Tumour-suppressive *microRNA-29s* inhibit cancer cell migration and invasion by targeting laminin-integrin signalling in head and neck squamous cell carcinoma

（頭頸部扁平上皮癌において *microRNA-29s* はラミニン・

インテグリン経路を制御する事により

癌細胞の遊走・浸潤を抑制する。）

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**Tumour-suppressive *microRNA-29s* inhibit cancer cell migration and invasion by targeting laminin-integrin signalling in head and neck squamous cell carcinoma**

**Running title:** *miR-29s* target laminin-integrin in HNSCC

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**Abstract**

**Background:** Our recent studies of microRNA (miRNA) expression signatures demonstrated that *microRNA-29s* (*miR-29s; miR-29a/b/c*) were significantly downregulated in head and neck squamous cell carcinoma (HNSCC) and were putative tumour suppressive miRNAs in human cancers. Our aim in this study was to investigate the functional significance of *miR-29s* in cancer cells and to identify novel *miR-29s*-mediated cancer pathways and responsible genes in HNSCC oncogenesis and metastasis.

**Methods:** Gain-of-function studies using mature *miR-29s* were performed to investigate cell proliferation, migration and invasion in two HNSCC cell lines (SAS and FaDu). To identify *miR-29s*-mediated molecular pathways and targets, we utilised gene expression analysis and *in silico* database analysis. Loss-of-function assays were performed to investigate the functional significance of *miR-29s* target genes.

**Results:** Restoration of *miR-29s* in SAS and FaDu cell lines revealed significant inhibition of cancer cell migration and invasion. Gene expression data and *in silico* analysis demonstrated that *miR-29s* modulated the focal adhesion pathway. Moreover, laminin γ2 (*LAMC2*) and α6 integrin (*ITGA6*) genes were candidate targets of *miR-29s'* regulation. Luciferase reporter assays showed that *miR-29s* directly regulated *LAMC2 and ITGA6*. Silencing of *LAMC2 and ITGA6* genes significantly inhibited cell migration and invasion in cancer cells.

**Conclusions:** Downregulation of *miR-29s* was a frequent event in HNSCC. *miR-29s* acted as tumour suppressors and directly targeted laminin-integrin signalling. Recognition of tumour suppressive miRNA-mediated cancer pathways provides new insights into the potential mechanisms of HNSCC oncogenesis and metastasis and suggests novel therapeutic strategies for the disease.

**Key words:** *miR-29s*, tumour suppressor, *LAMC2, ITGA6*, metastasis, HNSCC

**Introduction**

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world and approximately 500,000 cases are diagnosed every year (Jemal *et al*, 2010). In spite of considerable advances in multimodality therapy, including surgery, radiotherapy and chemotherapy, the overall five-year survival rate for patients with HNSCC is only 40 - 50% (Leemans *et al*, 2011). Local tumour recurrence and distant metastasis after conventional therapy appear to be major contributing factors for restricted survival of HNSCC patients. Therefore, understanding the molecular pathways of metastasis accompanying HNSCC would help to improve diagnosis, approaches to therapy and prevention of the disease.

The discovery of non-coding RNAs in the human genome was an important conceptual breakthrough in the post-genome sequencing era (Mattick, 2004). microRNAs (miRNAs) constitute a class of small, non-coding RNA molecules (19 – 22 nucleotides in length) that regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (Bartel, 2004). miRNAs are unique in their ability to regulate multiple protein-coding genes. Bioinformatic predictions indicate that miRNAs regulate more than 30% of the protein-coding genes in the human genome (Lewis *et al*, 2005).

A growing body of evidence suggests that miRNAs contribute significantly to HNSCC progression, development and metastasis (Esquela-Kerscher and Slack, 2006). In cancer pathways, normal regulatory mechanisms are disrupted by altered expression of tumour-suppressive or oncogenic miRNAs. Therefore, identification of differentially expressed miRNAs is an important step to understand human oncogenesis. Genome-wide miRNA expression signatures have been used to rapidly and precisely identify aberrant miRNA expression in HNSCC. Based on recent reports, including our own signatures, several down- or upregulated miRNAs have been discovered in HNSCC (Avissar *et al*, 2009; Hui *et al*, 2010; Kikkawa *et al*, 2010; Nohata *et al*, 2011).

Our expression signature of HNSCC demonstrated that the *microRNA-29* family (*miR-29s*) was downregulated in cancer tissues (Kikkawa *et al*, 2010; Nohata *et al*, 2011). The *miR-29s* include three members, *miR-29a*, *miR-29b* and *miR-29c* in the human genome. *miR-29b* includes two members, *miR-29b-1* and *miR-29b-2* (Wang *et al*, 2013). These miRNAs form clusters on two human genome loci: *miR-29b-1* and *miR-29a* in 7q32 and *miR-29b-2* and *miR-29c* in 1q32 (Kriegel *et al*, 2012). Although aberrant expression of *miR-29s* was reported in several types of human cancers, the expression status of *miR-29s* varied according to the cancer types (Fabbri *et al*, 2007; Fu *et al*, 2012; Gebeshuber *et al*, 2009; Han *et al*, 2010; Li *et al*, 2011; Mott *et al*, 2010; Presneau *et al*, 2013; Sengupta *et al*, 2008; Xiong *et al*, 2010; Zhao *et al*, 2010). Therefore, the aim of this study was to investigate the functional significance of the *miR-29s* in cancer cells and to identify novel *miR-29s*-regulated cancer pathways and

responsible genes in HNSCC oncogenesis and metastasis.

Genome-wide gene expression analysis of *miR-29a* transfectants and *in silico* database analysis showed that the focal adhesion pathway was a promising candidate target pathway. In this regard, laminin-integrin signalling is critically important. It occurs in the basal lamina and influences cell differentiation, migration and adhesion as well as proliferation and cell survival. Interestingly, laminin γ2 (coded by *LAMC2*), a member of laminin-332, and α6 integrin (coded by *ITGA6*) were listed as candidate genes in the focal adhesion pathway. Moreover, interaction between laminin-332 and α6β4 integrin triggers a number of signalling cascades, promoting both cell migration and cancer cell survival (Kariya *et al*, 2009; Marinkovich, 2007). We investigated the functional significance of *LAMC2 and ITGA6* in HNSCC and showed that these genes were regulated by *miR-29s*. The identification of novel cancer pathways regulated by tumour suppressive *miR-29s* provides new insights into the potential mechanisms of HNSCC oncogenesis and metastasis.

**Materials and Methods**

**Clinical HNSCC specimens**

Twenty-three pairs of primary HNSCC and corresponding normal epithelial samples were obtained from patients with HNSCC at Chiba University Hospital (Chiba, Japan) from 2005 to 2011. The samples considered normal were free of cancer cells by pathologic examination. The patients' backgrounds and clinico-pathological characteristics are summarised in Supplementary table 1. The patients were classified according to the 2002 Union for International Cancer Control (UICC) TNM staging criteria prior to treatment. Written consent for tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the Institutional Review Board of Chiba University. The specimens were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at -20°C until RNA was extracted.

**RNA isolation**

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically, and molecular integrity was checked by gel electrophoresis. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

**HNSCC cell culture**

The following human HNSCC cell lines were used: SAS (derived from a primary lesion of tongue SCC) and FaDu (derived from a primary lesion of hypopharyngeal SCC). Both cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37°C.

**Quantitative real-time RT-PCR (qRT-PCR)**

First-strand cDNA was synthesized from one μg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene-specific PCR products were assayed continuously using a 7900-HT Real-Time PCR System according to the manufacturer's protocol. The initial PCR step consisted of a ten min hold at 95°C, followed by 40 cycles consisting of a 15 sec denaturation at 95°C and a one min annealing/extension at 60°C. The expression levels of *miR-29a* (Assay ID: 002112), *miR-29b* (Assay ID: 000413) and *miR-29c* (Assay ID: 000587) were analysed by TaqMan quantitative real-time PCR (TaqMan® MicroRNA Assay; Applied Biosystems) and normalised to *RNU48* (Assay ID: 001006). TaqMan® probes and primers for, *LAMC2* (P/N: Hs01043711_m1), *ITGA6* (P/N: Hs01041011_m1) and *GUSB* (P/N: Hs99999908_m1) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products). The delta delta Ct method

was adopted and applied to calculate the relative quantities of subject genes. All reactions were performed in triplicate, and included negative control reactions that lacked cDNA.

**Mature miRNA transfection and small interfering RNA treatment**

The following mature miRNAs species were used in this study: mirVana[TM] miRNA mimic for hsa-miR-29a-3p (Product ID: MC12499), hsa-miR-29b-3p (Product ID: MC10103) and hsa-miR-29c-3p (Product ID: MC10518) (Applied Biosystems). The following siRNAs were used: Stealth Select RNAi™ siRNA; si-*LAMC2* (Cat# HSS105965), si-*ITGA6* (Cat# HSS179959) (Invitrogen) and negative control miRNA/siRNA (P/N: AM17111, Applied Biosystems). RNAs were incubated with OPTI-MEM (Invitrogen) and LipofectamineTM RNAiMax reagent (Invitrogen) as described previously (Ichimi *et al*, 2009). Transfection efficiency of miRNA in cell lines was confirmed based on downregulation of *TWF1*  (*PTK9*) mRNA following transfection with *miR-1* as previously reported (Ichimi *et al*, 2009).

**Cell proliferation, migration and invasion assays**

Cells were transfected with ten nM miRNA or siRNA by reverse transfection and plated in 96-well plates at $3 \times 10^3$ cells per well. After 72 h, cell proliferation was determined with the XTT assay, using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany) as previously reported (Chiyomaru *et al*, 2010).

A cell migration assay was performed using BD Falcon[TM] Cell Culture Inserts (BD Biosciences, Franklin Lakes, NJ, USA) that contained uncoated Transwell polycarbonate membrane filters with eight μm pores in 24-well tissue culture plates. Cells were transfected with ten nM miRNA or siRNA by reverse transfection and plated in ten cm dishes at $8 \times 10^5$ cells. After 48 h, the cells were collected and $2 \times 10^5$ cells were added to the upper chamber of each migration well and were allowed to migrate for 48 h. After gentle removal of the nonmigratory cells from the filter surface of the upper chamber, the cells that migrated to the lower side were fixed and stained with Diff-Quick (Sysmex Corporation, Japan). The number of cells that migrated to the lower surface was determined microscopically by counting four areas of constant size per well. A cell invasion assay was carried out using modified Boyden chambers containing Transwell-precoated Matrigel membrane filter inserts with eight μm pores in 24-well tissue culture plates at $2 \times 10^5$ cells per well (BD Biosciences). All experiments were performed in triplicate.

**_In silico_ analysis, genome-wide gene expression analysis and _miR-29s_ target search.**

To identify *miR-29s* target genes, we used *in silico* analysis and genome-wide gene expression analysis. First, we screened genes using TargetScan Release 6.2 (http://www.targetscan.org/).

These genes were then categorised into KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways using GENECODIS analysis (Tabas-Madrid *et al*, 2012) (http://genecodis.cnb.csic.es/). To identify upregulated genes in HNSCC we analysed a publicly available gene expression data set in GEO (accession number: GSE9844). To gain further insight into which genes were affected by *miR-29s*, we performed genome-wide gene expression analysis using *miR-29a* transfection of SAS. SurePrint G3 Human GE 8x60K Microarray (Agilent Technologies) was used for expression profiling of *miR-29a* transfectants in comparison with negative control miRNA transfectants.

**Western blotting**

Cells were harvested 72 h after transfection and lysates were prepared. Fifty µg of protein from each lysate were separated on a Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with    mouse LAMC2 antibody (1:500, MAB19562, Merck Millipore, Billerica, MA, USA) and rabbit ITGA6 antibody (1:1000, ab97760, Abcam, UK) with GAPDH antibody (1:1000, ab8245, Abcam) used as an internal control. The membrane was washed and incubated with anti-mouse IgG HRP-linked antibody (#7076, Cell Signaling Technology, Danvers, MA, USA) or anti-rabbit IgG HRP-linked antibody (#7074, Cell Signaling Technology). Complexes were visualized with an Immun-Star$^{TM}$ WesternC Chemiluminescence Kit (Bio-Rad), and the expression levels of these genes were evaluated by ImageJ software (ver.1.44; http://rsbweb.nih.gov/ij/).

**Plasmid construction and dual-luciferase reporter assay**

The partial wild-type sequences of the *LAMC2* 3'-untranslated region (3'-UTR) or those with deleted *miR-29s* target sites (Position 611-627 and 1430-1436 of *LAMC2* 3'-UTR) were inserted between the XhoI–PmeI restriction sites in the 3'-UTR of the hRluc gene in psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The same was done with the partial wild-type sequences of the *ITGA6* 3'-UTR or those with deleted *miR-29s* target site (Position 1570-1576 of *ITGA6* 3'-UTR). The sequences of the oligonucleotides were described in Supplementary document 1. SAS cells were transfected with five ng of vector and ten nM of *miR-29s* using Lipofectamine 2000 (Invitrogen). The activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalised data were calculated as the quotient of *Renilla*/firefly luciferase activities.

**Statistical analysis**

The relationships between two groups and the numerical values obtained by real-time RT-PCR were analysed using the paired t-test. Spearman's rank test was used to evaluate the correlation

between the expressions of *miR-29s*. The relationship among more than three variables and numerical values were analysed using the Bonferroni adjusted Mann-Whitney U test. All analyses were performed using Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA).

**Results**

**Expression of *miR-29s* in clinical HNSCC specimens**

The chromosomal locations of *miR-29s* in the human genome are shown in Supplementary figure 1. These miRNAs are clustered at two different human genomic loci, *miR-29b-1* and *miR-29a* at 7q32.3 and *miR-29b-2* and *miR-29c* at 1q32.2.

To validate our past miRNA profiling results, we evaluated *miR-29s* expression in 23 clinical HNSCC specimens. The expression levels of *miR-29a*, *miR-29b* and *miR-29c* were significantly lower in tumour tissues than in corresponding adjacent non-cancerous epithelia ($P = 0.029$, $P = 0.034$ and $P < 0.0001$, respectively, Figure 1A). Spearman's rank test showed positive correlation between the expression of *miR-29a* and that of *miR-29b* (R = 0.55 and $P = 0.0001$, Figure 1B). Similarly, the expression of *miR-29c* was positively correlated with that of *miR-29b* (R = 0.62 and $P < 0.0001$, Figure 1B).

**Effect of restoring *miR-29s* on cell proliferation, migration and invasion activities in HNSCC cell lines**

To investigate the functional effects of *miR-29s*, we performed gain-of-function studies using miRNA transfection of SAS and FaDu cell lines. The XTT assay demonstrated that cell proliferation was significantly inhibited in *miR-29s* transfectants in comparison with the mock or miR-control transfectant cells. Specifically, we observed the following growth, expressed as a percentage of the mock: (1) SAS -- Mock, 100.0 ± 2.4; miR-control, 110.8 ± 4.9; *miR-29a*, 48.6 ± 4.2; *miR-29b*, 56.4 ± 5.0; *miR-29c*, 60.7 ± 4.9; (2) FaDu -- Mock, 100.0 ± 1.9; miR-control, 104.5 ± 2.6; *miR-29a*, 82.3 ± 4.4; *miR-29b*, 84.5 ± 1.8; *miR-29c*, 62.7 ± 0.7, with $P < 0.005$ for both (Figure 2A).

The migration assay demonstrated that cell migration activity was significantly inhibited in *miR-29s* transfectants in comparison with the mock or miR-control transfectant cells. Specifically, we observed the following migration activities, expressed as a percentage of the mock: (1) SAS -- Mock, 100.0 ± 5.3; miR-control, 100.2 ± 4.8; *miR-29a*, 10.2 ± 1.3; *miR-29b*, 7.9 ± 0.9; *miR-29c*, 5.2 ± 0.8; (2) FaDu -- Mock, 100.0 ± 8.7; miR-control, 113.4 ± 13.8; *miR-29a*, 8.3 ± 4.4; *miR-29b*, 14.0 ± 4.0; *miR-29c*, 8.6 ± 2.3, with $P < 0.005$ for both (Figure 2B).

The Matrigel invasion assay demonstrated that cell invasion activity was significantly inhibited in *miR-29s* transfectants in comparison with the mock or miR-control transfectant cells. Specifically, we observed the following migration activities, expressed as a percentage of the mock: (1) SAS -- Mock, 100.0 ± 13.6; miR-control, 99.2 ± 11.4; *miR-29a*, 11.1 ± 2.8; *miR-29b*, 6.9 ± 1.8; *miR-29c*, 6.4 ± 1.8 and (2) FaDu -- Mock, 100.0 ± 16.3; miR-control, 94.9 ± 4.4; *miR-29a*, 5.8 ± 1.8; *miR-29b*, 10.9 ± 2.0; *miR-29c*, 8.3 ± 2.2, with $P < 0.005$ for both (Figure

2C).

**Selection of candidate genes targeted by *miR-29s***

To identify genes targeted by *miR-29s*, we used *in silico* analysis and genome-wide gene expression analysis. Our strategy for selection of *miR-29s*-targeted genes is shown in Supplementary figure 2. First, we screened *miR-29s*-targeted genes using the TargetScan database and identified 2,627 genes (Supplementary table 2). These genes were then categorised into KEGG pathways using GENECODIS analysis and 83 pathways were identified as significantly enriched pathways (Supplementary table 3). Among those pathways, we focused on the focal adhesion pathway because this pathway was implicated in cancer cell migration and invasion. A total of 58 genes was identified in this pathway (Supplementary table 4). The gene set was then analysed with a publicly available gene expression data set in GEO (accession number: GSE9844) and genes upregulated ($log_2$ ratio > 0.5) in HNSCC were chosen. To gain further insight into which genes were affected by *miR-29s*, we performed genome-wide gene expression analysis using SAS, and genes downregulated ($log_2$ ratio < -0.5) by *miR-29a* transfection were selected. Entries from the gene expression data were approved by GEO, and were assigned GEO accession number GSE47657. As a result, 16 candidate genes were identified as *miR-29s* targets in the focal adhesion pathway (Table 1). Among those candidates, we focused on *LAMC2* (a component of laminin-332), which is a specific ligand of *ITGA6* (a component of α6 β4 integrin). Laminin 332-α6β4 integrin interaction is known to promote tumour invasion and cell survival through PI3K and RAC1 activation. Therefore, we focused on *LAMC2* and *ITGA6* in further analyses.

**_LAMC2_ and _ITGA6_ were directly regulated by *miR-29s***

We performed qRT-PCR and Western blotting in SAS to investigate whether *LAMC2* and *ITGA6* expression was downregulated by restoration of *miR-29s*. The mRNA and protein expression levels of *LAMC2*/LAMC2 were significantly repressed in *miR-29s* transfectants in comparison with mock or miR-control transfectants ($P$ < 0.005, Figure 3A and 3B). Similarly, the mRNA and protein expression levels of *ITGA6*/ITGA6 were significantly repressed in *miR-29s* transfectants ($P$ < 0.005, Figure 4A and 4B).

We performed a luciferase reporter assay in SAS to determine whether *LAMC2* and *ITGA6* mRNA had target sites for *miR-29s*. The TargetScan database predicted that two putative *miR-29s* binding sites existed in *LAMC2* 3'-UTR (position 611 - 627 and 1430 - 1436, Figure 3C). We used vectors encoding either the partial wild-type sequence of the 3'-UTR of *LAMC2* mRNA, including the predicted *miR-29s* target sites or "deletion" vectors, i.e., those lacking the *miR-29s* target sites. We found that the luminescence intensity was significantly reduced by

transfection of *miR-29s* with the vector carrying the wild-type 3'-UTR of *LAMC2* whereas transfection with deletion vectors (positions 611 - 627 or 1430 - 1436 had been removed) blocked the decrease in luminescence ($P < 0.0083$, Figure 3D). As for *ITGA6*, the TargetScan database predicted a putative *miR-29s* binding site in *ITGA6* 3'-UTR (position 1570 - 1576). We constructed a vector encoding either the partial wild-type sequences of 3'-UTR of *ITGA6* or those with a deleted *miR-29s* target site (Figure 4C). The luminescence intensity was significantly reduced by transfection of *miR-29s* with the vector of wild-type 3'-UTR of *LAMC2* ($P < 0.0083$, Figure 4D). Although the luminescence intensity was also reduced by transfection of *miR-29s* with the deletion vector, the reduction was smaller than that with wild-type (Figure 4D).

**Effects of silencing *LAMC2* and *ITGA6* on cell proliferation, migration and invasion in SAS**

To investigate the functional role of *LAMC2* and *ITGA6*, we performed loss-of-function studies using si-*LAMC2* and si-*ITGA6* transfectants. First, we evaluated the knockdown efficiency of si-*LAMC2* and si-*ITGA6* treatments in SAS. qRT-PCR and Western blotting indicated that the two siRNAs effectively downregulated *LAMC2* and *ITGA6* expression in SAS (Supplementary figure 3).

The XTT assays demonstrated that cell proliferation was significantly inhibited in si-*LAMC2* transfectants in comparison with the mock or si-control transfectant in SAS (percentage of the mock: $64.7 \pm 2.2$, with $P < 0.0167$; Figure 5A). On the other hand, proliferation was not inhibited in si-*ITGA6* transfectants in comparison with the mock or si-control transfectants in SAS (Figure 5B).

The migration assays demonstrated that cell migration activity was significantly inhibited in both si-*LAMC2* and si-*ITGA6* transfectants in comparison with the mock or si-control transfectants in SAS (percentage of the mock: si-*LAMC2*, $14.3 \pm 7.2$; si-*ITGA6*, $15.8 \pm 12.5$, with $P < 0.0167$ for both; Figure 5C and 5D).

Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited in both si-*LAMC2* and si-*ITGA6* transfectants in comparison with the mock or si-control transfectants in SAS (percentage of the mock: si-*LAMC2*, $23.4 \pm 14.0$; si-*ITGA6*, $19.6 \pm 14.3$, with $P < 0.0167$ for both; Figure 5E and 5F).

**Discussion**

In the post-genome sequencing era, improved understanding of non-coding RNA is necessary for continued progress in cancer research. For elucidation of the molecular mechanisms underlying HNSCC, we have examined tumour suppressive miRNAs, focusing on their regulated molecular targets and novel cancer pathways based on HNSCC expression signatures (Kikkawa *et al*, 2010; Nohata *et al*, 2011). Our previous miRNA expression signatures demonstrated that the *miR-29s* family (*miR-29a/b/c*) was reduced in several types of cancers (Kikkawa *et al*, 2010; Nohata *et al*, 2011). Decreased expression of *miR-29s* was described in other types of cancers such as cholangiocarcinoma, nasopharyngeal cancer, non-small cell lung cancer, cervical cancer, hepatocellular carcinoma, malignant peripheral nerve sheath tumour and mantle cell lymphoma (Fabbri *et al*, 2007; Li *et al*, 2011; Mott *et al*, 2010; Presneau *et al*, 2013; Xiong *et al*, 2010; Zhao *et al*, 2010), all of which is consistent with our results. In contrast, upregulation of *miR-29s* was reported in breast cancer, colon cancer and acute myeloid leukaemia (Fu *et al*, 2012; Gebeshuber *et al*, 2009; Han *et al*, 2010). Therefore, the molecular mechanisms by which *miR-29s* expression is deregulated vary according to cancer types. In this study, our data showed that all members of the *miR-29s* family were significantly reduced in cancer tissues. Moreover, restoration of *miR-29s* strongly inhibited cancer cell migration and invasion. Thus, the *miR-29s* family functions as a group of tumour suppressors, and they might contribute to metastasis in HNSCC.

To elucidate the silencing mechanisms of *miR-29s* in HNSCC cells, we performed preliminary analysis of 5-Aza-dC treatment as a demethylation agent on HNSCC cells. Our data demonstrated that *miR-29s* were not recovered by the treatment (data not shown), suggesting that silencing of *miR-29s* were not caused by methylation of CpG islands on their genomic regions. Three annotated genes (*CR1L*, *CD46* and *C1orf32*) are located around the *miR-29b-2/miR-29c* cluster region on human chromosome 1q32.2 (Supplementary figure 1). Our microarray expression data of HNSCC (GEO accession number; GSE 36951) showed that expression changes of the three genes were not observed in cancer tissues. According to the UniGene database, expression levels of other genes shown in Supplementary figure 1 are extremely low.

Although the molecular mechanism by which *miR-29s* is silenced in HNSCC is still unknown, some important studies have examined the promoter region of *miR-29s* in the human genome. The human *miR-29b-1/miR-29a* promoter region contains two putative E-box sites (MYC-binding site), a Gli-binding site and four NF-kB-binding sites. Increased expression of MYC silenced *miR-29b-1/miR-29a* expression in cholangiocarcinoma cells (Mott *et al*, 2010). Furthermore, NF-kB signalling, which is known to be activated in inflammation-related cancers, directly repressed *miR-29b-1/miR-29a* promoter activity (Mott *et al*, 2010; Wang *et al*, 2008).

Recent data showed that GATA3, a transcription factor promotes differentiation, suppresses metastasis and alters the tumor microenvironment in breast cancer by inducing *miR-29b* expression (Chou *et al*, 2013). Thus, it will be necessary to identify the transcription factors contributing to HNSCC. Further detailed analysis is needed to understand the molecular mechanisms of *miR-29s* silencing in HNSCC cells.

Metastasis is the final stage of cancer progression and is the cause of most cancer deaths. The epithelial to mesenchymal transition (EMT) is considered to be an important step in cancer progression and metastasis, and TGF-β signalling contributes to EMT processes (Heldin *et al*, 2012). Recent data have suggested that TGF-β1 inhibits the expression of *miR-29s* and promotes the expression of extracellular matrix (ECM) components (Maurer *et al*, 2010; Roderburg *et al*, 2011). These data suggest that *miR-29s* could play an important role in modulating TGF-β signalling during cancer cell migration and invasion. It will be of interest to more fully identify the molecular targets and pathways of *miR-29s* in HNSCC, particularly those contributing to cancer cell migration and invasion.

miRNAs are unique in their ability to regulate many protein coding genes. Bioinformatic predictions indicate that miRNAs regulate more than 30% of protein coding genes (Lewis *et al*, 2005). We have taken the position that the identification of novel cancer pathways and responsible genes regulated by tumour suppressive *miR-29s* is an important first step in understanding HNSCC oncogenesis. Based on this view, we categorised *miR-29s* target genes into known pathways using the KEGG Pathway Database. These data have facilitated the understanding of tumour suppressive miRNA-regulated molecular pathways in human cancer. We devised this method of analysis and found that tumour suppressive miRNA could efficiently regulate cancer-associated pathways (Kinoshita *et al*, 2012; Nohata *et al*, 2013; Yoshino *et al*, 2013).

In this study, we focused on the "focal adhesion" pathway because restoration of *miR-29s* inhibited cancer cell migration and invasion in HNSCC cell lines. Cell adhesion is the binding of a cell to a surface, such as an extracellular matrix or another cell, and fundamental determinant of wide range of cell biology response and signaling (Parsons *et al*, 2010). Recent evidences suggested that several miRNAs including *miR-29s* regulated multiple molecular components of the cell adhesion machinery and contributed to functional key regulators of adhesion-associated processes (Valastyan and Weinberg, 2011). Our hypothesis is that downregulation of tumour suppressive miRNAs in cancer cells leads to upregulation of oncogenes. Therefore, we combined the gene expression data of *miR-29a* transfectants and the GEO expression data of upregulated genes in HNSCC, generating 16 candidate target genes for *miR-29s* in focal adhesion pathways. We focused on laminin γ2 (*LAMC2*), a member of laminin-332, and α6 integrin (*ITGA6*) as responsible genes that contribute to cancer cell

migration and invasion in HNSCC. Numerous clinical studies indicated that overexpression of *LAMC2* was positively correlated with invasiveness and poor survival in several types of cancer (Guess and Quaranta, 2009), suggesting *LAMC2* function as an oncogene and deeply contributes to cancer metastasis. Because of the small number of the HNSCC samples, no positive data was obtained between clinical significance of the HNSCC patients and the *LAMC2* and *ITGA6* expression levels in the present study. Further examination should be necessary to elucidate expression levels of *LAMC2* and *ITGA6* and clinico-pathological parameters and patient survival of HNSCC.

Integrins are a large family of cell surface receptors composed of two subunits (α and β) that bind to ECM components. Most types of cells require integrin-mediated signal pathways for proliferation, migration, invasion and survival. To date, a total of 18 different α and 8 different β subunits have been identified, accounting for at least 24 distinct integrin heterodimers (Gilcrease, 2007). Among those integrins, laminin-332 interacts with two major integrin receptors, α3β1 and α6β4, promoting the formation of focal adhesions and stable anchoring contacts (Marinkovich, 2007). α6β4 integrin is reportedly involved as a link between the cytoskeleton and ECM as well as in the activation of a variety of intercellular signalling processes in cooperation with growth factor receptors (Falcioni *et al*, 1997; Mariotti *et al*, 2001; Trusolino *et al*, 2001).

Of particular interest, laminin-332 and α6β4 integrin interaction triggers a number of signalling cascades in cancer cells, promoting both cell migration and cancer cell survival (Marinkovich, 2007). Furthermore, our previous data demonstrated that tumour suppressive *miR-218*, which was frequently reduced in HNSCC, directly regulated laminin-332, suggesting that *miR-218* contributed to cancer cell migration and invasion through regulating focal adhesion pathways (Kinoshita *et al*, 2012). Thus, the available data suggest that tumour suppressive *miR-29s* and *miR-218* contribute to cancer cell migration and invasion through their regulation of focal adhesion pathways, especially targeting laminin-332 and α6β4 integrin signalling (Figure 6). Evidence indicates that integrins crosstalk with receptor tyrosine kinases in an integrin type-dependent manner through a variety of specific mechanisms (Falcioni *et al*, 1997; Mariotti *et al*, 2001; Trusolino *et al*, 2001). Thus, the understanding of tumour suppressive miRNAs (*miR-29s* and *miR-218*) and their regulation of laminin-integrin signalling should shed light on HNSCC metastasis as well as delineate more effective strategies for future therapeutic interventions for this disease.

**Conclusions**

Our data showed that all members of the *miR-29s* group were frequently downregulated in HNSCC. They function as tumour suppressors and normally inhibit cancer cell migration and

invasion through their regulation of focal adhesion pathways, especially via laminin-332 and α6β4 integrin. Elucidation of cancer pathways regulated by tumour suppressive *miR-29s* should shed light on HNSCC metastasis as well as delineate more effective strategies for future therapeutic interventions for this disease.

**Disclosure Statement**

The authors have no conflict of interest.

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**Titles and legends to figures**

**Figure 1.**

**Expression levels of *miR-29s* in HNSCC clinical specimens**

(A) Expression levels of *miR-29a*, *miR-29b* and *miR-29c* in HNSCC clinical specimens were measured by qRT-PCR. *RNU48* was used for normalisation. (B) Correlation between *miR-29a-miR-29b* and *miR-29b-miR-29c* were determined in HNSCC specimens.

**Figure 2.**

**Effect of *miR-29s* transfection on cell proliferation, migration and invasion in SAS and FaDu.**

(A) Cell proliferation 72 h after transfection with *miR-29s* (ten nM) was determined with the XTT assay. (B) Cell migration activity 48 h after transfection with *miR-29s* (ten nM) was determined with the migration assay. (C) Cell invasion activity 48 h after transfection with *miR-29s* (ten nM) was determined with the Matrigel invasion assay. $*P < 0.005$.

**Figure 3.**

**Direct regulation of *LAMC2* by *miR-29s*.**

(A) *LAMC2* mRNA expression 72 h after transfection with ten nM *miR-29s* in SAS was evaluated with qRT-PCR. *GUSB* was used as an internal control. $**P < 0.005$ (B) LAMC2 protein expression 72 h after transfection with *miR-29s* in SAS was evaluated with Western blot analysis. GAPDH was used as a loading control. The expression ratio of LAMC2/GAPDH was calculated with ImageJ software. (C) TargetScan database analysis indicated two putative *miR-29s* binding sites existed in the *LAMC2* 3'-UTR (positions 611 - 627 and 1430 - 1436). With regard to these two sites, partial wild-type sequences of *LAMC2* 3'-UTR and those with the deleted *miR-29s* target sites were constructed and inserted between the XhoI – PmeI restriction sites in the 3'-UTR of the h*Rluc* gene in the psiCHECK-2 vector. (D) The vectors were co-transfected with *miR-29s* or miR-control into SAS cells. Firefly luciferase activity was normalised to *Renilla* luciferase activity. Relative luciferase activity in *miR-29s* transfectants was compared with that in miR-control cultures, which was set at 1, in cells transfected with wild type-3'UTR or deletion-3'UTR. $*P < 0.0083$

**Figure 4.**

**Direct regulation of *ITGA6* by *miR-29s*.**

(A) *ITGA6* mRNA expression 72 h after transfection with ten nM *miR-29s* in SAS was evaluated with qRT-PCR. *GUSB* was used as an internal control. $**P < 0.005$ (B) ITGA6 protein expression 72 h after transfection with *miR-29s* in SAS was evaluated with Western blot

analysis. GAPDH was used as a loading control. The expression ratio of ITGA6/GAPDH was calculated with ImageJ software. (C) TargetScan database analysis indicated a putative *miR-29s* binding site existed in the *ITGA6* 3'-UTR (positions 1570 - 1576). With regard to this site, partial wild-type sequences of *ITGA6* 3'-UTR and those with the deleted *miR-29s* target site were constructed and inserted between the XhoI – PmeI restriction sites in the 3'-UTR of the h*Rluc* gene in psiCHECK-2 vector. (D) The vectors were co-transfected with *miR-29s* or miR-control into SAS cells. Firefly luciferase activity was normalised to *Renilla* luciferase activity. Relative luciferase activity in *miR-29s* transfectants was compared with that in miR-control cultures, which was set at 1, in cells transfected with wild type-3'UTR or deletion-3'UTR. $*P < 0.0083$

**Figure 5.**

**Effect of si-*LAMC2* or si-*ITGA6* transfection on cell proliferation, migration and invasion in SAS.**

Cell proliferation 72 h after transfection with ten nM si-*LAMC2* (A) or si-*ITGA6* (B) in SAS was determined with the XTT assay. Cell migration activity 48 h after transfection with ten nM si-*LAMC2* (C) or si-*ITGA6* (D) in SAS was determined with the migration assay. Cell invasion activity 48 h after transfection with ten nM si-LAMC2 (E) or si-*ITGA6* (F) in SAS was determined with the Matrigel invasion assay. $*P < 0.0167$.
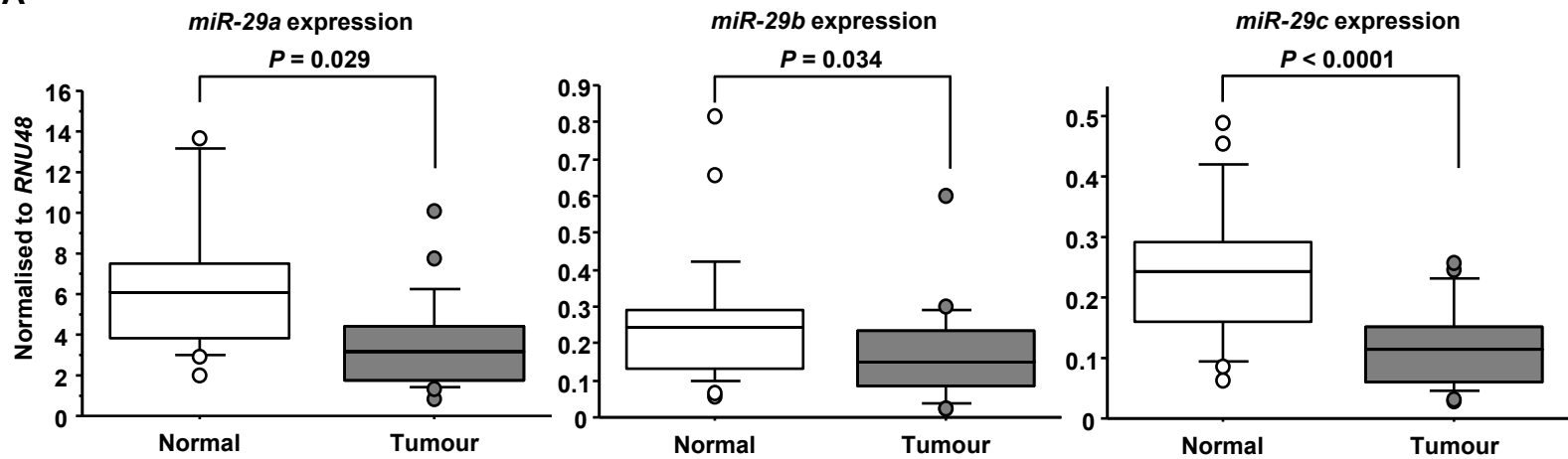
**Figure 6.**

**Illustration of tumour suppressive *miR-29s* and *miR-218* regulation of laminin-332 and α6ß4 integrin.**

Regulation of laminin-332 and α6ß4 integrin by *miR-29s* and *miR-218* is shown (modified a figure in Guess and Quaranta, 2009). Laminin-332, which consists of laminin α3, ß3 and γ2 chains, is a major component of basement membranes, but is also an important autocrine ligand produced by cancer cells to promote tumorigenesis. Laminin-332-α6ß4 integrin interaction promotes tumour invasion and survival by activation of PI3K and RAC1. *LAMC2* and *ITGA6* were directly regulated by tumour suppressive *miR-29s*, while *LAMB3* is directly regulated by tumour suppressive *miR-218* (our previous study). In addition, *ITGB4* has a putative *miR-218* binding site in the 3'-UTR region.
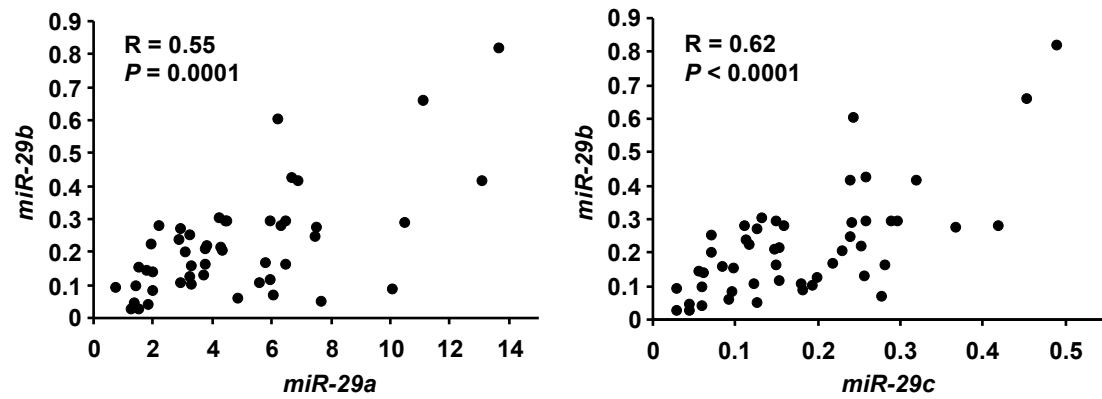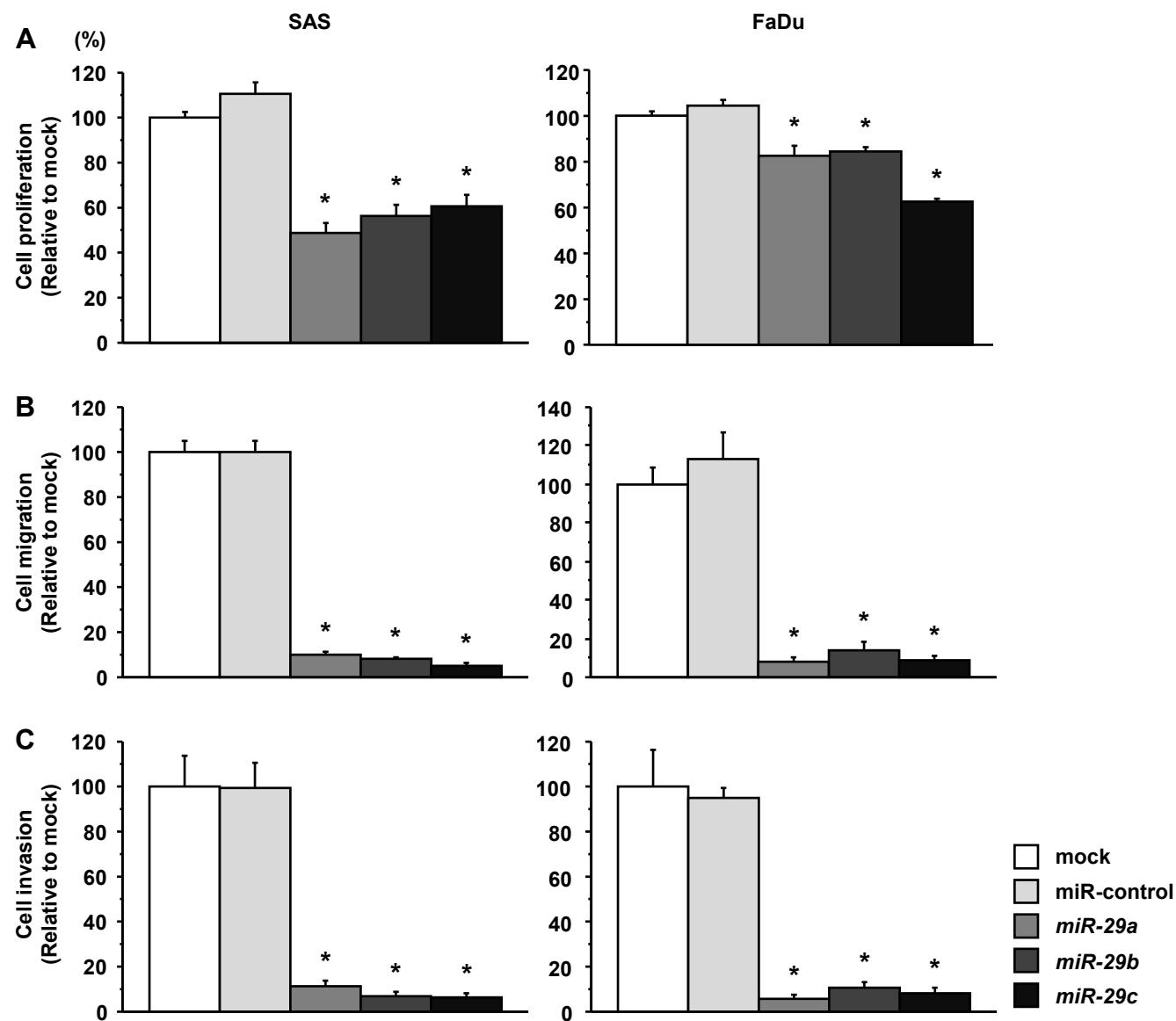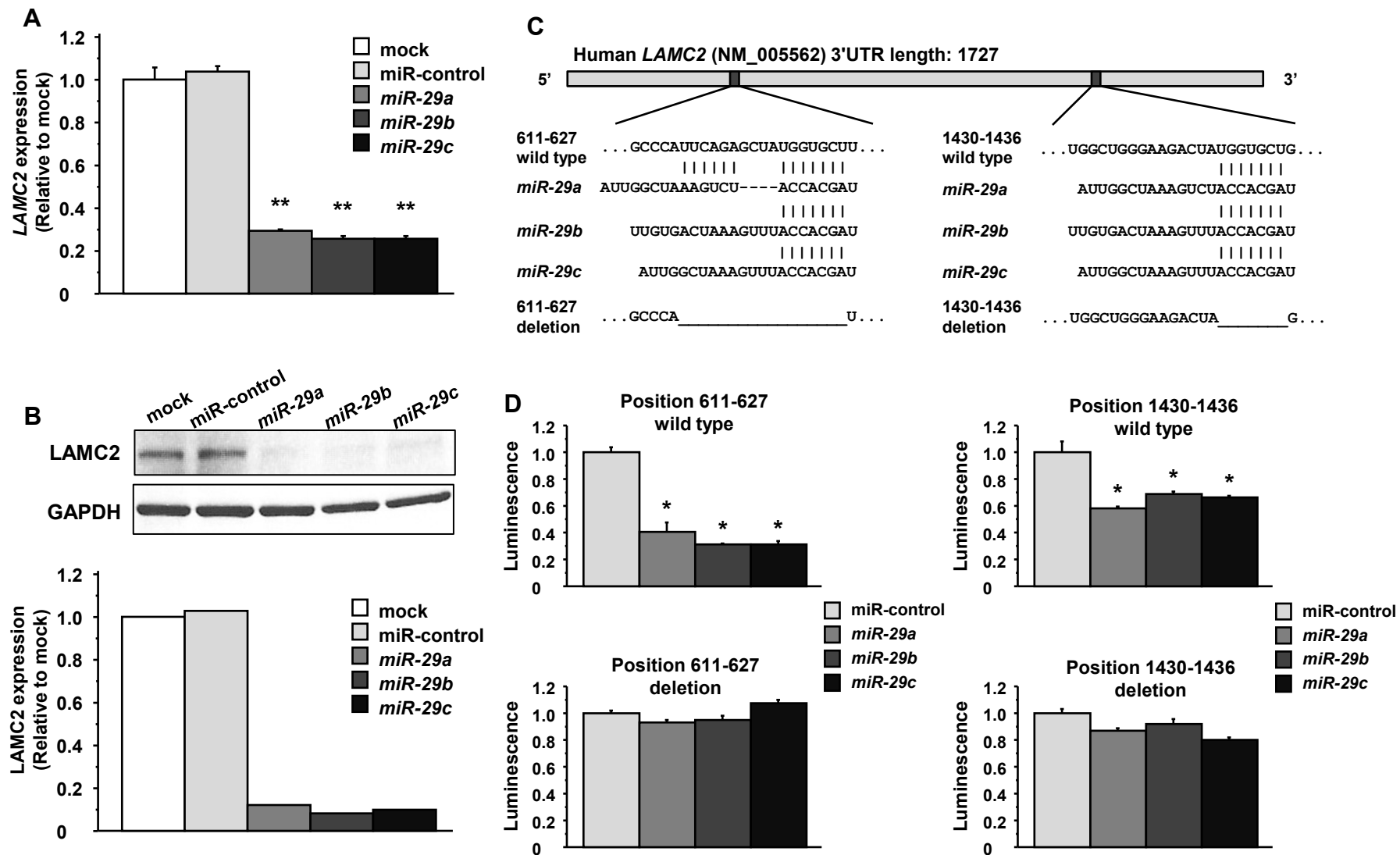
**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**



**A**

**B**

**C**

Human *ITGA6* (NM_000210) 3'UTR length: 2385

5'          3'

1570-1576
wild type ...GAGCAACAGCAAACAGGUGCUAA...
          | | | | | |
*miR-29a*  AUUGGCUAAAGUUUACCACGAU
          | | | | | |
*miR-29b*  UUGUGACUAAAGUUUACCACGAU
          | | | | | |
*miR-29c*  AUUGGCUAAAGUUUACCACGAU

1570-1576
deletion ...GAGCAACAGCAAACA_____A...

**D**

Position 1570-1576
wild type

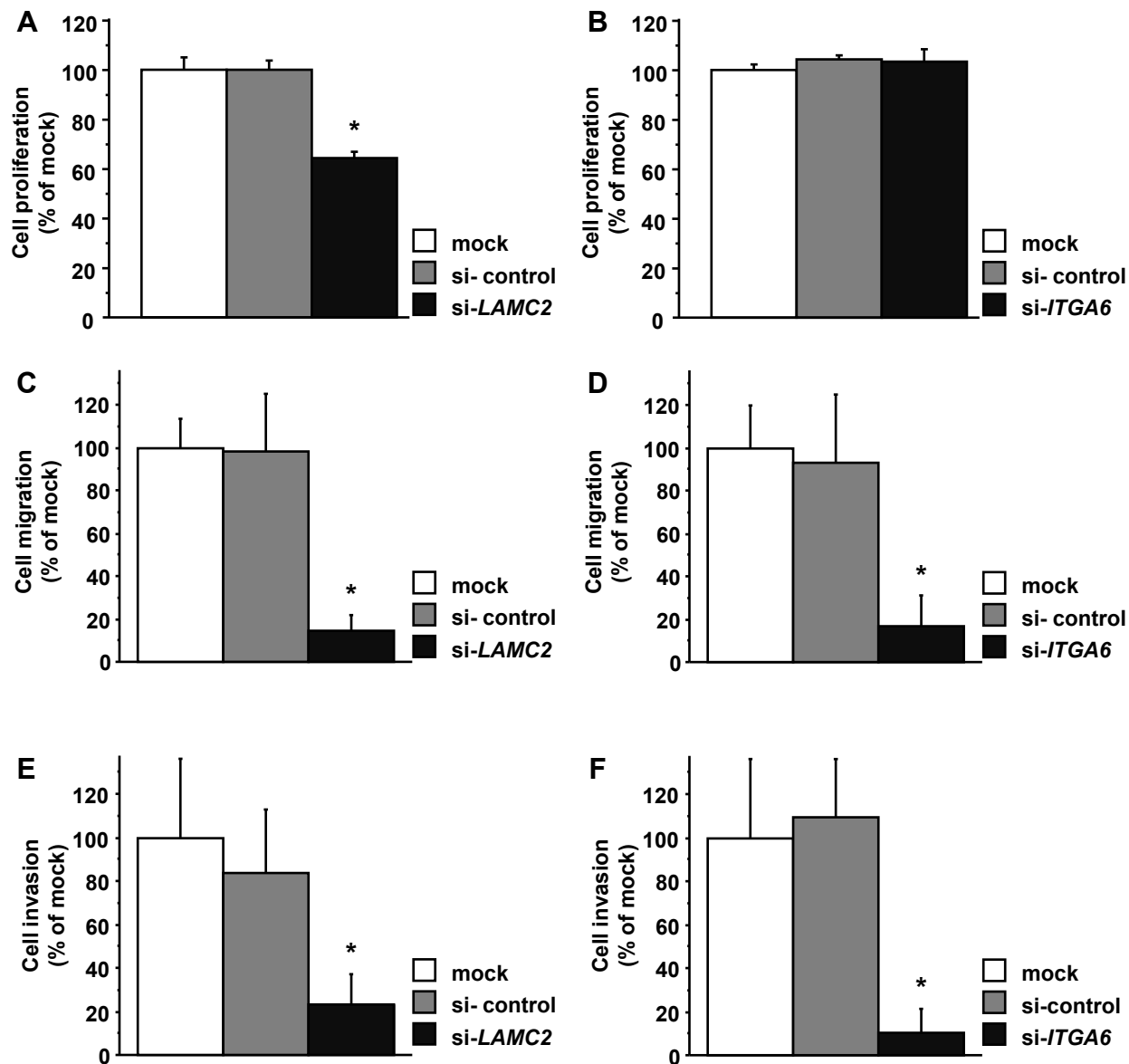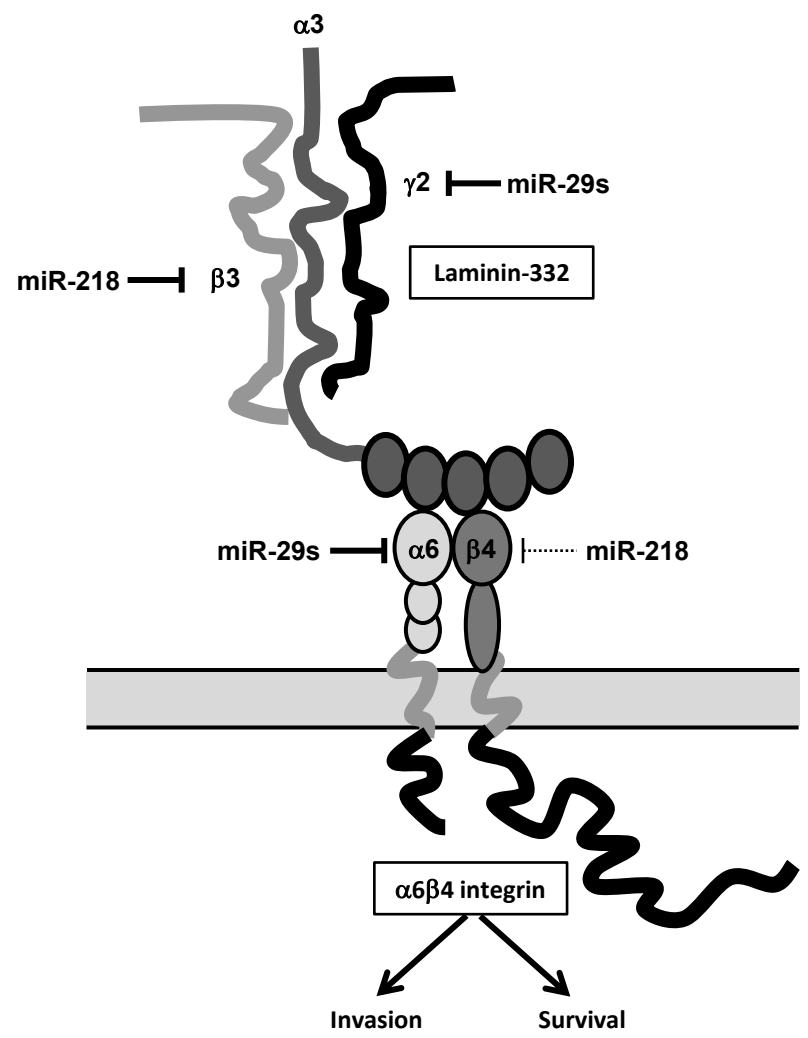Position 1570-1576
deletion
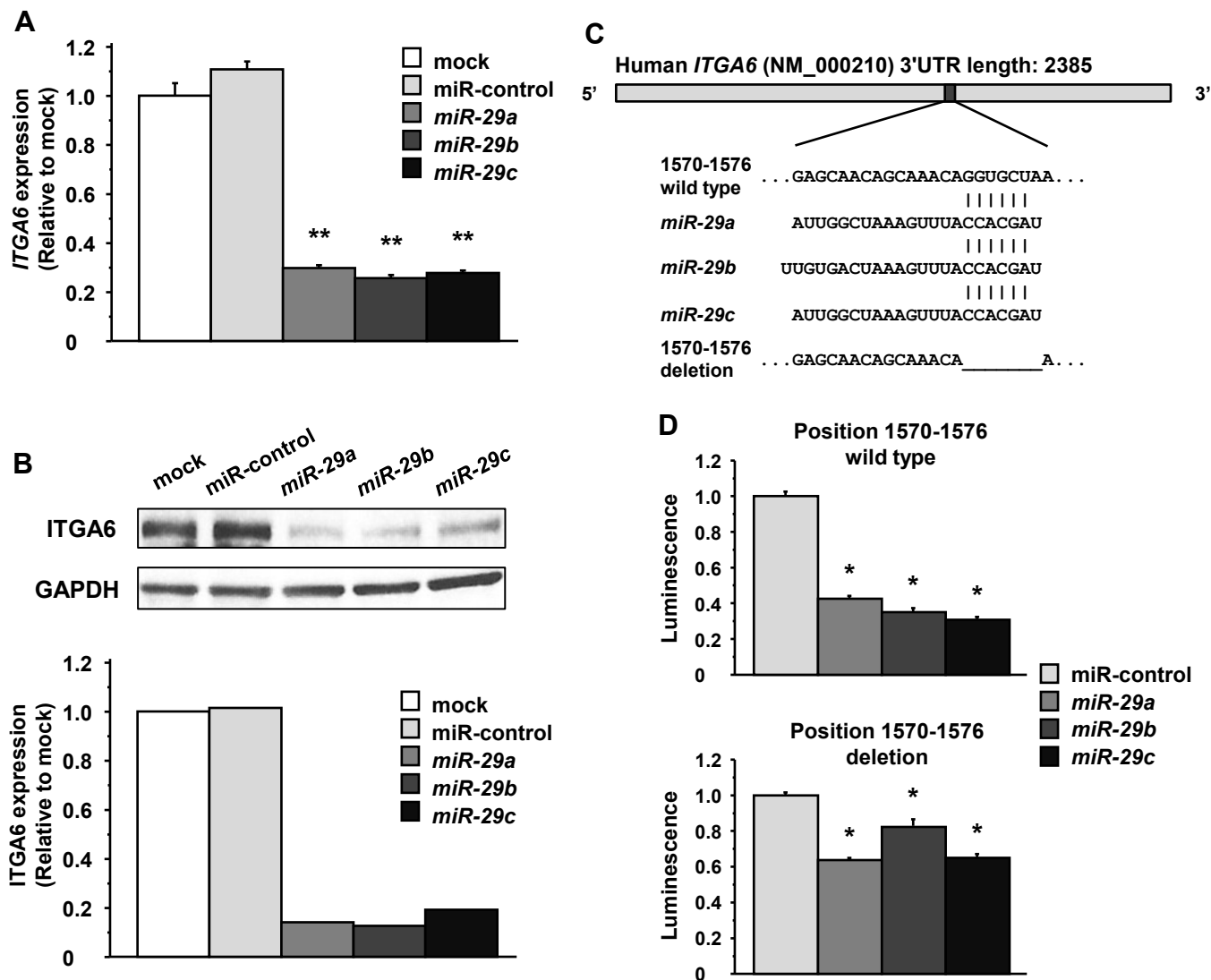
**Figure 5**

**Figure 6**

**Table 1. Candidate target genes for *miR-29s* in focal adhesion pathway.**

| Gene Symbol | Entrez Gene ID | GEO expression data (GSE9844) | | Gene expression analysis of SAS |
| --- | --- | --- | --- | --- |
| | | Log$_2$ ratio (Tumour /Normal) | *P* value | Log$_2$ ratio (*miR-29a* /miR-control) |
| LAMC2 | 3918 | 3.27 | 3.91E-06 | -1.18 |
| COL4A6 | 1288 | 2.60 | 2.12E-06 | -0.87 |
| COL4A1 | 1282 | 2.26 | 2.11E-04 | -1.78 |
| ITGA6 | 3655 | 2.07 | 7.10E-06 | -1.62 |
| COL11A1 | 1301 | 1.97 | 1.88E-03 | -0.75 |
| COL5A2 | 1290 | 1.74 | 3.87E-04 | -3.18 |
| COL4A2 | 1284 | 1.63 | 1.22E-03 | -1.76 |
| COL1A2 | 1278 | 1.45 | 6.95E-04 | -0.61 |
| COL6A3 | 1293 | 1.35 | 5.72E-03 | -0.80 |
| COL5A1 | 1289 | 1.23 | 1.69E-03 | -1.56 |
| COL3A1 | 1281 | 1.18 | 9.15E-03 | -0.78 |
| COL4A5 | 1287 | 1.14 | 5.52E-04 | -0.96 |
| COL5A3 | 50509 | 0.76 | 9.15E-03 | -0.70 |
| THBS2 | 7058 | 0.76 | 6.85E-02 | -1.14 |
| CAV2 | 858 | 0.55 | 5.15E-02 | -0.81 |
| CDC42 | 998 | 0.54 | 7.60E-03 | -0.70 |

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