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 the 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 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 in the invasion and anchorage-independent growth of HNSCC. (Cancer Res 2006; 66(14): 6928-35)

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common types of human cancer, with an annual incidence of >500,000 cases worldwide (1). HNSCC is associated with severe disease- and treatment-related morbidity and has a 5-year survival rate of ~50%; this rate has not improved in more than two 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 are 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 metastasis depends on the 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 lines 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 showed that periostin was involved in 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 10,458 gene probes, was used to compare the transcription proﬁles between parent cells and highly invasive clones. 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 clones. Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Preparations were quantiﬁed and their purity was determined by standard spectrophotometric 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 according to the manufacturer’s instructions. Preparations were quantiﬁed and their purity was determined by standard spectrophotometric methods. Data were expressed as the average differences between the perfect match and mismatch probes for the periostin gene (Supplemental Data 1).

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1. Ogawa and S. Kitajima contributed equally to this work.

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Periostin as an Invasion-Promoting Factor in HNSCC

Generation of periostin-overexpressing HNSCC cells. A periostin expression plasmid, pcDNA3.1, encoding a hexahistidine-tagged periostin cDNA, was kindly provided by Dr. X-F. Wang (Duke University, Durham, NC). 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 μg/mL; Life Technologies) in the culture medium. Cell transfections were done using FuGENE 6 (Roche, Castle Hill, Australia) according to the manufacturer’s instruction. Four periostin-expressing clones, and one control clone of each cell, were chosen for the subsequent experiments.

In vitro invasion assay. In vitro invasion assay was done as described previously (5). Briefly, invasion was measured by using a 24-well cell culture insert with 8 mm pores (Falcon, Becton Dickinson). The filter was coated with 20 μg 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 × 10^5 cells were resuspended in 100 μL of serum-free medium and placed in the upper compartment of the cell culture insert for 6 to 24 hours. For invasiveness after periostin small interfering RNA (siRNA) treatment, we used 50 μg of EHS extract in these 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 formalin-fixed and stained with H&E. The invasiveness of the cells was determined by counting the penetrating cells onto the lower side of the filter through the pores under a microscope at ×100 magnification. Assays were done thrice, and three fields were randomly selected and 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 siRNA. Stealth siRNA (Oligo ID, HSS16398, Invitrogen) is a 25-bp duplex oligoribonucleotide with a sense strand corresponding to nucleotides 62 to 86 of the reported human periostin mRNA sequence. The sense sequence of HSS16398 is 5'-CCCCAUAACGGCCACAAUCUUU-3'. MSCC-Inv1 cells were transfected with 150 pmol of siRNA in 1 mL of OPTI-MEM according to the manufacturer’s instructions. Following siRNA treatment (48 hours), 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, Cambridge, MA) were incubated overnight at 4°C with 10 μg/mL of periostin protein in the presence of anti-αvβ3 or anti-αvβ5 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 × 10^3 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 done 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. Cells (1 × 10^5) were then plated onto the previously prepared underlayers. The cells were kept wet by adding a small amount of RPMI 1640 with 10% FBS. The cells were incubated at 37°C in a humidified 5% CO2 atmosphere for 3 weeks. Afterwards, colonies were photographed and counted.

Xenograft assays in nude mice. To examine whether periostin expression in HNSCC cells affects anchorage-independent growth in vivo, periostin-overexpressing HSC2 cells (1 × 10^5 in 500 μL of HBSS) 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. Experimental protocols were approved by the Committee of Research. Cells were kept wet by adding a small amount of RPMI 1640 with 10% FBS. The cells were incubated at 37°C in a humidified 5% CO2 atmosphere for 3 weeks. Afterwards, colonies were photographed and counted.

Orthotopic implantation. Periostin-overexpressing and control HSC2 cells (5 × 10^5 in 50 μL of HBSS) were injected into the tongue of male athymic (nude) mice. The animals were monitored for tumor formation every 3 days 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-μm-thick sections and stained with H&E. 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. Buffered formalin-fixed and paraffin-embedded tissues (10%) 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 nonneoplastic tissues for reverse transcription-PCR (RT-PCR) analysis.

Immunohistochemical staining. Immunohistochemical detection of periostin in HNSCC cases was done on 4.5 μm sections mounted on silicion-coated glass slides, using a streptavidin-biotin peroxidase technique as described previously (11). The expression of periostin was graded as ++ (>30% of tumor cells showed strong or diffuse immunopositivity), + (10-30% of tumor cells showed moderate or patchy immunopositivity), and − (<10% of the tumor cells showed weak or focal immunopositivity or no staining). Three pathologists (Y. Kudo, I. Ogawa, and T. Takata) made all the assessments. Possible correlations between variables of the analyzed tumor samples were tested for association by the Fisher’s exact test. P < 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 (5). In the present study, we compared the transcriptional profile of parent

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 cells by RT-PCR was examined. Amplification was done 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 four stable clones each expressing periostin. Ectopic expression of periostin was examined by immunoblotting with anti-His antibody. The whole lysates from all samples were blotted with β-actin for loading control. C, cell proliferation (top) and invasion (bottom) 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 days. The invasiveness of the cells was determined by in vitro invasion assay. D, knockdown of periostin in MSCC-Inv1 cells. Periostin siRNAs were transfected. Top, the effectiveness of the expression of periostin at knockdown was validated at the mRNA level by RT-PCR, and at the protein level by immunoblotting with anti-periostin antibody. Bottom, knockdown of periostin inhibits the invasiveness of HNSCC cells. The invasiveness of the cells was determined by in vitro invasion assay.
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; Supplemental Data 1). Among these genes, the most overexpressed 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 in the medium compared 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 show the involvement of periostin in the invasion of HNSCC, we generated the periostin-overexpressing cells. At first, we examined the periostin mRNA in five HNSCC cell lines. Among the five 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). Although periostin overexpression did not promote cell proliferation, it dramatically enhanced the invasiveness of both HSC2 and HSC3 cells (Fig. 2C). Invasiveness was well correlated with ectopic expression levels of periostin (Fig. 2C). To confirm the correlation between periostin and invasion, we used clone no. 2 of HSC2 cells, which showed higher expression of periostin by siRNA treatment in MSCC-Inv1 cells (Fig. 2D). Periostin siRNA treatment remarkably inhibited the invasion. Overall, these results indicate that periostin plays an important role in the 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 fascinulin 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 β3 integrin, and two membrane proteins, FEEL-1 and FEEL-2. FAS1 of β3 integrin bears motifs interacting with integrins α3β1 and αvβ5 (14, 15), and mediates endothelial cell adhesion and migration via integrin αvβ3 (16). Similarly to β3 integrin, recombinant periostin supports the adhesion of ovarian epithelial cells that could be inhibited by monoclonal antibodies against αvβ3 or αvβ5 integrin, but not by anti-β3 integrin antibody (17). We also found that treatment of specific anti-αvβ3 and anti-αvβ5 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 the cell adhesion of HNSCC cells (Fig. 3B).

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 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 clone no. 2 of HSC2 cells, which showed higher invasiveness. The periostin-overexpressing cells formed considerably larger colonies in soft agar in comparison with control cells (Fig. 3C). In addition, the number of colonies with periostin-overexpressing cells was higher than those with control cells (Fig. 3C). To determine anchorage-independent growth in vivo,
periostin-overexpressing HNSCC cells were injected s.c. into nude mice. After 28 days, the growth characteristics of the resulting tumors were analyzed. Interestingly, transplantation of the periostin-overexpressing cells showed comparatively larger tumor volumes than that of the control cells (Fig. 3D, a-c). The volume of tumors derived from periostin-overexpressing cells was ~16-fold higher than that of tumors from control cells (Fig. 3D, d). Tumor volume was $187 \pm 57.1$ and $11.4 \pm 10.4 \text{mm}^3$ in the periostin overexpressed and control cells, respectively.

**Periostin-overexpressing cells frequently metastasize to lymph nodes and lung through their aggressive invasiveness.**

As shown in the 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. 4B, a and C, a). Strikingly, in mice implanted with periostin-overexpressing cells, the tumors were larger than in mice implanted with control cells. Histologically, periostin-overexpressing tumors showed a poorly differentiated phenotype characterized by a diffuse and trabecular growth pattern without keratinization. In contrast, control tumors showed characteristics consistent with a well-differentiated phenotype with tumor islands with keratin pearl formation (Fig. 4B, b and C, b).

Moreover, periostin-overexpressing tumors showed remarkable invasiveness, including destruction of mandibular bone and lymphocytic infiltration (Fig. 4C, a and c). Interestingly, the periostin-overexpressing cells spontaneously metastasized to cervical lymph nodes (Fig. 4C, d) and lung (Fig. 4C, e). 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 is associated with the invasiveness of HNSCC.**

To determine if the up-regulation of periostin is a common feature of HNSCC in human subjects, we did RT-PCR analysis on three normal tissues and nine HNSCC samples. As shown in Fig. 5A, a, the expression levels of periostin mRNA in cancer tissues was higher than in normal tissues. The average of periostin expression levels (periostin/GAPDH signal intensity ratio) was 3-fold higher in tumors than in normal tissues (Fig. 5A, b).

Next, we examined the expression of periostin in 12 normal oral mucosae and 102 HNSCC cases by immunohistochemistry. HNSCC cells showed high expression of periostin in comparison with normal oral mucosa (Fig. 5B, a and b; Table 1). In 102 HNSCC cases, 43 (42.2%) cases showed high expression of periostin. Then, we investigated the relationship between periostin expression and invasiveness in 102 HNSCC cases. For evaluation of invasiveness of HNSCC, we used the grading of mode of invasion, grades 1 to 4 as first described by Jacobsson et al. (21). We defined two groups, low

**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 the tongue of nude mice. After 2 weeks, tongue tumors, cervical lymph nodes, and lungs were dissected. B, representative H&E-stained images of histopathologic sections from mice injected with control cells: (a) histology of tumor mass in the tongue (original magnification, $\times 5$); (b) high-power magnification of (a). Tumor mass was enclosed with dotted line (original magnification, $\times 25$). C, representative H&E-stained images of histopathologic sections from mice injected with periostin-overexpressing cells: (a) histology of tumor mass in the tongue (original magnification, $\times 5$); (b) high-power magnification of (a); (c) histology of invading tumor in lymphatics; (d) histology of lymph node metastasis; (e) histology of lung metastasis (original magnification, $\times 25$). Arrow, invaded tumor (c) and metastasized tumor (d and e). D, summary of the metastasis of periostin-overexpressing and control cell–injected mice.
(grades 1 and 2) and high (grades 3 and 4). Interestingly, higher expression of peristin was significantly associated with the grading of mode of invasion ($P < 0.05$; Fig. 5B, c and d; Table 1). In particular, cancer cells at the invasive front expressed peristin at higher levels (Fig. 5B, e). We also examined the association between peristin expression and metastasis in 62 HNSCC cases with available clinical information. HNSCC cases with metastasis (56.2%) showed high expression of peristin (Table 1).

To further evaluate the expression of peristin in patients with HNSCC, we compared the expression in a previously published microarray data set of 41 HNSCC patients and 13 normal controls (9). Similar to our data, peristin was expressed at higher levels in HNSCC tissues, in comparison with normal oral mucosal tissues (Fig. 5C). Moreover, HNSCC cases with angiolymphatic invasion showed higher expression of peristin (Fig. 5C). To further explore the genes that are coordinately expressed with peristin in HNSCC tumors, we did 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 peristin in normal oral mucosal tissue and HNSCC tissues was then visualized by hierarchical clustering (Fig. 5D). FAR, SULF1, COL5A2, COL3A1, COL10A1, COL1A1, FN1, and INHBA were well correlated with peristin expression (Fig. 5D; Supplemental Data 2).

**Discussion**

It is believed that neoplasms contain a variety of subpopulations of cells with differing metastatic potentials, and the presence of highly metastatic clones may exist within a primary tumor (4). Recent data shows that re-injection of metastatic cell populations could lead to enrichment in the metastatic phenotype by work on mice model of experimental metastasis using cancer cell lines (22–25). Furthermore, 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 lines from metastatic cervical lymph nodes 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 showed that the 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, using a previous *in vitro* invasion assay method, we could obtain the highly invasive phenotype by isolation of cell populations. 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

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**Figure 5.** Higher expression of peristin is frequently observed in HNSCC. **A.** (a) expression of peristin mRNA in nine HNSCC tissues (T) and three normal tissues (N) by RT-PCR. Graph shows the peristin/GAPDH ratios which was measured by densitometry; (b) the average of peristin/GAPDH ratios in HNSCC cases (T) and in normal tissues (N) is 1.86 ± 0.40 and 0.51 ± 0.22, respectively. **B.** Immunohistochemical analysis of peristin. Expression of peristin was examined by immunohistochemistry in HNSCC (Cancer; a) and normal oral mucosa (Normal; b). To know the correlation between peristin expression and invasiveness of HNSCC, we evaluated the invasiveness of HNSCC by using invasion grading, as low (grades 1 and 2) and high (grades 3 and 4). Representative cases of low (c) and high (d) groups are shown. Representative case of higher expression of peristin in invasive front (e). **C.** Clustering of HNSCC samples to show relative expression of peristin. 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 signal intensity of peristin in 41 HNSCC and 13 normal tissues was labeled and hybridized to Affymetrix U133A Gene Chips as previously reported (9). The average signal intensity of peristin in normal and HNSCC tissues. **D.** Clustering of 41 HNSCC and 13 normal tissues to show relative expression of peristin. Using hierarchical clustering, we analyzed a filtered set of 6,800 variably expressed genes. The gene cluster that includes peristin is highlighted in blue in the horizontal dendrogram.
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, the 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 instead promoted soft agar and in vivo growth as tumor xenografts, demonstrating that periostin enhances the anchorage-independent growth of HNSCC cells.

Periostin contains an NH2-terminal secretory signal peptide, followed by a cysteine-rich domain, four internal homologous repeats, and a COOH-terminal hydrophilic domain. The four internal repeat regions of periostin share a homology with the axon guidance protein FAS1-containing sequences that allows binding of integrins and glycosaminoglycans in vivo (26). FAS1 domains of β3/β5, which share a significant structural homology with periostin, bear motifs interacting with integrins, α3/α5 and αv/α5 (14, 15), and mediate endothelial cell adhesion and migration via integrin αvβ3 (16). Similarly to β3/β5, we found that interference with the function of integrins by specific anti-αvβ3 and anti-αvβ5 integrin antibodies had an effect on the ability of periostin to mediate cell adhesion in HNSCC cells. Taken together, these data strongly suggest that the FAS1 domain of periostin binds to integrins. It is well known that integrin mediates cell–extracellular matrix interaction and that integrin-mediated adhesion regulates a variety of intracellular events (27). Therefore, we hypothesize that periostin-integrin interaction may inhibit the extracellular matrix–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 a recent report that periostin activated the Akt/PKB pathway via the αvβ3 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 important for cancer metastasis. Interestingly, periostin overexpression dramatically induced metastasis to the lymph nodes and to the lung in an orthotopic implant model of HNSCC, demonstrating spontaneous metastasis from the tongue. This orthotopic implant model of HNSCC seems clinically relevant because its tumor progression–containing metastasis mimics the clinical scenario. Moreover, periostin-overexpressing tumors became apparent, 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 in HNSCC cases. Moreover, 56.2% of HNSCC cases with metastasis showed higher expression of periostin, but we could not find any statistical significance between periostin expression and metastasis. In the near future, we will examine the correlation between periostin expression and metastasis in a large number of HNSCC cases. Interestingly, Bao et al. also showed 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 compared 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 yet to confirm this correlation, this finding gives us the impression that periostin expression seems 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

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

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<th>No. of cases</th>
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</tr>
<tr>
<td>High</td>
<td>80</td>
<td>11 (13.75)</td>
</tr>
<tr>
<td>Metastasis</td>
<td>30</td>
<td>5 (16.7)</td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>3 (9.4)</td>
</tr>
</tbody>
</table>

*The expression of periostin was graded as ++ (>$30\%$ of tumor cells showed strong or diffuse immunopositivity), + (10–30\% of tumor cells showed moderate or patchy immunopositivity), and − (<10\% of the tumor cells showed weak or focal immunopositivity or no staining).
Periostin as an Invasion-Promoting Factor in HNSCC

Mao L, Hong WK, Papadimitrakopoulou VA. Focus on metastatic potential, as shown in this study, raises the possibility that it could be used as a molecular target in the antimetastatic therapy of patients with HNSCC.

Acknowledgments

References

5. Kudo Y, Kitajima S, Ogawa I, et al. Invasion and anchorage-independent growth in the metastatic process. our studies have revealed a critical role of periostin for invasion and blood from patients with HNSCC in the future. In conclusion, Therefore, we will examine the detection of periostin in saliva fact, serum levels of periostin were elevated in patients with breast cancer (17). Taken together, increased expression of periostin may 2. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. Cancer Cell 2004;5:311–6.

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