* luciferase; RT-PCR, reverse transcriptase-PCR; SCLC, small cell lung cancer; siRNA, small interfering RNA; TPA, 4ß-12-*O*-tetradecanoylphorbol-13-acetate; wt-3′UTR, *PTCH1*-3′UTR wild type.

human malignancies, including breast carcinoma (Iorio *et al.*, 2005; Zhang *et al.*, 2006), primary glioblastoma (Chan *et al.*, 2005; Lu *et al.*, 2005), lung cancer (Takamizawa *et al.*, 2004), papillary thyroid carcinoma (He H *et al.*, 2005), colon carcinoma (Volinia *et al.*, 2006), and pancreatic tumors (Gaur *et al.*, 2007; Lee *et al.*, 2007).

MicroRNA-212 (miR-212) is located at chromosome 17p13.3 (Kozaki *et al.*, 2008), at which loss of heterozygosis has frequently been reported in human gastric cancer (Gleeson *et al.*, 1997; Choi *et al.*, 1998; Yustein *et al.*, 1999). In primary lung cancers, miRNA expression profiles have been analyzed in 104 pairs of primary lung cancers and corresponding noncancerous lung tissues, and miR-212 was reported to be up-regulated in lung cancer tissues in comparison with noncancerous lung tissues (Yanaihara *et al.*, 2006). Rabinowits and colleagues also reported that miR-212 was one of the up-regulated markers for lung cancer (Rabinowits *et al.*, 2009). It has been reported that miR-212 is involved in DNA methylation (Wada *et al.*, 2010), cell apoptosis (Incoronato *et al.*, 2010), cocaine intake (Hollander *et al.*, 2010), and mouse mammary gland development (Ucar *et al.*, 2010). Most recently, overexpressed miR-212 and miR-132 were reported targeting the retinoblastoma tumor suppressor, Rb1, in pancreatic adenocarcinoma tissues (Park *et al.*, 2011).

PTCH1 is a receptor of the hedgehog (Hh) signaling pathway. The Hh pathway plays a critical role in embryonic development and tissue polarity (Ingham, 1998). Secreted Hh molecules (Shh, Dhh, and Ihh) bind to the receptor (Hip1, PTCH1, and PTCH2), thereby activating a putative transmembrane protein *smoothened* (Smo). *Smo* initiates a cascade of events resulting in Gli entering the nucleus and acting as a transcription activator (Taipale and Beachy, 2001). Several inhibitors of the pathway, including Ptch and Hip1, are transcriptional target genes of *Gli*. The signaling pathway forms a negative feedback mechanism that maintains the pathway activity at an appropriate level in a given cell (Pasca and Hebrok, 2003). The Hh signaling pathway has been studied in small cell lung cancer (SCLC), and it was shown that the regeneration and carcinogenesis of SCLC was dependent on activation of Hh signaling (Watkins *et al.*, 2003). *Gli3* was found to be predominantly expressed in non–small cell lung cancer (NSCLC) when several members of the Hh signaling pathway were examined in a panel of 20 SCLC cell lines and four NSCLC cell lines. With regard to the expression pattern of Hh pathway members other than *Gli3*, no marked differences between SCLC and NSCLC were evident in the study (Vestergaard *et al.*, 2006). As a key member of the Hh pathway, *PTCH1* was reported to promote cell cycle progression and cell proliferation (Barnes *et al.*, 2001; Adolphe *et al.*, 2006), and the expression level of *PTCH1* was correlated with tumor metastasis potential in prostate cancer specimens (Sheng *et al.*, 2004) and colorectal cancer cell lines (You *et al.*, 2010). A limited number of articles have addressed the expression level of *PTCH1* in NSCLC or SCLC (Watkins *et al.*, 2003; Gialmanidis *et al.*, 2009; Singh *et al.*, 2011). However, the function of *PTCH1* has not been studied.

In the present study, we investigated the role of miR-212 in NSCLC cells. We found that exposure of A549, H1299, and BEAs-2B cells to 4ß-12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced increased expression of miR-212 and that miR-212 could potentiate cell proliferation and invasion. Our data also indicated that miR-212 negatively modulates *PTCH1* expression in NSCLC cell.

RESULTS

TPA induced the increased expression of miR-212

MicroRNA profiles have been reported in different type of tumors and in different drug-induced responses to cells. TPA is generally known for its tumor-promoting activity; however, miRNA expression of lung adenocarcinoma upon TPA treatment has not been explored. We initially analyzed miRNA expression in lung adenocarcinoma cell line A549, which was untreated (dimethyl sulfoxide [DMSO] only) or treated with 50 nm TPA at different time points (2, 12, and 24 h). Array data processing and analysis were performed using Illumina BeadStudio software. After normalization to control DMSO, 12 of the 739 miRNAs showed the greatly significant differential expression (Table 1).

Treated with 50 nm TPA compared with control	
Up-regulated (n = 10)	**Down-regulated (n = 2)**
miR-212	miR-148a
miR-10a	miR-223
miR-129	
miR-154*	
miR-363*	
miR-504	
miR-512-3p	
miR-517a	
miR-518a	
let-7c	

TABLE 1: Differential expression of microRNAs in A549 cells.

Among 12 differentially expressed miRNAs, miR-212 showed an increased expression at different time points, and its expression increased by fivefold when cells were treated with TPA for 24 h. To further validate the result, the change of miR-212 expression was confirmed by real-time reverse transcription PCR (RT-PCR) in two different lung cancer cell lines, A549 and H1299, as well as in human bronchial epithelial cell line BEAs-2B. As shown in Figure 1, the expression of miR-212 increased at different time points compared with the control. In A549 cells, the expression of miR-212 peaked at 12 h and remained stable until 24 h (Figure 1A); while in H1299 and BEAs-2B cells, the expression of miR-212 reached its peak at 24 h (Figure 1, B and C). To examine the influence of DMSO, which was used to dissolve TPA, on the expression of miR-212, we also detected miR-212 in cells treated with DMSO compared with untreated cells (blanks). The result showed that DMSO did not influence miR-212 expression (Supplemental Figure S1, A–C).

miR-212 mimics played a role in cell progress

Synthetic miR-212 mimic (miR-212m) was used for transient transfection to investigate the function of miR-212 compared with the cells transfected with a negative control mimic (miR-NC), which has no specific human gene product target. H1299, A549, and BEAs-2B cells were transfected with miR-212m or miR-NC for 48 h; a cell cycle assay was then performed. Cell cycle distribution was determined by flow cytometry analysis and is shown in Figure 2 as the percentage of cells in G1, S, and G2 phases. In H1299 cells, miR-212m caused a 9.7% decrease in G1 phase and a 9.3% increase in S phase, compared with miR-NC (Figure 2A). In A549 cells, miR-212m caused a 4% decrease in G1 phase and a 3% increase in S phase (Figure 2B). In BEAs-2B cells, miR-212m caused a 6.38% decrease in G1 phase and an 8% increase in S phase (Figure 2C).

Cell proliferation analysis was done using the Cell Counting Kit-8. We observed that when the cells were treated with miR-212m, cell proliferation was significantly increased in days 3, 4, and 5,

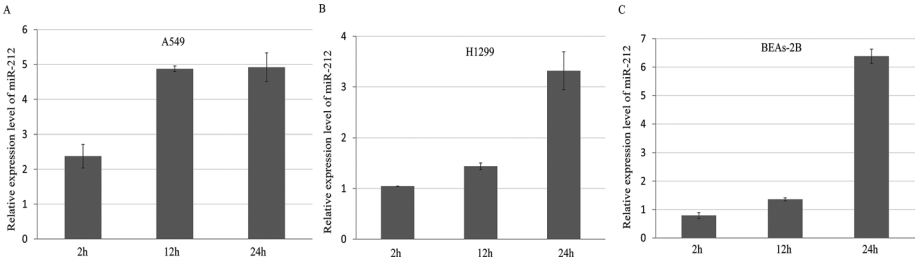

FIGURE 1: The expression of miR-212 in TPA treated NSCLC cells A549, H1299 and human bronchial epithelial cells BEAs-2B. Cells (A) 549, (B) H1299, and (C) BEAs-2B cells were treated with 50 nm TPA or control DMSO for 2, 12, and 24 h, respectively. The miR-212 expression was detected by real-time quantitative PCR. Each experiment was performed in triplicate. Bars represent mean ± SD.

compared with miR-NC in H1299 cells (Figure 2D). The miR-212m also increased cell proliferation in A549 and BEAs-2B cells (Figure 2, E and F).

We also performed migration and invasion assays. In H1299 cells, we found that miR-212m increased the ability of cells to migrate (Figure 3A), and also significantly increased the number of cells that penetrated the Matrigel-coated membrane (Figure 3B). The assays were also performed on A549 and BEAs-2B cells, and the results were consistent with those of H1299 cells (Figure 3, C–F).

H1299 cells stably expressing miR-212 altered the cell cycle status and promoted cell proliferation

To avoid clone-specific effects, we used bulk-selected cells for functional analysis. The bulk-selected clones stably expressing miR-212 were designated as "H1299-pEGP-miR-212," and the clones stably expressing GFP were designated as "H1299-pEGP-miR-null."

As in the mimic transient transfection, H1299-pEGP-miR-212 decreased the proportion of cells in G1 phase ~8.9% and increased the proportion of cells in S phase ~11.2% (Figure 4A). Cell proliferation analysis revealed that H1299-pEGP-miR-212 cells had a significant increase in cell growth rate compared with H1299-pEGP-miR-null cells (Figure 4B).

We used synthetic miR-212 inhibitor (anti-miR-212) to knock down the expression of miR-212, and performed a cell proliferation assay to examine whether anti-miR-212 could rescue the effect of miR-212 overexpression. As shown in Figure 4C, anti-miR-212 partially decreased cell proliferation in H1299-pEGP-miR-212 cells compared with negative control (anti-miR-NC) at days 3 and 4.

H1299 cells stably expressing miR-212 promoted anchorage-independent cell growth

We further investigated the role of miR-212 in anchorage-independent cell growth, a hallmark of tumor progression. After 3 wk of growth, ectopic expression of miR-212 in H1299-pEGP-miR-212 cells showed significantly increased numbers (~80-fold) of colonies and larger clones in soft agar (colony-formation assay; Figure 5) compared with H1299-pEGP-miR-null control cells. The result was confirmed in three independent experiments, suggesting that miR-212 overexpression promoted anchorage-independent cell growth.

H1299 cells stably expressing MiR-212 promoted the capacity of cell migration and invasion

We assessed the role of miR-212 on cell migration, a key determinant of malignant progression and metastases. A significant increase in cell migration was found in H1299-pEGP-miR-212 cells compared with H1299-pEGP-miR-null control cells (Figure 6A). We then determined the effect of miR-212 on cell invasion across the extracellular matrix. We found that the ectopic expression of miR-212 increased the ability of cells to invade (Figure 6B).

To further confirm the results, we transfected H1299-pEGP-miR-212 cells with anti-miR-212 or anti-miR-NC, and then detected the cell invasion and migration. The anti-miR-212 decreased the amount of cell migration and in--vasion compared with anti-miR-NC (Figure 6, C and D).

The results seen in transient transfection and bulk-selected cells supported a functional role for miR-212 in mediating cell proliferation, migration, and invasion and suggested a mechanism by which overexpression of miR-212 may contribute to tumor progression in human NSCLC.

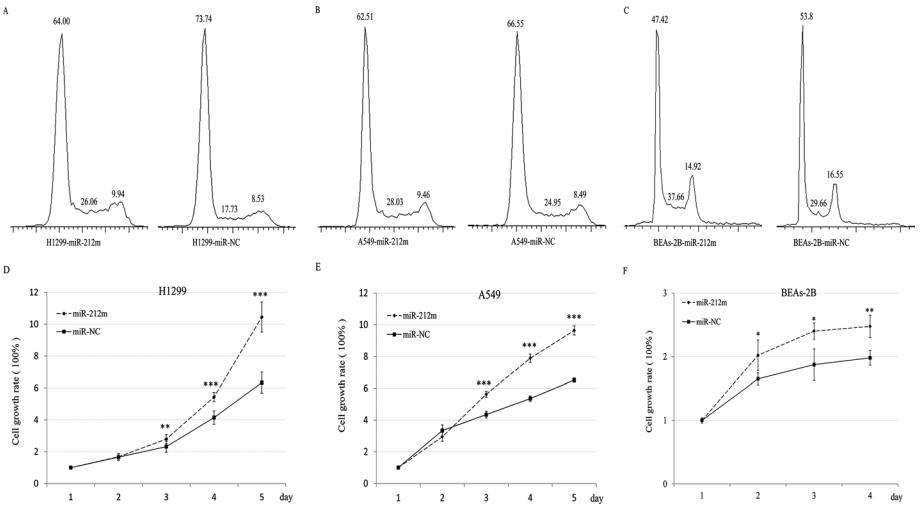

FIGURE 2: Overexpression of miR-212 altered cell cycle status and promoted cell proliferation. Cells were transfected with miR-212m or miR-NC for 48 h, and then subjected to cell cycle analysis. (A–C) miR-212 overexpression caused a decrease in G1 phase and an increase in S phase in H1299 (A), A549 (B), and BEAs-2B (C) cells. For cell proliferation analysis, cells were seeded into a 96-well plate at 5000 cells/well and examined at 24, 48, 72, 96, and 120 h. Each experiment was performed in sextuplicate (n = 6). (D–F) miR-212 overexpression promoted cell proliferation in H1299 (D), A549 (E), and BEAs-2B (F). The criterion for data significance is the p value. *, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$.

PTCH1 is a potential target of MiR-212

To identify possible miR-212 target genes, we performed a computational screen for genes with complementary sites of miR-212 in their 3′UTR using open-access software. The software included TargetScan (www.targetscan.org), PicTar (http://pictar.bio.nyu.edu), Sanger microRNA target (http://microrna.sanger.ac.uk), and Miranda (www

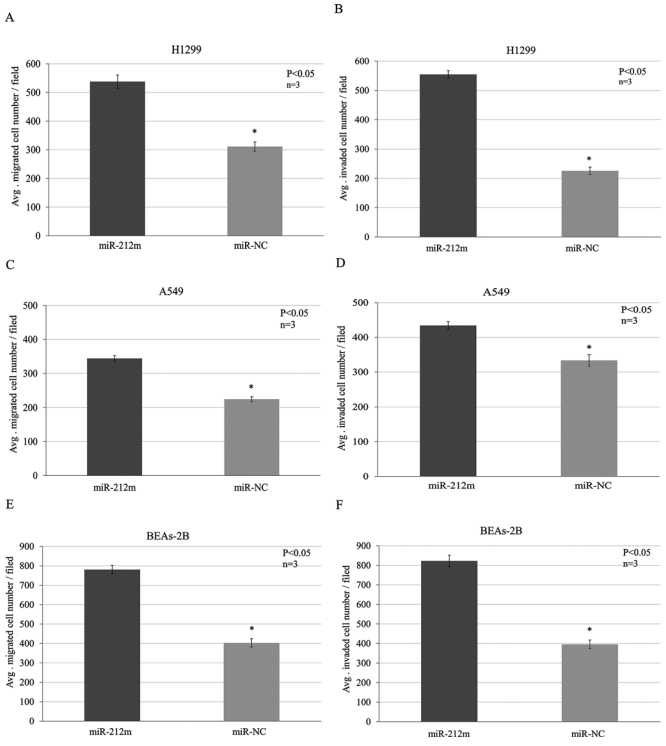

FIGURE 3: Overexpression of miR-212 enhanced cell migration and invasion. A sample of 5×10^4 cells overexpressing miR-212 or control cells grown in 0.1 ml media (1% FBS) were seeded into the upper chambers of Transwell uncoated (A, C, and E: migration assay) or Matrigel matrix–coated (B, D, and F: invasion assay). At 72 h, the cells that migrated through the bottom of the Transwell were stained and counted under a reverse microscope. miR-212 overexpression promoted cell migration and invasion in H1299 (A and B), A549 (C and D), and BEAs-2B (E and F) cells. Each experiment was performed in triplicate (n = 3). *, $p < 0.05$. Bars represent mean ± SD.

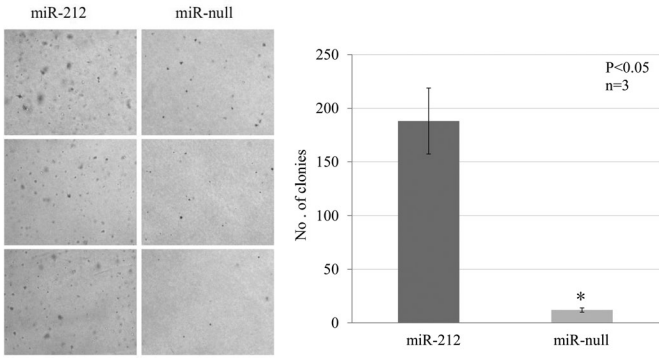

FIGURE 5: H1299 cells stably express miR-212 promoted anchorage-independent cell growth. Ten thousand cells were plated in soft agar in each well of a six-well plate. After incubation for 3 wk, colonies were examined under a microscope and colonies with a diameter greater than 3 mm were counted. Each experiment was performed in triplicate. *, $p < 0.05$. Bars represent mean ± SD.

.microrna.org). We focused our attention on *PTCH1*, a key member of the Hh pathway. *PTCH1* was reported as being involving in cancer cell cycle progress, as well as in tumor metastasis in prostate and colon cancer specimens (Sheng *et al.*, 2004; You *et al.*, 2010), but its role in mediating cell migration and invasion was unclear.

As shown in Figure 7A, a target sequence for miR-212 at nucleotides 488–494 was found in the 3′UTR of *PTCH1*, and it is highly conserved between humans and *Pan troglodytes*. The minimum free energy predicted for hybridization with the *PTCH1* 3′UTR and miR-212 hybridization is about –16.22 kcal/mol, as determined by Mfold analysis. The predicted minimum free energy is consistent with authentic miRNA targeting. Moreover, Hollander and coworkers reported that in HEK cell overexpression of miR-212, the expression of *PTCH1* was decreased ~53% (Hollander *et al.*, 2010).

To assess whether miR-212 can alter the expression of *PTCH1* in H1299 cells, we did a dual luciferase assay using miR-212m. The reporter activity was found to be decreased ~70% in the *PTCH1* 3′UTR wild-type (wt-3′UTR) cotransfections compared with that of the *PTCH1* 3′UTR mutant (mt-3′UTR) cotransfections (Figure 7B). We then used anti-miR-212 to knock down the expression of miR-212 and rescue the effect of miR-212m in *PTCH1* wt-3′UTR cotransfected cells. As show in Figure 7C, the decreased reporter activity induced by miR-212m could be specifically rescued by anti-miR-212, which led to almost equal luciferase activity upon anti-miR-212 treatment compared with that of negative control (anti-miR-NC).

We also performed the dual luciferase assay using the miR-212 expression construct pEGP-miR-212 instead of mimics. Cotransfection of pEGP-miR-212 with wild-type *PTCH1* 3′UTR and control *Renilla* luciferase (RL) reporter construct pRL-TK revealed a 90% fold decrease of firefly luciferase activity compared with that of the mutant *PTCH1* 3′UTR, indicating that miR-212 can modulate *PTCH1* gene expression via the miR-212 binding sites in its 3′UTR (Figure 7D).

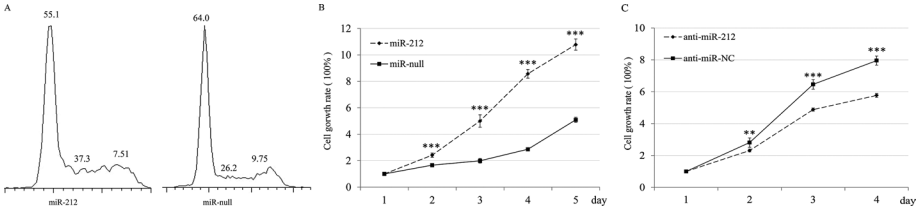

FIGURE 4: The effect of miR-212 in H1299 cells stably expressing miR-212. (A) The cell cycle profiles of H1299-pEGP-miR-212 and H1299-pEGP-miR-null cells. Stable expression of miR-212 caused a decrease in G1 phase and an increase in S phase. (B) Cell proliferation assays of H1299-pEGP-miR-212 and H1299-pEGP-miR-null, and stably expressed miR-212 promoted cell proliferation. (C) The effect of miR-212 on cell proliferation was partially reversed by anti-miR-212. H1299-pEGP-miR-212 cells were transfected with anti-miR-212 or anti-miR-NC for 48 h and then subjected to a proliferation assay. Each experiment was performed nine times (n = 9).**, $p < 0.01$; ***, $p < 0.001$. Bars represent mean ± SD.

Overexpression of MiR-212 influences endogenous PTCH1

To gain insight into the mechanism by which miR-212 inhibits luciferase expression, we then performed real-time PCR and Western blot analysis to examine the endogenous *PTCH1* mRNA and protein levels after miR-212 overexpression. We transiently transfected H1299, A549, and BEAs-2B cells with miR-212m or miR-NC. As shown in Figure 8, A and B, relative to miR-NC, miR-212m decreased the endogenous mRNA and protein level of *PTCH1*. The basal mRNA expression levels of *PTCH1* in H1299, A549, and BEAs-2B cells are shown in Figure S1D.

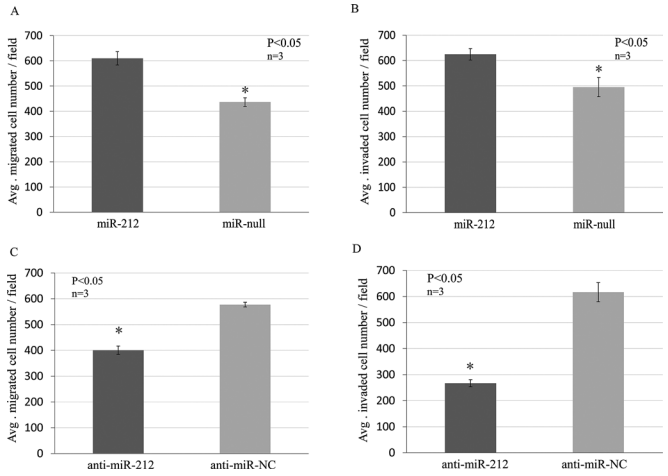

FIGURE 6: H1299 cells stably expressing miR-212 promoted cell migration and invasion. (A and B) 5×10^4 H1299-pEGP-miR-212 and H1299-pEGP-miR-null cells grown in 0.1 ml media (1% FBS) were seeded into the upper chambers of Transwell uncoated (A: migration assay) or Matrigel matrix–coated (B: invasion assay).At 72 h, the cells that migrated through the bottom of the Transwell were stained and counted under a reverse microscope. (C and D) The effect of miR-212 on cell migration and invasion were partially reversed by anti-miR-212. H1299-pEGP-miR-212 cells were transfected with anti-miR-212 or anti-miR-NC for 48 h, and then underwent cell migration (C) and invasion (D) assays. Each experiment was performed in triplicate. *, $p < 0.05$. Bars represent mean ± SD.

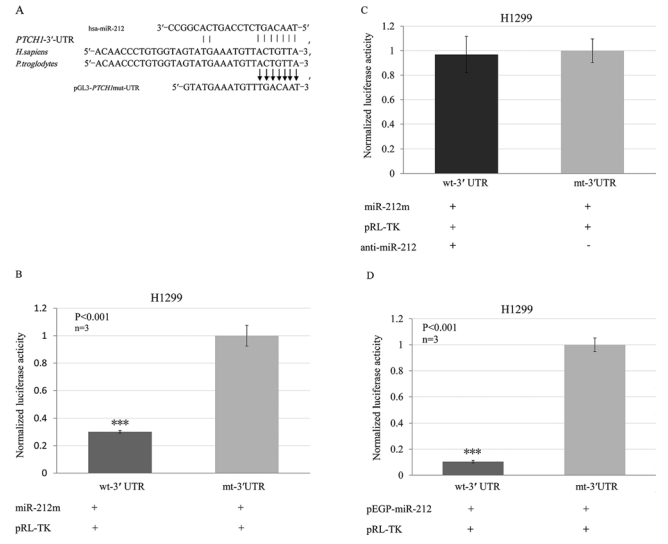

FIGURE 7: MiR-212 directly targeted *PTCH1*. (A) The binding site of miR-212 to the 3′-UTR of *PTCH1*. (B and D) miR-212 overexpression decreased the luciferase activity of *PTCH1*-3′UTR wild-type (wt-3′UTR), but not that of *PTCH1*-3′UTR-mutant (mt-3′UTR). H1299 cells were seeded into a 24-well plate. *PTCH1* reporter construct (wild-type or mutant) was cotransfected with miR-212m and pRL-TK (B), or cotransfected with pEGP-miR-212 and pRL-TK (D). (C) Anti-miR-212 reversed the effect of miR-212m compared with that of anti-miR-NC. Anti-miR-212 or anti-miR-NC was added to the cotransfection of wt-3′UTR, miR-212m, and pRL-TK. At 48 h, cells were lysed and detected in a microplate reader. Each experiment was performed in triplicate. Bars represent mean ± SD.

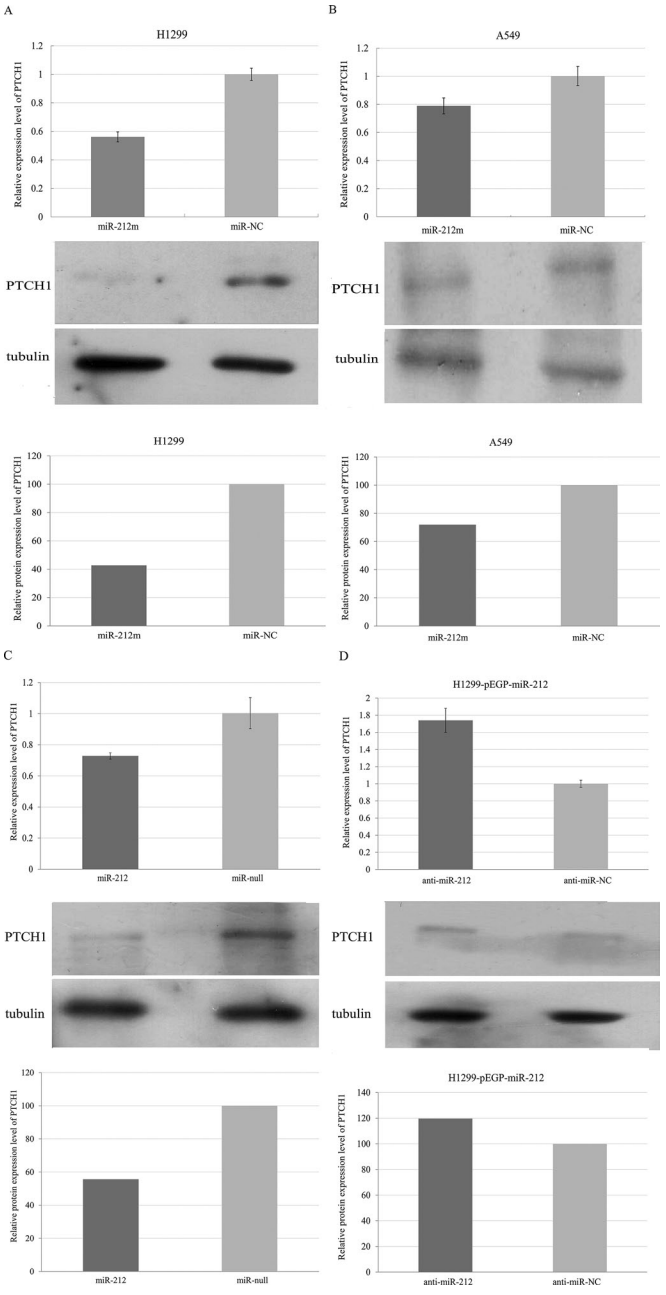

FIGURE 8: The endogenous expression of *PTCH1*. (A) Real-time PCR and Western blotting showed the endogenous expression of *PTCH1* in the following samples: (A) H1299 and (B) A549 cells transfected with miR-212m or miR-NC; (C) H1299-pEGP-miR-212 and H1299-pEGP-miR-null and (D) H1299-pEGP-miR-212 cells transfected with anti-miR-212 or anti-NC. The results revealed that overexpression of miR-212 repressed the endogenous expression of *PTCH1* compared with that of the control, and anti-miR-212 could partially restore the expression of *PTCH1*. Each experiment was performed in triplicate. Bars represent mean ± SD.

We then examined the *PTCH1* levels in H1299 cells stably expressing miR-212. Compared with H1299-pEGP-miR-null cells, the *PTCH1* mRNA and protein were decreased in H1299-pEGP-miR-212 cells (Figure 8C). When miR-212 activity was partially blocked by transfection with anti-miR-212 in H1299-pEGP-miR-212 cells, the expression of *PTCH1* protein was rescued compared with that of anti-miR-NC (Figure 8D).

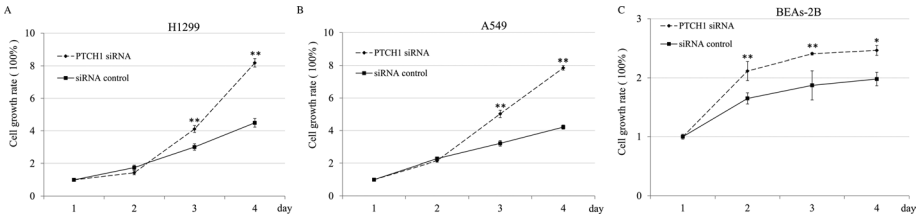

FIGURE 9: Knockdown *PTCH1* expression promoted cell proliferation. Cells were transfected with *PTCH1* siRNA or siRNA control for 48 h and then subjected to cell proliferation analysis. Cells were seeded into a 96-well plate at 5000 cells/well and examined at 24, 48, 72, and 96 h. *PTCH1* siRNA promoted cell proliferation in H1299 (A), A549 (B), and BEAs-2B (C) cells. Each experiment was performed in sextuplicate (n = 6). The criterion for data significance is the p value. *, $p < 0.1$; **, $p < 0.01$.

The result indicated miR-212 could decrease *PTCH1* expression by mRNA cleavage.

Knockdown *PTCH1* expression enhanced miR-212–dependent cell proliferation

We identified that *PTCH1* is a target of miR-212. To evaluate how *PTCH1* played in the functioning of miR-212, we used *PTCH1* small interfering RNA (siRNA) to knock down the expression of *PTCH1* and investigated the resulting cell proliferation. As with miR-212 overexpression, partially decreased *PTCH1* expression significantly increased cell growth rate in parental H1299, A549, and BEAs-2B cells (Figure 9, A–C).

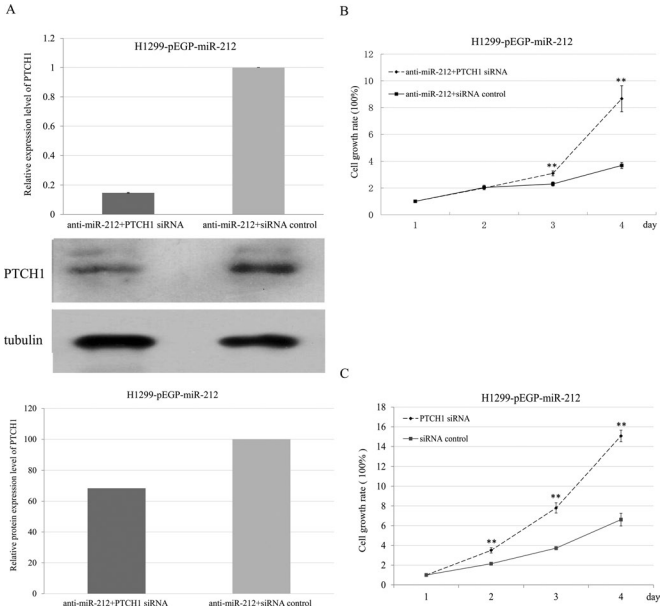

FIGURE 10: Knockdown *PTCH1* expression promoted miR-212–dependent cell proliferation. Real-time PCR and Western blotting (A) showed *PTCH1* siRNA inhibited the *PTCH1* expression in the presence of anti-miR-212 in H1299-pEGP-miR-212 cells. Each experiment was performed in triplicate. Bars represent mean ± SD. (B) *PTCH1* siRNA partially reversed the effect of anti-miR-212 on cell proliferation. Each experiment was performed in sextuplicate (n = 6). (C) Knockdown *PTCH1* in H1299-pEGP-miR-212 cells increased cell growth rate compared with that of siRNA control in the absence of anti-miR-212. Note that in the absence of anti-miR-212, the cell growth rate showed a significant increase in day 2, while in the presence of anti-miR-212, the significant increase started from day 3. Each experiment was performed in sextuplicate (n = 6). **, $p < 0.01$. Bars represent mean ± SD.

To further investigate the contribution of *PTCH1* to the biological effects of miR-212, we cotransfected the H1299-pEGP-miR-212 cells with *PTCH1* siRNA and anti-miR-212. As shown in Figure 10, A and B, *PTCH1* siRNA partially blocked the mRNA and protein expression of *PTCH1* in the presence of anti-miR-212 in H1299-pEGP-miR-212 cells. We then assessed the impact of *PTCH1* silencing on miR-212–dependent cell proliferation. Compared with siRNA control, in the presence of anti-miR-212, knockdown *PTCH1* promoted cell proliferation at days 3 and 4 (Figure 10C). Similar to those increases observed with enforced expression of miR-212 by transfection with miR-212m, *PTCH1* silencing by *PTCH1* siRNA increased cell growth rate at days 2–4 (Figure 10D), since the overexpression of miR-212 was not blocked.

The results seen with transient transfection and bulk-selected cells indicated *PTCH1* may be the effector that mediates the functional role of miR-212 on cell proliferation.

Knockdown of *PTCH1* expression did not enhance miR-212–dependent cell migration and invasion

We also explored whether *PTCH1* mediated the role of miR-212 in cell migration and invasion. We transfected H1299, A549, and BEAs-2B cells with *PTCH1* siRNA, and then performed cell migration and invasion assays. Contrary to our expectations, knockdown of *PTCH1* did not increase cell migration and invasion, but decreased the ability of cell to migrate or invade (Figure 11).

To confirm the results, we also detected the impact of *PTCH1* siRNA on cell migration and invasion in H1299-pEGP-miR-212 cells. In the presence of anti-miR-212, H1299-pEGP-miR-212 cells showed decreased cell migration and invasion. That is, *PTCH1* siRNA did not rescue the effect of miR-212 on cells' migration and invasion ability (Figure 12, A and B). Interestingly, as seen in H1299 parental cells, *PTCH1* silencing by *PTCH1* siRNA also decreased cell migration and invasion of H1299-pEGP-miR-212 cells. However, without the presence of anti-miR-212, the decrease in cell migration and invasion became more moderate (Figure 12, C and D).

The cell migration and invasion results of transient transfection and bulk-selected cells stably expressing miR-212 implied that *PTCH1* might not be the regulator that mediates the role of miR-212 in cell migration and invasion. Other target genes of miR-212 could play a role in tumor metastasis.

DISCUSSION

TPA is known for its tumor-promoting activity. Many signaling pathways, including those of phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, and protein kinase C (PKC), are thought to respond to TPA stimulation (Garg *et al.*, 2008). It is generally accepted that PKC is a major cellular receptor for diacylglycerol and TPA, and it is thought that many of its tumor-promoting, invasive, inflammatory, and proliferative effects are mediated through the activation of one or more *PKC* isoforms (Goel *et al.*, 2007). It also has been reported that many genes, such as *p18INK4c* (Matsuzaki *et al.*, 2004) and *PPARc* (Kim *et al.*, 2006), can be regulated by TPA. However, the role of TPA in miRNA expression in NSCLC has not been explored. We performed an miRNA profile analysis after TPA treatment and found expression of miR-212 was significantly increased.

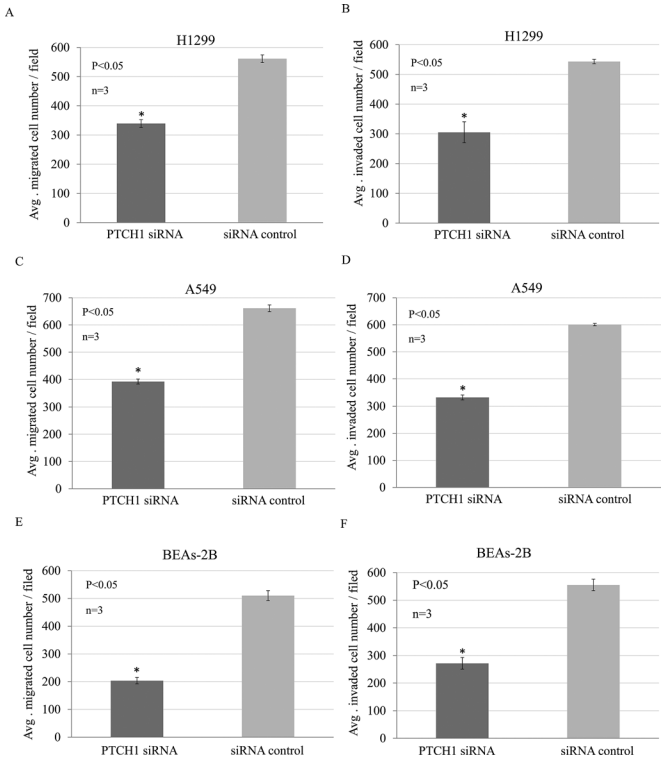

FIGURE 11: *PTCH1* silencing inhibited cell migration and invasion in H1299 (A and B), A549 (C and D), and BEAs-2B (E and F) cells. A sample of 5×10^4 cells transfected with *PTCH1* siRNA or control siRNA grown in 0.1 ml media (1% FBS) were seeded into the upper chambers of Transwell uncoated (A, C, and E: migration assay) or Matrigel matrix–coated (B, D, and F: invasion assay). At 72 h, the cells that migrated through the bottom of the Transwell were stained and counted under a reverse microscope. Each experiment was performed in triplicate (n = 3). Bars represent mean ± SD.

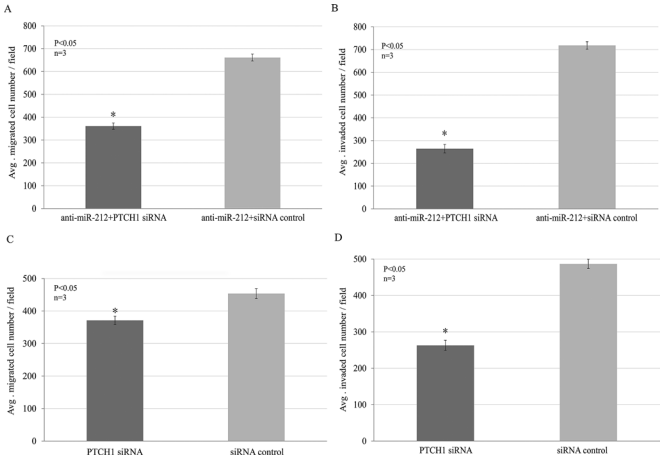

FIGURE 12: *PTCH1* siRNA inhibited cell migration and invasion in H1299-pEGP-miR-212 cells. Cotransfected anti-miR-212 and *PTCH1* siRNA into H1299-pEGP-miR-212, the cell ability of migration (A) and invasion (B) were not rescued by *PTCH1* siRNA. Transfection of *PTCH1* siRNA alone in H1299-pEGP-miR-212 cells inhibited the capacity of cells to migrate (C) or invade (D). Each experiment was performed in triplicate (n = 3). Bars represent mean ± SD.

In 2010, several studies about the role of miR-212 were reported. miR-212 down-regulated and suppressed *MeCP2* in human gastric cancer (Wada *et al.*, 2010), which implied that miR-212 expression might be down-regulated through DNA methylation in some gastric carcinomas. Homeostatic interactions between *MeCP2* and miR-212 in dorsal striatum may be important in regulating vulnerability to cocaine addiction through the CREB signaling pathway (Hollander *et al.*, 2010). It was also reported that miR-212 negatively modulated *PED/PEA-15* expression and sensitized NSCLC cells to tumor necrosis factor–related induced apoptosis (Incoronato *et al.*, 2010) and that miR-212 decreased the protein level, but not the mRNA level, of *Zonulaoccludens 1* (Tang *et al.*, 2008) and *C-terminal binding protein 1* (Fiedler *et al.*, 2008). Recently it also has been reported that miR-132 and miR-212 were increased in pancreatic cancer and targeted the retinoblastoma tumor suppressor (Park *et al.*, 2011).

PTCH1 is a member of the Hh signaling pathway. The Hh pathway regulates cell proliferation, tissue polarity, and cell differentiation during normal development (Ingham, 1998). Abnormal signaling of this pathway has been reported in a variety of human cancers, including basal cell carcinomas, medulloblastomas, SCLCs, and gastrointestinal tract cancers (Hahn *et al.*, 1996; Johnson *et al.*, 1996; Berman *et al.*, 2002, 2003; Taylor *et al.*, 2002; Thayer *et al.*, 2003; Watkins *et al.*, 2003; You *et al.*, 2010). Constitutive activation of Hh signaling, such as *shh, Smo* and *Gli*, has been identified in human cancers (Kinzler *et al.*, 1987; Fan *et al.*, 1997; Oro *et al.*, 1997), demonstrating that the most downstream components of the pathway are sufficient to initiate tumor growth (Dahmane *et al.*, 1997; Grachtchouk *et al.*, 2000; Nilsson *et al.*, 2000). In addition, loss-of-function mutations in negative regulators of the pathway, such as *PTCH1* and *SUFU*, have been shown to be associated with tumorigenesis, which indicates that inhibitors of Hh signaling act as tumor suppressors (Hahn *et al.*, 1996; Johnson *et al.*, 1996; Goodrich *et al.*, 1997; Taylor *et al.*, 2002). Moreover, misregulation of the Hh pathway was found in familial cancers, indicating that the dysregulation is sufficient to cause tumor formation (Hahn *et al.*, 1996; Johnson *et al.*, 1996).

MicroRNAs are important regulators of gene expression; however, little is known about miRNA-mediated targeting of the Hh pathway. It has been reported that *sufu* was targeted by miR-214 in zebrafish (Flynt *et al.*, 2007) and *smo, cos2*, and *fu* were directly repressed by the miR-12/miR-283/miR-304 cluster in *Drosophila* (Friggi-Grelin *et al.*, 2008). In human medulloblastoma cells, the expression of *Smo* was inhibited by miR-326 (Ferretti *et al.*, 2008). In this report, we detected the change of miR-212 expression in cells treated with TPA and predicted that *PTCH1* might be the target of miR-212. We confirmed the binding of *PTCH1* to miR-212 with a dual luciferase assay, and the endogenous expression of *PTCH1* could be inhibited by overexpression of miR-212. It is the first evidence that miR-212 targets the inhibitor of Hh pathway *PTCH1* in NSCLC cells.

The functions of *PTCH1* have been studied in different cell lines and tumors. It has been reported the overexpression of *PTCH1* alone prevents cell growth (Barnes *et al.*, 2001). The loss of *PTCH1* function is likely to induce a tumorigenic phenotype by promoting cell cycle progression through G1–S and G2–M phases via direct regulation of the nuclear accumulation of cyclinB1, which indicates *PTCH1* functions as a "gatekeeper" (Adolphe *et al.*, 2006). We first studied the role of miR-212 in cell cycle status and cell growth rate and showed the overexpression of miR-212 promoted cell proliferation. We also explored whether *PTCH1* is the mediator of miR-212 effects on cell proliferation. *PTCH1* silencing promoted cell

proliferation in H1299, A549, and BEAs-2B cells. Similar results were found in H1299-pEGP-miR-212 cells. Transfection of *PTCH1* siRNA alone in H1299-pEGP-miR-212 cells reinforced the effect of miR-212 on cell growth rate. Moreover, *PTCH1* siRNA partially reversed the effect of anti-miR-212 on cell proliferation in H1299-pEGP-miR-212 cells. Thus, *PTCH1* may be the mediator of miR-212 effects in cell proliferation.

We then explored the role of miR-212 in cell migration and invasion and found that overexpression of miR-212 increased the cells' capacity to migrate and invade. We also examined whether *PTCH1* is a mediator of this behavior. However, unlike cell proliferation, decreased expression of *PTCH1* in H1299, BEAs-2B, and A549 cells by *PTCH1* siRNA inhibited cell migration and invasion, which was contrary to the effects of miR-212. In H1299-pEGP-miR-212 cells, *PTCH1* siRNA did not rescue the effect of miR-212 on cell migration and invasion, but decreased the capacity of the cell to migrate or invade. A similar result was seen in H1299-pEGP-miR-212 cells transfected with *PTCH1* siRNA alone. We conjectured that *PTCH1* is not the mediator of miR-212 effects on cell migration and invasion. *PTCH1* has been shown to be related with tumor metastasis in a few studies. There were results showing that *PTCH1* was overexpressed in metastatic prostate cancer compared with normal tissue (Sheng *et al.*, 2004), but that *PTCH1* was negatively correlated with metastatic potential of colorectal cancer (You *et al.*, 2010). These two reports used human specimens with no mechanism study. Thus, little was known about the role of *PTCH1* in tumor migration and invasion.

Why is *PTCH1* the target of miR-212 if its function in cell migration and metastasis is not in tune with miR-212? The possible reason is that *PTCH1* regulates the Hh pathway and most target genes (such as *PDGFRα*, *BCL2*, *MUS5AC, CDK2*, *follistatin*, *Nanog, basonuclin, Myf5, snail*) of *Gli*, a downstream transcription factor of the Hh pathway that is involved in cell proliferation, rather than cell migration and invasion (Xie *et al.*, 2001; Gustafsson *et al.*, 2002; Cui *et al.*, 2004; Regl *et al.*, 2004; Li *et al.*, 2006; Eichberger *et al.*, 2008; Brandner, 2010; Rizvi *et al.*, 2010; Inaguma *et al.*, 2011). Other targets of miR-212 could be responsible for cell migration and invasion.

Incoronato and colleagues studied the expression of miR-212 and *PED* in 14 NSCLC-affected individuals and four normal lung tissues. In normal lung samples, the levels of miR-212 were high, whereas *PED* was expressed at low levels. On the contrary, in the majority of lung cancer samples, miR-212 was expressed at low levels, and *PED* was overexpressed (Incoronato *et al.*, 2010). The protein PED/PEA-15 has been demonstrated to increase cell migration in lung cancer (Zanca *et al.*, 2010), which is also a target of miR-212 (Incoronato *et al.*, 2010). It was concluded that miR-212 should be considered a tumor suppressor, because it negatively regulates the anti-apoptotic protein PED. However, in a study using 104 pairs of samples, it also was reported that miR-212 is up-regulated in lung cancer tissues compared with noncancerous lung tissues. The result of miR-212 expression was confirmed by real-time RT-PCR in 32 independent samples of adenocarcinoma tissues/squamous cell carcinoma tissues versus noncancerous lung tissues (Yanaihara *et al.*, 2006). miR-212 was also demonstrated to be one of up-regulated markers for lung cancer in 27 patients with lung adenocarcinoma (Rabinowits *et al.*, 2009).

MiRNAs can function both as oncogenes and as tumor suppressors (Esquela-Kerscher and Slack, 2006). MiRNAs that are amplified or overexpressed in cancer, such as miR-155, can act as oncogenes (Kluiver *et al.*, 2006; Ventura and Jacks, 2009). Several miRNAs have been implicated as tumor suppressors based on their physical deletion or reduced expression in human cancer. Functional studies of a subset of these miRNAs indicate that their overexpression can limit cancer cell growth or induce apoptosis in cell culture or upon transplantation in suitable host animals (Ventura and Jacks, 2009). A representative microRNA is the miR-15a~16-1 cluster (Calin *et al.*, 2002). miRNAs also can act as either oncogenes or tumor suppressors, depending on the context, as seen in the miR-29 and miR-17-19b cluster (He L *et al.*, 2005; O'Donnell *et al.*, 2005; Gebeshuber *et al.*, 2009).

The expression of miR-212 has been reported in different kinds of tumors. In human gastric cancer, miR-212 was down-regulated (Wada *et al.*, 2010). In human lung cancer and pancreatic adenocarcinoma tissues, miR-212 was up-regulated (Yanaihara *et al.*, 2006; Rabinowits *et al.*, 2009; Park *et al.*, 2011). An miRNA can have different expression in different cancers, as seen in the miR-17-19b cluster, which demonstrates loss of heterozygosis in hepatocellular carcinoma (He L *et al.*, 2005) but is up-regulated in B-cell lymphomas (O'Donnell *et al.*, 2005).

We showed that overexpressing miR-212 promoted cell cycle progress and cell proliferation, migration, and invasion and that the functional role of miR-212 could be partially reversed by anti-miR-212 in H1299-pEGP-miR-212 cells stably expressing miR-212. Thus, we inferred that miR-212 may have tumor-promoting properties in NSCLC cells.

Our findings, together with published papers (Incoronato *et al.*, 2010; Zanca *et al.*, 2010), suggest that miR-212 can control opposing cellular functions. A possible explanation for this phenomenon might rely on the large number and diverse nature of miR-212 target genes; different algorithms predicted hundreds of conserved targets for miR-212. In general, the net effect of changes in the level of an miRNA will be the sum of all impacts on its targets in a cell type–specific manner. In addition, owing to the large number of mRNA targets, overexpression and functional inhibition of an miRNA might have diverse effects (Gebeshuber *et al.*, 2009). Similar observations were found in other miRNAs, including the miR-17–92 cluster (Calin and Croce, 2006).

PTCH1, the inhibitor of Hh pathway, is itself also the target gene of GLI1, which is the downstream transcription factor of the pathway. It has been reported that all the Hh signaling molecules, including PTCH1, are overexpressed in NSCLC compared with the adjacent nonneoplastic lung parenchyma. Hh pathway activity and expression of *PTCH1* and *SMO* were significantly higher in squamous cell carcinomas compared with other NSCLC histological types (Gialmanidis *et al.*, 2009). In human tumors with *PTCH1* mutations, mutational inactivation of *PTCH1* resulted in a pathological activation of its signaling pathway, with consecutive expression of high levels of *GLI1* and *PTCH1* mRNA. Similar to most *PTCH1*-associated tumors in humans, all tumors of *Ptch1* mutant mice overexpressed *Gli1* and *Ptch1* transcripts (Goodrich *et al.*, 1997; Hahn *et al.*, 1998; Toftgard, 2000). A reasonable explanation for these results is that *PTCH1/Ptch1* regulates itself via a negative-feedback mechanism. Loss of *PTCH1/Ptch1* function, as is caused by a mutation or suppression by miR-212 or both, could lead to a transcriptional activation of *GLI1/Gli1*, the protein product of which binds to *GLI1/Gli1* binding sites in the *PTCH1/Ptch1* promoter, resulting in a transcriptional activation of the *PTCH1/Ptch1* gene and an overexpression of *PTCH1/Ptch1* transcripts (Hahn, 2006). The reduced expression of *PTCH1* by miR-212 was compensated for by the transcription activation of *GLI1* on *PTCH1*.

An unexpected result is that *PTCH1* silencing promoted cell proliferation while inhibiting cell migration and invasion. This phenomenon was found in H1299, A549, and BEAs-2B cells. Several genes, such as *SPRR3*, *VSSC b-subunits*, and *Rho GTPase*, have

also been reported to have opposite functions in cell proliferation and cell migration and invasion (Sequeira *et al.*, 2008; Gribben *et al.*, 2011; Jansson *et al.*, 2011; Kim *et al.*, 2011). It seems that the ability of a molecule to have different effects on tumor formation and metastasis is not uncommon (Jansson *et al.*, 2011). A more detailed study of *PTCH1* will help in understanding the roles of *PTCH1* and miR-212.

In summary, we have shown that miR-212 may act as an oncogene in human NSCLC cells to promote cell proliferation, migration, and invasion. The down-regulation of Hh signaling member *PTCH1* might be responsible for the cell proliferation effect of miR-212.

MATERIALS AND METHODS

Cell culture

All cell lines were obtained from the American Type Culture Collection (Manassas, VA). The medium, fetal bovine serum (FBS), HEPES, nonessential amino acids, and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). BEAS-2B cells were cultured in serum-free bronchial epithelial growth media (Clonetics/Lonza, Walkersville, MD). WI-38 cells were cultured in DMEM supplemented with 10% FBS. NCI-H1299 and A549 cells were cultured with RPMI 1640 medium supplemented with 10% FBS, 2.383 mg/ml HEPES, and 0.11 mg/ml sodium pyruvate at 37°C in a humidified atmosphere of 5% CO_2.

Microarray analysis

Human NSCLC cell line A549 cells were treated with 50 nm TPA for 2, 12, and 24 h; total RNA from each sample was then isolated with Trizol (Invitrogen) according to the manufacturer's instructions. The purity and quantity of the isolated small RNAs were assessed using 1% formaldehyde-agarose gel electrophoresis and spectrophotometry (Bio-Rad Laboratories, Hercules, CA) and then applied onto an Illumina ChIP (Illumina, San Diego, CA) according to the manufacturer's instructions. On the Illumina ChIP, each detection probe consisted of a chemically modified nucleotide-coding segment complementary to and able to target all 739 mature human miRNA sequences from miRBase version 10.0 (Sanger Institute, Cambridge, UK). After RNA hybridization, microarrays were stained with streptavidin-Cy3. Image data were analyzed using BeadStudio software (Illumina). Data normalization and differential analysis were carried out with GeneSpring GX software (Agilent Technologies, Santa Clara, CA).

Expression construct

A genomic fragment of hsa-miR-212 precursor from human chromosome 17 was amplified by PCR. The PCR primers were: 5′- CGCGGATCCCGGCGCCTGCGTTGATCAGCAC-3′ and 5′- CTAGCTAGCAGGGGAGGCGGAGCAGCAGAGC-3′.The PCR product was digested with *Bam*HI and *Nhe*I restriction enzymes and cloned into the pEGP-miR cloning and expression vector (Cell Biolabs, San Diego, CA) and designated pEGP-miR-212.

A modified pGL-3-promoter vector was used for the construction of the 3′UTR luciferase reporter. Several restriction endonuclease sites (*Xba*I-*Nde*I-*Apa*I-*Pst*I-*Eco*RI-*Xba*I) had been inserted into pGL-3-promoter vector. The wild-type 3′UTR of *PTCH1* was amplified by PCR from WI-38 cell line and then inserted into the modified vector. The primers: 5′–CCCATATGTGCCAGGACAGCAGTTCATT-3′ and 5′-CGGAATTCGAGCCTACTACAGGTTACAGACAG-3′ were used. The amplified fragment was digested with *Nde*I and *Eco*RI, and inserted immediately downstream from the stop codon of luciferase. The mutant 3′UTR luciferase reporter of *PTCH1* containing the mutant binding sites of miR-212 was created using the same procedure with the primers: 5′-TGTGGTAGTATGAAATGTTTGACAATACTTTCAAACACGCTATGCGTGATAATT-3′, and 5′-ATTGTCAAACATTTCATACTACCACAGGGTTGTGATAT- 3′, with primers used for wide-type 3′UTR of *PTCH1* to amplify the mutant 3′UTR of *PTCH1*.

Transient transfection and bulk-selected H1299 cells stably expressing miR-212

Transfection was carried out with *Lipofectamine 2000* Transfection Reagent in accordance with the manufacturer's procedure (Invitrogen). The day before transfection, cells were seeded in six-well plates. A 100-pmol sample of miR-212m or miR-NC in 250 µl Opti-MEM medium (Gibco, Grand Island, New York) was mixed with 5 µl Lipofectamine 2000 dissolved in 250 µl of the same medium and allowed to stand at room temperature for 20 min. The resulting 500 µl transfection solutions were then added to each well, which already contained 1.5 ml of Opti-MEM. Six hours later, the cultures were replaced with 2 ml fresh RPMI 1640 medium. The same procedure was performed for the transfection of miR-212 inhibitor or inhibitor negative control (Table 2), which were synthesized by GenePharma (Shanghai, China).

H1299 cells were transfected with pEGP-miR-212 or control vector pEGP-miR-null (Cell Biolabs). At 48 h after transfection, the cells were harvested and seeded into six-well plates and then selected with 5 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO) for 2 wk. The GFP fluorescence was monitored under a fluorescence microscope. For 2 wk, the bulk-selected H1299 cells stably expressing miR-212 or null were maintained in RPMI 1640 with 2.5 µg/ml puromycin. Bulk cultures were used to avoid clone-specific effects.

Quantitative RT-PCR

Total RNA was extracted using Trizol (Invitrogen), and reverse transcription was performed according to the manual of PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). Real-time quantitative PCR was performed using an ABI Prism 7900HT (Applied Biosystems, Foster City, CA). miR-212–specific reverse transcription (RT) primer was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCCGT-3′. Quantitative real-time PCR was utilized to quantify the gene expression levels with 2X HotSybr PCR Reaction Mix (Nustar). PCRs consisted of a hot start (10 min at 95°C), followed by 40 cycles (15 s at 95°C and 60 s at 60°C). Transcript levels for each gene were normalized to 18srRNA levels by using comparative threshold cycle (Ct) method, in which fold difference = $2^{-(\Delta Ct \text{ of target gene} - \Delta Ct \text{ of reference})}$. Each sample was run in triplicate to ensure quantitative accuracy. Expression levels of miR-212, *PTCH1*, and 18srRNA were detected. The primers used for real-time PCR are listed in Table 2.

RNA interference

The online software siRNA Target Finder (Applied Biosystems) was used to design siRNA against *PTCH1* (www.ambion.com/techlib/misc/siRNA_finder.html), followed by a BLAST search

Genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
miR-212	CGGCGGTAACAGTCTCCAGTC	GTGCAGGGTCCGAGGT
PTCH1	CGCTTTCTACATCTACCTGA	AAGGGAACTGGGCATACTC
18srRNA	TTGGTGGAGCGATTTGTCTG	ATCTCGGGTGGCTGAACG

TABLE 2: Primers used for real-time PCR amplification.

Oligonucleotides	Sense (5′ to 3′)	Anti-sense (5′ to 3′)
miR-212m	UAACAGUCUCCA-GUCACGGCC	CCGUGACUGGA-GACUGUUAUU
Negative control	GCGACGAU-CUGCCUAA-GAUdTdT	AUCUUAGGCA-GAUCGUCGCdTdT
miR-212 inhibitor	GGCCGUGACUG-GAGACUGUUA	
Inhibitor negative control	CAGUACUUUUGU-GUAGUACAA	
PTCH1 siRNA	GAUUGGAGAA-GAGGCUAUGUU	CAUAGCCUCUU-CUCCAAUCUU

TABLE 3: Sequences of synthetic oligonucleotides.

to evaluate the uniformity of the siRNA sequences in the human transcriptome. siRNA targeting nucleotides 624–644 (Table 2) was selected to be chemically synthesized by GenePharma. An siRNA lacks homology to the genome used as a negative control. Mature miR-212m and miR-NC, as well as miR-212 inhibitor (anti-miR-212) and negative control inhibitor (anti-miR-NC) were also synthesized by GenePharma. All sequences are listed in Table 3. Transient transfection of siRNA and miRNA mimics was performed as described in *Transient transfection and bulk-selected H1299 cells stably expressing miR-212*.

Cell cycle assay

A sample of 1×10^6 bulk-selected H1299 cells stably expressing miR-212 were typsinized, washed twice with phosphate-buffered saline (PBS), permeabilized overnight in 70% ethanol at –20°C, and incubated with propidium iodide (10 μg/ml) containing RNase at 4°C for 30 min. The percentages of cells in different phases of the cell cycle were measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA) and analyzed with ModFit software (Verity Software House, Topsham, ME). The same cell cycle assay procedure was applied for transiently transfected A549, BEAS-2B, and H1299 cells.

Colony-formation assay

The anchorage-independent colony-formation assays were performed in soft agar in a six-well plate. Each well was covered with a layer (3 ml) of 0.7% agar in a medium supplemented with 20% FBS. Two thousand viable cells were prepared in 3 ml of 0.35% agar (Cat.A9045; Sigma-Aldrich), poured into the wells, and laid with medium supplemented with 0.7% agar. The plate was cultured at 37°C at 5% CO_2, incubated for 20 d, and then photographed using a light microscope. The number of colonies with diameters ≥0.3 mm were counted. Each value was derived from three independent experiments and results were expressed as the mean ± SE.

Cell proliferation assay

Cell proliferation analysis was performed with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manual of the manufacturer. Briefly, 5000 cells were seeded into each well of 96-well plate. Cells were examined at 24, 48, 72, 96, and 120 h. CCK-8 (10 μl) was added to each well at different time points. After an incubation of 1.5 h at 37°C, absorbance was measured at 450 nm with a Microplate Reader ELx808 (Bio-Tek Instruments, Winooski, VT). The absorbance at 630 nm was used as a reference. Each experiment was performed at least in sextuplicate.

Cell migration and invasion assay

The migration and invasiveness potential of transient transferred cells and bulk-selected H1299 cells were examined. The cell invasion assay were performed in Transwell plates (8-μm pore size, 6.5-mm diameter; Corning Life Sciences, Lowell, MA) precoated with Matrigel Basement Membrane Matrix (coating concentration: 1 mg/ml; BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, a total of 5×10^4 cells in 0.1 ml media (with 1% FBS) were seeded into the upper chamber, with 0.6 ml of medium (10% FBS) under the upper chamber. Plates were incubated in a humidified incubator at 37°C and 5%CO_2. At 72 h, chambers were removed, and a cotton swab was used to scrape the noninvading cells from the upper side of the chamber. The cells under the chamber were fixed with methanol for 10 min, stained with 5% Giemsa solution for 5 min, and washed twice with PBS. The cells that migrated through the Matrigel were counted under the microscope. Migration assays were performed with the same procedure, except that the Transwell chamber inserts were not coated with Matrigel, and medium containing 10% FBS was used for the cell suspensions.

Dual luciferase assay

H1299 cells were cotransfected in 24-well plates with 50 pmol miR-212m, together with 1 μg firefly luciferase report construct containing the wild-type or mutant *PTCH1*-3′-UTR and 150 ng control vector pRL-TK (Promega, Madison, WI) containing *Renilla* luciferase. Firefly and *Renilla* luciferase activities were measured 48 h after transfection using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Cotransfection of pEGP-miR-212; luciferase report construct and pRL-TK and anti-miR-212; wild-type *PTCH1*-3′-UTR; miR-212m and pRL-TK were analyzed using the same procedure.

Immunoblotting

Cells were grown to 80% confluence, and whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride [Sigma-Aldrich]; Complete Protease Inhibitor Cocktail [Roche, Indianapolis, IN]). Samples were centrifuged at 4°C for 15 min to pellet insolubles. Protein concentrations were measured by a modified Lowry assay (SpectraMax M5 plate reader; Molecular Devices). Samples of 20 μg were resolved by 12% SDS–PAGE and electroblotted onto polyvinylidenedifluoride plus membrane (Micron Separation). Membranes were blocked in a solution of TBS containing 5% nonfat milk and 0.1% Tween 20 for 2 h on a rotary shaker. Blots were incubated with a 1:1000 dilution of rabbit anti-PTCH1 (Proteintech Group, Chicago, IL) or 1:5000 dilution of mouse anti-tubulin (Sigma-Aldrich) overnight at 4°C, washed three times with TBS containing 0.1% Tween-20 at room temperature, and then incubated in horseradish peroxidase–coupled secondary antibodies against rabbit or mouse immunoglobulin G (1:5000; KPL, Gaithersburg, MD) for 1.5 h at room temperature. The membranes were washed three times and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Bremen, Germany).

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