

## **Periostin promotes invasion and anchorage-independent growth in the metastatic process of head and neck cancer**

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**Abstract**

Head and neck squamous cell carcinoma (HNSCC) is one of the most common types of human cancer. Typically HNSCC cells show persistent invasion that frequently leads to local recurrence and distant lymphatic metastasis. However, molecular mechanisms associated with invasion and metastasis of HNSCC remain poorly understood. Here we identified Periostin as an invasion promoting factor in HNSCC by comparing the gene expression profiles between parent HNSCC cells and a highly invasive clone. Indeed, Periostin overexpression promoted the invasion and anchorage independent growth both *in vitro* and *in vivo* in HNSCC cells. Moreover, Periostin overexpressing cells spontaneously metastasized to cervical lymph nodes and to the lung through their aggressive invasiveness in an orthotopic mouse model of HNSCC. Interestingly, Periostin was highly expressed in HNSCCs in comparison with normal tissues, and the level of Periostin expression was well correlated with the invasiveness of HNSCC cases. In summary, these findings suggest that Periostin plays an important role for invasion and anchorage independent growth in the metastatic process of HNSCC.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common types of human cancer, with an annual incidence of more than 500,000 cases worldwide (1).

HNSCC is associated with severe disease- and treatment-related morbidity and has a 5-year survival rate of approximately 50%; this rate has not improved in more than 2 decades (2).

Review of the literature indicates that the most important factor for the high mortality rate is the advanced stage of the disease at the time of diagnosis and treatment. In the prognosis of HNSCC, the extent of lymph node metastasis is a major determinant. Like most epithelial cancers, HNSCC develops through the accumulation of multiple genetic and epigenetic alterations in a multistep process. Recent molecular studies have advanced our understanding of the disease and provided a rationale to develop novel strategies for early detection, classification, prevention and treatment. Attempts to identify the genes involved in the metastasis is pivotal for the early prediction of HNSCC behavior. However, the identity and time of onset of the alterations that endow cancer cells with these metastatic functions are largely unknown. The process of metastasis consists of sequential and selective steps including proliferation, induction of angiogenesis, detachment, motility, invasion into circulation, aggregation and survival in the circulation, cell arrest in distant capillary beds and extravasation into organ parenchyma (3). The development of a metastasis depends on interplay between host factors and intrinsic characteristics of cancer cells, and the metastatic lesion represents the end point of many destructive events that only a few cells can survive (4). Moreover, neoplasms contain a variety of subpopulations of cells with differing metastatic potential, and the possible existence of highly metastatic clones may exist within a primary tumor (4). Indeed, we previously isolated highly invasive clones from parent HNSCC cell line by using an *in vitro* invasion assay method (5).

Here we compared the transcriptional profile of parent cells and a highly invasive clone by microarray analysis in order to identify genes that differ in their expression. We identified Periostin (osteoblast specific factor 2 (fasciclin I-like)) as the gene demonstrating the highest fold change expression in the invasive clone. Periostin is a secreted protein, which was originally identified from osteoblasts (6, 7). In the present study, we demonstrated that Periostin was involved in the invasion and anchorage independent growth in HNSCC.

## **Materials and Methods**

**Gene array analysis.** The Amersham CodeLink system using the UniSet Human I Expression Bioarray, containing 10458 genes probes, was used to compare the transcription profiles between parent cells and high invasive clone. This array contains a broad range of genes derived from publicly available, well-annotated mRNA sequences. The CodeLink array is unique in being capable of detecting minimal differences in gene expression, as low as 1.3-fold with 95% confidence, because of the novel three-dimensional aqueous gel matrix, which the empirically tested 30-mer oligonucleotides are deposited on (8). This substantially reduces background, enhances sensitivity, and allows for the detection and quantification of subtle regulatory relationships among genes in parent HNSCC cells and highly invasive clone. Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen) according to the manufacture's instructions. Preparations were quantified and their purity was determined by standard spectrophometric methods. Data were expressed as the average differences between the perfect match and mismatch probes for the Periostin gene (Supplemental data 1).

Total RNA from 41 primary HNSCC and 13 normal tissues was labeled and hybridized to Affymetrix U133A Gene Chips as previously reported (9). To explore the genes that are

coordinately expressed with Periostin in HNSCC tumors, we performed a similarity search using a Pearson correlation metric as implemented in tGeneData Analyst Pro 1.0 software (Supplemental data 2).

**Cell culture.** MSCC-1 and MSCC-Inv1 cells were previously established in our laboratory (5, 10). These cells were maintained in Keratinocyte-SFM (Invitrogen) under a condition of 5 % CO<sub>2</sub> in air at 37 °C. Other HNSCC cell lines HSC2, HSC3 and Ca9-22 were provided by Japanese Collection of Research Bioresources Cell Bank. They were maintained in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10 % heat-inactivated FBS (Invitrogen) and 100 U/ml penicillin-streptomycin (Gibco) under conditions of 5 % CO<sub>2</sub> in air at 37 °C. For growth assay, cells were plated onto 24 well plates (Falcon), and trypsinized cells counted by Cell Counter (Coulter Z1).

**RT-PCR.** Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen). Preparations were quantified and their purity was determined by standard spectrophometric methods. cDNA was synthesized from 1 µg total RNA according to the ReverTra Dash (Toyobo Biochemicals, Tokyo, Japan). Primer sequences included the following; human Periostin, 5'-gatggagtgcctgtgaaat-3' (forward) and 5'-aactcctcacgggtgtgtc-3' (reverse) (product size, 239bp); human GAPDH, 5'-tccaccaccctgttgctgta-3' (forward) and 5'-accacagtccatgccatcac-3' (reverse) (product size, 450bp). Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen), and amplifications were performed in a PC701 thermal cycler (Astec, Fukuoka, Japan) for 30-35 cycles after an initial 30 sec denaturation at 94 °C, annealed for 30 sec at 60 °C, and extended for 1 min at 72 °C in all primers. The amplification reaction products were resolved on 1.5% agarose/TAE gels

(Nacalai tesque, Inc., Kyoto, Japan), electrophoresed at 100 mV, and visualized by ethidium-bromide staining.

**Western blot analysis.** Western blotting was carried out as we described previously (11). A polyclonal anti-Periostin antibody was generated by immunizing the rabbits with specific peptides (EGEPEFRLIKEGETC) for Periostin and purified through an affinity column. An anti-His polyclonal antibody (Cell Signaling Technology Inc.) and  $\beta$ -actin monoclonal antibody (Sigma) were also used. Thirty  $\mu$ g of protein was subjected to 10 % polyacrylamide gel electrophoresis followed by electroblotting onto a nitrocellulose filter. For detection of the immunocomplex, the ECL western blotting detection system (Amersham) was used

**Generation of Periostin-overexpressing HNSCC cells.** A Periostin expression plasmid, pcDNA3.1 encoding a hexa-histidine tagged Periostin cDNA, was kindly provided by Dr. X-F. Wang (Duke University). The periostin/pcDNA3.1 plasmid or the vector alone was introduced into HSC2 and HSC3 cells, and the stable clones were obtained by G418 selection (500  $\mu$ g/ml, Gibco) in the culture medium. Cell transfections were performed using FuGENE 6 (Roche) according to the manufacture's instruction. Four Periostin-expressing clones and one control clones of each cells were chosen for the subsequent experiments.

**In vitro invasion assay.** *In vitro* invasion assay was performed as described previously (5).

Briefly, invasion was measured by use of a 24 well cell culture insert with 8 mm pores (3097, Falcon, Becton Dickinson, Franklin Lakes, NJ). The filter was coated with 20  $\mu$ g of EHS extract (Iwaki Garasu, Tokyo, Japan), which was reconstituted basement membrane substance. The lower compartment contained 0.5 ml of serum-free medium. After

trypsinization,  $1.5 \times 10^5$  cells were resuspended in 100  $\mu$ l of serum-free medium and placed in the upper compartment of the cell culture insert for 6-24 hours. ~~Cells were placed in the upper compartment of the cell culture insert for 6-24 hours.~~ For invasiveness after Periostin siRNA treatment, we used 50  $\mu$ g of EHS extract in this experiments, because MSCC-Inv1 cells have a high invasive activity. After incubation, we collected the penetrating cells onto the lower side of the filter to isolate highly invasive clones by the method of Kalebic et al. with minor modification (12). To examine the invasiveness, cells were fixed with formalin and stained with hematoxylin and eosin. The invasiveness of the cells was determined by counting of the penetrating cells onto the lower side of the filter through the pores under a microscope at x100 magnification. We assayed 3 times and randomly selected 3 fields were counted for each assay.

**Generation of recombinant Periostin.** Full-length human Periostin cDNA was subcloned into pIZ/V5-His vector (Invitrogen). pIZ/V5-His vector containing Periostin was transfected into High-Five insect cells by using Cellfectin reagent (Invitrogen). Stable clones were obtained by Zeocin selection in the culture medium. A Ni-nitrilotriacetic acid column was used to purify recombinant Periostin according to the manufacturer's instructions (Invitrogen).

**Silencing by Small Interfering RNA.** Stealth siRNA (Oligo ID, HSS116398, Invitrogen) is a 25-bp duplex oligoribonucleotide with a sense strand corresponding to nucleotides 62-86 of the reported human Periostin mRNA sequence. The sense sequence of HSS116398 is 5'-CCCUAUAAACGCCAACAAUCAUUUAU-3'. MSCC-Inv1 cells were transfected by 150 pmol of siRNA in 1 ml of OPTI-MEM per the instruction of the manufacturer. Following siRNAs treatment (48 h), MSCC-Inv1 cells were used for *in vitro* invasion assay as described above.

A scrambled sequence that does not show significant homology to rat, mouse or human gene sequences was used as a control.

**Cell adhesion assay.** Flat-bottomed 96-well ELISA plates (Costar) were incubated overnight at 4°C with 10 µg/ml of Periostin protein in the presence of anti- $\alpha$ v $\beta$ 3 or anti- $\alpha$ v $\beta$ 5 integrin antibody (10 µg/ml) and blocked for 1 hour at room temperature with PBS containing 2% bovine serum albumin. Cells were suspended in medium at a density of  $3 \times 10^5$  cells/ml, and 0.1 ml of the cell suspension was added to each well of the coated plates. After incubation for 1 hour at 37°C, unattached cells were removed by rinsing with PBS. Attached cells were then trypsinized and counted by Cell Counter.

**Soft-Agar Colony Formation Assay.** Assays of colony formation in soft agar were performed using standard methods. Briefly, 4 ml underlayers consisting of 0.5 % agar medium with 20 % FBS were prepared in 60 mm dishes. Periostin overexpressing cells and control cells were trypsinized, centrifuged, and resuspended in 0.2 % agar medium with 20 % FBS.  $1 \times 10^4$  cells were then plated onto the previously prepared underlayers. The cells were kept wet by adding a small amount of RPMI-1640 medium with 10 % FBS. The cells were incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 3 weeks. Then, colonies were photographed and counted.

**Xenograft assays in nude mice.** To examine whether Periostin expression in HNSCC cells affects the anchorage independent growth *in vivo*, Periostin-overexpressing HSC2 cells ( $1 \times 10^7$  in 500 µl of Hanks' balanced salt solution) were injected s.c. into multiple sites in athymic (nude) mice. The control groups were injected with the same number of vector-transfected



HSC2 cells. The protocol of the experiment was approved by the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University. The animals were monitored for tumor formation every week and sacrificed 1 month later. Tumor length (L) and width (W) were measured at the end of the experiment, and tumor volume was calculated by the formula of  $(L \times W^2)/2$ .

**Orthotopic implantation.** Periostin-overexpressing and control HSC2 cells ( $5 \times 10^5$  in 50  $\mu$ l of Hanks' balanced salt solution) were injected into the tongue of male athymic (nude) mice. The animals were monitored for tumor formation every 3 day and sacrificed 2 weeks later. Tongue tumors, cervical lymph nodes and lungs were removed and fixed in 4 % formalin. Specimens were embedded in paraffin, cut into 4  $\mu$ m-thick sections and stained with hematoxylin and eosin. They were histologically evaluated for diagnosis, regional lymph node metastasis and pulmonary metastasis.

**Tissue samples.** Tissue samples of HNSCC were retrieved from the Surgical Pathology Registry of Hiroshima University Hospital from 1998 to 2004, after approval by the Ethical Committee of our institutions. 10 % buffered-formalin fixed and paraffin embedded tissues were used for immunohistochemical examination. The histological grade and stage of tumor were classified according to the criteria of the Japan Society for Head and Neck Cancer. Fresh samples were taken from the neoplastic tissues and non-neoplastic tissues for RT-PCR analysis.

**Immunohistochemical staining.** Immunohistochemical detection of Periostin in HNSCC cases was performed on 4.5  $\mu$ m sections mounted on silicon-coated glass slides, using a

streptavidin-biotin peroxidase technique as described previously (11). The expression of Periostin was graded as ++ (over 30 % of tumor cells showed strong or diffuse immunopositivity), + (10-30 % of tumor cells showed moderate or patchy immunopositivity) and - (less than 10 % of the tumor cells showed weak or focal immunopositivity or no staining).

Three pathologists (Y.K., I.O., and T.T.) made all the assessments. Possible correlation between variables of the analyzed tumor samples was tested for association by the Fisher's exact test. A  $P$  value < 0.05 was required for significance.

## Results

### Identification of Periostin as an overexpressed gene in a highly invasive HNSCC cell

**line.** We previously established a HNSCC cell line, MSCC-1, from lymph node metastasis (10). Moreover, we isolated a highly invasive clone MSCC-Inv1 from MSCC-1 cells by using an *in vitro* invasion assay device (5). In the present study, we compared the transcriptional profile of parent cells (MSCC-1) and a highly invasive clone (MSCC-Inv1) by microarray analysis in order to identify genes that differ in their expression (Fig.1A). Several genes were selectively overexpressed in the highly invasive clone (Fig. 1A and Supporting Data 1). Among these genes, the most overexpressed gene was Periostin. The finding of higher expression of Periostin in the highly invasive clone than the parent cells was confirmed by RT-PCR and immunoblotting (Fig. 1B and C). For immunoblotting, we generated a polyclonal Periostin antibody. Moreover, MSCC-Inv1 cells secreted significant amounts of Periostin to media in comparison with MSCC-1 cells (Fig. 1D). In the present study, Periostin was chosen to investigate its ability to promote the invasion of HNSCC.

**Overexpression of Periostin promotes invasion of HNSCC cells in vitro.** To demonstrate the involvement of Periostin in the invasion of HNSCC, we generated the Periostin overexpressing cells. At first, we examined the Periostin mRNA in 5 HNSCC cell lines. Among above 5 cell lines, only MSCC-1 and MSCC-Inv1 cells highly expressed Periostin (Fig. 2A). Then, we transfected Periostin into HSC2 and HSC3 cells with slight expression of Periostin mRNA and obtained stable clones expressing Periostin (Fig. 2B). ~~In the following analyses, we used clone #2 of HSC2 and clone #3 of HSC3 cells, which showed Periostin overexpression.~~ Although Periostin overexpression did not promote the cell proliferation (Fig. 2C), it dramatically enhanced the invasiveness of both HSC2 and HSC3

cells (Fig. 2D2C). Invasiveness was well correlated with ectopic expression levels of Periostin (Fig. 2C). Moreover, treatment with recombinant Periostin protein also enhanced the invasiveness of HSC2 cells in a concentration dependent manner (Fig. 2E). To confirm the correlation between Periostin and invasion, we reduced the expression of Periostin by small interference RNA (siRNA) treatment in MSCC-Inv1 cells (Fig. 2F2D). Periostin siRNA treatment remarkably inhibited the invasion (Fig. 2G2D). Overall, these results indicate that Periostin plays an important role in invasion of HNSCC.

### **Periostin enhanced anchorage independent growth of HNSCC cells both in vitro and in vivo.**

Similar to Periostin transfected HSC2 cells, treatment with recombinant Periostin protein also enhanced the invasiveness of HSC2 cells in a concentration dependent manner (Fig. 3A). Periostin contains the four internal repeats of fasciclin I (FAS1) domain that represents an ancient cell adhesion domain common to plants and animals (13). In mammals, there are four proteins containing FAS1 domains, specifically, two secretory proteins, Periostin and  $\beta$ ig-h3, and two membrane proteins, FEEL-1 and -2. FAS1 of  $\beta$ ig-h3 bears motifs interacting with integrins  $\alpha$ 3 $\beta$ 1 and  $\alpha$ v $\beta$ 5 (14, 15) and mediates endothelial cell adhesion and migration via integrin  $\alpha$ v $\beta$ 3 (16). Similarly to  $\beta$ ig-h3, recombinant Periostin supports adhesion of ovarian epithelial cells that can be inhibited by monoclonal antibodies against  $\alpha$ v $\beta$ 3 or  $\alpha$ v $\beta$ 5 integrin, but not by anti- $\beta$ 1 integrin antibody (17). We also found that treatment of specific anti- $\alpha$ v $\beta$ 3 and anti- $\alpha$ v $\beta$ 5 integrin antibodies inhibited the adhesion of HSC2 cells to the culture wells precoated with Periostin, indicating that interference with the function of integrins has an effect on the ability of Periostin to mediate cell adhesion of HNSCC cells (Fig. 3A3B).

It has been suggested that alterations in cell–cell adhesion molecules, integrins or integrin-associated signaling molecules in cancer cells may be involved in anchorage-independent growth (18). Therefore, we hypothesize that Periostin may affect the anchorage-independent growth of HNSCC through the interaction with integrins. To test this hypothesis, we examined the anchorage independent growth of Periostin overexpressing cells *in vitro* by using soft-agar colony formation assay. In the following analysis, we used a clone #2 of HSC2 cells which showed higher invasiveness. The Periostin overexpressing cells formed considerably larger colonies in the soft agar in comparison with control cells (Fig. 3B3C). In addition, the number of colonies with Periostin overexpressing cells was higher than those with control cells (Fig. 3C). To determine the anchorage independent growth *in vivo*, Periostin overexpressing HNSCC cells were injected subcutaneously into nude mice. After 28 days, the growth characteristics of resulting tumors were analyzed. Interestingly, transplantation of the Periostin overexpressing cells showed comparatively large tumor volume than that of the control cells (Fig. 3Da-c). The volume of tumors derived from Periostin overexpressing cells was about sixteen fold higher than that of tumors from control cells (Fig. 3E3Dd). Tumor volume was  $187 \pm 57.1$  and  $11.4 \pm 10.4$  in the Periostin overexpressed and control cells, respectively.

**Periostin overexpressing cells frequently metastasize to lymph nodes and lung through their aggressive invasiveness.** As demonstrated above experiments, Periostin enhanced invasion and anchorage independent growth of HNSCC cells. To further evaluate if Periostin overexpression affects metastasis *in vivo*, we orthotopically implanted the Periostin overexpressing cells into the tongues of nude mice (Fig. 4A). An orthotopic implant technique has been previously used to examine the lymphatic metastatic activity of human

HNSCC derived from different patients (19, 20). After 2 weeks of implantation, mice were sacrificed. Tumors were observed in the tongues of mice implanted with both Periostin overexpressing cells and control cells (Fig. 4Ba and Ca). Strikingly, in mice implanted with Periostin overexpressing cells the tumors were larger than in mice implanted with control cells. Histologically, Periostin overexpressing tumors demonstrated a poorly differentiated phenotype characterized by a diffuse and trabecular growth pattern without keratinization. In contrast, control tumors demonstrated characteristics consistent with a well differentiated phenotype with tumor islands with keratin pearl formation (Fig. 4Bb and Cb). Moreover, Periostin overexpressing tumors showed remarkable invasiveness including destruction of mandibular bone and lymphocytic infiltration (Fig. 4Ca and Cc). Interestingly, the Periostin overexpressing cells spontaneously metastasized to cervical lymph nodes (Fig. 4Cd) and lung (Fig. 4Ce). Overall, 6 of 11 mice implanted with Periostin overexpressing cells showed metastasis to regional lymph nodes and/or lung, but no metastasis was observed in mice implanted with control cells (0 of 10) (Fig. 4D). These findings suggest that Periostin overexpression may be involved in metastasis through aggressive invasiveness.

### **Overexpression of Periostin is frequently found in HNSCC cases and associated with**

**the invasiveness of HNSCC.** To determine if the upregulation of Periostin is a common feature of HNSCC in human subjects, we performed RT-PCR analysis on 3 normal tissues and 9 HNSCC samples. As shown in Fig. 5Aa, the expression levels of Periostin mRNA in cancer tissues was higher than in normal tissues. The average of Periostin expression levels (Periostin/Gapdh ratio) was 3-fold higher in tumors than normal tissues (Fig. 5B5Ab).

Next, we examined the expression of Periostin in 12 normal oral mucosae and 62 HNSCC cases by immunohistochemistry. HNSCC cells showed highly expression of

Periostin in comparison with normal oral mucosae (Fig. [5C-5Ba and b](#) and Table 1). In 62 HNSCC cases, 26 (41.9 %) cases showed high expression of Periostin. Then, we investigated the relationship between Periostin expression and the invasiveness in [62-102](#) HNSCC cases. For evaluation of invasiveness of HNSCC, we used the grading of mode of invasion (Grade 1 has a well defined borderline, Grade 2 has a less-marked borderline, Grade 3 has groups of cells and no distinct borderline, and Grade 4 has diffuse growth) as firstly described by Jacobsson et al. (21). We defined two groups, low (Grade 1 and 2) and high (Grade 3 and 4). Interestingly, higher expression of Periostin was significantly associated with the grading of mode of invasion ( $P<0.05$ ) (Fig. [5D-5Bc and d](#) and Table 1). In particular, cancer cells at invasive front expressed Periostin at higher levels (Fig. [5E5Be](#)). We also examined the association between Periostin expression and metastasis in [40-62](#) HNSCC cases with available clinical information. [60-56.2](#) % of HNSCC cases with metastasis showed high expression of Periostin (Table 1).

To further evaluate the expression of Periostin in patients with HNSCC we compared the expression in a previously published microarray dataset of 41 HNSCC patients and 13 normal controls (9). Similar to our data, Periostin was expressed at higher levels in HNSCC tissues, in comparison with normal oral mucosal tissues (Fig. [5F5C](#)). Moreover, HNSCC cases with angiolymphatic invasion showed higher expression of Periostin (Fig. [5F5C](#)). To further explore the genes that are coordinately expressed with Periostin in HNSCC tumors, we performed a similarity search using a Pearson correlation metric as implemented in tGeneData Analyst Pro 1.0 software. The expression of selected genes demonstrating the highest coordinate expression with periostin in normal oral mucosal tissue and HNSCC tissues was then visualized by hierarchical clustering (Fig. [5G5D](#)). FAP, SULF1, COL5A2,

COL3A1, COL10A1, COL4A1, FN1 and INHBA were well correlated with Periostin expression (Fig. [5G-5D](#) and Supplemental data 2).

## Discussion

It is believed that neoplasms contain a variety of subpopulations of cells with differing metastatic potential, and the presence of highly metastatic clones may exist within a primary tumor (4). Recent data demonstrate that reinjection of metastatic cell populations can lead to enrichment in the metastatic phenotype by work on experimental metastasis with cancer cell lines (22-25). Further, metastasis related genes were identified by comparing the gene expression profiles between parent and metastatic cell populations using microarray analysis (22-25). We previously established HNSCC cell line from metastatic cervical lymph node of HNSCC and then isolated highly invasive clones from this cell line by *in vitro* invasion assay (5, 10). By using these cell lines, we demonstrated that methylation of E-cadherin and degradation of  $\beta$ -catenin were involved in the invasion of HNSCC through the loss of cell-cell adhesion (5). Thus, we previously could obtain the highly invasive phenotype by isolation of cell populations using an *in vitro* invasion assay. Therefore, we thought that comparing the gene expression profile of the parent and highly invasive clone could be a good approach to identify genes that influence the invasion of HNSCC. Here, we identified several genes, which encode secretory or cell surface proteins implicated in invasion, cell adhesion, angiogenesis and growth factor as candidate genes for the invasion of HNSCC by comparing the gene expression profiles between parent HNSCC cells and a highly invasive clone using microarray analysis. Among these genes, Periostin was found to be the most highly expressed gene in invasive HNSCC cells. As expected, overexpression of Periostin dramatically promoted the invasion of HNSCC cells *in vitro*. Moreover, invasive phenotype



was abolished by Periostin siRNA treatment. These observations strongly indicate that Periostin plays an important role in the invasion of HNSCC. In addition, we found that Periostin overexpression did not promote proliferation, but growth in soft agar and *in vivo* growth as tumor xenografts, demonstrating that Periostin enhances anchorage independent growth of HNSCC cells.

Periostin contains an N-terminal secretory signal peptide, followed by a cysteine-rich domain, four internal homologous repeats, and a C-terminal hydrophilic domain. The four internal repeats region of Periostin shares a homology with the axon guidance protein FAS1 containing sequences that allows binding of integrins and glycosaminoglycans *in vivo* (26). FAS1 domains of  $\beta$ ig-h3, which shares a significant structural homology with Periostin, bear motifs interacting with integrins  $\alpha$ 3 $\beta$ 1 and  $\alpha$ v $\beta$ 5 (14, 15) and mediate endothelial cell adhesion and migration via integrin  $\alpha$ v $\beta$ 3 (16). Similarly to  $\beta$ ig-h3, we found that interference with the function of integrins by specific anti- $\alpha$ v $\beta$ 3 and anti- $\alpha$ v $\beta$ 5 integrin antibodies had an effect on the ability of Periostin to mediate cell adhesion in HNSCC cells. Taken together, these data strongly suggest that FAS1 domain of Periostin binds to integrins. It is well known that integrin mediates cell-extracellular matrix (ECM) interaction and that integrin-mediated adhesion regulates a variety of intracellular events (27). Therefore, we hypothesize that Periostin-integrin interaction may inhibit the ECM-integrin interaction and trigger the intracellular signaling and activation of certain genes that are involved in invasion and anchorage independent growth of HNSCC. This hypothesis is supported by recent report that Periostin activated the Akt/PKB pathway via the  $\alpha$ v $\beta$ 3 integrin to promote cellular survival in colon cancer (28). We suggest that overexpression of Periostin may confer on HNSCC cells the ability to survive in the absence of anchorage by inhibiting anoikis-related apoptotic pathways, thus allowing Periostin overexpressing HSC2 cells to form colonies in

soft agar and tumors in nude mice. However, in order to clarify the underlying mechanism of invasion and anchorage independent growth by Periostin, further studies are required.

As mentioned above, Periostin promoted invasion and anchorage independent growth in HNSCC cells. These striking phenotypes seem to be an important for cancer metastasis.

Interestingly, Periostin overexpression dramatically induced metastasis to lymph nodes and to the lung in an orthotopic implant model of HNSCC, demonstrating spontaneous metastasis from tongue. This orthotopic implant model of HNSCC seems clinically relevant because its tumor progression containing metastasis mimic the clinical scenario. Moreover, Periostin overexpressing tumors showed forming a larger tumor mass and remarkable invasiveness including destruction of mandibular bone and lymphatic infiltration. Therefore, we suggest that aggressive invasiveness and anchorage independent growth by Periostin overexpression may consequently lead to metastasis. To confirm the role of Periostin in metastasis as

described above, we would like to examine the effect of Periostin siRNA *in vivo* in the future.

Indeed, immunohistochemical analysis revealed that Periostin expression was well associated with the pattern of invasion ~~and lymph node metastasis~~ in HNSCC cases.

Moreover, 56.2 % of HNSCC cases with metastasis showed higher expression of Periostin, but we could not find statistical significance between Periostin expression and metastasis. In near future, we will examine the correlation between Periostin expression and metastasis in a large number of HNSCC cases. Interestingly, Bao et al. also demonstrated that a colon

cancer cell line with low metastatic potential engineered to overexpress Periostin displayed a striking phenotype of greatly accelerated tumor metastatic growth as xenografts in the animal model system of metastasis (28). Importantly, higher expression of Periostin was frequently observed in HNSCC tissues in comparison with normal tissues. By evaluation of Periostin expression in HNSCC patients, a strong correlation of Periostin expression with FAP, SULF1,

COL5A2, COL3A1, COL10A1, COL4A1, FN and INHBA was observed. Although we have to confirm this correlation, this finding gives an imagination that Periostin expression appears to be correlated with a stromal reaction likely related to invasion and metastasis. In addition, previous studies have shown that Periostin expression is up-regulated in various types of tumor including HNSCC (29), colon (28, 30), breast (31), lung (32) and ovarian cancer (17). Taken together, highly expression of Periostin may be a common event of tumor development in various types of cancer and can be a useful marker to predict its malignancy. In fact, serum levels of Periostin were elevated in patients with breast cancer, non-small cell lung cancer and thymoma (33-35). Therefore, we will examine the detection of Periostin in saliva and blood from the patients with HNSCC in the future. In conclusion, our studies have revealed a critical role of Periostin for invasion and anchorage independent growth in the metastatic process. These findings provide new and important information on the progression of HNSCC. The finding that Periostin influences metastatic potential demonstrated in this study raises the possibility that it could be used as a molecular target in anti-metastasis therapy of HNSCC patients.

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## Figure legends

**Figure 1.** Periostin is identified as an invasion related gene in HNSCC. (A) Schematic representation of the isolation process of highly invasive clone. Highly invasive clone (MSCC-Inv1) was isolated from parent cells (MSCC-1) by *in vitro* invasion assay. The transcriptional profile of MSCC-1 cells and MSCC-Inv1 cells were compared by microarray analysis. Highly expressed genes in the highly invasive clone are listed. Among these genes, the mostly overexpressed gene in the highly invasive clone is Periostin (**redbold**). (B) Confirmation of higher expression of Periostin mRNA in the highly invasive clone by RT-PCR. Amplification was performed for 30 cycles. (C) Confirmation of higher expression of Periostin protein in the highly invasive clone by Western blot analysis.  $\beta$ -actin expression was used as a loading control. (D) The production of Periostin by MSCC-1 and MSCC-Inv1 cells. The production of Periostin was examined by Western blot analysis with conditioned culture media.

**Figure 2.** Periostin promotes the invasion of HNSCC cells. (A) Expression of Periostin mRNA in HNSCC cell lines. Expression of Periostin mRNA in HSC2, HSC3, Ca9-22, and MSCC-1 and MSCC-Inv1 cells by RT-PCR was examined. Amplification was performed for 35 cycles. (B) Generation of Periostin overexpressing cells. HSC2 and HSC3 cells were engineered to overexpress Periostin by transfection with pcDNA3.1-His tagged Periostin. We obtained each 4 stable clones expressing Periostin. Ectopic expression of Periostin was examined by immunoblotting with anti-His antibody. The whole lysates from all samples were blotted with  $\beta$ -actin for loading control. (C) Cell proliferation (upper panel) and invasion (lower panel) of Periostin overexpressing HNSCC cells. Cells were plated on 24 well plates and trypsinized cells were counted by Cell Counter at 0, 2 and 4 day. ~~(D) Invasion of~~

~~Periostin overexpressing HNSCC cells.~~ The invasiveness of the cells was determined by *in vitro* invasion assay. ~~(E) Treatment with Recombinant Periostin enhanced invasiveness in HNSCC. Recombinant Periostin protein (0, 1 and 10 mg/ml) was treated in HSC2 cells. The invasiveness of the cells was determined by in vitro invasion assay.~~ (FD) Knockdown of Periostin in MSCC-Inv1 cells. Periostin siRNA were transfected. Upper panel shows that ~~T~~he effectiveness at knockdown the expression of Periostin was validated at the mRNA by RT-PCR and at the protein level by immunoblotting with anti-Periostin antibody. ~~(G) Lower panel shows that~~ Knockdown of Periostin inhibits the invasiveness of HNSCC cells. The invasiveness of the cells was determined by *in vitro* invasion assay.

**Figure 3.** Periostin is involved in the anchorage independent growth of HNSCC *in vitro* and *in vivo*. (A) Treatment with Recombinant Periostin enhanced invasiveness in HNSCC. Recombinant Periostin protein (0, 1 and 10 mg/ml) was treated in HSC2 cells. The invasiveness of the cells was determined by in vitro invasion assay. (B) Interaction between Periostin and Integrin in HNSCC cells. HSC2 cells ( $3 \times 10^4$  cells/well) were cultured in a 96-well plate precoated with 10  $\mu$ g/ml of recombinant Periostin in the presence of anti- $\alpha$ v $\beta$ 3 or anti- $\alpha$ v $\beta$ 5 integrin antibody (10  $\mu$ g/ml) for 1 hour. Non-coated wells were used as a control. Adhesive cells were counted after washing. (BC) Colony formation of Periostin overexpressing HNSCC cells in soft agar. After 3 weeks, colonies were photographed. Upper panel shows Rrepresentative photographs of colonies ~~are shown.~~ ~~(G) Lower panel shows~~ Nnumber of colonies of Periostin overexpressing HNSCC cells. The colonies under a phase contrast microscope were counted at x10 magnification. We assayed twice and randomly selected 5 fields were counted for each assay. (D) Representative results showing the tumor growth of xenografts in immunocompromised mice. Periostin overexpressing and

control HSC2 cells ( $1 \times 10^7$  cells) were individually injected subcutaneously into 2 sites in each 5 nude mice. After 1 month, the growth characteristics of the resulting tumors were analyzed. (a) Representative photographs of tumors. (b) Higher magnification of photographs of tumors. (c) Gross appearance of the tumor masses. (d) The volume of tumors. Tumor length (L) and width (W) were measured and tumor volume was calculated by the formula of  $(L \times W^2)/2$ .

**Figure 4.** Periostin promotes metastasis of HNSCC mediated by aggressive *invasiveness in vivo*. (A) Schema of orthotopic implantation of HNSCC cells with or without Periostin overexpression. Periostin overexpressing and control cells ( $5 \times 10^5$  cells) were orthotopically implanted into tongue of nude mice. After 2 weeks, tongue tumors, cervical lymph nodes and lungs were dissected. (B) Representative hematoxyline and eosin-stained images of histopathological sections from mice injected with control cells. (a) Histology of tumor mass in the tongue. (b) High power of (a). Tumor mass is enclosed with dotted line. Original magnifications: x5 (a); x25 (b). (C) Representative hematoxyline and eosin-stained images of histopathological sections from mice injected with Periostin overexpressing cells. (a) Histology of tumor mass in the tongue. (b) High power of (a). (c) Histology of invading tumor in lymphatics. (d) Histology of lymph node metastasis. (e) Histology of lung metastasis. Arrow indicates invaded tumor (c) and metastasized tumor (d and e). Original magnifications: x5 (a); x25 (b-e). (D) Summary of the metastasis of Periostin overexpressing and control cells injected mice.

**Figure 5.** Higher expression of Periostin is frequently observed in HNSCC. (A) (a) Expression of Periostin mRNA in 9 HNSCC tissues (T) and 3 normal tissues (N) by RT-PCR.

Graph shows the Periostin/GAPDH ratios which was measured by densitometry. (Bb) The average of Periostin/GAPDH ratios. The average of Periostin/GAPDH ratios in HNSCC cases (T) and in normal tissues (N) is  $1.86 \pm 0.40$  and  $0.51 \pm 0.22$ , respectively. (CB)

Immunohistochemical analysis of Periostin. Expression of Periostin was examined by immunohistochemistry in HNSCC (Cancer) (a) and normal oral mucosae (Normal) (b).—(D)

To know the Cc correlation between Periostin expression and Invasiveness-invasiveness of HNSCC, we.—To evaluated d the invasiveness of HNSCC by using invasion grading, we defined two groups as as low (Grade 1 and 2) and high (Grade 3 and 4). Representative cases of low (c) and high (d) groups s are shown. (E) Periostin expression in invasive front of HNSCC.—Representative case of higher expression of Periostin in invasive front is shown (e).

(FC) Clustering of HNSCC samples to demonstrate relative expression of Periostin. Total RNA from 41 primary HNSCC and 13 normal tissues was labeled and hybridized to Affymetrix U133A Gene Chips as previously reported (9). The average of signal intensity of Periostin in 41 HNSCC and 13 normal tissues in microarray analysis. Left graph shows signal intensity of Periostin in normal and HNSCC tissues. Right graph shows signal intensity of Periostin in HNSCC cases with or without angiolymphatic invasion. The average of signal intensity of Periostin is shown at the bottom of the graph. (GD) Clustering of 41 HNSCC and 13 normal tissues to demonstrate relative expression of Periostin. We analyzed by hierarchical clustering using a filtered set of 6800 variably expressed genes. The gene cluster that includes Periostin is highlighted in blue in the horizontal dendrogram.

### Supplemental data

Microsoft Excel file 1. Raw expression data of microarray analysis between parent cells and highly invasive clone.

~~Microsoft Excel file 2. Raw data of correlation analysis for Periostin.~~

**Table 1.** Periostin expression in normal oral mucosae and HNSCC and correlation with invasion and metastasis.

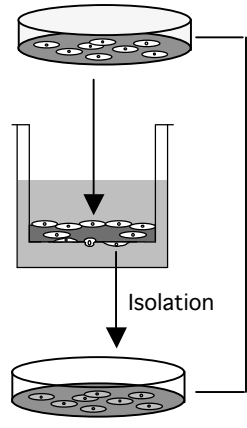
	No. of cases	Periostin expression (%)*			P value
		-	+	++	
Normal	12	9 (75.0)	3 (25.0)	0 (0)	0.0001
HNSCC	102	20 (19.6)	39 (38.2)	43 (42.2)	
Invasion grading					0.01
Low	22	9 (40.9)	8 (36.4)	5 (22.7)	
High	80	11 (13.75)	31 (38.75)	38 (47.5)	
Metastasis					0.28
Negative	30	5 (16.7)	14 (46.7)	11 (36.6)	
Positive	32	3 (9.4)	11 (34.4)	18 (56.2)	

\*The expression of Periostin was graded as ++ (over 30 % of tumor cells showed strong or diffuse immunopositivity), + (10-30 % of tumor cells showed moderate or patchy immunopositivity) and - (less than 10 % of the tumor cells showed weak or focal immunopositivity or no staining).

**Fig. 1**

**A**

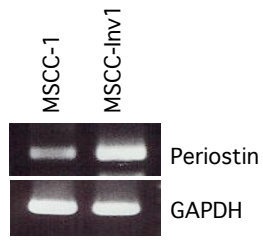
Parent oral cancer cell  
(MSCC-1)



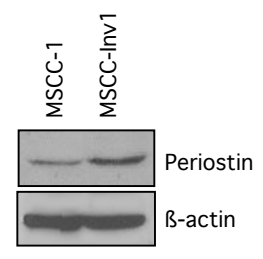
Microarray analysis  
MSCC-1 vs MSCC-Inv1

Probe_Name	Ratio	Description
NM_006475.1	65.175345	<b>OSTEOBLAST SPECIFIC FACTOR 2 (FASCICLIN I-LIKE) (OSF-2/Periostin)</b>
NM_003641.1	18.556444	INTERFERON INDUCED TRANSMEMBRANE PROTEIN 1 (9-27) (IFITM1)
NM_032642.1	18.173555	WINGLESS-TYPE MMTV INTEGRATION SITE FAMILY, MEMBER 5B (WNT5B), TRANSCRIPT VARIANT 1
NM_001387.1	16.965932	DIHYDROPYRIMIDINASE-LIKE 3 (DPYSL3)
AA810805	13.177289	603639245F1 HOMO SAPIENS CDNA, 5' END
NM_005397.1	12.508605	PODOCALYXIN-LIKE (PODXL)
X16323	11.012348	MRNA FOR HEPATOCYTE GROWTH FACTOR (HGF)
NM_007036.2	9.4544153	ENDOTHELIAL CELL-SPECIFIC MOLECULE 1 (ESM1)
NM_002575.1	9.446	SERINE (OR CYSTEINE) PROTEINASE INHIBITOR, CLADE B (OVALBUMIN), MEMBER 2 (SERPINB2)
NM_000055.1	8.7671114	BUTYRYLCHOLINESTERASE (BCHE)
NM_001920.1	8.5095798	DECORIN (DCN)
NM_006528.1	7.0713753	TISSUE FACTOR PATHWAY INHIBITOR 2 (TFPI2)
AK023450	6.958723	CAPILLARY MORPHOGENESIS PROTEIN 2 (CMG2)
NM_007363.2	6.6625375	NON-POU-DOMAIN-CONTAINING, OCTAMER-BINDING (NONO)
NM_032495.1	6.2201358	HYPOTHETICAL PROTEIN SMAP31 (SMAP31)
NM_001257.1	6.0081838	CADHERIN 13, H-CADHERIN (HEART) (CDH13)
BC009269	5.8917143	MRNA FOR PUTATIVE OXIDOREDUCTASE
NM_002192.1	5.5939091	INHIBIN, BETA A (ACTIVIN A, ACTIVIN AB ALPHA POLYPEPTIDE) (INHBA)
NM_000627.1	5.4214464	LATENT TRANSFORMING GROWTH FACTOR BETA BINDING PROTEIN 1 (LTBP1)

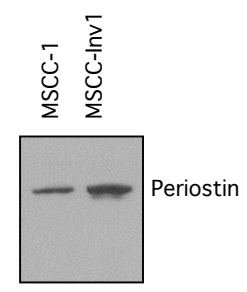
**B**



**C**

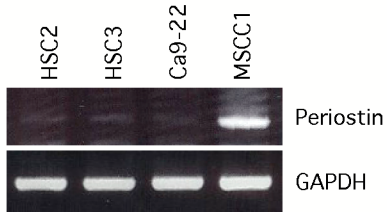


**D**

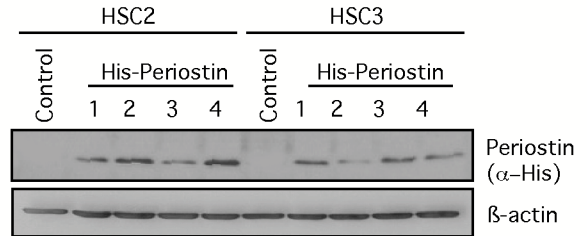


**Fig. 2**

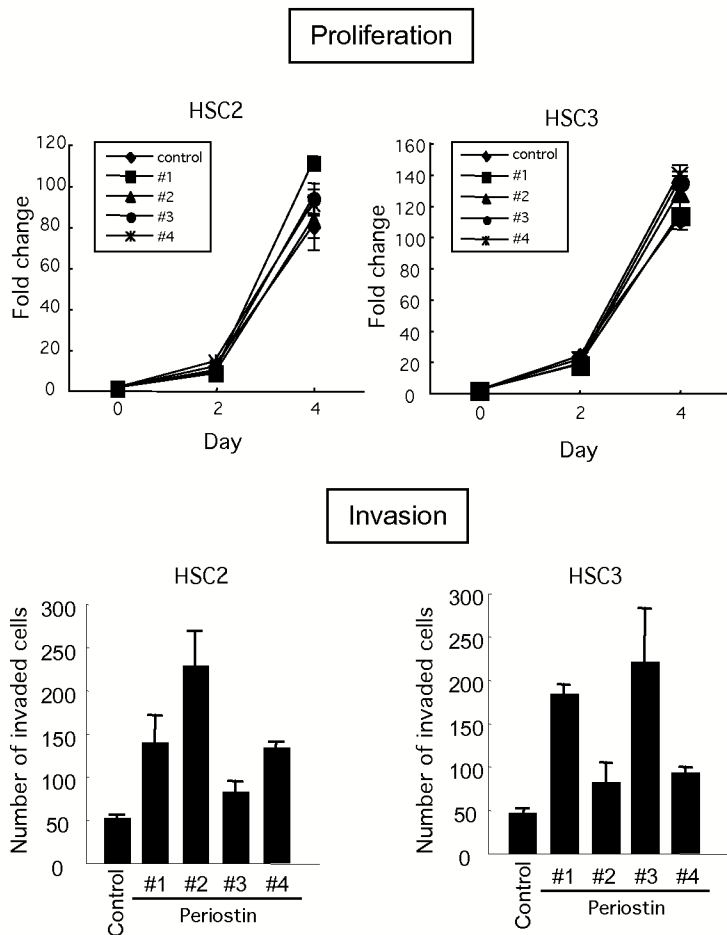
**A**



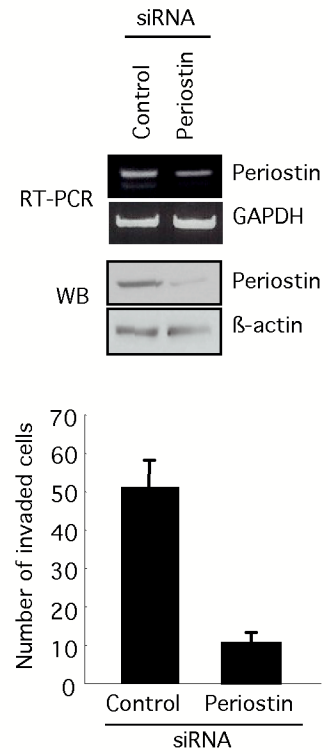
**B**



**C**

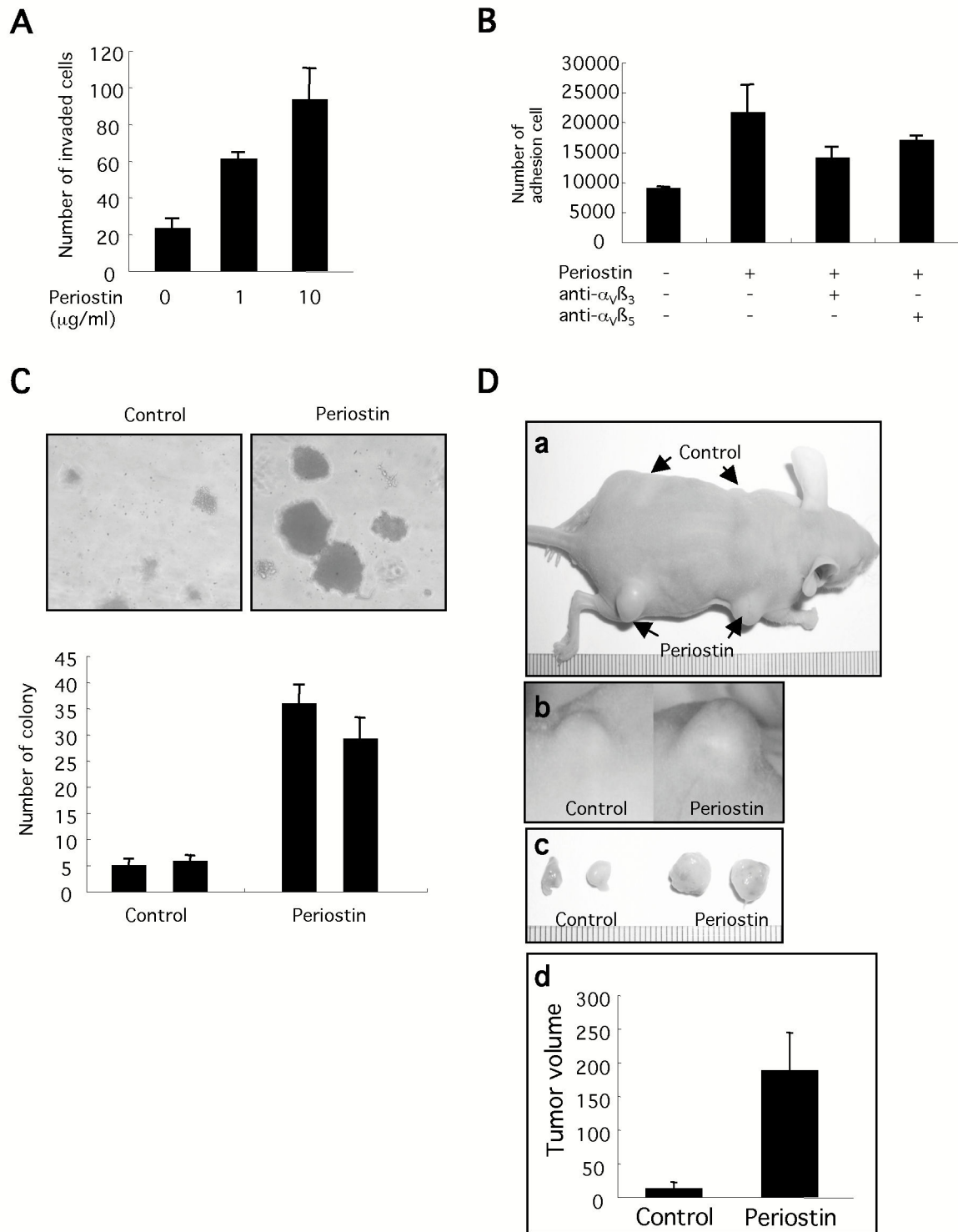


**D**

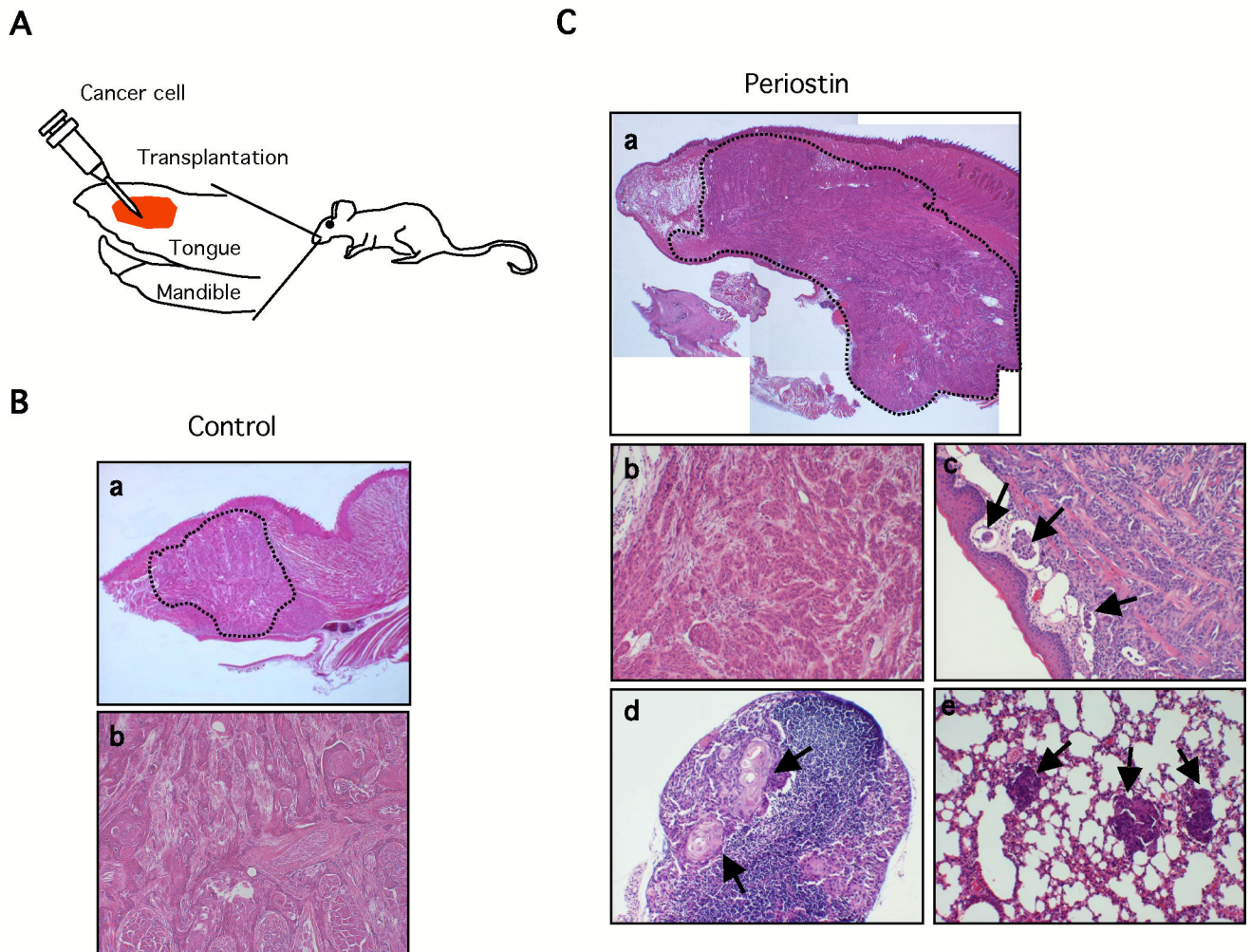




**Fig. 3**



**Fig. 4**



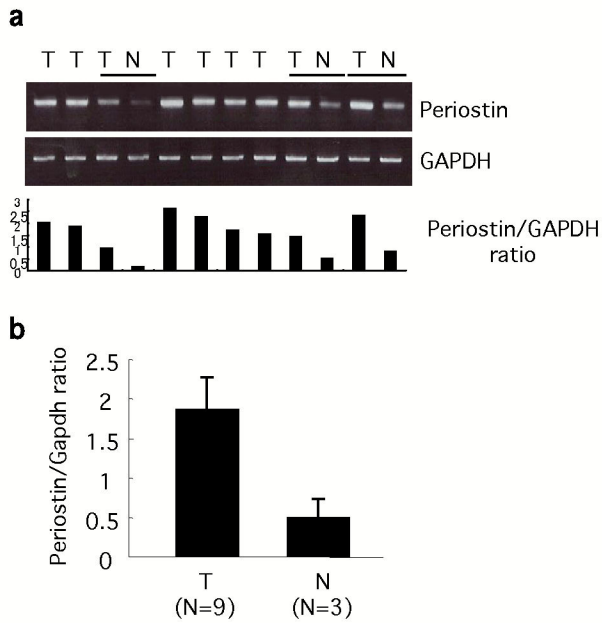
**D**

HSC2 cell	N	Metastasis		Total
		LN	Lung	
Control	10	0	0	0
Periostin	11	3	4	6*

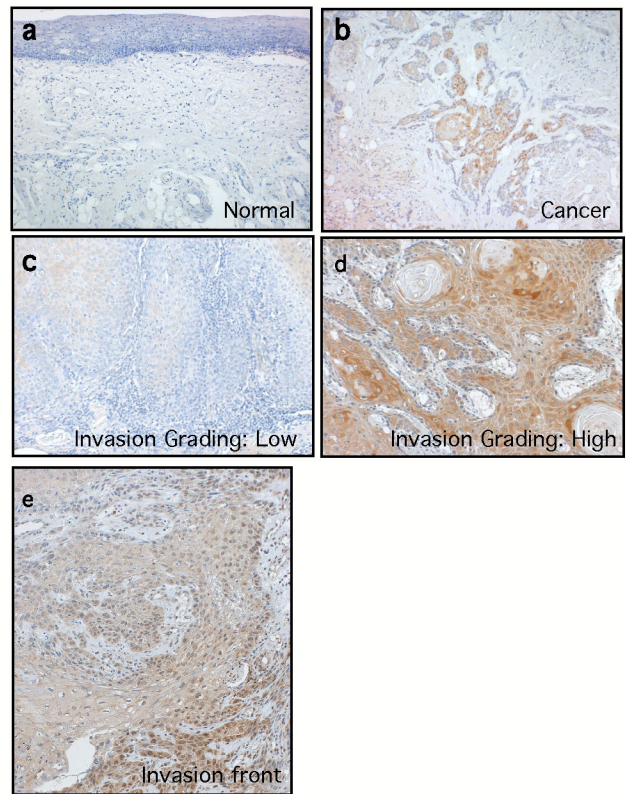
\*One case showed metastatic tumors in both lymph node and lung. N; number, LN; lymph node.

**Fig. 5**

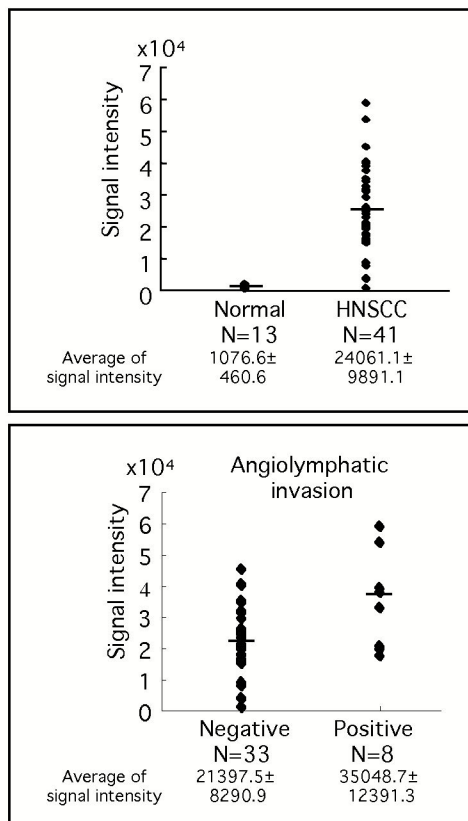
**A**



**B**



**C**



**D**

