Abstract: Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor whose expression level is positively correlated with tumor aggressiveness and metastatic potential. However, the mechanism underlying SLPI-induced enhancement of malignant phenotype is not completely understood. The malignancy of cancer cells is highly dependent on cell migration activity. Our previous study revealed that gingival carcinoma Ca9-22 cells, but not colorectal adenocarcinoma HT-29 cells, expressed SLPI. Therefore, we investigated the migration activity of these two cell types to understand the nature of SLPI-mediated tumor aggressiveness and metastatic potential. In vitro wound healing assay indicated that HT-29 cells and SLPI-deleted Ca9-22 cells showed lower migration activity than wild-type Ca9-22 cells, suggesting that SLPI-induced cell migration plays an important role in tumor aggressiveness and metastatic potential. In addition, our gene expression profiling study based on microarray data for the three cell types identified a number of candidates, including LCP1 and GLI, that could be key molecules in the mechanism of SLPI-induced cell migration.

Keywords: HT-29; Ca9-22; SLPI; migration; gene expression profiling.

Introduction
Secretory leukocyte protease inhibitor (SLPI), a serine protease inhibitor, plays an important role in the protection of the skin and mucosa (1,2). In normal tissues, SLPI is found in large quantities in fluids lining the mucosal surfaces, including those of the respiratory tract, inhibiting bacterial infection and inflammation, and promoting wound healing and epithelial proliferation independent of its anti-protease activity (3-6). Although a previous report has suggested that DNase I hypersensitivity sites in the 5’ flanking region of SLPI are responsible
for its transcription (3), little is known about how SLPI expression is controlled. SLPI expression is increased in various human malignancies, including breast, lung, and ovarian cancers, colorectal carcinoma, and glioblastoma (7-9). Furthermore, several studies have shown that the level of SLPI expression is positively correlated with tumor aggressiveness and metastatic potential (10,11). Devoogdt et al. (10) reported that SLPI was expressed more strongly in highly malignant Lewis lung carcinoma (LLC) cells than in less malignant LLC cells, and that forced expression of exogenous SLPI in LLC cells increased both their tumorigenicity and capacity for lung metastasis. The mechanism underlying SLPI-induced enhancement of malignant phenotype is still not completely understood. The malignancy of cancer cells is highly dependent on their migration activity (12,13). Therefore, assessment of the role played by SLPI in the migration activity of cancer cells is important for understanding the mechanisms underlying their malignancy. One approach to this is microarray-based gene expression profiling.

HT-29 is a human colorectal adenocarcinoma cell line (14), and Ca9-22 is a cell line derived from carcinoma of the gingiva (15). These cells exhibit an epithelial morphology and have been used as in vitro models of cancer cells in numerous cancer research projects (16,17). Furthermore, our previous study showed that Ca9-22 cells, but not HT-29 cells, expressed SLPI under standard culture conditions (18). Therefore, we performed microarray-based gene expression profiling of HT-29 and Ca9-22 cells for high-throughput analysis and for screening of differentially expressed genes (DEGs). In addition, we performed the same analysis using SLPI-deleted (ΔSLPI) Ca9-22 cells (18). Our study aimed to identify candidate molecules that might be important mediators of SLPI-induced cell migration by comparing the gene expression profiles of these cells.

**Materials and Methods**

**Cells and reagents**

HT-29 and Ca9-22 cells were purchased from RIKEN BRC (Tsukuba, Japan). The cells were cultured in minimal essential medium (Wako, Tokyo, Japan) supplemented with 1% penicillin-streptomycin (Wako) and 10% fetal bovine serum (Japan Bio Serum, Tokyo, Japan) at 37°C under 5% CO₂. ΔSLPI Ca9-22 cells were generated as described previously (18). Briefly, the fragment containing SLPI was amplified by PCR using genomic DNA from wild-type Ca9-22 (wtCa9-22) cells. For the SLPI-disruption construct, the neomycin resistance gene under control of the TK promoter was inserted between Ndel sites in the SLPI fragment. In total, 1 × 10⁷ Ca9-22 cells were transfected with the plasmid containing the SLPI-disruption construct. After transfection, the cells were selected on a medium containing G418 (Sigma-Aldrich, St. Louis, MO, USA).

**In vitro wound healing assay**

The cells were seeded in 3-cm culture dishes (cell density, 5 × 10⁴ cells/dish) and grown to confluence. Cell monolayers were scratched with a 2-mm-wide silicone tip of CELL Scratcher (AGC Techno Glass, Shizuoka, Japan) in the center of the dishes, and the cells were washed once with the culture medium to remove detached cells. After incubation for 0 or 24 h, the cells were fixed with 4% paraformaldehyde for 15 min and then stained with 0.05% toluidine blue. Stained cells were imaged using a GTX-890 scanner (Epson, Nagano, Japan).

**Cell counting assay**

The cells were seeded in 10-cm culture dishes (cell density, 1 × 10⁵ cells/dish) and cultured for 2, 4, 6, 8, 10, and 12 days in the growth medium as described above. The cells were then trypsinized and resuspended, and counted using a hemocytometer under a microscope.

**Analysis of mRNA expression**

Analysis of mRNA expression was performed as described previously (19). Briefly, total RNA was isolated using RNAiso Plus (Takara Bio, Shiga, Japan), in accordance with the manufacturer’s instructions. First-strand cDNA was synthesized from 1.5 μg of total RNA using a SuperScript III RNase H reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA obtained was amplified by performing real-time RT-PCR using a SYBR Premix Ex Taq II (Takara Bio) in CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). Bioinformatic analysis using microarray data showed that, among the migration-associated genes, expression of mRNA for the lymphocyte cytosolic protein 1 gene (LCP1) and NADPH oxidase 1 gene (NOX1) was markedly up- and down-regulated in HT-29 and ΔSLPI Ca9-22 cells, respectively, to wtCa9-22 cells. The expression levels of these genes were confirmed by real-time RT-PCR. The levels of mRNA were normalized to those of β-actin (ACTB). Primers used for PCR were: SLPI (5'-GAG ATG TTG TTC TCC CAC TTA GT -3' and 5'-AGG CTT CCT CCT TGT TGG GT -3'), LCP1 (5'-GAT CAG TGT CCG ATG AGG AAA TG -3' and 5'-CCA GAT CAT CAC TAG CCA TC -3'), NOX1 (5'-CTG CTT CCT GTG TGT CCG A -3' and 5'-AGG CAG ATC ATA TAG GCC AC -3'), and ACTB (5'-CTT TCT
ACATGAGCTGCTG-3′, and 5′-ATGGCTGGGTGTTAAGG-3′).

Western blotting

The cells were lysed using a sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol). Next, samples containing 10 μg protein were separated by SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies against SLPI (dilution, 1:500; Dako Japan, Tokyo, Japan) and β-actin (dilution, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were incubated with HRP-conjugated secondary antibodies (dilution, 1:10,000; Cell Signaling Technology, Beverly, MA, USA) for 45 min. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA).

Gene expression profiling

Gene expression profiling was performed using microarray data for HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells. Total RNA was extracted from each cell type using a NucleoSpin RNA kit (Takara Bio). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and 50 ng of total RNA from each sample was subjected to microarray analysis using the SurePrint G3 Human 8×60K Version 1.0 Array (Agilent Technologies) in accordance with the manufacturer’s instructions. Raw data are available in the NCBI GEO database (accession no.: GSE83104). Microarray data were analyzed using GeneSpring GX software (Tomy Digital Biology, Tokyo, Japan). All data obtained from the same chip were normalized to the 75th percentile of all values on that chip. DEGs were screened using the formula |2-fold change| > 1 (2-fold change = log2 [normalized intensity of sample/normalized intensity of control]). A Web Gene Ontology Annotation Plot (WEGO) was used for plotting the gene ontology (GO) annotation results for DEGs. KEGG pathway analysis based on hypergeometric testing for DEGs was performed using the EMA package.

Statistical analysis

Quantifiable results are presented as mean ± standard deviation (SD) of triplicate cultures. The significance of differences between samples was assessed using Student’s t-test. Differences at $P < 0.05$ were considered statistically significant.

Results

SLPI expression in HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells

Because our previous study had shown that SLPI was expressed in Ca9-22 cells but not in HT-29 cells, we verified this in the present study by performing real-time RT-PCR and Western blotting with anti-SLPI antibody. We found that wtCa9-22 cells had marked expression of SLPI mRNA and protein (Fig. 1A, B), whereas expression of SLPI mRNA in HT-29 cells was much lower than that in wtCa9-22 cells. Moreover, expression of SLPI protein was negligible in HT-29 cells (Fig. 1A, B).
ΔSLPI Ca9-22 cells were generated by replacing SLPI with the neomycin resistance gene (18), then examined by real-time RT-PCR and Western blotting to confirm the inhibition of SLPI expression. The results confirmed that SLPI expression was completely inhibited at both the mRNA and protein levels in the ΔSLPI Ca9-22 cells (Fig. 1A, B).

### Table 1  Top 10 upregulated and downregulated genes in ΔSLPI Ca9-22 compared to wtCa9-22 cells

| Gene symbol | Description | Fold change |
|-------------|-------------|-------------|
| SPINK1      | serine peptidase inhibitor, Kazal type 1 | 11607.1     |
| S100P       | S100 calcium binding protein P | 1965.0      |
| EMC10       | ER membrane protein complex subunit 10 | 1577.9      |
| TESC        | tescalcin | 1464.7      |
| ABCC3       | ATP-binding cassette, sub-family C member 3 | 1274.3      |
| NPDC1       | neural proliferation, differentiation and control, 1 | 865.9      |
| CLDN3       | claudin 3 | 836.5       |
| HSD17B2     | hydroxysteroid dehydrogenase 2 | 819.6       |
| HTATIP2     | HIV-1 Tat interactive protein 2 | 780.0       |
| FBP1        | fructose-1,6-bisphosphatase 1 | 777.6      |

| Gene symbol | Description | Fold change |
|-------------|-------------|-------------|
| ANXA8L1     | annexin A8-like 1 | 0.00006     |
| MIR205HG    | MIR205 host gene | 0.00016     |
| ACKR3       | atypical chemokine receptor 3 | 0.00020     |
| BASP1       | brain abundant, membrane attached signal protein 1 | 0.00027     |
| HIST1H1A    | histone cluster 1 | 0.00069     |
| CA1         | caveolin 1 | 0.00103     |
| EFEMP1      | EGF containing fibulin-like extracellular matrix protein 1 | 0.00126     |
| ADIRF       | adipogenesis regulatory factor | 0.00143     |
| BMP7        | bone morphogenetic protein 7 | 0.00163     |
| PTPRU       | protein tyrosine phosphatase, receptor type, U | 0.00167     |

### Table 2  Top 10 upregulated and downregulated genes in HT-29 cells compared to wtCa9-22 cells

| Gene symbol | Description | Fold change |
|-------------|-------------|-------------|
| SPINK1      | serine peptidase inhibitor, Kazal type 1 | 10826.0     |
| S100P       | S100 calcium binding protein P | 2143.2      |
| PHGR1       | proline/histidine/glycine-rich 1 | 1485.8      |
| TESC        | tescalcin | 1130.1      |
| ABCC3       | ATP-binding cassette, sub-family C member 3 | 1049.0      |
| EMC10       | ER membrane protein complex subunit 10 | 1025.7      |
| HSD17B2     | hydroxysteroid (17-beta) dehydrogenase 2 | 912.5       |
| NPDC1       | neural proliferation, differentiation and control 1 | 897.4       |
| CLDN3       | claudin 3 | 830.6       |
| HPDL        | 4-hydroxyphenylpyruvate dioxygenase-like | 667.0       |

| Gene symbol | Description | Fold change |
|-------------|-------------|-------------|
| MIR205HG    | MIR205 host gene | 0.00019     |
| ANXA8L1     | annexin A8-like 1 | 0.00029     |
| ADIRF       | adipogenesis regulatory factor | 0.00047     |
| BASP1       | brain abundant, membrane attached signal protein 1 | 0.00054     |
| KRT6C       | keratin 6C | 0.00071     |
| WDR66       | WD repeat domain 66 | 0.00125     |
| COL5A1      | collagen, type V, alpha 1 | 0.00138     |
| LMO1        | LIM domain only 1 | 0.00140     |
| EFS         | embryonal Fyn-associated substrate | 0.00143     |
| CALD1       | caldesmon 1 | 0.00171     |
Migration and proliferation of HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells

We next examined the migration of HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells using the in vitro wound healing assay. The results indicated that HT-29 and ΔSLPI Ca9-22 cells had lower migration from the border of the original scratch zone than wtCa9-22 cells in 24-h cultures (Fig. 1C). No marked difference in migration level was observed between HT-29 and ΔSLPI Ca9-22 cells. We then examined the proliferation of HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells (Fig. 1D). For this, the cells were cultured for the indicated number of days, and then counted at each time point. The three cell types proliferated rapidly from days 6 to 10. However, the proliferation of wtCa9-22 cells was slightly higher than that of HT-29 and ΔSLPI Ca9-22 cells on day 10 (Fig. 1D).

Summary of DEG analyses based on microarray data

Gene expression profiling was performed using microarray data for HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells. Approximately 36,000 transcriptomes (63,000 probes) were investigated. The increase or decrease in the expression of genes in HT-29 and ΔSLPI Ca9-22 cells was compared with that in wtCa9-22 cells. The number of upregulated and downregulated genes in HT-29 cells was 4,344 (>2 fold) and 4,625 (<0.5 fold), respectively, and that in ΔSLPI Ca9-22 cells was 4,396 (>2 fold) and 4,571 (<0.5 fold), respectively (Fig. 2A). In all, 2,917 and 3,788 genes were commonly upregulated and downregulated, respectively, in both HT-29 and ΔSLPI Ca9-22 cells. The top 10 genes with increased or decreased mRNA expression in ΔSLPI Ca9-22 and HT-29 cells are listed in Tables 1 and 2, showing that 60% of them were common to both cell lines. Scatter plot analysis showed that the difference in gene expression status between HT-29 and ΔSLPI Ca9-22 cells was much lower than that between these two cell types and wtCa9-22 cells (Fig. 2B).

Gene expression profiling: GO and KEGG pathway analyses

GO analysis was performed for the genes that were commonly upregulated and downregulated in HT-29 and ΔSLPI Ca9-22 cells relative to those in wtCa9-22 cells. Among them, 1,941 upregulated and 2,399 downregulated genes were assigned to 49 and 50 GO terms, respectively (Fig. 3A). The dominant terms for the upregulated genes were “cell”, “cell part”, “cellular process”, “binding”, “metabolic process”, “response to stimulus”, “biological regulation”, “cell movement”, “negative regulation of cellular process”, “membrane”, “membrane part”, “cytoplasm”, “plasma membrane”, “extracellular”, “extracellular region”, “extracellular matrix”, “extracellular space”, “plasma membrane part”, and “extracellular region part”. The dominant terms for the downregulated genes were “cell”, “cell part”, “cellular process”, “biological regulation”, “cell movement”, “positive regulation of cellular process”, “membrane”, “membrane part”, “cytoplasm”, “plasma membrane”, “extracellular”, “extracellular region”, “extracellular matrix”, “extracellular space”, “plasma membrane part”, and “extracellular region part”. The scatter plot analysis showed that the difference in gene expression status between HT-29 and ΔSLPI Ca9-22 cells was much lower than that between these two cell types and wtCa9-22 cells (Fig. 2B).

Fig. 2 Summary of DEG analyses based on microarray data. (A) The numbers of DEGs in HT-29 and ΔSLPI Ca9-22 cells compared with those in wtCa9-22 cells. The numbers of DEGs were counted based on microarray data. (B) Scatter plot analysis showing the expression levels of DEGs against the background of all transcripts for the indicated cell types on the x-axis and y-axis.

A

B

Gene expression profiling was performed using microarray data for HT-29, wtCa9-22, and ΔSLPI Ca9-22 cells. Approximately 36,000 transcriptomes (63,000 probes) were investigated. The increase or decrease in the expression of genes in HT-29 and ΔSLPI Ca9-22 cells was compared with that in wtCa9-22 cells. The number of upregulated and downregulated genes in HT-29 cells was 4,344 (>2 fold) and 4,625 (<0.5 fold), respectively, and that in ΔSLPI Ca9-22 cells was 4,396 (>2 fold) and 4,571 (<0.5 fold), respectively (Fig. 2A). In all, 2,917 and 3,788 genes were commonly upregulated and downregulated, respectively, in both HT-29 and ΔSLPI Ca9-22 cells. The top 10 genes with increased or decreased mRNA expression in ΔSLPI Ca9-22 and HT-29 cells are listed in Tables 1 and 2, showing that 60% of them were common to both cell lines. Scatter plot analysis showed that the difference in gene expression status between HT-29 and ΔSLPI Ca9-22 cells was much lower than that between these two cell types and wtCa9-22 cells (Fig. 2B).

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and “organelle” (Fig. 3A). The top five dominant terms for the downregulated genes were similar to those for the upregulated genes (Fig. 3A).

As the principal aim of this study was to identify candidate molecules that might play an important role in SLPI-induced cell migration, we focused on the GO
Our results showed that SLPI was weakly expressed in ht-29 cells relative to its level in wtCa9-22 cells, and that the migration and proliferation of HT-29 and ΔSLPI Ca9-22 cells were less marked than those of wtCa9-22 cells. These results suggest that SLPI regulates the proliferation and migration of HT-29 and wtCa9-22 cells. Both cell proliferation and migration are closely associated with tumor aggressiveness and metastatic potential (12,13). SLPI is highly expressed in various cancers, including inflammatory breast cancer, which has high angiogenic and metastatic capacities (11,21,22). Furthermore, forced expression of exogenous SLPI in lung carcinoma cells has been reported to markedly increase their tumorigenicity and capacity for lung metastasis after subcutaneous inoculation (10). These findings suggest that SLPI promotes tumor aggressiveness and metastatic potential in part by regulating cell proliferation and migration. However, in the present study, a small difference in proliferation was observed between wtCa9-22 cells and the other two cell types. In contrast, the migration capacity of wtCa9-22 cells was completely different from that of the other two cell types. These results suggest that SLPI promotes tumor aggressiveness and metastatic potential by regulating the migration of cancer cells, rather than their proliferation. Furthermore, our results suggest that the molecular mechanism underlying SLPI-induced cell migration differs from that of cell proliferation.

We next examined DEGs that were common to both HT-29 and ΔSLPI Ca9-22 cells relative to those in wtCa9-22 cells. The color chart showing the expression levels of genes associated with “cell migration” indicated that the gene expression pattern in HT-29 and ΔSLPI Ca9-22 cells was different from that in wtCa9-22 cells (Fig. 3B). Particularly, expression levels of LCP1 mRNA were much lower in both HT-29 and ΔSLPI Ca9-22 cells than in wtCa9-22 cells (Fig. 3B). In contrast, the levels of NOX1 mRNA were much higher in both HT-29 and ΔSLPI Ca9-22 cells than in wtCa9-22 cells (Fig. 3B).

We next compared the gene expression profile of wtCa9-22 cells with those of the other two cell lines to identify candidate genes that might play an important role in SLPI-induced cell migration. In all, 4,344 and 4,396 genes were upregulated in HT-29 and ΔSLPI Ca9-22 cells, respectively, relative to those in wtCa9-22 cells. Of these genes, 2,917 were common to both HT-29 and ΔSLPI Ca9-22 cells. Similarly, many downregulated genes (approximately 80% in each cell line) were common to both cell lines. Scatter plot analysis showed that the gene expression patterns of HT-29 and ΔSLPI Ca9-22 cells were more similar than those of wtCa9-22 cells. These results suggest that the lower migration capacity of HT-29 and ΔSLPI Ca9-22 cells relative to that of wtCa9-22 cells was promoted by the same molecular mechanism. Therefore, we analyzed the DEGs that were common to both HT-29 and ΔSLPI Ca9-22 cells by performing GO and KEGG pathway analyses.

GO analysis showed that the expression pattern of genes associated with “cell migration” was more similar between HT-29 and ΔSLPI Ca9-22 cells than between these two cell lines and wtCa9-22 cells, being consistent with the results of the scatter plot analysis. Among the genes associated with “cell migration”, we focused on LCP1 and NOXI because their mRNA expression
levels were mostly up- or downregulated in HT-29 and ΔSLPI Ca9-22 cells relative to wtCa9-22 cells. Nox1 is a catalytic subunit of the superoxide-generating enzyme NADPH oxidase in phagocytes (23,24). Previously it had been reported that Nox1 contributes to cell migration (23). For example, Nox1-dependent superoxide production enhanced the migration of colon adenocarcinoma cells (23). In contrast to these previous reports, our data showed that expression of Nox1 mRNA was downregulated in HT-29 and SLPI Ca9-22 cells relative to that in wtCa9-22 cells, although the migration capacity of the former was lower than that of the latter. Thus, Nox1 may not be a key molecule for SLPI-induced migration of wtCa9-22 cells.

In contrast, LCP1 was abundantly expressed in wtCa9-22 cells but scarcely expressed in both HT-29 and ΔSLPI Ca9-22 cells, and this was confirmed by real-time RT-PCR. LCP1 belongs to a family of actin-binding proteins that are expressed in most tissues of higher eukaryotes (25). Under normal conditions, LCP1 is expressed mainly by cells of the hematopoietic lineage (25); however, under pathological conditions, it is expressed in various malignant human cells of non-hematopoietic origin, and enhances the migration and metastasis of cancer cells (25,26). A previous study showed that glioma-associated oncogene family zinc finger (GLI), a zinc finger transcription factor, upregulated LCP1 expression in human breast cancer cells (26) (Fig. 5). GLI activity is regulated by its phosphorylation through the cAMP signaling pathway and is thought to be crucial for the development and progression of various human cancers, including those of the lung, pancreas, prostate and breast (26-28). In addition, the results of our KEGG analysis showed that 45 downregulated genes were associated with the “cAMP signaling pathway”, whereas no upregulated gene was associated with this pathway, suggesting an inactivated status of the cAMP signaling pathway in HT-29 and ΔSLPI Ca9-22 cells. Together, these results suggest that SLPI enhances LCP1 expression by upregulating GLI activity through the cAMP signaling pathway, resulting in SLPI-induced cell migration (Fig. 5).

In conclusion, our results indicate that SLPI plays an important role in the migration of cancer cells, suggesting that it may be crucial for tumor aggressiveness and metastatic potential. In addition, it is suggested that LCP1 and GLI2 might be the key molecules involved in SLPI-induced cell migration. However, we cannot rule out the possibility that other molecules contribute to SLPI-induced cell migration. For example, BAMBI, RHBDF, and STYK1 may be the potential candidates because they also belong to the GO term “cell migration” and exhibit differences in expression between wtCa9-22 cells and the other two cell lines (Fig. 3B). The dataset generated in this study will provide a substantial transcriptome-level resource for the study of cell migration and further aid understanding of the molecular mechanisms underlying the malignancy of cancer cells.

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Conflict of interest
There are no conflicts of interest with regard to our study.

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