

ORIGINAL ARTICLE

# microRNA-107 functions as a candidate tumor-suppressor gene in head and neck squamous cell carcinoma by downregulation of protein kinase C $\epsilon$

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide with about 600 000 new cases diagnosed each year. Understanding the molecular pathways that lead to HNSCC is crucial to identify new targets for anti-cancer drug development. Protein kinase C $\epsilon$  (PKC $\epsilon$ ) is elevated in HNSCC and regulates the activation of Akt, Stat3 and Rho GTPases. To date, the molecular mechanism of PKC $\epsilon$  dysregulation in HNSCC remains to be elucidated. *In silico* analysis identified three putative microRNA-107 (miR-107) binding sites in the 3'-untranslated region (UTR) of PKC $\epsilon$ . An inverse relationship was revealed between miR-107 and PKC $\epsilon$  in HNSCC cell lines. Delivery of miR-107 reduced PKC $\epsilon$  levels in SCC15, SCC25 and CAL27, three HNSCC cell lines with high PKC $\epsilon$  and low miR-107. The activity of a luciferase reporter construct containing the 3'-UTR of PKC $\epsilon$  was downregulated by miR-107 and mutations in the three cognate miR-107 binding sites completely ablated the regulation by miR-107. Treatment with miR-107 significantly blocked cell proliferation, DNA replication, colony formation and invasion in SCC25 and CAL27 cells. Ectopic expression of miR-resistant PKC $\epsilon$  was sufficient to partially rescue the loss-of-function phenotype in miR-107-overexpressing SCC25 cells. Tumor growth in nude mice was retarded by 93  $\pm$  7% in CAL27/miR-107 cells compared with CAL27/miR-control cells. Last, human primary HNSCC tumors with elevated PKC $\epsilon$  had reduced miR-107 expression. Our results demonstrate that PKC $\epsilon$  is directly regulated by miR-107 and, moreover, suggest that miR-107 may be a potential anti-cancer therapeutic for HNSCC.

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**Keywords:** head and neck cancer; protein kinase C; microRNA; miR-107

## Introduction

Approximately 600 000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed worldwide each year (Parkin, 2001; Kamangar *et al.*, 2006; Leemans *et al.*, 2011). Curative treatment is often achieved in patients presented with early-stage disease through surgery or radiation. Palliative chemotherapy is the standard approach for patients with recurrent/metastatic disease. The majority, greater than two-thirds, of HNSCC patients present with locally advanced disease that requires a modern multidisciplinary approach involving surgery, radiation and pharmacotherapy. However, patients with locally advanced HNSCC frequently develop locoregional recurrence and/or distant metastasis after definitive treatment. The risk of failure is particularly high in patients with inadequate resection margins, extra-nodal spread or multiple lymph node involvement (Bonner *et al.*, 2010). In these high-risk patients, primary surgery is often followed by adjuvant radiation therapy. In spite of this intense treatment regimen, locoregional recurrence occurs in about 30% of patients, distant metastasis occurs in about 25% of patients and the 5-year survival rate is a dismal 40% (Laramore *et al.*, 1992). Thus, a critical need remains to identify and characterize genes that are responsible for promoting an aggressive HNSCC phenotype prone to relapse and metastasis to allow for better clinical management.

Protein kinase C (PKC) is a family of serine/threonine kinases known to have critical roles in the signal transduction pathways involved in proliferation, differentiation, apoptosis and migration (Dempsey *et al.*, 2000; Jaken and Parker, 2000; Gutcher *et al.*, 2003; Aziz *et al.*, 2010). Published work showed that PKC $\alpha$ ,  $\epsilon$  and  $\zeta$  are dysregulated in HNSCC and contribute to HNSCC development and/or progression. PKC $\zeta$  was reported to control epidermal-growth-factor-receptor-dependent proliferation of HNSCC cells (Cohen *et al.*, 2006). A recent study showed that PKC $\alpha$  downregulates microRNA (miR)-15a to enhance DNA synthesis and cell-cycle progression in HNSCC (Cohen *et al.*, 2009). Moreover, elevated PKC $\alpha$  expression was found to be associated with poor prognosis in HNSCC (Cohen *et al.*, 2009). Our laboratory demonstrated that PKC $\epsilon$  is elevated in HNSCC and promotes an invasive and

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motile phenotype through modulation of RhoA and RhoC GTPases (Pan *et al.*, 2006). In primary HNSCC tumors, PKC $\alpha$ ,  $\beta$ ,  $\epsilon$ ,  $\gamma$  and  $\zeta$  levels were shown to be elevated but only PKC $\epsilon$  was found to be a prognostic biomarker, even better than the traditional gold standard of TNM staging (Martinez-Gimeno *et al.*, 1995). This prospective study indicated that elevated PKC $\epsilon$  is significantly associated with an increase in disease recurrence and a decrease in overall survival (Martinez-Gimeno *et al.*, 1995).

It is clear that PKC $\epsilon$  is elevated in HNSCC; however, the molecular mechanisms leading to increased PKC $\epsilon$  levels remain to be elucidated. In this study, we demonstrate that miR-107 directly regulates PKC $\epsilon$  in HNSCC. An inverse association was found for miR-107 and PKC $\epsilon$  in HNSCC cell lines and tumor specimens. Overexpression of miR-107 decreased PKC $\epsilon$  levels, and reduced the tumorigenic properties of HNSCC cells *in vitro* and *in vivo*. Moreover, enforced expression of miR-resistant PKC $\epsilon$  partially reversed the loss-of-function phenotype in miR-107-overexpressing HNSCC cells. Our work revealed a novel mechanism for PKC $\epsilon$  dysregulation in HNSCC and demonstrated miR-107 as a candidate tumor suppressor in this patient population.

## Results

### *Inverse association between miR-107 and PKC $\epsilon$ in HNSCC cell lines*

Our laboratory and others reported that PKC $\epsilon$  is elevated in HNSCC (Martinez-Gimeno *et al.*, 1995; Cohen *et al.*, 2006; Pan *et al.*, 2006). However, the molecular mechanism of this observation remains to be elucidated. Several miRs were predicted by TargetScan to bind to the 3'-untranslated region (UTR) of PKC $\epsilon$ , suggesting that miRs may be a negative regulator of PKC $\epsilon$ . We focused our initial experiments on miR-103a and miR-107 as both miRs have three putative binding sites in the 3'-UTR of the PKC $\epsilon$  gene. As shown in Figure 1, three HNSCC cell lines, SCC15, SCC25 and CAL27, have significantly elevated PKC $\epsilon$  protein levels and reduced miR-107 expression compared with primary keratinocytes. In contrast, the level of miR-103a, which has the same seed sequence as miR-107, was variable in the three HNSCC cell lines (Figure 1c). Compared with primary keratinocytes, miR-103a expression was higher in SCC15, unchanged in SCC25 and lower in CAL27. These results provide evidence to demonstrate an inverse association between miR-107 and PKC $\epsilon$  in HNSCC.

### *PKC $\epsilon$ is a target of miR-107*

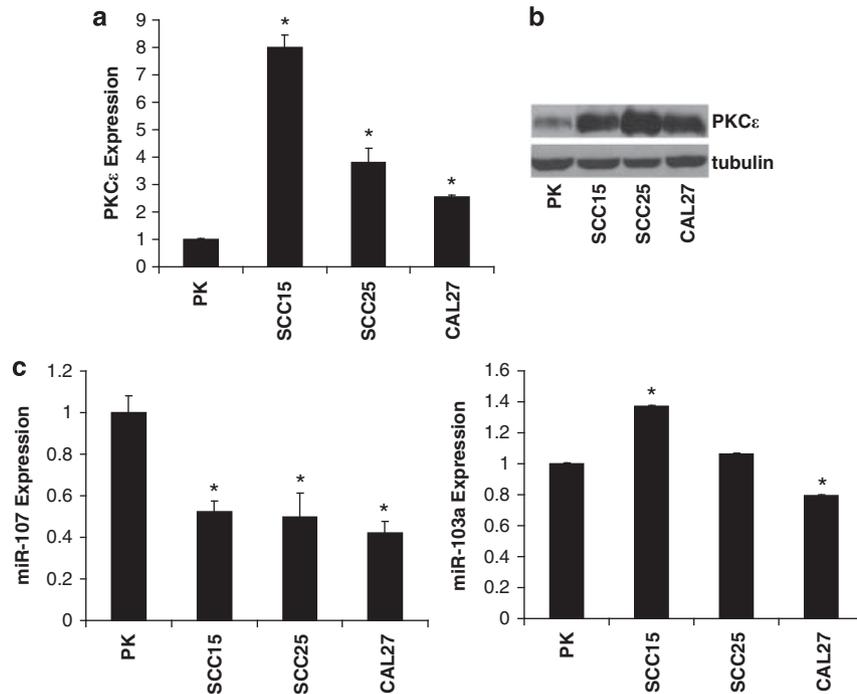
To further provide evidence that miR-107 regulates PKC $\epsilon$ , HNSCC cells were transiently transfected with pre-miR-control or pre-miR-107 (50 nmol/l for 48 h). Transfection with pre-miR-107 enhanced the expression of mature miR-107 by 329  $\pm$  8-fold, 386  $\pm$  4-fold, and 227  $\pm$  11-fold in SCC15, SCC25 and CAL27 cells, respectively ( $P < 0.001$ ). In comparison with pre-miR-control, delivery of pre-miR-107 resulted in a dramatic

decrease in PKC $\epsilon$  levels in SCC15, SCC25 and CAL27 cells confirming that PKC $\epsilon$  is a target of miR-107 (Figure 2a). Next, we determined whether miR-107 directly binds to their predicted sites in the 3'-UTR of PKC $\epsilon$  to control PKC $\epsilon$  levels. *In silico* analysis with TargetScan, identified three putative miR-107 binding sites in the 3'-UTR of PKC $\epsilon$  located at nucleotides 27–33 (site 1), 1517–1523 (site 2), and 1564–1570 (site 3). CAL27 cells were co-transfected with pre-miR-control or pre-miR-107 and a reporter expression vector containing wild-type or mutant 3'-UTR of PKC $\epsilon$  cloned downstream of a luciferase gene (Figure 2c). Pre-miR-107 blocked luciferase activity by 55  $\pm$  2% ( $P < 0.01$ ) in cells transfected with the full-length wild-type 3'-UTR of PKC $\epsilon$ . Mutations in one of the three miR-107 sites dampened the inhibitory effect of pre-miR-107 on luciferase activity; 38  $\pm$  2% inhibition for mutant 1, 32  $\pm$  4% inhibition for mutant 2 and 30  $\pm$  3% inhibition for mutant 3. Interestingly, pre-miR-107 had no effect on luciferase activity in cells transfected with the 3'-UTR of PKC $\epsilon$  containing mutations in all three miR-107 sites (Mut 1–3). Our work confirmed that miR-107 regulates PKC $\epsilon$  through direct binding in the 3'-UTR of the PKC $\epsilon$  mRNA and moreover, optimal suppression of PKC $\epsilon$  requires occupation of all three miR-107 sites.

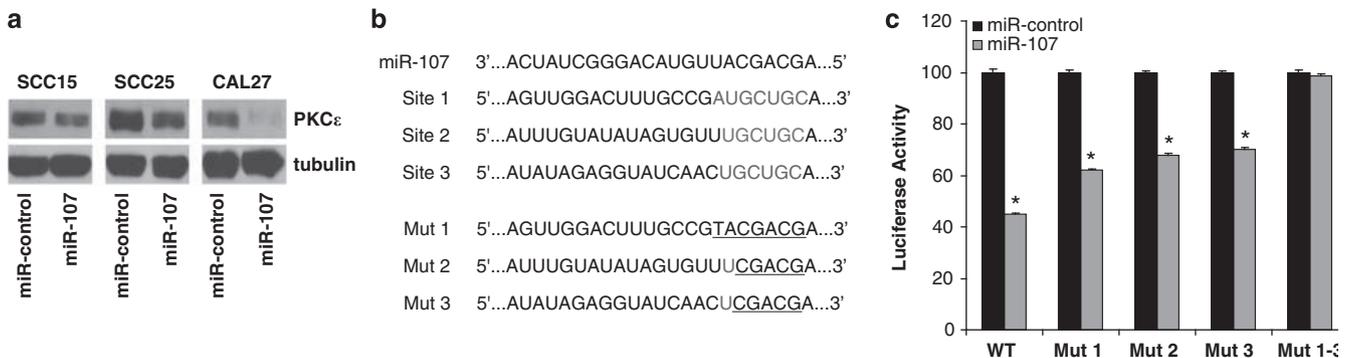
### *miR-107 reduces the tumorigenic potential of HNSCC in vitro and in vivo*

Our previous work demonstrated that PKC $\epsilon$  has a critical role in promoting an aggressive phenotype in HNSCC (Pan *et al.*, 2006). As miR-107 was sufficient to dramatically reduce PKC $\epsilon$  levels, the anti-cancer effects of miR-107 on HNSCC cells were assessed. As shown in Figure 3, cell proliferation, DNA replication, colony formation and cell invasion were determined in SCC25 and CAL27 cells following treatment with pre-miR-control or pre-miR-107 (50 nmol/l). Cell proliferation was reduced in SCC25 cells by 20  $\pm$  2% ( $P < 0.01$ ) and in CAL27 cells by 28  $\pm$  2% ( $P < 0.01$ ) following pre-miR-107 treatment. Pre-miR-107 blocked DNA replication and colony formation by 72  $\pm$  5 and 70  $\pm$  3% in SCC25 cells and 67  $\pm$  3 and 45  $\pm$  6% in CAL27 cells, respectively ( $P < 0.01$ ). Treatment with pre-miR-107 inhibited cell invasion by 93  $\pm$  5% in SCC25 cells and 84  $\pm$  8% in CAL27 cells ( $P < 0.01$ ).

Stable polyclonal miR-control or miR-107 overexpressing CAL27 cells were implanted in athymic nude mice to determine the effect of miR-107 on *in vivo* tumorigenicity (Figure 4). In Figure 4a, CAL27/miR-107 cells had a dramatic increase in miR-107 expression compared with CAL27/miR-control cells ( $P < 0.005$ ). The levels of PKC $\epsilon$  and two other confirmed miR-107 targets, hypoxia-inducible factor 1- $\beta$  (HIF1- $\beta$ ) and cyclin-dependent kinase 6 (CDK6), were decreased in miR-107 overexpressing CAL27 cells. Interestingly, Dicer1, a confirmed miR-107 target in breast cancer, was unchanged in CAL27/miR-107 cells compared with CAL27-miR-control cells. Compared with anti-miR-control transfection, primary keratinocytes transfected with anti-miR-107 (PK/anti-miR-107) had lower mature



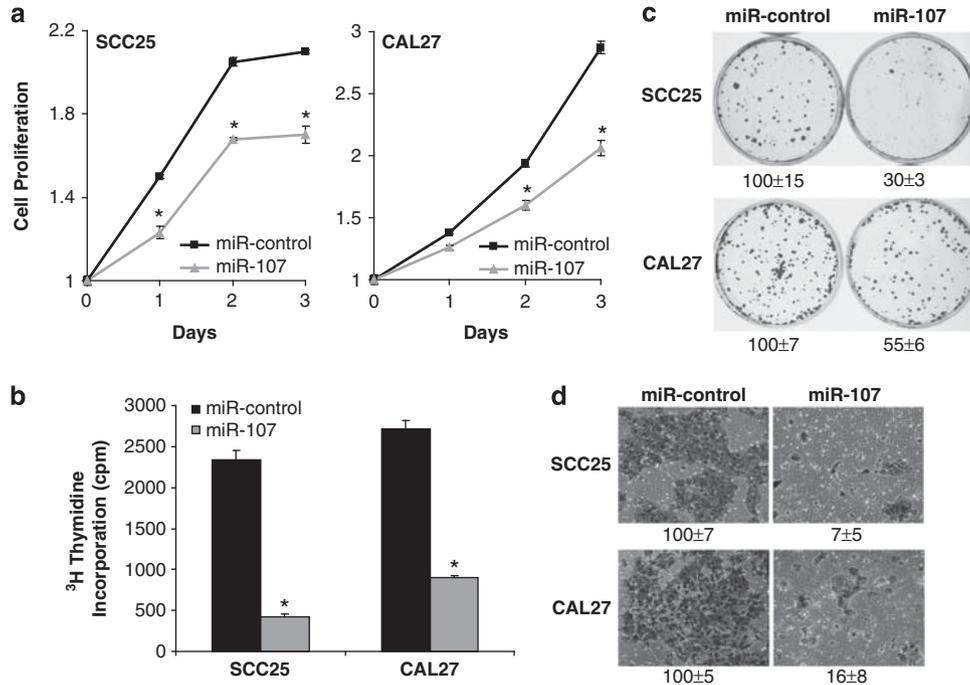
**Figure 1** miR-107 is downregulated and inversely associated with PKC $\epsilon$  in HNSCC cell lines. (a) PKC $\epsilon$  mRNA expression is elevated in HNSCC cell lines. Quantitative PCR (qPCR) analysis of PKC $\epsilon$  and GAPDH was performed with total RNA isolated from human primary keratinocytes and HNSCC cell lines using validated TaqMan assays. Data are presented as mean  $\pm$  s.e.m. \**P*-value < 0.005. (b) PKC $\epsilon$  protein levels are elevated in HNSCC cell lines. Western blot analysis of PKC $\epsilon$  and tubulin was performed with whole-cell lysates isolated from human primary keratinocytes and HNSCC cell lines. (c) miR-107 expression levels are lower in HNSCC cell lines. qPCR analysis of miR-103 and miR-107 in primary keratinocytes and HNSCC cell lines was performed using validated TaqMan assays. Data are presented as mean  $\pm$  s.e.m. \**P*-value < 0.005.



**Figure 2** PKC $\epsilon$  is a downstream target of miR-107. (a) Ectopic expression of miR-107 reduced PKC $\epsilon$  levels in HNSCC. SCC15, SCC25 and CAL27 cells were transfected with pre-miR-control or pre-miR-107 (50 nmol/l) using Lipofectamine 2000 for 48 h. Whole-cell lysates were extracted and western blot analysis was performed using anti-PKC $\epsilon$  and anti-tubulin antibodies. (b) Wild-type and mutant miR-107 binding sites in the 3'-UTR of PKC $\epsilon$ . (c) Luciferase activity controlled by 3'-UTR of PKC $\epsilon$  is inhibited by ectopic expression of miR-107. HEK293 T cells were co-transfected with wild-type or mutant PKC $\epsilon$ -3'UTR and pre-miR-control or pre-miR-107. Renilla and Firefly luciferase activities were measured using the Dual-Luciferase Reporter assay system. Renilla luciferase is normalized to Firefly luciferase and data are presented as mean  $\pm$  s.e.m. of four independent experiments. \**P*-value < 0.01.

miR-107 expression (70  $\pm$  1% inhibition, *P* < 0.001). Importantly, PK/anti-miR-107 cells demonstrated an increase in PKC $\epsilon$ , HIF1- $\beta$  and CDK6 protein levels providing further evidence that miR-107 is a key regulator of these genes (Figure 4c). Tumor growth was significantly reduced in the mice implanted with CAL27/miR-107 cells compared with CAL27/miR-control cells. Mice bearing CAL27/miR-control tumors

had a mean tumor volume of 1691  $\pm$  455 mm<sup>3</sup> at 6 weeks post implantation. In contrast, CAL27/miR-107 cells were less tumorigenic and had a mean tumor volume of 108  $\pm$  39 mm<sup>3</sup> (*P* < 0.005, *n* = 7). Moreover, mean tumor weight was significantly lowered by 93  $\pm$  2% in CAL27/miR-107 tumors compared with CAL27/miR-control tumors (*P* < 0.005, *n* = 7). Importantly, PKC $\epsilon$  protein levels were dramatically lower in tumors from CAL27/



**Figure 3** Ectopic expression of miR-107 reduces the tumorigenic properties of SCC25 and CAL27 cells *in vitro*. SCC25 and CAL27 cells were transfected with pre-miR-control or pre-miR-107 (50 nmol/l) using Lipofectamine 2000. After 48 h, cells were used for the phenotypic assays. (a) Cell proliferation. Cell proliferation was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value < 0.01,  $n = 3$ . (b) DNA synthesis. Incorporation of  $^3\text{H}$ -thymidine into DNA was monitored after 4 h in a scintillation counter. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value < 0.01,  $n = 3$ . (c) Colony formation. Colonies were stained with crystal violet and counted. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value < 0.01,  $n = 3$ . (d) Cell invasion. Cell invasion was assessed using the modified Boyden chamber invasion assay with Matrigel basement membrane. Invasive cells were counted. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value < 0.01,  $n = 3$ .

miR-107 cells than in tumors from CAL27/miR-control cells. Taken together, these results indicate that down-regulation of PKC $\epsilon$  by miR-107 is an effective approach to suppress the tumorigenicity of HNSCC *in vitro* and *in vivo*.

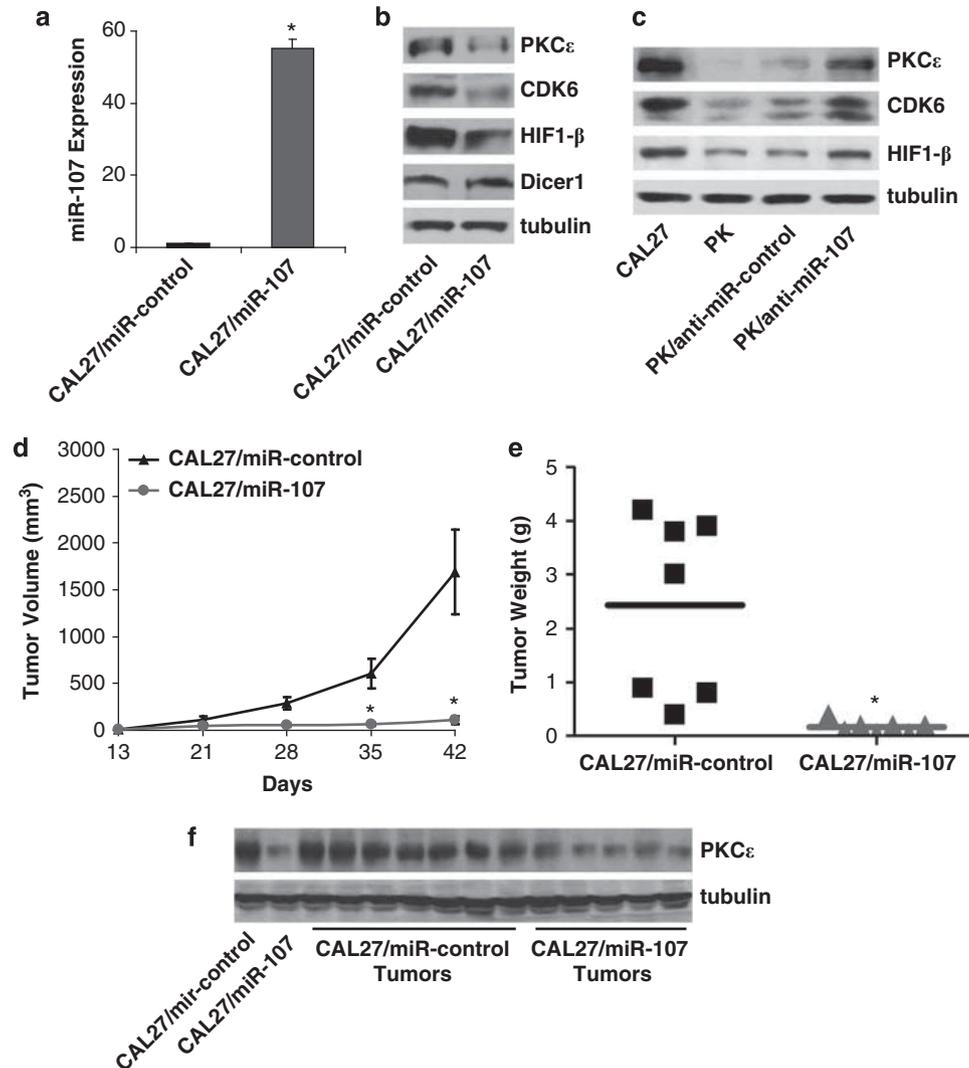
#### miR-resistant PKC $\epsilon$ reverses the anti-tumorigenic effects of miR-107

In addition to targeting PKC $\epsilon$ , other groups have reported that miR-107 directly regulates HIF1- $\beta$  and CDK6 (Lee *et al.*, 2009; Yamakuchi *et al.*, 2010; Feng *et al.*, 2011). To provide direct evidence that down-regulation of PKC $\epsilon$  is required for the anti-tumorigenic effects of miR-107, miR-resistant PKC $\epsilon$  was over-expressed in miR-107 expressing SCC25 (SCC25/miR-107) cells to determine whether restoring PKC $\epsilon$  would be sufficient to rescue the miR-107 loss-of-function phenotype. SCC25/miR-107 cells were transfected with a wild-type PKC $\epsilon$  expression or empty vector without the 3'-UTR of PKC $\epsilon$ . In Figure 5a, the SCC25/miR-107/PKC $\epsilon$  cells had the proper genetic alterations and had elevated PKC $\epsilon$  levels in a high miR-107 background. Empty vector-transfected SCC25/miR-107 cells had minimal effect on cell phenotype as these cells maintained their miR-107 loss-of-function defect compared with SCC25/miR-control cells. Importantly, ectopic overexpression of PKC $\epsilon$  partially rescues the defect of SCC25/miR-107 cells and resulted in a

significant increase in cell proliferation ( $P < 0.005$ ), DNA replication ( $56 \pm 8\%$ ,  $P < 0.005$ ), colony formation ( $130 \pm 18\%$ ,  $P < 0.005$ ) and cell invasion ( $392 \pm 39\%$ ,  $P < 0.005$ ) compared with SCC25/miR-107/empty cells. These results reveal that the tumor-suppressive actions of miR-107 are, at least, partially through modulation of PKC $\epsilon$  levels.

#### miR-107 is reduced and inversely associated with PKC $\epsilon$ in primary HNSCC tumors

Our studies with HNSCC cell lines demonstrated an inverse association between miR-107 expression and PKC $\epsilon$  protein levels. Next, we examined miR-107 and PKC $\epsilon$  expression in primary tumors and matched normal adjacent epithelium from previously untreated HNSCC patients by quantitative PCR. As shown in Figure 6a, a majority (12/17) of HNSCC patients had significantly lower miR-107 expression in the tumor compared with normal tissue. A 37.5% reduction in miR-107 expression in the tumor compared with the normal tissue was demonstrated for this patient cohort ( $P < 0.008$ ,  $n = 17$ ); mean miR-107 expression was  $0.005 \pm 0.0008$  for the primary HNSCC tumors and  $0.008 \pm 0.0007$  for the adjacent normal epithelium. These observations are consistent and further support our results demonstrating that miR-107 has tumor-suppressive actions in HNSCC. In this cohort, four patients were found to have significantly higher PKC $\epsilon$  expression



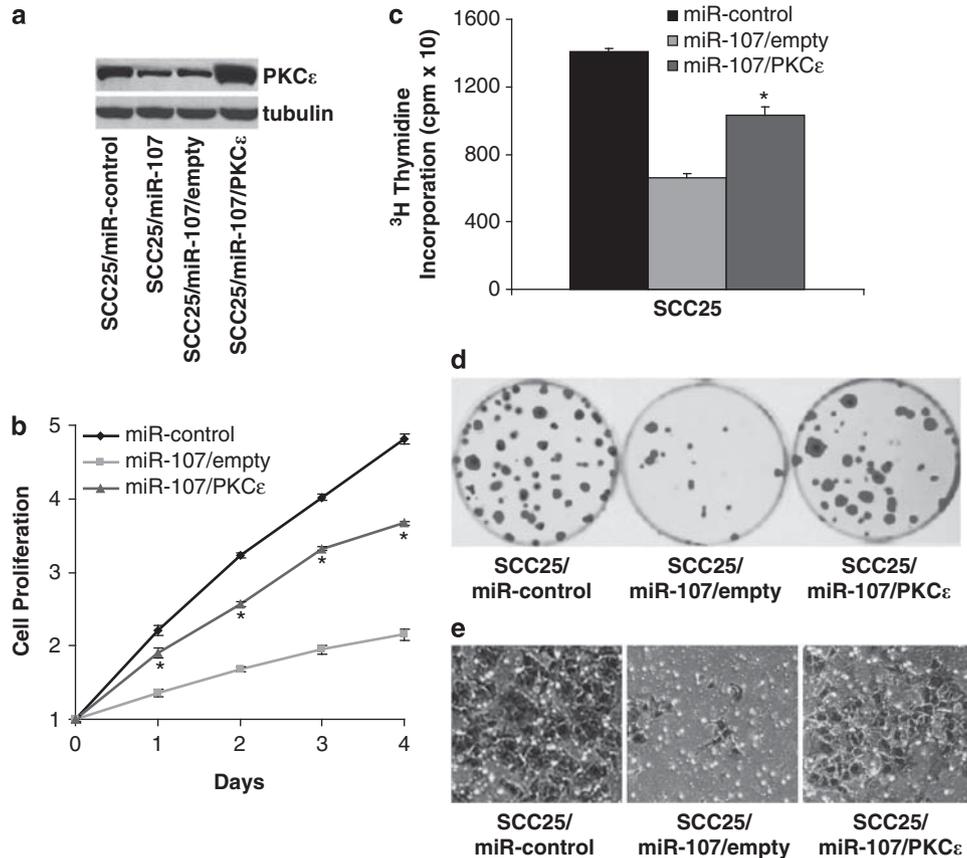
**Figure 4** miR-107 reduces tumorigenicity in nude mice. (a) miR-107 expression. Total RNA was isolated from CAL27/miR-control and CAL27/miR-107 cells. miR-107 expression was measured using quantitative PCR. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value  $< 0.005$ ,  $n = 3$ . (b) Protein levels of miR-107 target genes in miR-107 overexpressing CAL27 cells. Whole-cell lysates were extracted and western blot analysis was performed. (c) Protein levels of miR-107 target genes in primary keratinocytes transfected with anti-miR-107. Whole-cell lysates were extracted and western blot analysis was performed. (d) Tumor growth. CAL27/miR-control or CAL27/miR-107 cells were injected subcutaneously to the flanks of the nude mice. Tumors were measured weekly using a caliper and tumor volumes were calculated. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value  $< 0.005$ ,  $n = 7$ . (e) Tumor weight. Tumors were weighed and data are presented as mean  $\pm$  s.e.m. for each group. \* $P$ -value  $< 0.005$ ,  $n = 7$ . (f) Tumor PKC $\epsilon$  levels. Whole-cell lysates were isolated from the resected tumors and PKC $\epsilon$  levels were measured by western blot analysis.

in their primary tumor compared with normal adjacent epithelium. All four patients with high PKC $\epsilon$  were part of the cohort of 12 patients with reduced miR-107 expression in the tumor. We were unable to determine PKC $\epsilon$  protein levels in this population due to insufficient and/or unavailable patient specimen. As miRs are known to regulate mRNA stability and translation, assessment of PKC $\epsilon$  protein may reveal additional patients in the low miR-107 population with elevated PKC $\epsilon$  levels in the tumor. Nonetheless, even with this small sample size, miR-107 expression was found to be significantly lower ( $73 \pm 13\%$  reduction,  $P < 0.01$ ) in patients with high PKC $\epsilon$  expression than in patients with low PKC $\epsilon$  (Figure 6c). Our results demonstrate

that miR-107 is reduced in HNSCC tumors and is associated with elevated PKC $\epsilon$  expression in HNSCC.

## Discussion

miRs are short (20–25 nucleotides) single-stranded non-coding RNAs that have a critical role in the regulation of global gene expression. miRs negatively regulate target protein levels by suppressing gene mRNA translation and/or enhancing gene mRNA degradation (Filipowicz *et al.*, 2008). Published work has clearly shown that miRs are involved in the regulation of different signaling pathways including cellular

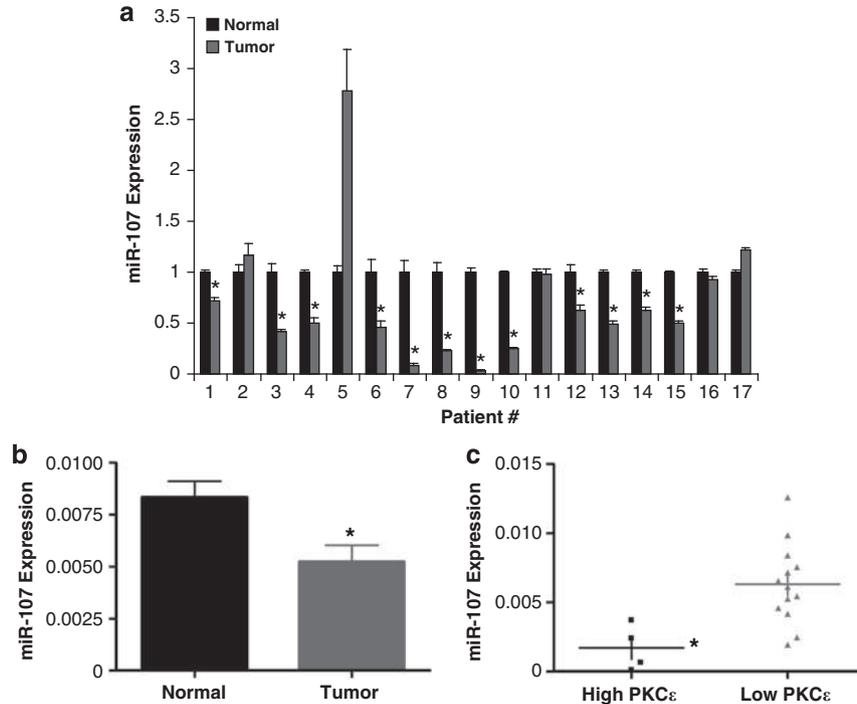


**Figure 5** miR-resistant PKC $\epsilon$  reverses the tumor-suppressive actions of miR-107. SCC25/miR-107 cells were transfected with an empty vector or a PKC $\epsilon$  expression vector to generate SCC25/miR-107/empty and SCC25/miR-107/PKC $\epsilon$  cells. **(a)** PKC $\epsilon$  levels. Whole-cell lysates were extracted and western blot analysis was performed using anti-PKC $\epsilon$  and anti-tubulin antibodies. **(b)** Cell proliferation. Cell proliferation was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value < 0.005,  $n = 3$ . **(c)** DNA synthesis. Incorporation of <sup>3</sup>H-thymidine into DNA was monitored after 4 h in a scintillation counter. Data are presented as mean  $\pm$  s.e.m. \* $P$ -value < 0.005,  $n = 3$ . **(d)** Colony formation. Colonies were stained with crystal violet and counted. **(e)** Cell invasion. Cell invasion was assessed using the modified Boyden chamber invasion assay with Matrigel basement membrane. Invasive cells were counted.

differentiation, proliferation, migration and apoptosis (Calin and Croce, 2006; Hwang and Mendell, 2006; Datta *et al.*, 2008; Nasser *et al.*, 2008; Avissar *et al.*, 2009; Croce, 2009; Liu *et al.*, 2009). Thus, it is not surprising that aberrant expression of certain miRs is associated with cancer development and progression, including HNSCC (Calin and Croce, 2006; Hwang and Mendell, 2006; Datta *et al.*, 2008; Nasser *et al.*, 2008; Avissar *et al.*, 2009; Croce, 2009; Liu *et al.*, 2009). In the present study, our results revealed that miR-107 is under-expressed and functions as a tumor suppressor in HNSCC. Ectopic expression of miR-107 is sufficient to dampen the tumorigenic potential of HNSCC cells *in vitro* and *in vivo*. PKC $\epsilon$  was confirmed as a direct target of miR-107 and had an inverse relationship with miR-107. Our study indicates that suppression of miR-107 leads to an increase in PKC $\epsilon$  resulting in the development of HNSCC.

Consistent with our work, other groups have reported that miR-107 functions as a tumor suppressor in bladder, colon and pancreatic cancer. miR-107 was dramatically reduced in bladder carcinoma *in situ* compared with normal bladder (Ayala de la Pena

*et al.*, 2011). An inverse association between miR-107 and HIF1- $\beta$  was demonstrated in human colon cancer specimens (Yamakuchi *et al.*, 2010). Overexpression of miR-107 inhibits HIF1- $\beta$  levels and retards tumor growth in HCT116 colon cancer cells (Yamakuchi *et al.*, 2010). Enforced expression of miR-107 in pancreatic cancer cells, MiaPACA-2 and PANC-1, repressed CDK6 levels and inhibited cell proliferation (Lee *et al.*, 2009). In contrast, several reports have shown that miR-107 functions as an oncogene in breast and gastric cancer. Elevated miR-107 levels were associated with distant metastasis and poorer clinical outcome in the breast cancer (Martello *et al.*, 2010). Overexpression of miR-107 in MCF10A mammary epithelial cells promotes epithelial-to-mesenchymal transition resulting in a highly metastatic phenotype thorough modulation of Dicer1 (Martello *et al.*, 2010). In line with this report, knockdown of miR-107 leads to an increase in Dicer1 level, and inhibition of cell invasion and migration in the gastric cancer cells (Li *et al.*, 2010). Taken together, these results indicate that the biology of miR-107 is complex and highly cell-type dependent.



**Figure 6** miR-107 is significantly downregulated and associated with elevated PKC $\epsilon$  in HNSCC tumors. (a) miR-107 expression in individual HNSCC patients. Primary tumors and adjacent normal tissue from HNSCC patients were measured for miR-107 expression using quantitative PCR. \* $P < 0.05$ ,  $n = 3$ . (b) miR-107 expression is reduced in HNSCC tumors. Data are presented as mean  $\pm$  s.e.m. for each group. \* $P < 0.008$ ,  $n = 17$ . (c) HNSCC patients with elevated PKC $\epsilon$  expression have reduced miR-107 expression. Data are presented as mean  $\pm$  s.e.m. for each group. \* $P < 0.01$ .

Our data showed that CDK6 and HIF1- $\beta$  levels are decreased whereas Dicer1 levels were unchanged in CAL27/miR-107 cells compared with CAL27/miR-control cells. This indicates that miR-107 target genes are not regulated in concert and raises the possibility that other factors, including additional genetic alterations, may dictate which set of miR-107 targets is regulated in a particular cell type. Moreover, it is possible that downregulation of Dicer1 may be a prerequisite for miR-107 to function as an oncogene instead of a tumor suppressor. Expression of miR-resistant PKC $\epsilon$  was sufficient, at least partially, to rescue the loss-of-function phenotype observed for SCC25/miR-107 cells. This key observation indicates that a critical event for the tumor-suppressive actions of miR-107 in HNSCC is to negatively control the levels of PKC $\epsilon$ .

Global miR expression analysis showed that miR-107 was significantly downregulated in HNSCC tumors compared with matched normal tissue (Wong *et al.*, 2008). In addition, miR expression profiling between 18 HNSCC cell lines and oral keratinocytes indicates that miR-107 is decreased at high frequency in HNSCC (Kozaki *et al.*, 2008). Our work showed that a majority (12/17) of HNSCC patients have reduced miR-107 expression in the primary tumor and, thus, provide additional support that loss of miR-107 is a frequent event in the development of HNSCC. In this cohort, HNSCC patients with high PKC $\epsilon$  mRNA expression had a dramatic 73% reduction in miR-107 expression compared with patients with low PKC $\epsilon$  mRNA expres-

sion. This observation is in line with our *in vitro* results and provide further evidence that miR-107 is inversely associated with PKC $\epsilon$  in HNSCC.

In summary, our results showed that miR-107 functions as a tumor suppressor in HNSCC through regulation of PKC $\epsilon$ . Reconstitution of miR-107 is sufficient to retard the tumorigenicity of HNSCC cells. Our study indicates that the reduction in miR-107 expression is a pathogenetic event in HNSCC and suggests that miR-107 may be a potential anti-cancer therapeutic for this patient population.

## Materials and methods

### Cell culture

Human HNSCC cell lines (SCC15, SCC25 and CAL27) were obtained from ATCC (Rockville, MD, USA). CAL27 cells were cultured in DMEM medium containing 10% fetal bovine serum. SCC25 and SCC15 cells were grown in DMEM/F12 (1:1) medium containing 10% fetal bovine serum. Primary keratinocytes were obtained from ATCC and cultured in EpiLife medium (Invitrogen, Carlsbad, CA, USA) supplemented with Ca<sup>2+</sup> and other growth factors in a 5% CO<sub>2</sub> incubator at 37°C. SCC15, SCC25 and CAL27 cell lines were authenticated using short tandem repeat profiling (ATCC).

### Generation of transient and stable cell lines

Transient miR-107 and miR-control overexpressing SCC15, SCC25 and CAL27 cells were generated by transfection with pre-miR-107 or pre-miR-control (50 nmol/l) using Lipofectamine

2000. Cells were harvest after 48 h for gene expression, protein levels and functional assays. Stable CAL27/miR-107, CAL27/miR-control, SCC25/miR-107 and SCC25/miR-control were generated by transduction using shMIMIC miR-107 or miR-control lentiviral particles (Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated with viral particles (multiplicity of infection = 1:20) mixed with polybrene (at a final concentration of 10  $\mu$ g/ml) for 12 h. Subsequently, cells were cultured in complete growth media for 72 h and polyclonal populations were selected with 1  $\mu$ g/ml puromycin. SCC25/miR-107 cells were transiently transfected with an empty vector or a PKC $\epsilon$  expression vector (Ex-A1280-MO2; GeneCopoeia, Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested after 48 h for protein levels and functional assays. Primary keratinocytes were transiently transfected with anti-miR-control or anti-miR-107 (100 nmol/l) using Lipofectamine 2000. Cells were harvested after 48 h for gene expression and protein levels.

#### Real-time PCR

Total RNA was isolated from HNSCC cells and primary keratinocytes with TRIzol (Invitrogen). Expression of PKC $\epsilon$ , GAPDH, mature miR-103a, mature miR-107 and RNU44 were determined using the Applied Biosystems 7900HT Fast Real-Time PCR System with validated TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). PKC $\epsilon$  expression was normalized to GAPDH, and mature miR-103a/miR-107 expression was normalized to RNU44 using the  $\Delta\Delta$ Ct method.

#### Luciferase reporter assay

Wild-type or mutant PKC $\epsilon$ -3'UTR psiCHECK-2 plasmid was transiently transfected into H293T cells. Cells were transfected with 50 ng of psiCHECK-2 along with pre-miR-107 or pre-miR-control (100 nmol/l) using Lipofectamine 2000 (Invitrogen). After 48 h, cells were washed with phosphate buffered saline, resuspended in the lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 0.5 mM dithiothreitol) and measured for Firefly/Renilla luciferase activities in a luminometer using the Dual-Light System (Applied Biosystems). Renilla luciferase activities were normalized to Firefly luciferase activities to control for transfection efficiency.

#### Western blot analysis

Whole-cell lysates were mixed with Laemmli loading buffer, boiled, separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Subsequently, immunoblot analyses were performed using antibodies specific to PKC $\epsilon$  (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CDK6 (Thermo Fisher Scientific), HIF1- $\beta$  (Cell Signaling Technology, Danvers, MA, USA), Dicer1 (Abcam, Cambridge, MA, USA) or tubulin (Cell Signaling Technology). The signal was developed with ECL (Thermo Fisher Scientific) after incubation with appropriate secondary antibodies.

#### Cell invasion and proliferation

Cell invasion was determined as described from the cell invasion assay kit (BD Biosciences, Bedford, MA, USA). Cells were harvested and resuspended in serum-free medium. An aliquot ( $1 \times 10^5$  cells) of the prepared cell suspension was added into the chamber and incubated for 24 h at 37  $^{\circ}$ C in a 10% CO<sub>2</sub> tissue culture incubator. Non-invading cells were gently removed from the interior of the inserts with a cotton-tipped swab. Invasive cells were stained and counted. Cell

proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent to detect metabolic active cells (Roche Molecular Biochemicals, Nutley, NJ, USA). Absorbance was measured at 570 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA, USA) after overnight incubation.

#### Colony formation

Cells (500) were plated in complete growth media and allowed to grow until visible colonies formed (10–12 days). Cell colonies were fixed with cold methanol, stained with 0.25% crystal violet in 25% methanol, washed and air dried.

#### DNA replication

Cells (10 000 cells per well) were plated in 24-well plates and allowed to grow for 24 h. Subsequently, cells were incubated with serum-free media for 16 h and then incubated with complete growth media containing 1  $\mu$ Ci of <sup>3</sup>H-thymidine for 6 h. Cells were washed with phosphate buffered saline and <sup>3</sup>H-thymidine incorporation into DNA was measured using a Hitachi scintillation counter.

#### Tumor growth in nude mice

CAL27/miR-control or CAL27/miR-107 cells ( $1 \times 10^6$  cells) were implanted into the flanks of nude mice (6–8 weeks). After 2 weeks, tumors were measured once a week using a digital caliper and tumor volumes were calculated using the formula  $d1 \times d2 \times d3 \times 0.5236$ , where d represents the three orthogonal diameters. In addition, tumors were resected and weighed at the end of the 6-week study protocol. Whole-cell lysates were extracted from tumor tissues and determined for PKC $\epsilon$  protein levels.

#### Analysis of primary HNSCC tissues

Seventeen primary tumors were collected at The Ohio State University James Cancer Hospital from HNSCC patients at the time of surgical resection between 1997 and 2000. All tissues were diagnosed histologically as HNSCC by a board certified pathologist. Written informed consent, as required by the institutional review board, was obtained from all patients. Collected samples were stored immediately in liquid nitrogen at –80  $^{\circ}$ C until analysis. Total RNA was isolated from the frozen tumors with TRIzol (Invitrogen). Expression of PKC $\epsilon$ , GAPDH, mature miR-107 and RNU44 were determined using the Applied Biosystems 7900HT Fast Real-Time PCR System with validated TaqMan gene expression assays (Applied Biosystems). PKC $\epsilon$  and mature miR-107 expression were normalized to GAPDH and RNU44, respectively, using the  $\Delta\Delta$ Ct method.

#### Conflict of interest

The authors declare no conflict of interest.

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