博士論文

Functional cross-talks between heterogeneous cancer cells accelerate tumor malignant progression

不均一ながん細胞の相互作用による腫瘍悪性化機構の解明

2013 年度

富山大学大学院

医学薬学教育部　生命薬科学専攻

加藤　真一郎

Table of Contents

Preface……………………………………………………………………3

Chapter1: Mesenchymal-transitioned cancer cells instigate the invasion of epithelial cancer cells through secretion of WNT3 and WNT5B.

1. Abstract…………………………………………………………..6

2. Introduction……………………………………………………...7

3. Materials and Methods……………………………………......10

4. Results…………………………………………………………..16

5. Discussion……………………………………………………...39

Chapter2: ***Unpublished study***

Chapter3: Procyanidin C1 from Cinnamomi Cortex inhibits TGF-β-induced epithelial-to-mesenchymal transition in A549 lung cancer cell lines.

11. Abstract……………………………………………………….....46

12. Introduction……………………………………………………..48

13. Materials and Methods…………………………………………..50

14. Results…………………………………………………………...56

15. Discussion……………………………………….………………64

16. General Conclusion……………………………………………68

17. References……………………………………………………70

18. Acknowledgement……………………………………………81

Preface

This thesis is submitted for the degree of Doctor of Philosophy (PhD) to the Faculty of Pharmaceutical Life Sciences, University of Toyama, Japan. This work was carried out in the Division of Pathogenic Biochemistry, Institute of Natural Medicine. The results within this thesis were published in two papers as follow:

1) Mesenchymal-transitioned cancer cells instigate the invasion of epithelial cancer cells through the secretion of WNT3 and WNT5B, Kato S, Hayakawa Y, Sakurai H, Saiki I, Yokoyama S, *Cancer Sci.*, 2014, *in press*

2) Procyanidin C1 from Cinnamomi Cortex inhibits TGF-β-induced epithelial-to-mesenchymal transition in the A549 lung cancer cell line, Kin R, Kato S, Kaneto N, Sakurai H, Hayakawa Y, Li F, Tanaka K, Saiki I, Yokoyama S, Int. J Oncol., 2013; 43(6): 1901-1906

※All the data in chapter 2 is unpublished and in preparation for submission in 2014, March.

The thesis is divided into three chapters:

We focus on the tumor heterogeneity of mesenchymal-transitioned cancer cells (M-cell) within tumor microenvironment and the role of the cross-talk between M-cell and surrounding cancer cells during tumor malignant progression.

Chapter 1 identifies the cross-talk with TGF-β-induced M-cell instigates the invasion and metastasis of epithelial cancer cells (E-cell) through the secretion of WNT3 and WNT5B. This finding highlights the significance of cancer heterogeneities and the interaction between E- and M-cells within tumor microenvironment in promoting metastatic disease through WNT-dependent mechanism.

Chapter 2 focuses on the cross-talk between M-cells each other. Since the data in chapter 2 is unpublished March, 2014 now, chapter 2 is going to disclose when this study is published.

Regarding to chapter 1 and 2, TGF-β-induced M-cell promotes tumor malignancy by mediating the cross-talk between surrounding E- and M-cells. The aim of chapter 3 is exploration of an active component which prevents TGF-β-induced EMT from a Japanese traditional medicine, *Juzentaihoto*. Procyanidin C1, from *Cinnamoni Crtex*, is newly characterized as an inhibitor of

TGF-β-induced EMT.

Chapter 1: Mesenchymal-transitioned cancer cells instigate the invasion of epithelial cancer cells through secretion of WNT3 and WNT5B.

1. Abstract

Although the heterogeneities of epithelial and mesenchymal-transitioned cancer cells are often observed within tumor microenvironment, the biological significance of interaction between epithelial cancer cells and mesenchymal-transitioned cancer cells is not yet understood. In this study, we showed that the mesenchymal-transitioned cancer cells instigate the invasive ability and metastatic potential of the neighboring epithelial cancer cells *in vitro* and *in vivo*. We further identified WNT3 and WNT5B as critical factors secreted from TGF-β-induced mesenchymal cancer cells for instigating to epithelial cancer cell invasion along with the induction of secondary-EMT phenotype. These results shed the light on the significance of cancer heterogeneities and the interaction between epithelial and mesenchymal-transitioned cancer cells within tumor microenvironment in promoting metastatic disease through WNT-dependent mechanism.

Key Words: EMT, heterogeneity, cross-talk, WNT, metastasis

2. Introduction

Tumor tissue consists of not only cancer cells, but also various stromal cells such as cancer-associated fibroblasts (CAFs), immune inflammatory cells, myeloid progenitor cells and vascular endothelial cells [1]. Such heterogeneity of complicated environment within tumor tissue has been considered as the tumor microenvironment and known to play important roles in cancer malignant progression [1]. Recent evidences further suggest that not only the tumor microenvironment but also cancer cells themselves within tumor tissue are also heterogeneous by representing numerous subpopulations with both genetic and non-genetic variations [2, 3].

Cancer metastasis disease, one of the major causes of the high mortality rate in cancer patients, involves multiple biological steps such as intravasation, attachment to vessel, extravasation, angiogenesis, and subsequent growth in distal tissues to primary tumor. Amongst those steps, the initial acquisition of cellular invasiveness is likely key steps of metastatic disseminations from the primary tumor site and the process called epithelial-to-mesenchymal transition (EMT) is known to play an important role during this process [4-7]. In accordance with the dynamic yet transient morphological and phenotypic alteration of cancer

cells during EMT process, such mesenchymal-transitioned cancer cells are often seen at an invasive front of a tumor tissue by neighboring epithelial cancer cells [8-13]. Even though the importance of TGF-β in the initiation of EMT has been shown [14, 15], there are several reports that primary cancer specimens acquire mesenchymal features and develop metastatic disease even in the presence of the deletion in Smad4 which is a key component of TGF-β signaling pathway [16, 17]. These observations might imply that there are alternative pathways to maintain the EMT phenotype other than TGF-β pathway within tumor microenvironment. Interestingly, the cooperation of mesenchymal-transitioned and surrounding epithelial cancer cells for establishing spontaneous metastasis in a mouse model was reported [18]. It was also studied that the heterogeneity of cancer cells and the intra-tumoral cross-talk between distinct types of cancer cells might contribute to induce abnormal proliferation and metastasis [19-21], however the exact mechanism how those distinct cancer cell types interact with each other is not yet understood.

In the present study, we demonstrated that the co-existence of mesenchymal-transitioned cancer cells with epithelial cancer cells induces invasive ability and metastatic potential of epithelial cancer cells *in vitro* and *in*

vivo. Furthermore, we identified WNT3 and WNT5B are the secretory factors from TGF-β-induced mesenchymal-transitioned cancer cells to induce invasion of neighboring epithelial cancer cells and secondary-EMT phenotype. Collectively, those results strongly implicate that secretory WNT ligands are critical soluble factors to mediate invasion instigation of epithelial cancer cells derived from mesenchymal-transitioned cancer cells and further targeting those secretory WNT proteins could be a new approach to prevent cancer invasion and subsequent metastasis.

3. Materials and Methods

3.1 Cell culture and inhibitors

Human lung adenocarcinoma A549 and human pancreatic ductal adenocarcinoma Panc-1 cells were obtained from American Type Culture Collection (ATCC). A549 cells were maintained in RPMI1640, and Panc-1 cells were maintained in DMEM, containing 10% FBS, 1 mM L-glutamine and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. To establish labeled A549 and Panc-1 cells, A549 or Panc-1 cells were transfected with pGL4.50/Luc2 (Promega, Madison, WI, USA) or pEGFP-C1 (Clontech, Palo Alto, CA, USA), selected, and cloned in growth medium containing 200 μg/ml hygromycin B or 1 mg/ml G418, respectively. The reagents used were: WNT secretion inhibitor, IWP-2 (Sigma-Aldrich) and TGF-β receptor kinase inhibitor (TβRI) (Calbiochem, Dermstadt, Germany).

3.2 Preparation of E-cells and M-cells

Non-stimulated epithelial A549 and Panc-1 cells were used as E-cells in this

study. To prepare mesenchymal-transitioned cancer cells (M-cells), A549 or Panc-1 cells were treated with 5 ng/ml recombinant TGF-β (Pepro Tech, New Jersey, USA) for 48 hr then washed with fresh culture medium twice and harvested for subsequent experiments.

3.3 Direct and separated co-culture experiment

For direct co-culture experiment, E-cells and M-cells were re-seeded into 60 mm culture dish by indicated cell number and co-cultured for 24 hr. For separated co-culture experiment, 1×10^5 cells of E-cells and 3×10^5 cells of M-cells were seeded into lower or upper compartment of transwell chamber with 1 μm pore diameter (BD Falcon, Bedford, MA, USA) for 24 h.

3.4 Generation of conditioned medium (CM)

After preparation of E- or M-cells, the cells were further cultured in fresh growth medium for additional 48 hr. Finally, these culture supernatants were collected and diluted with fresh growth medium by 2:1. The freshly prepared supernatant was utilized as conditioned medium (CM) in each experiment.

For preparation of WNT3- and WNT5B-depleted CM by siRNA, parental

A549 and Panc-1 cells were transfected with siControl (siGENOME Control Pool Non-targeting siRNA#2), siWNT3 and/or siWNT5B (ON-TARGETplus SMARTpool siRNA) (Thermo Fisher scientific) by Lipofectamine™ RNAiMAX (Invitrogen) 48 hr prior to induction of M-cells. For preparation of WNTs-depleted CM by IWP-2, E- and M-cells were cultured in fresh medium containing IWP-2 for 48 hr. The protein level of WNT3 and WNT5B in CMs was determined by specific ELISA (CUSABIO Biotech Co., Ltd., Wuhan, China) according to manufacturer's protocol.

3.5 Matrigel invasion assay

Cancer cell invasion through reconstituted basement membrane (Matrigel; BD Biosciences) was assayed as previously described [22]. After fixed the filter and stained with hematoxylin and eosin, the invaded cells were counted manually under a microscope at x100. For detection of luciferase activity in invaded A549/Luc2 cells, the filters were soaked into passive lysis buffer (Promega) and luciferase activity was determined. For detection of $EGFP^+$ invaded cells, filters were fixed with 4% paraformaldehyde and stained with VECTASHIELD mounting media with DAPI (Vector Laboratories, Burlingame, CA).

3.6 Western blotting

Whole cell lysates and nuclear protein extracts were prepared as described previously [23]. Primary antibodies used were Epithelial-Mesenchymal transition (EMT) Antibody Sampler Kit (#9782, Cell Signaling Technology), antibodies against WNT3 (ab32249) and WNT5B (ab94914) from Abcam, and antibodies against PCNA (PC10), β-actin (C11), Lamin B (C-20), and α-tubulin (D-10) from Santa Cruz biotechnology.

3.7 Experimental lung metastasis experiment (in vivo invasion assay)

C.B-17/IcrHsd-*Prkdc*^{scid} mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). All experiments were approved and performed according to the guidelines of the Care and Use of Laboratory Animals of University of Toyama. Cells were inoculated intravenously ($2x10^6$ cells/200 μl PBS/mouse) into mice and the lungs were removed 24 hr after the tumor inoculation. Mice were intra-peritonealy injected with 200 μl of luciferin (1.5 mg/ml, VivoGlo™, Promega, Madison, WI, USA) 20 min prior to subject bioluminescent assay by using an *in vivo* imaging system (IVIS Lumina II, Caliper Life Sciences, Hopkinton, MA,

USA). The data was presented as the mean luminescence ± SEM.

3.8 Microarray data analysis

The data sets (GSE17708 and GSE23952) were reanalyzed on GenePattern [24]. Briefly, the differential expression level of all genes between TGF-β-treated samples and non-treated samples was computed and the top 5% upregulated genes in TGF-β-treated cells compared with control cells were selected by using "Comparative Marker Selection" tool from each data set. Finally, the genes coding the secreted proteins were picked up from the commonly upregulated genes in both data sets.

Gene set enrichment analysis (GSEA) was performed using javaGSEA application v2.0.13 (GSEA, Broad Institute, Boston). These pathway gene sets were provided by the Molecular Signatures Database (MSigDB; www.broadinstitute.org/gsea/msigdb).

3.9 Quantitative RT-PCR

Total RNA was purified using RNeasy plus mini kit (QIAGEN) and then 50 ng RNA of each sample was amplified using the One Step SYBR PrimeScript

RT-PCR kit (TaKaRa) and appropriate primers. The primers used were: 5’-TGT GAG GTG AAG ACC TGC TG-3’ (sense) and 5’-AAA GTT GGG GGA GTT CTC GT-3’ (antisense) for WNT3 and 5’-ACG CTG GAG ATC TCT GAG GA-3’ (sense) and 5’-CGA GGT TGA AGC TGA GTT CC-3’ (antisense) for WNT5B.

3.10 Statistical analysis

Statistical significance was calculated using Excel software (Microsoft). More than three means were composed using one-way analysis of variance (ANOVA) with Bonferroni correction, and two means were composed using unpaired Student’s *t*-test. $p<0.05$ were considered statistically significant.

4. Results

4.1 Epithelial cancer cells acquire metastatic potential by the co-culture with mesenchymal-transitioned cancer cells.

To directly evaluate the role of heterogeneity of cancer cells in their invasive potentials, we performed Matrigel invasion assay in human epithelial lung adenocarcinoma A549 and pancreatic ductal adenocarcinoma Panc-1 cell co-culture at various ratios of epithelial cancer cells (E-cells) and TGF-β-induced mesenchymal-transitioned cancer cells (M-cells), because A549 and Panc-1 are previously reported to be occurred EMT by the stimulation with TGF-β (Fig. 1), which is the most potent EMT-inducing cytokine secreted from cancer-associated fibroblast and cancer cells [25, 26]. After 24 hr co-culture of E- and M-cells, the invasive potentials of E- and M-cells mixture were enhanced in both A549 and Panc-1 cell lines in M-cell dose-dependent manner than that of M-cells alone (Fig. 2A). To determine exact cell population, E- or M-cells, is responsible for the enhanced invasive potential after the co-culture, we employed luminescence-labeled E-A549 (E-A549/Luc2) or fluorescent-labeled E-Panc-1 (E-Panc/EGFP) to distinguish E-cells from M-cells within the co-culture.

By measuring the invasiveness of labeled E-cells in either A549 or Panc-1, we have found that the invasive potentials of E-cells co-cultured with M-cells were higher than that of E-cells alone in both A549 and Panc-1 (Fig. 2B) and such induction of invasiveness was observed in M-cell dose-dependent manner (Fig. 2B). We have also found that the invasion potential of E-cells was not altered without the co-culture with M-cells (Fig. 2C). Of note, the invasive potential of M-Panc cells was not affected by the co-culture with E-Panc cells as compared with M-Panc cells alone (data not shown). These results clearly indicate that the co-culture of E-cells with M-cells selectively enhances the invasiveness of E-cells in both A549 and Panc-1 cell lines.

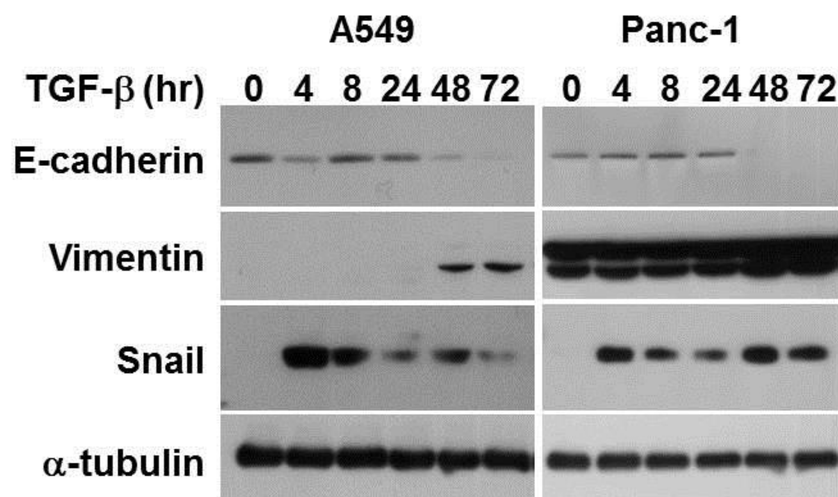
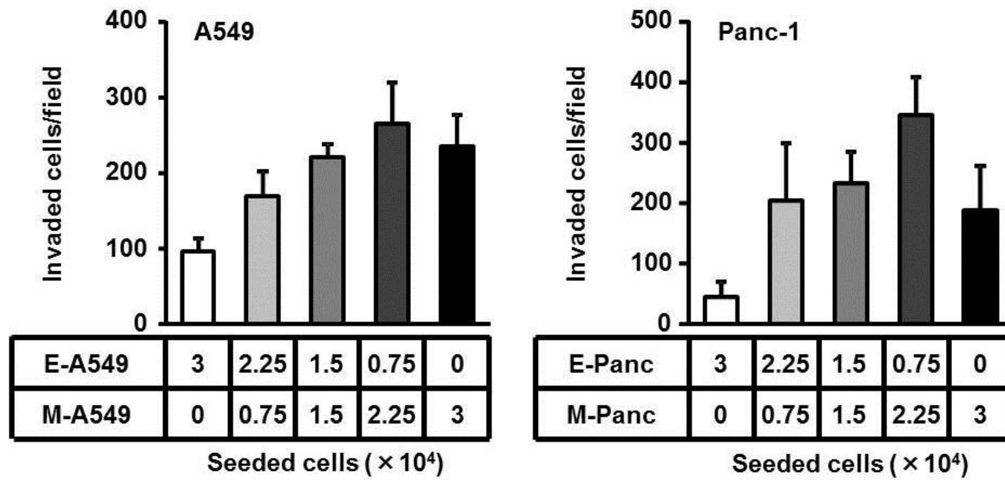


Figure 1. Expression of EMT-related proteins following TGF-β-induced EMT. A549 and Panc-1 cells were treated with 5 ng/ml TGF-β up to 72 hr. EMT-related protein expression were determine by western blotting.

A



B

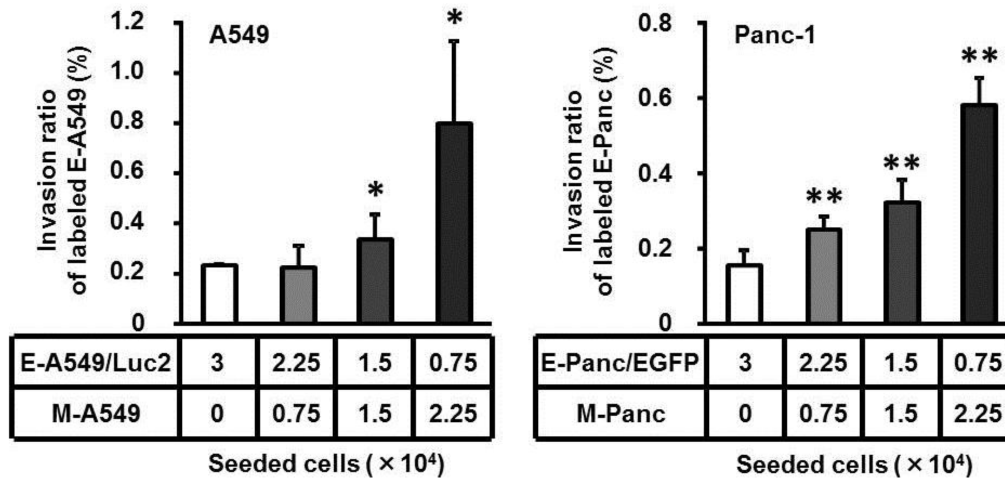


Figure 2. Epithelial cancer cells acquire metastatic potential upon co-culture with mesenchymal-transitioned cancer cells.

(A) A549 (left) or Panc-1 (right) cells were subjected to Matrigel invasion assay after 24 hr-co-culture of epithelial cancer cells (E-cells) with mesenchymal-transitioned cancer cells (M-cells) at the indicated cell numbers. Total invaded cells were counted after H & E staining. Data represented as the mean ± SD of four independent experiments. (B) Labeled E-cells (E-A549/Luc2; left panel or E-Panc/EGFP; right panel) were subjected to Matrigel invasion assay after 24 hr-co-culture with M-cells. Invasive abilities were determined by measuring luciferase activity (A549) or counting invaded $EGFP^{+}$ cells (Panc-1), respectively. Invasion ratio was calculated by the division of invaded E-cells by total E-cells applied. Data represented as the mean ± SD of triplicate experiment. *p<0.05, **p<0.01 vs E-cells alone group by Dunnett's

To further investigate the significance of the interaction between E-cells and M-cells *in vivo*, the metastatic potentials of E-A549 cells to lung were examined by injecting E-A549/Luc2 cells upon co-culture with either E-A549 or M-A549 for 24 hr. The metastatic spread of E-A549/Luc2 in the lung were much higher after the co-culture with M-A549 cells compared to with E-A549 cells (Fig. 2D), therefore indicating the potential of M-cells to promote metastatic ability of neighboring E-cells *in vivo.*

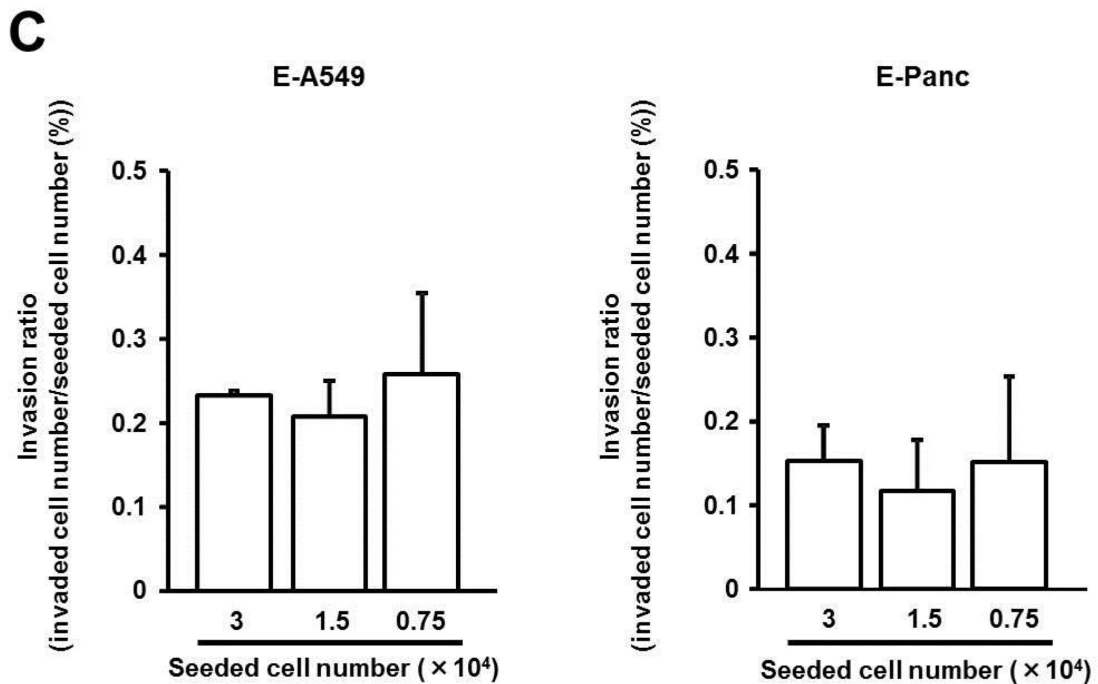


Figure 2. Epithelial cancer cells acquire metastatic potential upon co-culture with mesenchymal-transitioned cancer cells. (continued)

(C) To check whether the decrease of seeded cell number itself affect invasion potentials, E-A549 or E-Panc cells were subjected to matrigel invasion assay at indicated cell numbers. Invasion potentials were calculated based on the following formulation. (Invasion potential) = (invaded cell numbers) / (seeded cell numbers). Data represented as the mean ± SD of triplicate experiment.

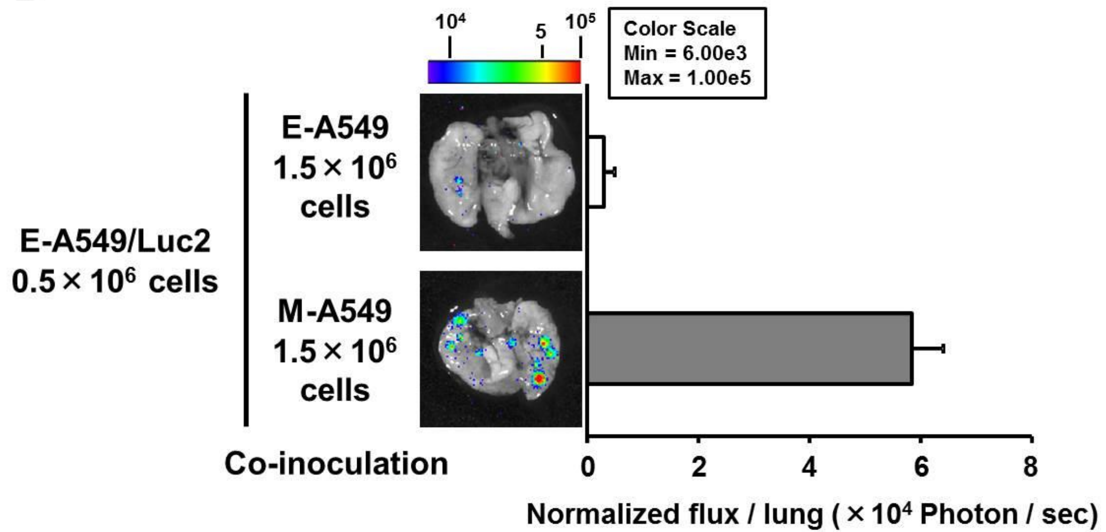


Figure 2. Epithelial cancer cells acquire metastatic potential upon co-culture with mesenchymal-transitioned cancer cells. (continued)

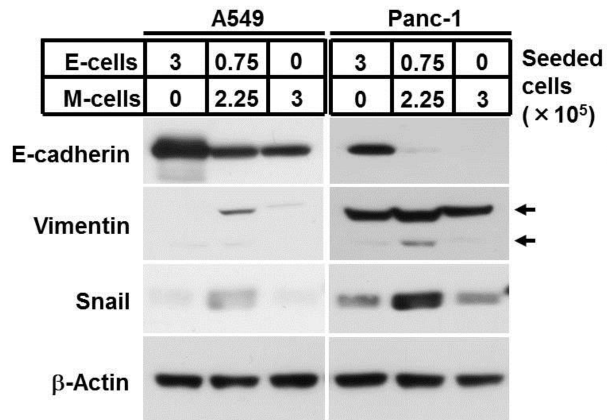
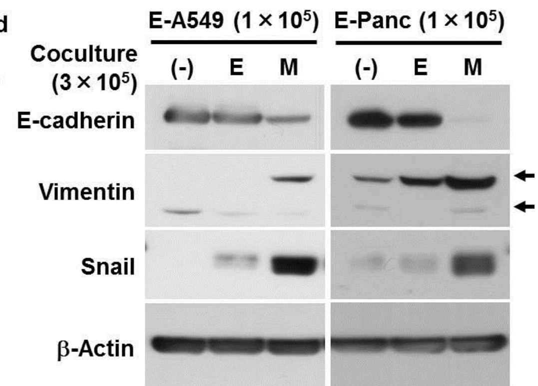
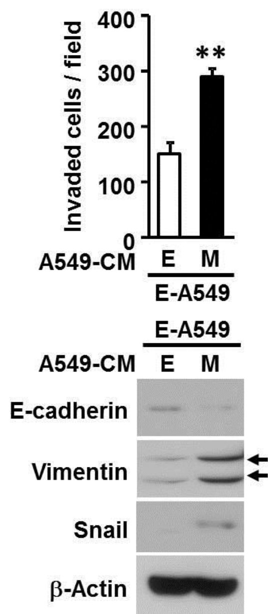
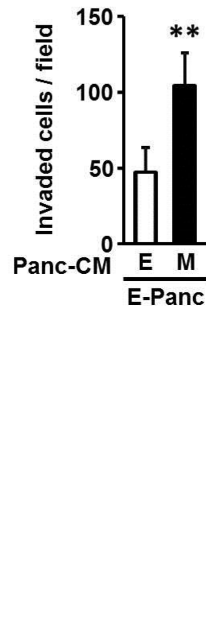
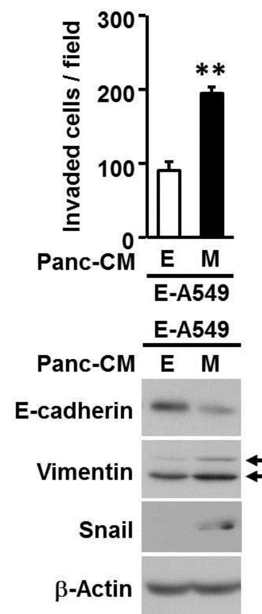
(D) Epithelial A549 cells overexpressing Luc2 gene (E-A549/Luc2) were co-cultured with either epithelial A549 (E-A549) cells or mesenchymal-transitioned A549 (M-A549) cells and i.v. inoculated into mice. Mice were sacrificed 24 hr after the tumor inoculation and lungs were subjected to bioluminescent imaging to determine total flux (photon/sec) for lung metastasis quantification. The representative *ex vivo* images are shown. Data represented as the mean ± SEM (n=5).

4.2 Mesenchymal-transitioned cancer cells induce metastatic potential of the neighboring epithelial cancer cells in cell-cell contact independent mechanism.

To determine whether the direct cell-cell interaction is required for promoting metastatic potential of E-cells by neighboring with M-cells, we first examined the expression of EMT-related proteins in A549 or Panc-1 cells under the co-culture of E-cells with M-cells in the presence or absence of direct cell-cell contact (Fig. 3A and 3B). Given the up-regulation of mesenchymal marker (Snail and Vimentin) and the down-regulation of epithelial marker (E-cadherin) were markedly induced by both direct and separated co-culture of E- and M-cells, the soluble factor(s) derived from M-cells was at least sufficient for the induction of secondary-EMT phenotype of E-cells upon co-culture with M-cells. Vimentin has two isoforms (full-length and C-terminal-deletion form) that show distinct roles on the bundle formation, subsequent cell morphology and motility (Chou YH, et al, J. Cell Sci., 1996; **109**: 817-826). In this study, both vimentin isoforms were up-regulated by the treatment with M-CM, and indeed affect functional phenotypes of E-cells. We further confirmed that the invasion of E-A549 cells was remarkably enhanced after the culture with M-A549-derived conditioned

medium (M-A549-CM) compared with E-A549-CM (Fig. 3C). In concert with the enhanced invasive ability of E-A549 cells after the cultivation with M-A549-CM, the secondary-EMT phenotype in E-A549 cells were also induced after the culture with M-A549-CM. Similar results were obtained in the invasion of E-Panc cells after the cultivation with M-Panc-CM (Fig. 3D).

Importantly, the CM from M-Panc cells was able to introduce enhanced invasive ability and secondary-EMT phenotype in E-A549 cells (Fig. 3E) indicating the common soluble factor(s) derived from M-A549 cells and M-Panc cells are likely to be involved in this process. Considering the induction of secondary-EMT in E-cells by M-cell-CM did not affected by TGF-β receptor kinase inhibitor (Fig. 3F), the involvement of TGF-β signaling pathway is less likely. Collectively, these data indicate that mesenchymal-transitioned cancer cells-derived soluble factor(s), which is shared by A549 and Panc-1 cells, play a significant role in the induction of invasive ability and secondary-EMT phenotype in the neighboring epithelial cancer cells.


A
Direct coculture
A549
Panc-1
E-cells
M-cells
3
0.75
0
2.25
Seeded cells
(×10⁵)
E-cadherin
Vimentin
Snail
β-Actin

B
Separated coculture
E-A549 (1×10⁵)
E-Panc (1×10⁵)
Coculture (3×10⁵)
(-)
E
M
E-cadherin
Vimentin
Snail
β-Actin

C
Invaded cells / field
400
300
200
100
0
**
A549-CM
E
M
E-A549
E-cadherin
Vimentin
Snail
β-Actin

D
Invaded cells / field
150
100
50
0
**
Panc-CM
E
M
E-Panc

E
Invaded cells / field
300
200
100
0
**
Panc-CM
E
M
E-A549
E-cadherin
Vimentin
Snail
β-Actin

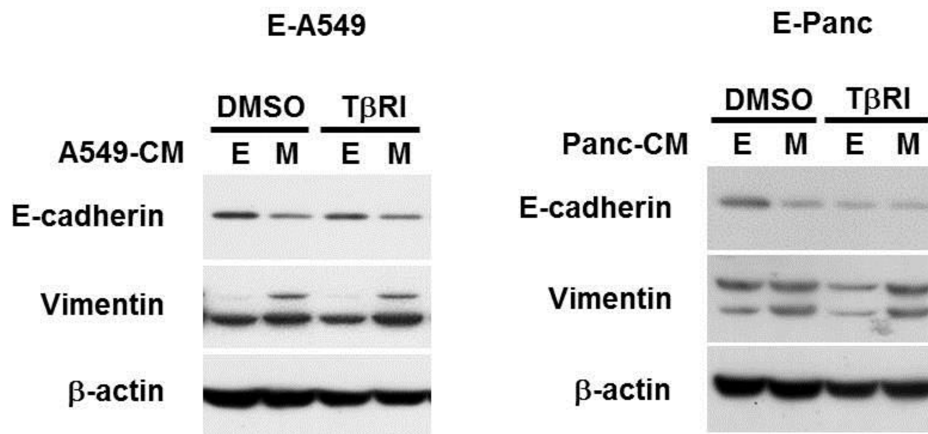


Figure 3. Cell-cell contact independent induction of secondary-EMT phenotype and invasiveness in neighboring epithelial cancer cells by mesenchymal-transitioned cancer cells.

(A, B) A549 or Panc-1 cells were co-cultured directly (A) or separately in transwell cell culture chamber (B) at the indicated cell numbers for 24 hr and EMT-related protein expression were determined by western blotting. Both arrows indicate vimentin expression. No cells (-), E-cells (E) or M-cells (M) were seeded in upper compartment of transwell chamber. In the separated co-culture, the total protein from E-cells in lower compartment was examined. (C, D, E) E-A549 or E-Panc cells were treated with conditioned mediums (CM) from E-/M-A549 cells (C) or E-/M-Panc cells (D, E) for 48 hr and subjected to Matrigel invasion assay or western blotting. Both arrows indicate vimentin expression. Total invaded cells were counted after H & E staining. Data represented as the mean ± SD of triplicate experiment. **$p<0.01$ vs E-CM group by two-tailed student's *t* test.

4.3 WNT3 and WNT5B derived from mesenchymal-transitioned cancer cells are the soluble factors to induce metastatic potential in the neighboring epithelial cancer cells.

In order to identify the common soluble factor(s) which is secreted from mesenchymal-transitioned A549 and Panc-1 cells, we analyzed the published cDNA microarray datasets (GSE17708 and GSE23952), which represent A549 and Panc-1 gene expression following after the TGF-β stimulation for 72 or 48 hr, respectively. There are 55 candidate genes as top 5% of encoding secretory proteins that are commonly up-regulated in both A549 and Panc-1 cells (Fig. 4A and Table 1). By using Gene Set Enrichment Analysis, we further selected candidate pathway gene sets that are significantly enriched in phenotype of TGF-β as shown in Table 2. Amongst those candidate pathways, WNT pathway was commonly enriched in both M-A549 and M-Panc. Thus, we further focused on WNT3 and WNT5B molecules in the induction of secondary-EMT in epithelial cancer cells. WNT3 and WNT5B are known to be a ligand for activating both canonical, that is mediated by β-catenin/TCF transcription factor complex, and non-canonical WNT pathways, that is mediated by JNK or ROCK kinases [27]. As shown in Fig. 4B, we confirmed the higher expression of WNT3 and WNT5B

at protein level in both M-cells compared to E-cells. Consistent with the upregulation of WNT3 and WNT5B, the secretion of these WNT ligands was detected in CMs of M-A549 by ELISA (Fig. 4C). We also confirmed higher nuclear β-catenin expression and β-catenin transcriptional activity in E-cells with M-cell-CM, indicating that E-cells received the WNT signals from M-cells (Fig. 4D and 4E).

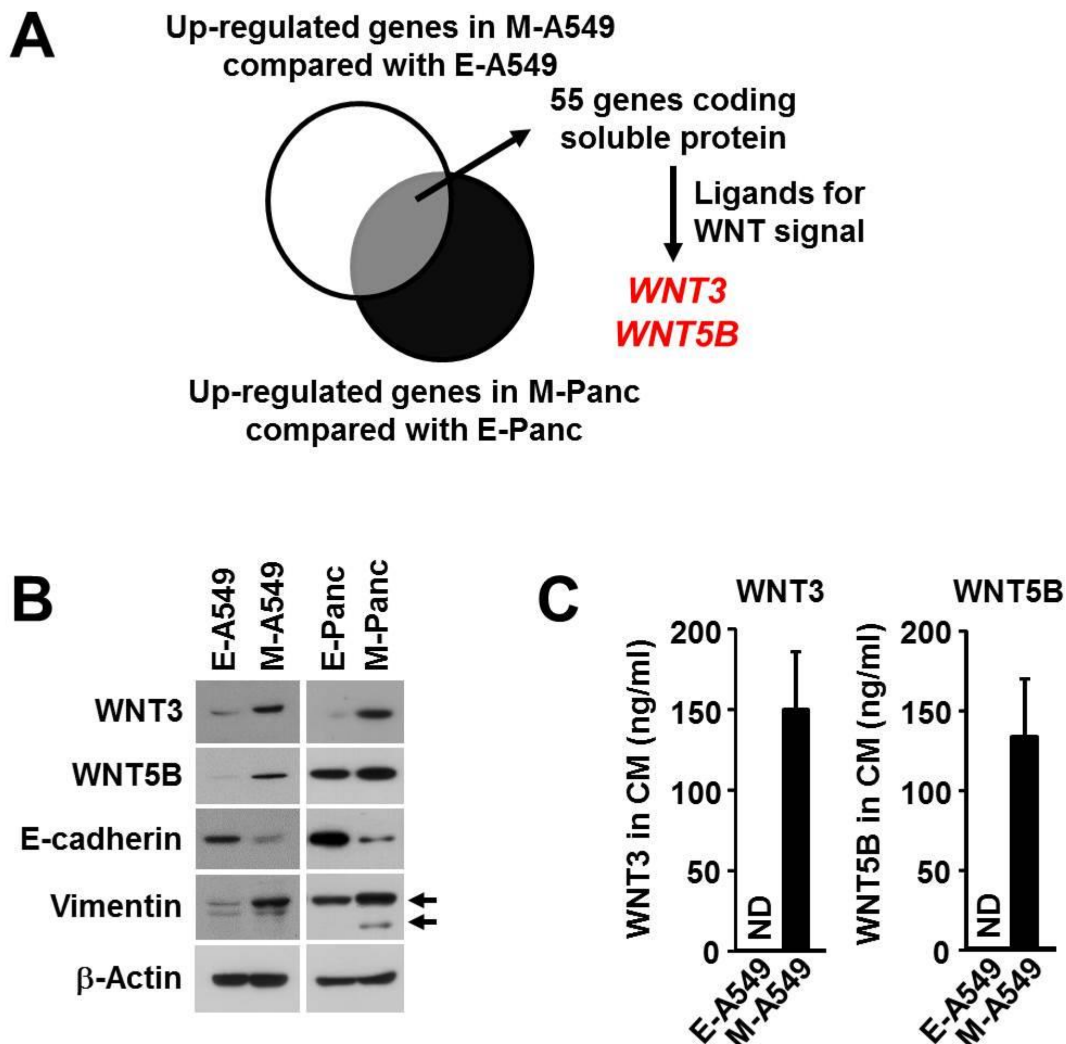


Figure 4. Secretory WNT3 and WNT5B from mesenchymal-transitioned cancer cells induce secondary-EMT phenotype in epithelial cancer cells.

(A) Commonly up-regulated genes encoding soluble protein in the top 5% in GSE17708 (Panc-1) and GSE23952 (A549) datasets were shown as Benn diagram. (B) Epithelial or mesenchymal-transitioned A549 or Panc-1 cells were subjected to western blotting to determine the expression of indicated proteins. Arrows indicate vimentin band. (C) Conditioned mediums from E-cells or M-cells were subjected to ELISA for detecting WNT3 or WNT5B. ND; not detected.

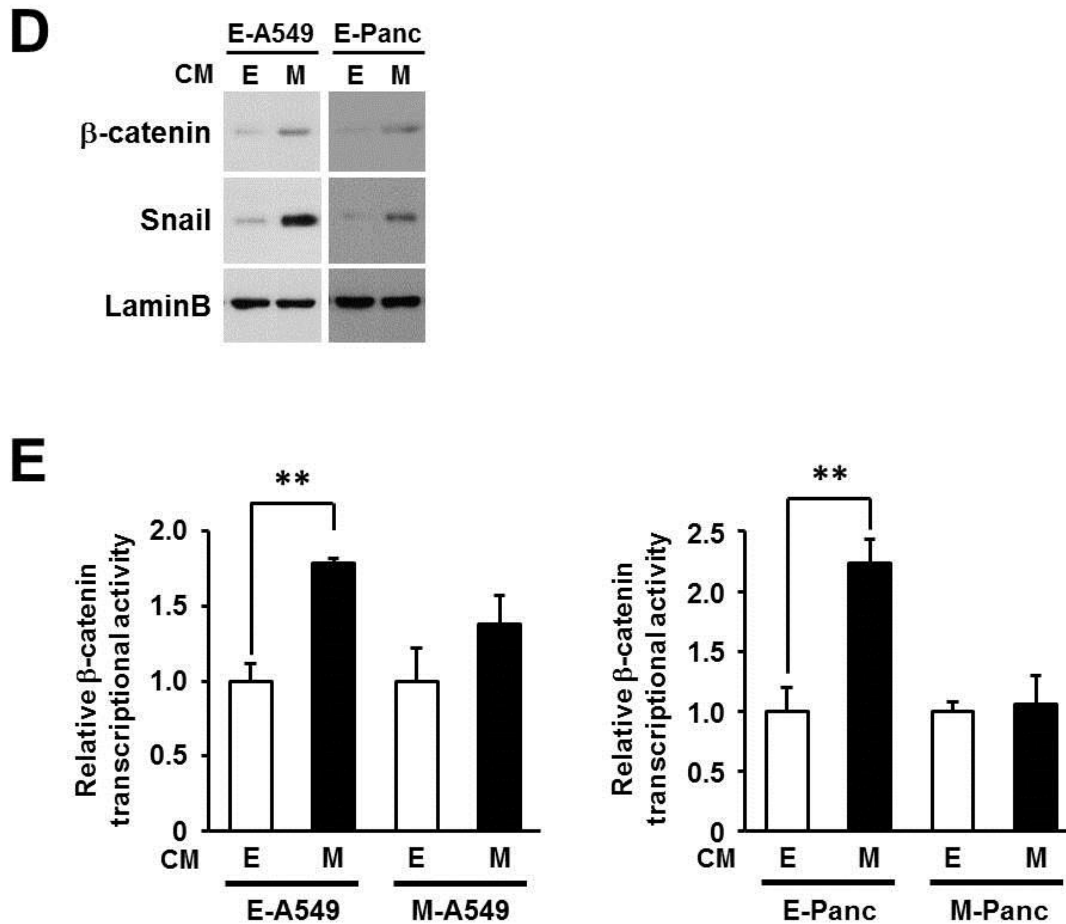


Figure 4. Secretory WNT3 and WNT5B from mesenchymal-transitioned cancer cells induce secondary-EMT phenotype in epithelial cancer cells. (continued) (D) 48 hr after treatment with E- or M-CM, nuclear protein was fractionated and subsequently subjected to western blotting for the detection of nuclear β-catenin and Snail expression. LaminB was used as an internal control of nuclear protein. (E) 48 hr after treatment with E- or M-CM, transcriptional activity of β-catenin was determined by luciferase assay using TOPFlash, which contains β-catenin/TCF binding site, and FOPFlash, which contains mutation at β-catenin/TCF binding site of TOPFflash construct. Data represented as the mean ± SD of triplicate experiment. **p<0.01 vs E-CM group by two-tailed student's *t* test.

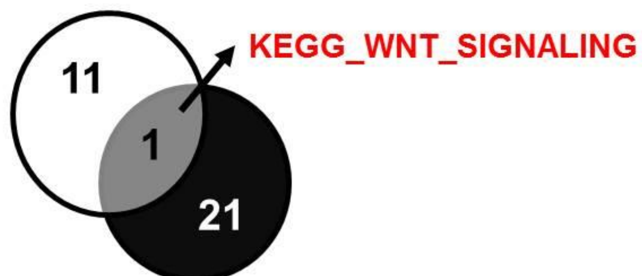
Table1. Common up-regulated genes (top 5%) in both mesenchymal-transitioned A549 and Panc-1 cells by the stimulation of TGF-b for 72 h and 48h ,respectively.

Gene name	Ranking	Full name
ADMTS15	54	ADAM metallopeptidase with thrombospondin type 1 motif, 15
CLEC18A	55	C-type lectin domain family 18, member A
CD59	37	CD59 molecule, complement regulatory protein
EDIL3	17	EGF-like repeats and discoidin-I-like domains 3
TIMP2	33	TIMP metallopeptidase inhibitor 2
B4GALT1	43	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
GALNT1	52	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1
GALNT2	29	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2
ULBP2	19	UL16 binding protein 2
ANGPTL4	2	Angiopoetin-like 4
CLCF1	49	Cardiotrophin-like cytokine factor 1
CCL2	34	chemokine (C-C motif) ligand 2
CXCL12	45	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
CKLF	38	chemokine-like factor
COL1A1	6	collagen, type I, alpha 1
COL4A2	51	collagen, type IV, alpha 2
COL5A1	5	collagen, type V, alpha 1
COL7A1	21	collagen, type VII, alpha 1
COL27A1	36	collagen, type XXVII, alpha 1
CTGF	22	connective tissue growth factor
CYR61	44	cysteine-rich, angiogenic inducer, 61
EGFR	39	epidermal growth factor receptor
FST	28	follistatin
FSTL3	25	follistatin-like 3 (secreted glycoprotein)
GAL	14	galanin prepropeptide
HBEGF	12	heparin-binding EGF-like growth factor
HYAL3	35	hyaluronoglucosaminidase 3

Table1. (Continued)

Gene name	Ranking	Full name
IGFBP3	48	insulin-like growth factor binding protein 3
IL11	11	interleukin 11
IL32	18	interleukin 32
LAMC2	3	Laminin, gamma 2
LTBP2	4	latent transforming growth factor beta binding protein 2
LIF	32	leukemia inhibitory factor (cholinergic differentiation factor)
LOX	9	lysyl oxidase
LOXL2	15	lysyl oxidase-like 2
MMP10	13	matrix metallopeptidase 10 (stromelysin 2)
MMP2	26	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
METRNL	53	meteorin, glial cell differentiation regulator-like
NOG	7	noggin
PLAU	31	plasminogen activator, urokinase
PLAUR	24	plasminogen activator, urokinase receptor
PDGFB	41	platelet-derived growth factor beta polypeptide
PVR	30	poliovirus receptor
RSPRY1	50	ring finger and SPRY domain containing 1
SCG2	10	secretogranin II (chromogranin C)
SERPINE1	16	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SERPINE2	23	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
SPOCK1	1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1
STC1	8	stanniocalcin 1
STX1A	42	syntaxin 1A (brain)
TGFBI	40	transforming growth factor, beta-induced, 68kDa
TUFT1	46	tuftelin 1
VEGFA	47	vascular endothelial growth factor A
WNT3	27	wingless-type MMTV integration site family, member 3
WNT5B	20	wingless-type MMTV integration site family, member 5B

Table 2. Common pathway gene sets enriched in both mesenchymal-transitioned A549 and Panc-1.



Gene Set enriched in M-A549
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_KERATAN_SULFATE
KEGG_NOTCH_SIGNALING_PATHWAY
KEGG_TYPE_I_DIABETES_MELLITUS
KEGG_COLORECTAL_CANCER
KEGG_ALLOGRAFT_REJECTION
KEGG_AXON_GUIDANCE
KEGG_TGF_BETA_SIGNALING_PATHWAY
KEGG_WNT_SIGNALING_PATHWAY
KEGG_HEDGEHOG_SIGNALING_PATHWAY
KEGG_VIRAL_MYOCARDITIS
KEGG_ABC_TRANSPORTERS
KEGG_MELANOGENESIS

Gene Set enriched in M-Panc
KEGG_DORSO_VENTRAL_AXIS_FORMATION
KEGG_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT
KEGG_ARGININE_AND_PROLINE_METABOLISM
KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION
KEGG_LONG_TERM_DEPRESSION
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM
KEGG_PATHWAYS_IN_CANCER
KEGG_GAP_JUNCTION
KEGG_GLYCEROLIPID_METABOLISM
KEGG_P53_SIGNALING_PATHWAY
KEGG_ERBB_SIGNALING_PATHWAY
KEGG_ECM_RECEPTOR_INTERACTION
KEGG_FOCAL_ADHESION
KEGG_CYSTEINE_AND_METHIONINE_METABOLISM
KEGG_BASAL_CELL_CARCINOMA
KEGG_COLORECTAL_CANCER
KEGG_PATHOGENIC_ESCHERICHIA_COLI_INFECTION
KEGG_TERPENOID_BACKBONE_BIOSYNTHESIS
KEGG_LYSOSOME
KEGG_PURINE_METABOLISM
KEGG_WNT_SIGNALING_PATHWAY

To further examine whether WNT3 and WNT5B are responsible molecules in M-cell-derived CMs for the induction of invasiveness and secondary-EMT phenotype in E-cells, we used the siRNAs of WNT3 and WNT5B or the chemical WNT-ligand secretion inhibitor, IWP-2, during the preparation of CMs. IWP-2 directly suppresses porcupine that is required for the secretion of WNT ligands through palmitoylation (Chen B, et al. Nat. Chem. Biol., 2009; **5**: 100-107). The knockdown efficiencies or the inhibition of secretion were confirmed by qRT-PCR, western blotting and ELISA (Fig. 5A, 5B and 5C). While the M-cell-CM derived from single knockdown of either WNT3 or WNT5B did not completely diminish the induction of invasive potential and vimentin/Snail expression of E-A549 and E-Panc cells, the knockdown of both WNT3 and WNT5B completely abrogated the activity of M-cell-CM in the induction of invasive potential and secondary-EMT phenotype of E-A549 and E-Panc cells (Fig. 5D). Importantly, the M-cell-CMs prepared in the presence of IWP-2 also completely diminished its activity to induce invasion and secondary-EMT phenotype in both E-A549 and E-Panc cells (Fig. 5E and 5F). These results strongly indicate that WNT3 and WNT5B are likely to be key soluble factors produced by mesenchymal-transitioned cancer cells to instigate the metastatic

potential of neighboring epithelial cancer cells by enhancing their invasiveness and inducing secondary-EMT phenotype.

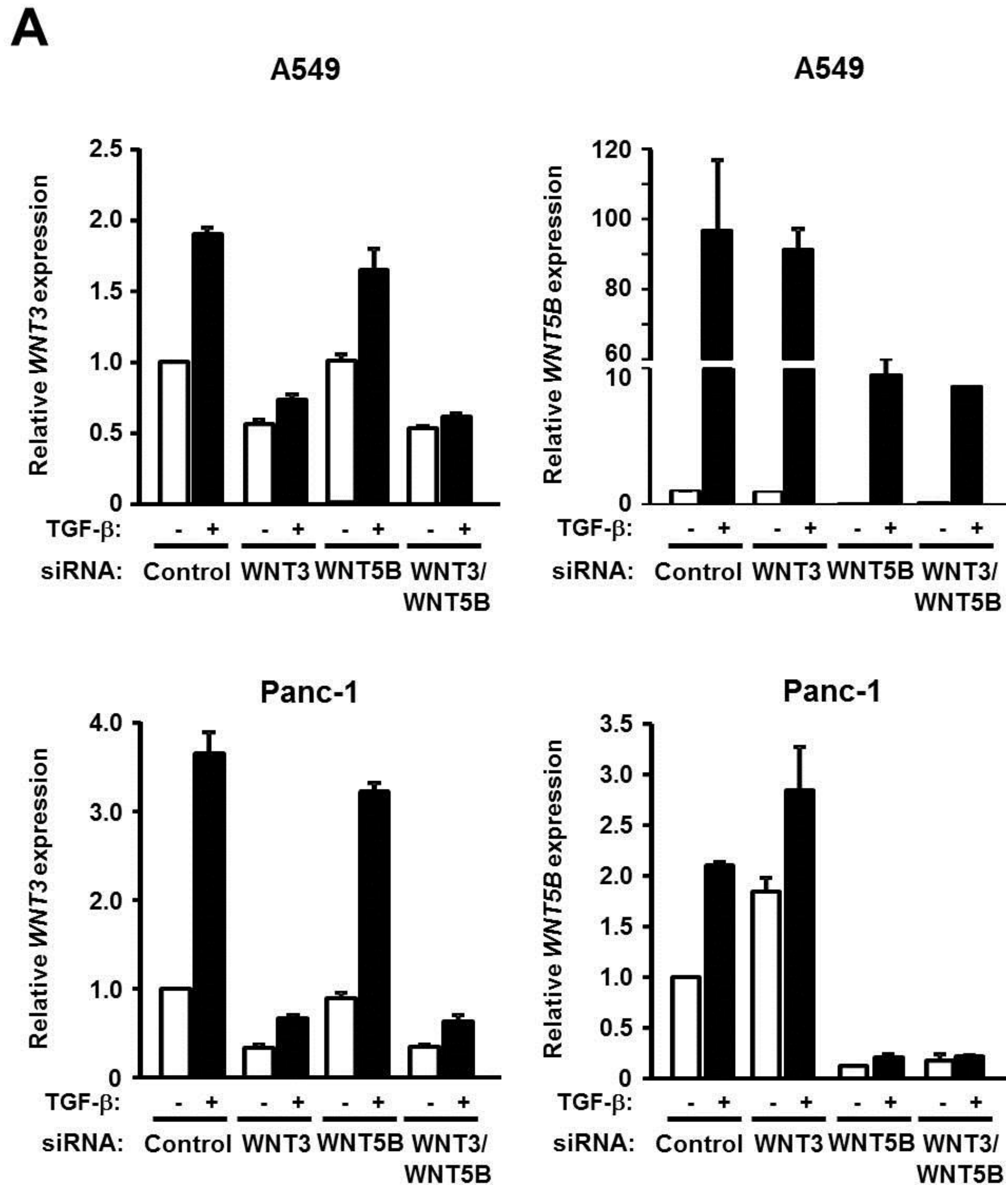
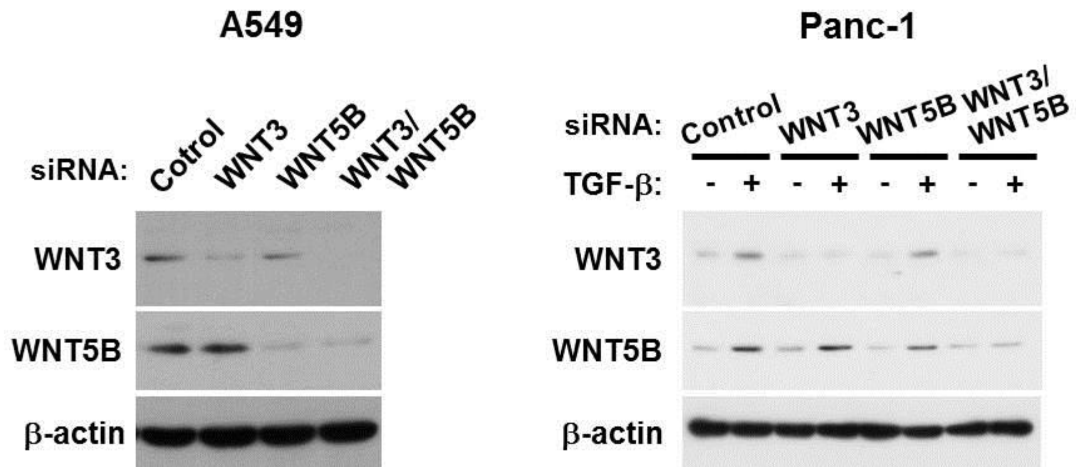
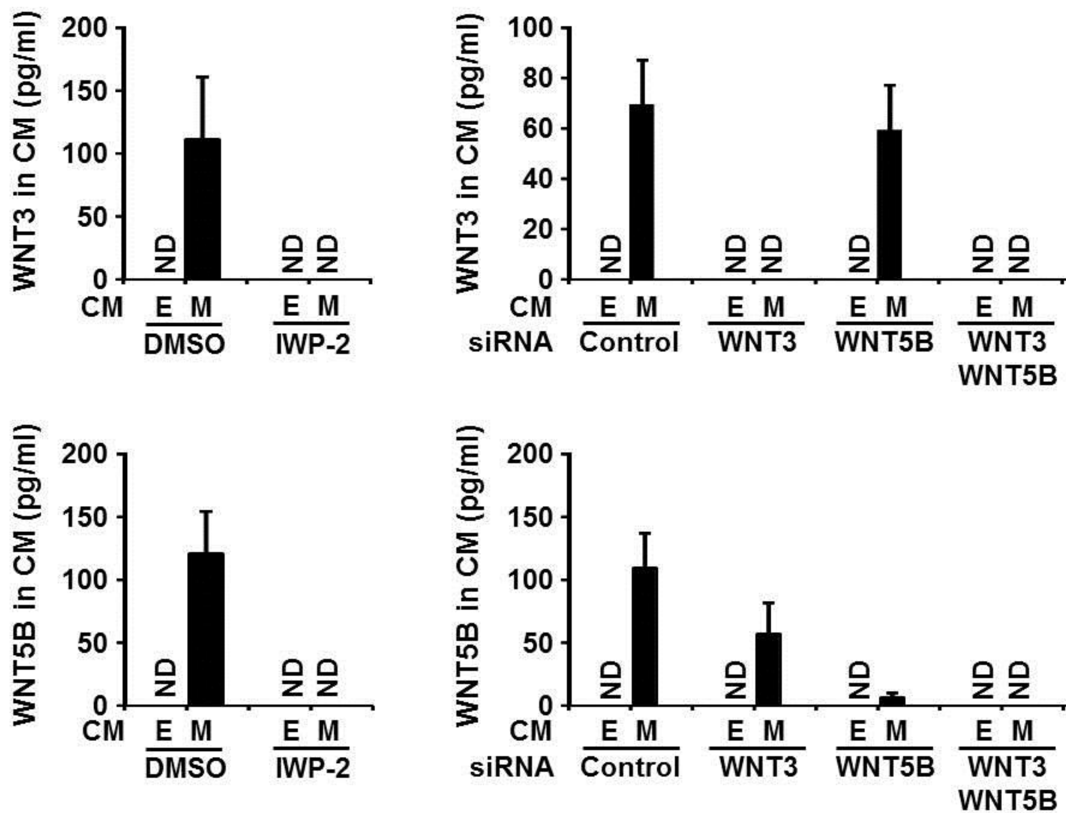


Figure 5. Critical requirement of secretory WNT3 and WNT5B from mesenchymal-transitioned cancer cells for inducing secondary-EMT phenotype of epithelical cancer cells.

(A) mRNA and protein expression in (B) cell lysate and (C) CM of WNT3 and WNT5B upon siRNA and TGF-β treatment was determined by qPCR western blotting and ELISA, respectively. siRNA-treated cells were prepared according to "Material and Methods". Data represented as the mean ± SD of triplicate experiments. ND; not detected


B
A549
siRNA:
Cotrol
WNT3
WNT5B
WNT3/
WNT5B
WNT3
WNT5B
β-actin
Panc-1
siRNA:
Control
WNT3
WNT5B
WNT3/
WNT5B
TGF-β:
- + - + - + - +
WNT3
WNT5B
β-actin


C
WNT3 in CM (pg/ml)
200
150
100
50
0
ND
ND ND
CM
E M
E M
DMSO
IWP-2
WNT3 in CM (pg/ml)
100
80
60
40
20
0
ND
ND ND
ND
ND ND
CM
E M
siRNA
Control
WNT3
WNT5B
WNT3
WNT5B
WNT5B in CM (pg/ml)
200
150
100
50
0
ND
ND ND
CM
E M
DMSO
IWP-2
WNT5B in CM (pg/ml)
ND
siRNA
Control
WNT3
WNT5B
WNT3
WNT5B

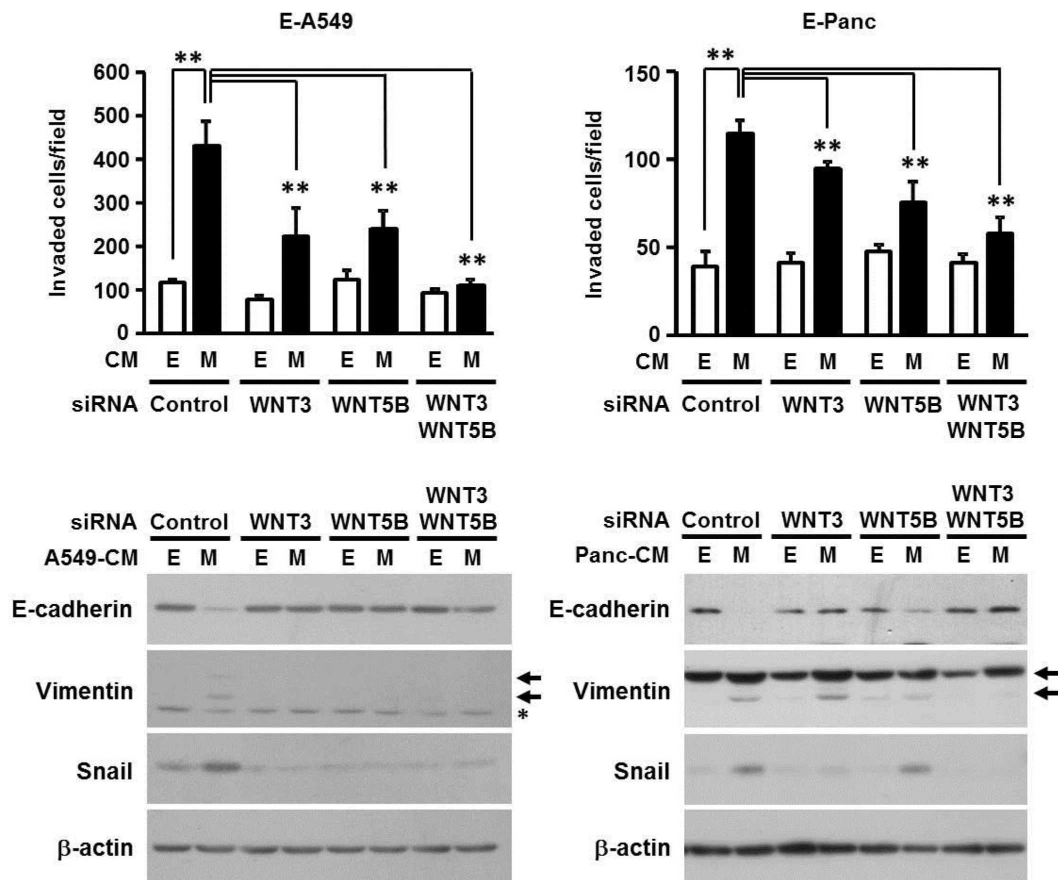


Figure 5. Critical requirement of secretory WNT3 and WNT5B from mesenchymal-transitioned cancer cells for inducing secondary-EMT phenotype of epithelical cancer cells. (Continued)

(D) E-A549 (left) and E-Panc (right) cells were treated with WNT-depleted CMs derived from E- or M-cells either by siRNAs against WNT3/WNT5B for 48 hr. The cells were subjected to Matrigel invasion assay and western blotting. **p<0.01 by one-way ANOVA with Bonferroni correction. Arrows and asterisk in western blotting indicate vimentin and non-specific bands, respectively.

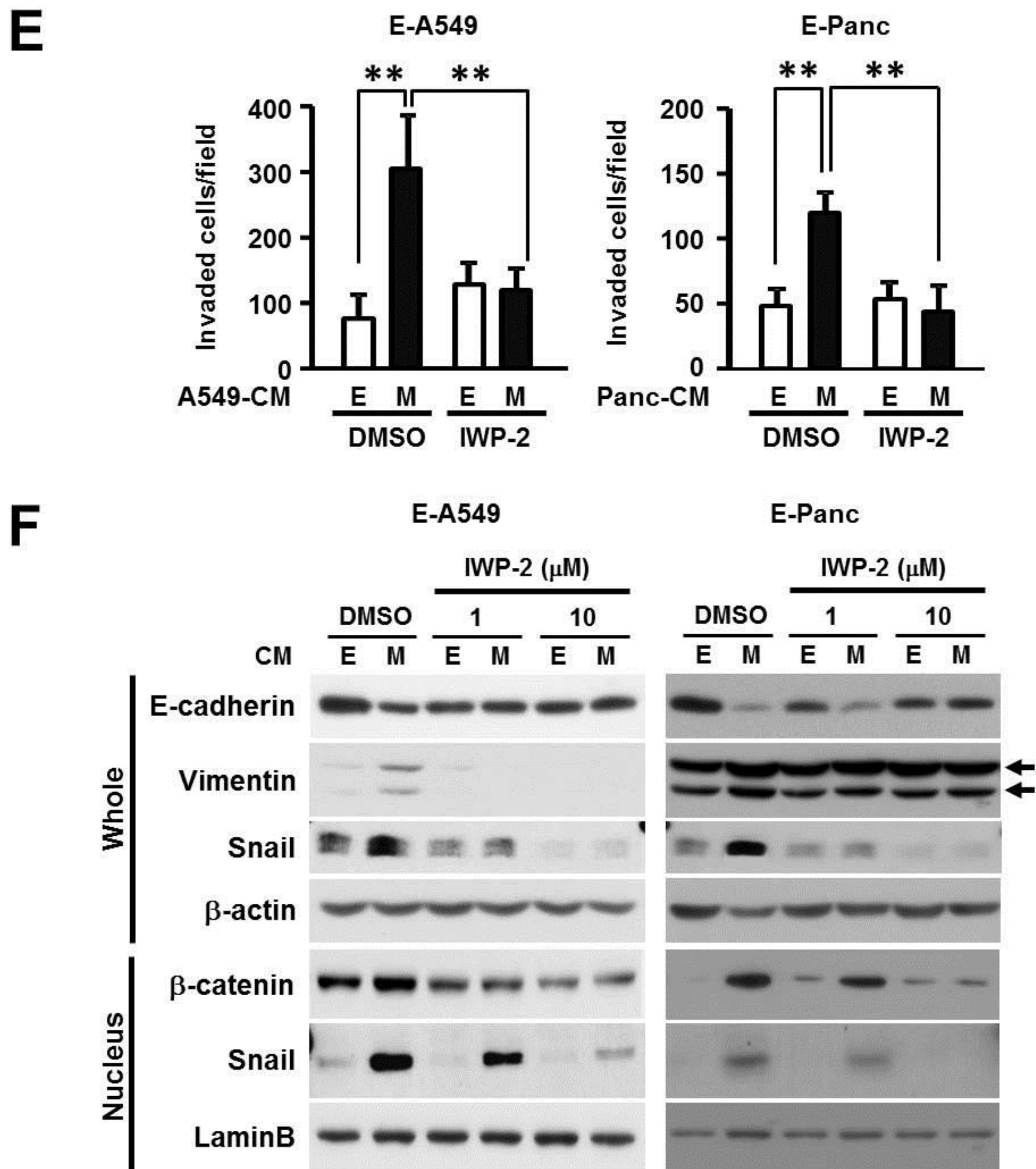


Figure 5. Critical requirement of secretory WNT3 and WNT5B from mesenchymal-transitioned cancer cells for inducing secondary-EMT phenotype of epithelical cancer cells. (Continued)

(E) E-A549 (left) and E-Panc (right) cells were treated with WNT-depleted CMs derived from E- or M-cells either by 10 μM WNT secretion inhibitor (IWP-2) for 48 hr. The cells were subjected to Matrigel invasion assay and western blotting. **$p<0.01$ by one-way ANOVA with Bonferroni correction.(F) E-A549 or E-Panc cells were treated with WNT-depleted CMs derived from E-cells or M-cells by the indicated dose of IWP-2 for 48 hr and the expression of proteins were determined by western blotting. Both arrows in western blotting indicate vimentin expression

Finally, we have tested whether the mesenchymal-transitioned cancer cells can instigate metastatic spread of neighboring epithelial cancer cells through providing secretory WNT3 and WNT5B ligands. Consistent with the induction of invasiveness and secondary-EMT phenotype *in vitro*, the lung colonization of E-A549/Luc2 cells co-cultured with M-A549 cells was largely enhanced compare to that of E-A549/Luc2 cells co-cultured with E-A549 cells (Fig. 6). Such increased lung colonization was fully abrogated when E-A549/Luc2 cells co-cultured with M-A549 cells in the presence of IWP-2.

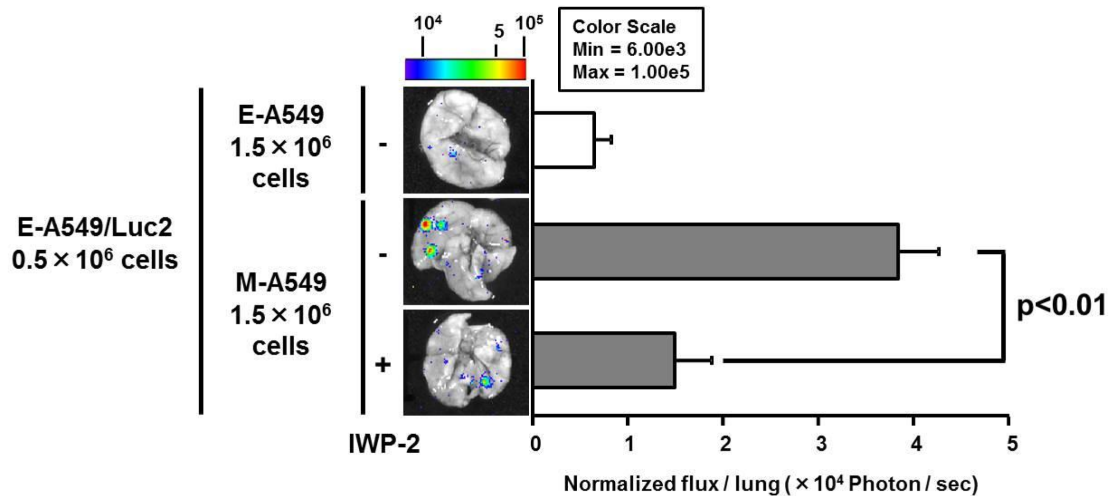


Figure 6. Secretory WNT-dependent metastasis instigation of epithelial cancer cells by mesenchymal-transitioned cancer cells.
E-A549 cells were co-cultured with either control (E-A549) cells or mesenchymal-transitioned A549 (M-A549) cells in the presence or absence of IWP-2 for 48 hr and i.v. inoculated into mice. Mice were sacrificed 24 hr after the tumor inoculation and lungs were subjected to bioluminescent imaging to determine total flux (photon/sec) for lung metastasis quantification. The representative *ex vivo* images are shown. Data represented as the mean ± SEM (n=3-5).

Collectively, these results strongly indicate that the secretory WNT3 and WNT5B derived from mesenchymal-transitioned cancer cells are able to enhance the metastatic potential of neighboring epithelial cancer cell *in vivo*.

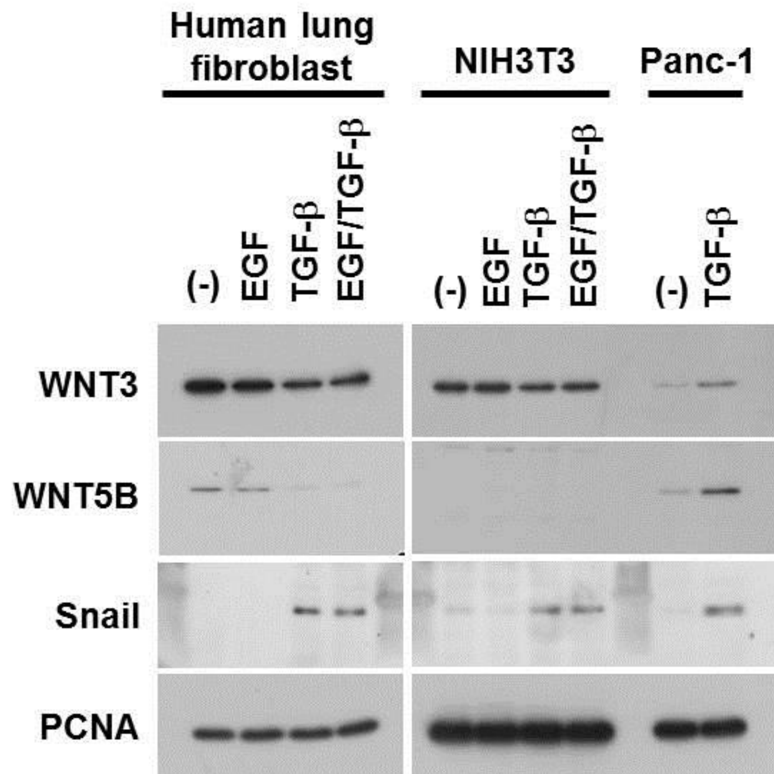


Figure S1. The up-regulation of WNT3 and WNT5B depends on TGF-β-induced EMT in cancer cells.

Human lung fibroblast, mouse fibroblast (NIH3T3) and Panc-1 cells were treated with EGF (20 ng/ml) or/and TGF-β (5 ng/ml) for 48 hr. Whole cell lysate was harvested and subjected to western blotting for detection WNT3, 5B and Snail protein expression. PCNA was used as loading control. EGF is also known as EMT-inducer [25].

5. Discussion

In this study, we demonstrated the evidence that the mesenchymal-transitioned cancer cells instigate invasive ability and metastatic potential of the neighboring epithelial cancer cells *in vitro* and *in vivo*. Furthermore, we determined that WNT3 and WNT5B are critical factors secreted from TGF-β-induced mesenchymal cancer cells for such metastasis instigation in the epithelial cancer cells along with the introduction of secondary-EMT phenotype. These results shed the light on the significance of cancer heterogeneities within tumor microenvironment and the interaction between epithelial and mesenchymal-transitioned cancer cells in promoting metastasis disease.

Considering the significance of *in vivo* metastatic ability of epithelial cancer cells upon co-culture with mesenchymal-transitioned cancer cells (Fig. 2 and Fig. 6), there are additional effects on epithelial cancer cells other than inducing invasive ability and secondary-EMT phenotype through the secretion of WNT ligands by neighboring mesenchymal-transitioned cancer cells. Firstly, it is reported that mesenchymal-transitioned cancer cells play a unique role in escorting epithelial cancer cells to metastatic organ *in vivo* [18]. Secondly, the

interaction with other host stromal cells, such as platelets or fibroblasts, might be involved in cancer metastasis by preventing cancer cells from cellular stresses, inducing EMT within the blood stream [28], or leading to collective cell invasion through gap junctions or integrins [29, 30]. Thirdly, WNT-ligands are known to not only enhance the invasiveness or secondary-EMT of epithelial cancer cells but also affect the multiple steps in cancer metastasis and progression. Along with the paracrine WNT signaling as seen in this study, the autocrine WNT signaling of mesenchymal-transitioned cancer cells has been reported to contribute to the maintenance of mesenchymal phenotypes and stem cell-like states in breast cancer [31]. It has also reported that WNT-signaling is involved in the expression of matrix metalloproteinases for digesting extracellular matrix during intra- or extra-vasation of metastasis process [32], niche formation [33], and enhancement of anchorage-independent sphere formation to increase metastatic ability of pancreatic cancer cells [34]. Collectively, those evidences might implicate that not only the cell-contact independent interaction but also the involvement of direct cell-cell interaction between epithelial cancer cells and mesenchymal-transitioned cancer cells play important roles in the regulation of metastasis process.

Although WNT3 and WNT5B secreted from mesenchymal-transitioned cancer cells are indispensable to induce invasiveness and secondary EMT in neighboring epithelial cancer cells (Fig. 5), we have also identified other gene candidates of secretory proteins those include other known-inducers of cancer cell invasiveness and EMT such as CXCL12 [35], LOX/LOXL2 [36], and HB-EGF [37]. Those candidates of secretory proteins from mesenchymal-transitioned cancer cells might also contribute to induce invasiveness and secondary-EMT in epithelial cancer cells by cooperating with WNT-ligands therefore further studies should be required in this context.

Figure 1 shows that the behavior of Snail expression over time in culture with TGF-β is different between A549 and Panc-1 cells, suggesting that the mechanism of the maintenance of Snail expression and leading EMT phenotype could be different between both cell lines. Although the regulation of EMT through snail is still unclear, the paracrine system driven by the secretion of WNT3 and WNT5B is a common event between both TGF-β-induced M-A549 and Panc-1 cells. TGF-β is one of the most potent inducer of EMT (Fig. 1) and metastasis; however, TGF-β pathway could be often genetically abrogated in relatively late-stage tumors because of the deletion or mutation of Smad4

(*DPC4*) or TGF-β receptors [16, 17, 38]. In addition, TGF-β-induced EMT is reversible unless long-time exposure to TGF-β as reported previously [39, 40]. We also clarified that M-cell-CM-dependent EMT was also reversible process at least in vitro (preliminary data). Thus, the heterogeneity of epithelial and mesenchymal-transitioned cancer cells might be maintained within tumor microenvironment through such dynamic cellular transition between E- and M-cell states. Considering TGF-β receptor kinase inhibitor did not affect in the induction of invasive ability and secondary-EMT phenotype in this study (Fig. 3F), the paracrine WNT stimulation can be an alternative inducer of EMT and metastasis to TGF-β in the cross-talk between mesenchymal-transitioned cancer cells and epithelial cancer cells. In this study, we focused on the WNT-ligands secreted from mesenchymal-transitioned cancer cells; however other stromal cells in cancer microenvironment might also produce WNT-ligands and therefore be involved in cancer metastasis process. Although we did not observe the induction of WNT3 and WNT5B by TGF-β stimulation in mouse NIH3T3 fibroblast or primary human lung fibroblasts (Fig. S1), it has been reported that up-regulation of WNT3A in cancer-associated fibroblasts (CAFs) could result in the aggressive progression of prostate tumor [41]. Besides secretion of

WNT-ligands, the hyperactivation of WNT signaling pathway has been observed in highly metastatic lung, colon adenocarcinoma [42, 43], and pancreatic cancer [34]. In the context of the clinical significance, WNT3 was reported to associate with poor prognosis of non-small cell lung cancer [44], and to promote EMT in HER2-overexpressing breast cancer cells [45]. Although we cannot exclude the possibility that WNT5B need to be coordinated with WNT3 to induce cellular invasion, we suggest WNT5B can solely responsible for impaired instigation considering non-canonical WNT pathway through WNT5B is reported to be involved in inducing tumor invasion [46, 47]. Furthermore, WNT5A, a paralog of WNT5B, and its receptor (FZD3) are known to be involved in the promotion of cell motility through the activation of paracrine non-canonical WNT signaling in skin cancer [48]. Even though IWP-2 is a pan WNT ligand secretion inhibitor, our presented data by knock down both WNT3 or 5B with siRNA almost completely diminished the activity of M-cell CM to induce invasion of E-cells therefore these results strongly support the critical importance of both WNT3 and 5B as WNT ligands secreted from M-cells to induce of E-cell invasion. Given E-cell CM in the presence of IWP-2 downregulated Snail or nuclear β-catenin expression in E-A549 cells (Fig. 5D), we speculate that even E-cells may produce substantial

level of WNT ligand by which maintains Snail or β-catenin expression of E-cells in an autocrine manner. Collectively, WNT-ligands derived from cancer stromal cells as well as mesenchymal-transitioned cancer cells and subsequent activation of WNT-signaling pathway may play a significant role in the malignant behavior of cancer cells including metastatic spread to distant organ.

In conclusion, the intra-tumoral heterogeneity has been considered to be one of hallmarks in cancer malignancy and we newly identified secretory WNT-ligands from mesenchymal-transitioned cancer cells instigate the invasion of neighboring epithelial cancer cells. This novel function of WNT-signaling in cancer microenvironment could be an attractive target not only for the new therapeutic opportunity but also for the new biomarker candidate in metastatic disease.

Chapter 2: ***Unpublished study***

Chapter 2 focuses on the cross-talk between M-cells each other. Since the data in chapter 2 is unpublished March, 2014 now, chapter 2 is going to disclose when this study is published.

Chapter 3: Procyanidin C1 from Cinnamomi Cortex inhibits TGF-β-induced epithelial-to-mesenchymal transition in A549 lung cancer cell lines.

11. Abstract

Cancer metastasis is one of the most critical events in cancer patients, and the median overall survival of Stage-IIIb or -IV patients with metastatic lung cancer in the TNM classification is only 8 or 5 months, respectively. We previously identified that *Juzentaihoto*, a Japanese traditional medicine, can inhibit cancer metastasis through the activation of macrophages and T cells in mouse cancer metastatic models; however, it is not well known how *Juzentaihoto* directly affects tumor cells during the metastasis process and which herbal medicines from *Juzentaihoto* inhibit metastatic potential. In this study, we focus on epithelial-to-mesenchymal transition (EMT), which plays an important role in the formation of cancer metastasis. We newly determined that only Cinnamomi Cortex (CC) extract, one of 10 herbal medicines of *Juzentaihoto*, inhibits TGF-β-induced EMT. Moreover, the contents of catechin trimer in CC extracts were significantly correlated with the efficacy of inhibiting TGF-β-induced EMT. Finally, the structure of the catechin trimer from CC extract was chemically

identified as procyanidin C1 and the compound showed inhibitory activity against TGF-β-induced EMT. This illustrates that procyanidin C1 is the main active compound in CC extract for EMT inhibition and that procyanidin C1 could be useful as a lead compound to develop inhibitors of cancer metastasis and other diseases related to EMT.

Key words: Cinnamomi cortex, epithelial-to-mesenchymal transition, lung cancer, procyanidin C1

12. Introduction

The essential characteristics of cancer are the ability to invade surrounding tissues and metastasize to distal tissues, known as cancer metastasis, which is the major cause of mortality in cancer patients. In non-small-cell lung cancer (NSCLC) patients, the median overall survival of patients with metastatic lung cancer (Stage IIIb or IV in the TNM classification) is limited (only 8 or 5 months, respectively) [66]; therefore, suppression of cancer metastasis results in the improved survival of lung cancer patients.

Cancer metastasis consists of several steps: intravasation, attachment to a vessel, extravasation, angiogenesis, and growth in distal tissues [67-70]. Among them, epithelial-to-mesenchymal transition (EMT) is involved in an early step of metastasis [71, 72]. EMT is a phenomenon in which cobblestone-like epithelial cells change into spindle-like mesenchymal cells with downregulation of epithelial markers such as E-cadherin and also upregulation of mesenchymal markers such as N-cadherin [73]. In addition to metastasis, this physiological phenomenon is important for resistance to apoptosis, maintenance of cancer stem cells and production of extracellular matrix. Thus, EMT would be an

attractive therapeutic target in metastatic cancers.

Juzentaihoto, which is a Japanese Kampo medicine, has been widely used for the decline of physical strength, general debility, cold hands and feet, fatigue, night sweats, circulatory problems, and anemia [74]. Interestingly, our previous studies showed that *Juzentaihoto* indirectly inhibited cancer metastasis through the activation of macrophages and T cells in mouse models [75-77]. Although *Juzentaihoto* could suppress cancer metastasis, it is not well known how *Juzentaihoto* can directly affect tumor cells during the metastasis process and which herbal medicines of *Juzentaihoto* are involved in the regulation of EMT.

Here we firstly report that Cinnamomi cortex (CC), one of the herbal medicines from *Juzentaihoto*, inhibits TGF-β-induced EMT phenotypes. After fractionation of CC extract, the content of catechin trimer was well-associated with the inhibitory activities of CC extract. Finally, procyanidin C1 from CC extract is newly identified as the responsible molecule for the EMT inhibition.

13. Materials and Methods

13.1 Herbal medicine extraction

Astagali Radix (AsR), Glycyrrhizae Radix (GlR), Cinnamomi Cortex extract (CC), Rehmanniae Radix (RR), Paeoniae Radix (PR), Cnidii Rhizoma (CR), Angelicae Radix (AnR), Ginseng Radix (GiR), Hoelen (Ho), Atractylodis Lanceae Radix (ALR), and Spatholobi Caulis (SC) were purchased from Uchida-Wakanyaku Co.,Ltd (Tokyo, Japan). In this study, Cinnamomi Cortex was added to an appropriate volume of distilled water (w/v, 1:10) and extracted at 100 °C for 1 hour. The extracted solution was filtered and then freeze dried to obtain dried powder. A voucher sample of this extract (INM 10000007, University of Toyama) was preserved in the Research Promotion Office, Institute of Natural Medicine, University of Toyama, Toyama, Japan. Six other Cinnamomi Cortex samples were collected from different regions of Vietnam and China, coded as CC-1 (Vietnam), CC-2 (Guang Xi, China), CC-3 (Guang Xi, China), CC-4 (Guang Dong, China), CC-5 (Guang Xi, China), CC-6 (Vietnam). Water extracts of the above-mentioned six CC samples were prepared and provided by the National Institute of Biomedical Innovation, Osaka, Japan, and a part of each extract was

deposited at our institute (voucher specimen no. INM 10000001-10000006).

13.2 Cell Cultures

Human lung adenocarcinoma A549 cells were cultured in RPMI Medium 1640 (Invitrogen, Carlsbad, CA, USA) with 10 % fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5 % CO_2 at 37 ˚C. The cells were treated with recombinant human TGF-β (5 ng/ml) (Peprotech, London, UK) for various times as indicated, after pretreatment with TGF-β receptor I kinase inhibitor (10 μM) (Merck, Whitehouse, NJ, USA), each herbal medicine from *Juzentaihoto*, or Cinnamomi Cortex extracts (CC, or CC-1 to CC-6) for 30 min.

13.3 Protein Preparation and Western Blotting

Whole cell lysates were collected in lysis buffer supplemented with some protease and phosphatase inhibitors as described previously {Sakurai, 2003}. Equal amounts of protein were resolved by electrophoresis on acrylamide gels and transferred to PVDF membranes. Antibodies against phospho-specific Smad-2 (Ser 465/467), E-cadherin, and N-cadherin were purchased from Cell

Signaling Technology (Beverly, MA, USA) and an antibody against PCNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

13.4 Cell morphology and cell migration

Cell morphology was determined by BZ-8000 (Keyence, Osaka, Japan) after staining with hematoxylin and eosin. For the migration assay, Transwell cell culture chambers were used as described previously [78]. Briefly, the filters were precoated with 1.25 μg fibronectin on the lower surfaces. The cell suspension ($3x10^4$ cells/100 μl) in serum-free medium was added to the upper compartment and incubated for 6 h. The migrated cells were stained with hematoxylin and eosin, and counted under the microscope in three predetermined fields at a magnification of x400.

13.5 Fractionation of CC and identification of procyanidin C1 from CC

CC extract 2.0 g was subjected to reversed-phase silica gel (Cosmosil $75C_{18}$-OPN; Nacalai Tesque Inc., Kyoto, Japan) using medium pressure liquid chromatography (MPLC; Buchi, Flawil, Switzerland) with a H_2O–CH_3CN gradient system (98:2→96:4→92:8→90:10→80:20→60:40→40:60→10:90) to obtain

eight fractions (fr. 1, 435 mg; fr. 2, 282 mg; fr. 3, 123 mg; fr. 4, 212 mg; fr. 5, 105 mg; fr. 6, 305 mg; fr. 7, 205 mg; fr. 8, 105 mg). The bioactive fraction 4 (fr. 4, 200 mg) was further subjected to preparative HPLC (Discovery C18 column; 10 × 250 mm i.d., 5 μm particle size; Supelco, PA,USA) with H_2O–CH_3CN (92:8) containing 0.01% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min to yield a procyanidin trimer (**1**, 5.5 mg, t_R 23.3 min). The molecular formula of compound **1** was determined by HR-TOF-MS to be $C_{45}H_{38}O_{18}$ [*m/z* 865.2003 $(M - H)^+$]. Its chemical structure was further identified to be epicatechin-(4*β*→8)-catechin-(4*β*→8)-epicatechin (procyanidin C1, Fig. 4C) by comparing the [^{1}H] nuclear magnetic resonance (NMR), [^{13}C] NMR, and circular dichroism (CD) spectral data with those in the literature [79].

13.6 Liquid chromatography-mass spectrometry (LC-MS) analysis

For chemical profiling of 7 CC extracts, liquid chromatography-mass spectrometry (LC-MS) analysis was performed with a Shimadzu LC-IT-TOF mass spectrometer (Kyoto, Japan) equipped with an ESI interface (Shimadzu). The ESI parameters were as follows: source voltage +4.5 kV, capillary temperature 200 °C and nebulizer gas 1.5 l/min. The mass spectrometer was

operated in positive ion mode scanning from m/z 200 to 2000. A Waters Atlantis T3 column (2.1 mm i.d. × 150 mm, 3 m; Milford, MA, USA) was used and the column temperature was maintained at 40°C. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution and (B) CH3CN under the following gradient conditions: 0-30 minute linear gradient from 10 % to 100 % B, 30-40 min isocratic at 100 % B. The flow rate was 0.15 ml/min. Mass spectrometry data obtained from the extract were deposited in the MassBank Database and stored with pharmacological information on the extract in the Wakan-Yaku Database System, Institute of Natural Medicine, University of Toyama.

14. Results

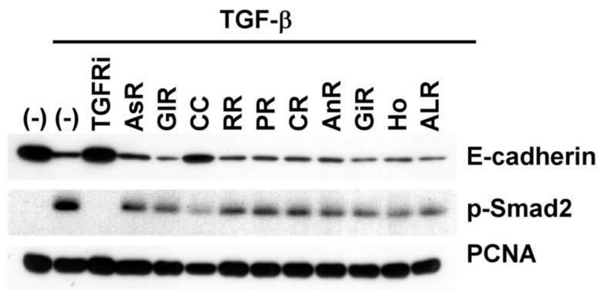
14.1 Cinnamomi Cortex (CC) extract suppresses TGF-β-induced EMT

Our previous studies showed that *Juzentaihoto*, a Japanese Kampo medicine, prevents metastasis in mouse models [75-77]. Because *Juzentaihoto* contains ten (“Ju" means “ten” in Japanese) kinds of herbal medicines, we thus investigated whether each herbal medicines can suppress TGF-β-induced EMT. We firstly screened the expression of an epithelial marker, E-cadherin, in a human non-small-cell lung cancer cell line, A549 cells, treated with TGF-β, TGF-β receptor kinase inhibitor (TGFRi) ,which used as a positive control, or herbal medicines (Fig. 11A). Known as EMT, the reduction of E-cadherin expression in A549 cells was detected after TGF-β treatment in A549 cells. Among the ten herbal medicines, only Cinnamomi Cortex (CC) extract strikingly suppressed TGF-β-induced EMT, as indicated by the restoration of E-cadherin. We could not detect any inhibition of the cell viabilities by CC extract

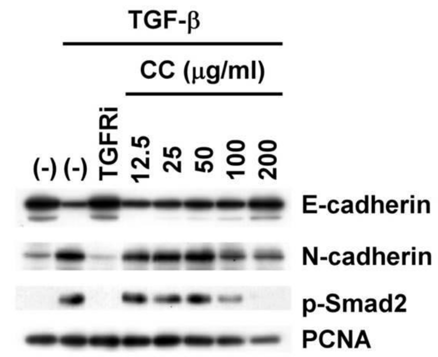
In order to further determine the inhibition of TGF-β-induced EMT by CC extract, E-cadherin, N-cadherin, and phosphorylated Smad-2 were examined. Similarly to Fig. 11A, restoration of E-cadherin expression by CC extract was

detected in a concentration/time-dependent manner (Fig. 11B and 11C). Interestingly, phosphorylation of Smad-2 by TGF-β, a downstream molecule of TGF-β signaling, was suppressed by CC extract after the early phase (10 min, 20 min) as well as the late phase (24 h, 48 h). We also detected the inhibition of TGF-β-induced EMT by CC extract at mRNA levels of *snail*, *E-cadherin*, and *fibronectin* (data not shown). In addition to expression levels of EMT markers, we observed morphological changes with CC extract (Fig. 11D). A549 cells treated with TGF-β showed spindle-like shapes as compared with untreated cells. On the other hand, TGF-β-stimulated cells treated together with CC extract showed cobblestone-like shapes similar to the untreated cells or the cells with TGFRi and TGF-β. Moreover, consistent with cell morphology, TGF-β-induced cell migration was also suppressed with CC extract (Fig. 11E). These results support that CC extract suppresses TGF-β-induced EMT in A549 cells and consequently inhibits cell migration.

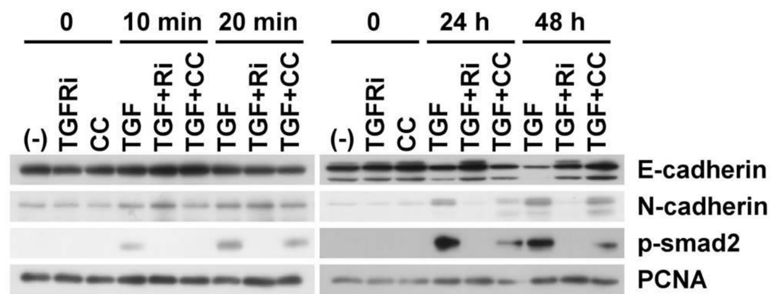
A



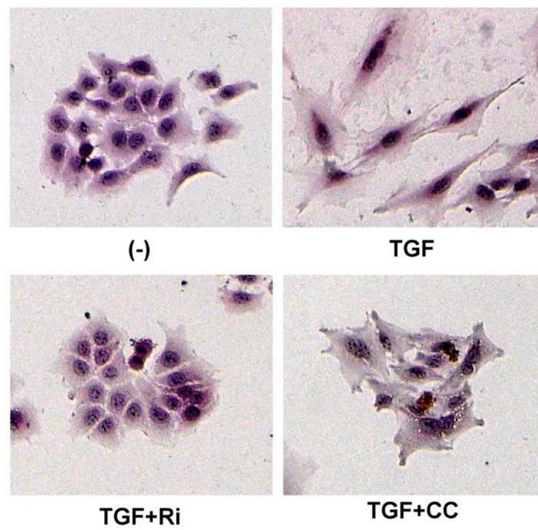
B



C



D



E

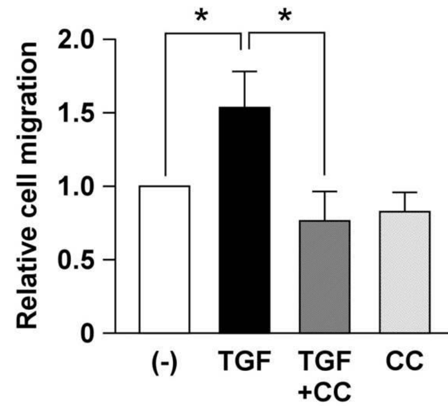


Figure 11. Cinnamomi Cortex (CC) suppresses TGF-β-induced EMT. (Left)

(A) Effects of ten herbal medicines from Juzentaihoto. AsR: Astagali Radix, GlR: Glycyrrhizae Radix, CC: Cinnamomi Cortex extract, RR: Rehmanniae Radix, PR: Paeoniae Radix, CR: Cnidii Rhizoma, AnR: Angelicae Radix, GiR: Ginseng Radix, Ho: Hoelen, ALR: Atractylodis Lanceae Radix. A549 cells were pretreated with various herbal medicines (100 μg/ml) or TGF-β receptor I kinase inhibitor (TGFRi; 10 μM) for 30 min, and stimulated with TGF-β (5 ng/ml) for 48 h. Expression of each protein was detected by Western blotting.

(B) Dose-dependent inhibition of TGF-β-induced EMT with CC treatment. A549 cells were pretreated with various concentrations of CC (12.5, 25, 50, 100, 200 μg/ml) or TGF-β receptor I kinase inhibitor (TGFRi; 10 μM) for 30 min, and stimulated with TGF-β (5 ng/ml) for 48 h. Expression of each protein was detected by Western blotting.

(C) Time-dependent inhibition of TGF-β-induced EMT with CC treatment. A549 cells were pretreated with CC (100 μg/ml) for 30 min and stimulated with TGF-β (5 ng/ml) for the indicated times. Expression of each protein was detected by Western blotting.

(D) Cell morphologies with CC treatment. A549 cells were stained by H&E after treatment as in Fig. 11C for 48 hr.

(E) Migration with CC treatment. A549 cells treated as in Fig. 11D were seeded in Transwell chambers for 6 h. No. of migrated cells per field (x400) was counted. Relative cell migration with TGF-β (TGF: filled bar), TGF-β with CC extract (TGF+CC: shadow bar), or CC extract (CC: dotted bar) was normalized by untreated cell migration (open bar). Data are the mean ± S.D. of four independent experiments. *$p<0.01$, compared among each pair, by one-way ANOVA and Bonferroni's multiple comparision test.

14.2 Identification of an active fraction inhibiting TGF-β-induced EMT

To gain insight into the variations of CC extracts in EMT-inhibitory activities, we examined six additional CC (CC-1 to -6) extracts, which differ according to the harvest location (Fig. 12A). CC-2, CC-3, and CC-5 extracts showed restoration of E-cadherin expression stronger than CC-1, CC-4, and CC-6 extracts; therefore, an active component(s) for the inhibition of EMT might be included in CC-2, CC-3, and CC-5 extracts more than in CC-1, CC-4, and CC-6.

To identify the active chemical compound(s) that is able to inhibit TGF-β-induced EMT, total ion chromatogram analysis was performed in CC and CC-1 to CC-6 extracts. Notably, the peaks of catechin-catechin-catechin trimer (catechin trimer) significantly overlapped with EMT inhibitory activities in CC-2, CC-3, and CC-5 extracts, but were not detected in CC-1, CC-4, and CC-6 extracts (Fig. 12B), suggesting that the catechin trimer may have some crucial roles in suppressing TGF-β-induced EMT.

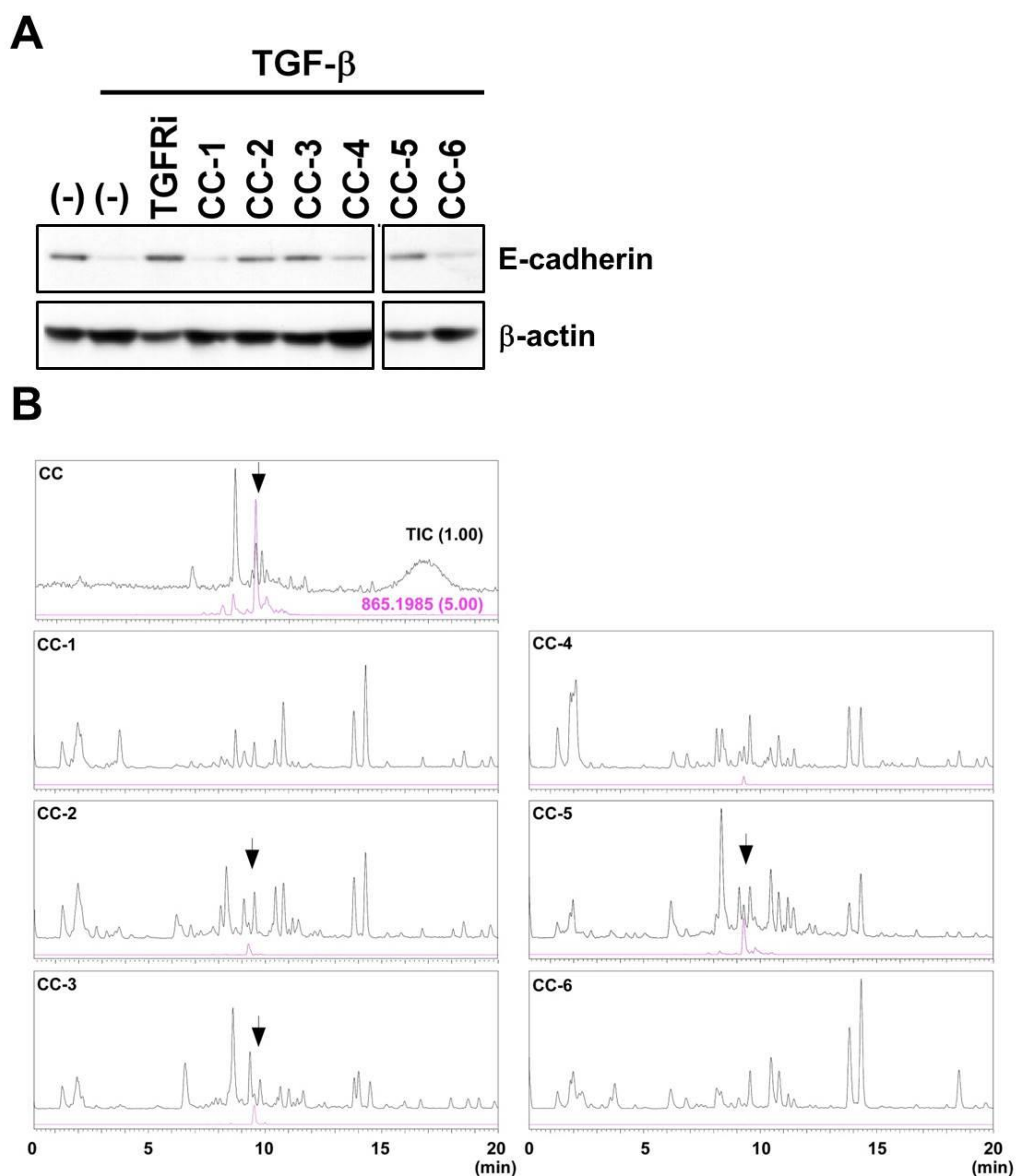


Figure 12. CCs show variability in the activity of TGF-β-induced EMT.

(A) Effects of various CCs. A549 cells were pretreated with various CCs (100 μg/ml), but other conditions were similar to Fig. 11C. Expression of each protein was detected by Western blotting.

(B) Inhibition activities of CCs correlated with the content of catechin trimer. Total ion chromatograms (TIC) profiles of various CC extracts are shown. Arrows show the catechin trimer fractions.

14.3 Procyanidin C1 can suppress TGF-β-induced EMT

Next to assess whether catechin trimer fraction of CC extract has activity to inhibit EMT, we examined the protein expression levels with the catechin trimer fraction from CC (Fig. 13A). As predicted, the catechin trimer fraction inhibited E-cadherin reduction and N-cadherin induction. Similarly to protein levels, the change of cell morphology (Fig. 13B) also supported the inhibition of TGF-β-induced EMT.

We finally purified the catechin trimer from CC and identified its structure, which was an epicatechin-epicatechin-epicatechin trimer, procyanidin C1 (Fig. 13C). To check the inhibition of EMT by procyanidin C1, the protein expression and cell migration activities were examined (Fig. 13D and 13E). Strikingly, EMT marker represented the inhibition of EMT in Western blotting, and TGF-β-induced cell migration was suppressed by procyanidin C1.

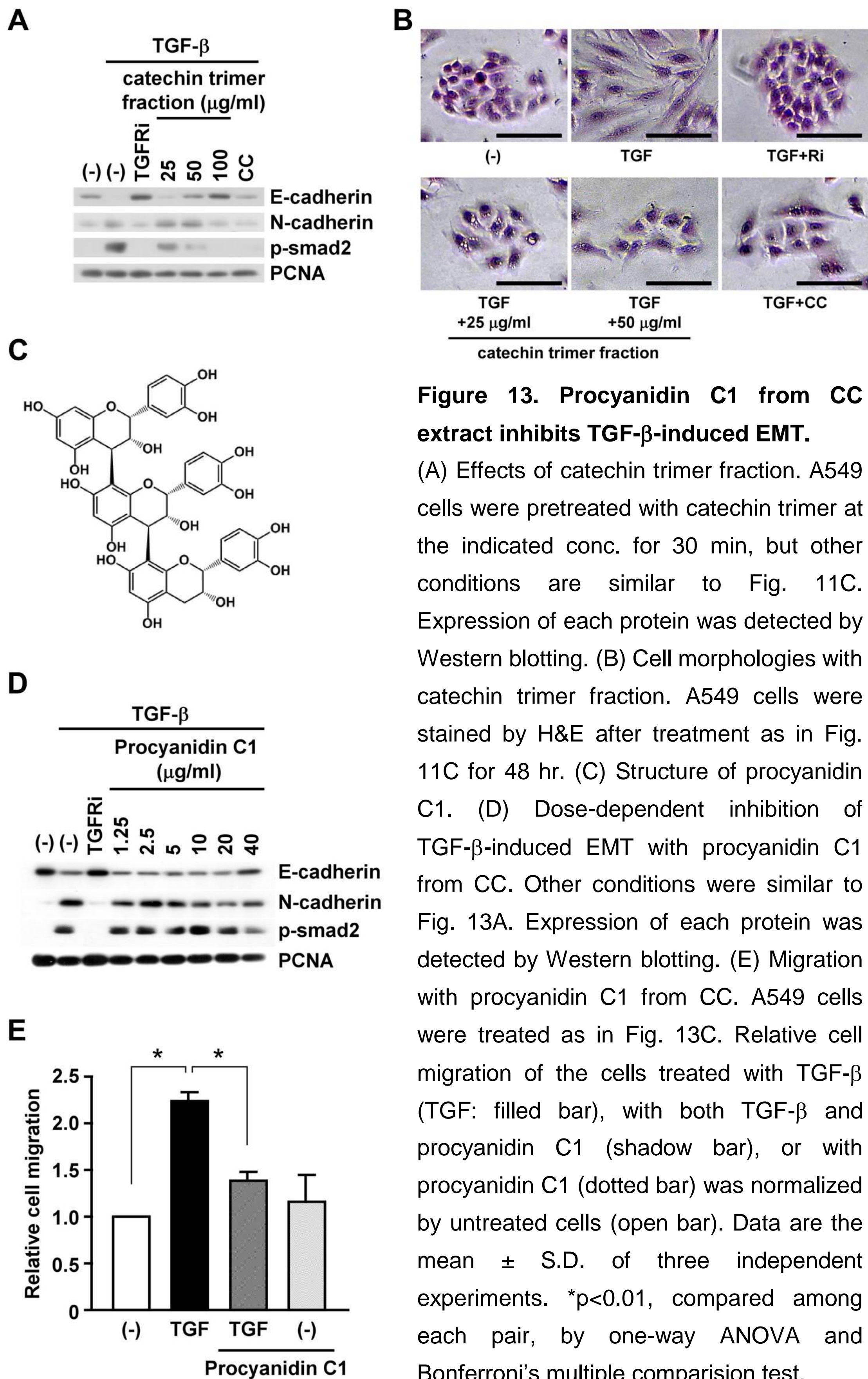


Figure 13. Procyanidin C1 from CC extract inhibits TGF-β-induced EMT.

(A) Effects of catechin trimer fraction. A549 cells were pretreated with catechin trimer at the indicated conc. for 30 min, but other conditions are similar to Fig. 11C. Expression of each protein was detected by Western blotting. (B) Cell morphologies with catechin trimer fraction. A549 cells were stained by H&E after treatment as in Fig. 11C for 48 hr. (C) Structure of procyanidin C1. (D) Dose-dependent inhibition of TGF-β-induced EMT with procyanidin C1 from CC. Other conditions were similar to Fig. 13A. Expression of each protein was detected by Western blotting. (E) Migration with procyanidin C1 from CC. A549 cells were treated as in Fig. 13C. Relative cell migration of the cells treated with TGF-β (TGF: filled bar), with both TGF-β and procyanidin C1 (shadow bar), or with procyanidin C1 (dotted bar) was normalized by untreated cells (open bar). Data are the mean ± S.D. of three independent experiments. *$p<0.01$, compared among each pair, by one-way ANOVA and Bonferroni's multiple comparision test.

15. Discussion

In this study, we firstly identified Cinnamomi Cortex (CC) extract in 10 herbal medicines from *Juzentaihoto* as the only inhibitory herbal medicine of TGF-β-induced epithelial-to-mesenchymal transition (EMT) in a human non-small-cell lung cancer cell line, A549 cells. The inhibitory effects of CC extracts could be derived from the procyanidin C1 because of the correlation of the content of procyanidin C1 in CC extract with their inhibitory activities.

The inhibitory effect of CC extract on EMT phenotypes we showed here might be supported by the evidences of *Ninjinyoeito* and *Keishibukuryogan*, both of which are other Kampo medicines containing CC. *Ninjinyoeito* has also been reported to suppress cancer metastasis [80]. On the other hand, *Keishibukuryogan* has been shown to suppress renal fibrosis, which is caused by TGF-β-induced EMT [81]. Despite the inhibitory activity of CC extract, the possibility cannot be excluded that other herbal medicines might play a role in concert with CC extract, because of the harmonization effects among various herbal medicines [76, 77].

Even though we identified procyanidin C1 as an active compounds for the inhibition of EMT, but CC extract contains various chemical compounds,

such as phenyl propanoids (cinnamic acid, cinnamaldehyde), terpenoids (cinnamonol, cassioside), and tannin (epicatechin), etc [82]. There are many reports about the biological activities of cinnamic acid or cinnamaldehyde, for example, induction of ROS-mediated apoptosis [83]. In addition, the procyanidin C1 we purified is known for its anti-inflammatory effects [84, 85]; however, it is the first time to show that procyanidin C1 has anti-metastatic or anti-EMT effects. Among the catechin sub-family, epigallocatechin-3 gallate in green tea has been reported to inhibit EMT in human melanoma cells [86, 87]. These reports raise the possibility that another catechin sub-family is involved in the inhibition of EMT besides procyanidin C1. The inhibition of cell migration by procyanidin C1 (Fig. 13E) and the clear linkage of the amount of procyanidin C1 to the inhibition of TGF-β-induced EMT (Fig. 13A and B) suggest that procyanidin C1 is still the main compound in CC extracts to inhibit cell migration and EMT.

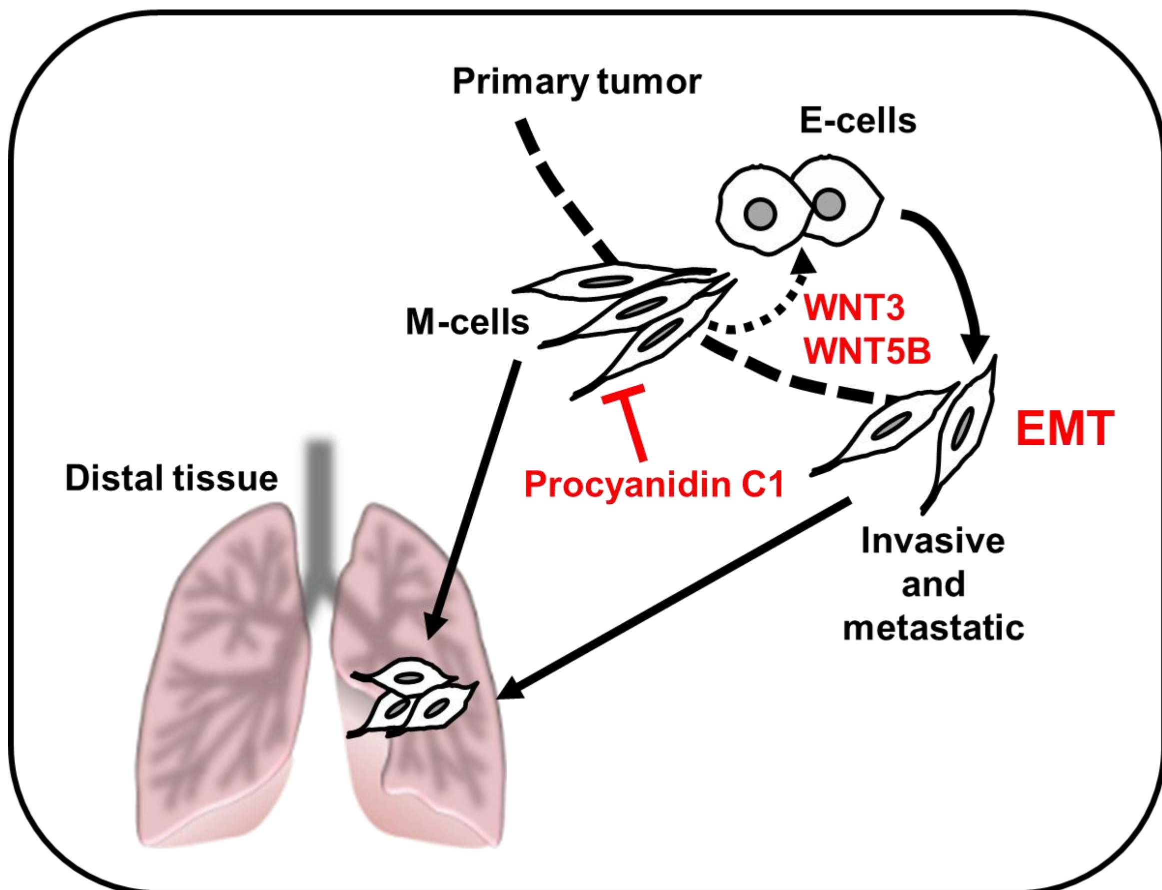
Although we need more efforts to identify the target molecule of procyanidin C1, the phosphorylation of Smad-2 (Fig. 13D) and its transcriptional activity (data not shown) were suppressed. Indeed, CC extract and procyanidin C1 did not affect basal cell migration in A549 cells (CC alone or procyanidin C1 alone in Fig. 11E and 13E), suggesting that the inhibition of EMT by CC extract

and procyanidin C1 is dependent on inhibition of the TGF-β signal pathway and that mesenchymal-to-epithelial transition is not induced by CC extract or procyanidin C1, which might affect basal levels of cell migration.

Collectively, our data firstly identified CC in *Juzentaihoto* as the only herbal medicine inhibiting TGF-β-induced EMT, which could be worthy of clinical study in a variety of settings associated with EMT, including cancer metastasis and/or tissue fibrosis. As procyanidin C1 showed inhibition of EMT, it is suggested that the utilization of procyanidin C1 as a lead compound would be attractive for the development of cancer metastasis inhibitors. Also shown in chapter 1 and 2, inhibition of TGF-β-induced EMT by procyanidin C1 could prevent the cross-talk between mesenchymal-transitioned cancer cells and surrounding cancer cells.

16. General conclusion

This study focused on biological role of the cross-talk between mesenchymal-transitioned cancer cells and surrounding cancer cells on tumor malignant progression. Based on the results, this study is summarized below.



***Chapter 1*:**

Mesenchymal-transitioned cancer cells (M-cell) instigate epithelial cancer cells (E-cell) to invasion and subsequent metastasis in association with secondary-EMT phenotypes in WNT-dependent manner.

***Chapter 2*:**

Unpublished

***Chapter 3*:**

Procyanidin C1 inhibits TGF-β-induced EMT and could prevent subsequent malignant progression through cross-talks between M-cell and E-cell/M-cell.

Overall, these novel findings demonstrate the dynamic regulation of EMT/MET within tumor microenvironment through the cross-talk between heterogeneous cancer cells, which leads to tumor malignant progression.

17. References

[1] Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009; **9**: 239-52.

[2] Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer. 2003; **3**: 453-8.

[3] Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer. 2012; **12**: 323-34.

[4] Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002; **2**: 442-54.

[5] Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell. 2012; **22**: 725-36.

[6] Ledford H. Cancer theory faces doubts. Nature. 2011; **472**: 273.

[7] Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science. 2012; **331**: 1559-64.

[8] Brabletz T, Jung A, Reu S, et al. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment.

Proc Natl Acad Sci U S A. 2001; **98**: 10356-61.

[9] Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. Nat Cell Biol. 2009; **11**: 1287-96.

[10] Bonnomet A, Syne L, Brysse A, et al. A dynamic in vivo model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer. Oncogene. 2012; **31**: 3741-53.

[11] Massague J. TGFbeta in Cancer. Cell. 2008; **134**: 215-30.

[12] Franci C, Takkunen M, Dave N, et al. Expression of Snail protein in tumor-stroma interface. Oncogene. 2006; **25**: 5134-44.

[13] Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer. 2005; **5**: 744-9.

[14] Rhim AD, Mirek ET, Aiello NM, et al. EMT and dissemination precede pancreatic tumor formation. Cell. 2012; **148**: 349-61.

[15] Morris JPt, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nat Rev Cancer. 2010; **10**: 683-95.

[16] Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev. 2006; **20**: 1218-49.

[17] Rasheed ZA, Yang J, Wang Q, et al. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. J Natl Cancer Inst. 2010; **102**: 340-51.

[18] Tsuji T, Ibaragi S, Shima K, et al. Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth. Cancer Res. 2008; **68**: 10377-86.

[19] Axelrod R, Axelrod DE, Pienta KJ. Evolution of cooperation among tumor cells. Proc Natl Acad Sci U S A. 2006; **103**: 13474-9.

[20] Grunewald TG, Herbst SM, Heinze J, Burdach S. Understanding tumor heterogeneity as functional compartments--superorganisms revisited. J Transl Med. 2011; **9**: 79.

[21] Calbo J, van Montfort E, Proost N, et al. A functional role for tumor cell heterogeneity in a mouse model of small cell lung cancer. Cancer Cell. 2011; **19**: 244-56.

[22] Saiki I, Murata J, Watanabe K, Fujii H, Abe F, Azuma I. Inhibition of

tumor cell invasion by ubenimex (bestatin) in vitro. Jpn J Cancer Res. 1989; **80**: 873-8.

[23] Sakurai H, Suzuki S, Kawasaki N, et al. Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappaB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. J Biol Chem. 2003; **278**: 36916-23.

[24] Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. Nat Genet. 2006; **38**: 500-1.

[25] Horiguchi K, Shirakihara T, Nakano A, Imamura T, Miyazono K, Saitoh M. Role of Ras signaling in the induction of snail by transforming growth factor-beta. J Biol Chem. 2009; **284**: 245-53.

[26] Kim JH, Jang YS, Eom KS, et al. Transforming growth factor beta1 induces epithelial-to-mesenchymal transition of A549 cells. J Korean Med Sci. 2007; **22**: 898-904.

[27] Benhaj K, Akcali KC, Ozturk M. Redundant expression of canonical Wnt ligands in human breast cancer cell lines. Oncol Rep. 2006; **15**: 701-7.

[28] Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes

metastasis. Cancer Cell. 2011; **20**: 576-90.

[29] Friedl P, Locker J, Sahai E, Segall JE. Classifying collective cancer cell invasion. Nat Cell Biol. 2012; **14**: 777-83.

[30] Gaggioli C, Hooper S, Hidalgo-Carcedo C, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol. 2007; **9**: 1392-400.

[31] Scheel C, Eaton EN, Li SH, et al. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell. 2011; **145**: 926-40.

[32] Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer. 2013; **13**: 11-26.

[33] Malanchi I, Santamaria-Martinez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. Nature. 2011; **481**: 85-9.

[34] Yu M, Ting DT, Stott SL, et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. Nature. 2012; **487**: 510-3.

[35] Li X, Ma Q, Xu Q, et al. SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and epithelial-mesenchymal transition in vitro through

non-canonical activation of Hedgehog pathway. Cancer Lett. 2012; **322**: 169-76.

[36] Peinado H, Portillo F, Cano A. Switching on-off Snail: LOXL2 versus GSK3beta. Cell Cycle. 2005; **4**: 1749-52.

[37] Yagi H, Yotsumoto F, Miyamoto S. Heparin-binding epidermal growth factor-like growth factor promotes transcoelomic metastasis in ovarian cancer through epithelial-mesenchymal transition. Mol Cancer Ther. 2008; **7**: 3441-51.

[38] Yanagisawa K, Uchida K, Nagatake M, et al. Heterogeneities in the biological and biochemical functions of Smad2 and Smad4 mutants naturally occurring in human lung cancers. Oncogene. 2000; **19**: 2305-11.

[39] Gregory PA, Bracken CP, Smith E, et al. An autocrine TGF-beta/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition. Mol Biol Cell. 2011; **22**: 1686-98.

[40] Dong C, Wu Y, Yao J, et al. G9a interacts with Snail and is critical for Snail-mediated E-cadherin repression in human breast cancer. J Clin Invest. 2012; **122**: 1469-86.

[41] Li X, Placencio V, Iturregui JM, et al. Prostate tumor progression is mediated by a paracrine TGF-beta/Wnt3a signaling axis. Oncogene. 2008; **27**:

7118-30.

[42] Nguyen DX, Chiang AC, Zhang XH, et al. WNT/TCF signaling through LEF1 and HOXB9 mediates lung adenocarcinoma metastasis. Cell. 2009; **138**: 51-62.

[43] Singh A, Sweeney MF, Yu M, et al. TAK1 inhibition promotes apoptosis in KRAS-dependent colon cancers. Cell. 2012; **148**: 639-50.

[44] Nakashima N, Liu D, Huang CL, Ueno M, Zhang X, Yokomise H. Wnt3 gene expression promotes tumor progression in non-small cell lung cancer. Lung Cancer. 2012; **76**: 228-34.

[45] Wu Y, Ginther C, Kim J, et al. Expression of Wnt3 activates Wnt/beta-catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. Mol Cancer Res. 2012; **10**: 1597-606.

[46] Deraz EM, Kudo Y, Yoshida M, et al. MMP-10/stromelysin-2 promotes invasion of head and neck cancer. PLoS One. 2011; **6**: e25438.

[47] Klemm F, Bleckmann A, Siam L, et al. beta-catenin-independent WNT signaling in basal-like breast cancer and brain metastasis. Carcinogenesis. 2011; **32**: 434-42.

[48] Pourreyron C, Reilly L, Proby C, et al. Wnt5a is strongly expressed at the leading edge in non-melanoma skin cancer, forming active gradients, while canonical Wnt signalling is repressed. PLoS One. 2012; **7**: e31827.

[49] Nieto MA. Epithelial plasticity: a common theme in embryonic and cancer cells. Science. 2013; **342**: 1234850.

Reference [55]-[65] are contained in chapter 2.

[66] Ou SH, Zell JA. Validation study of the proposed IASLC staging revisions of the T4 and M non-small cell lung cancer descriptors using data from 23,583 patients in the California Cancer Registry. J Thorac Oncol. 2008; **3**: 216-227.

[67] Fidler IJ. The evolution of biological heterogeneity in metastatic neoplasms. In: Cancer Invasion and Metastases: Biologic and Therapeutic Aspects, Nicolson GL and Milas L (ed.) Raven Press, New York. 1984: pp. 5-26.

[68] Nicolson GL. Tumour cell instability, diversification, and progression to the metastatic phenotype from oncogene to oncofetal expression. Cancer Res. 1987; **47**: 1473-1487.

[69] Hart IR. Seed and soil revisited: mechanisms of site specific metastasis. Cancer Metastasis Rev. 1982; **1**: 5-16.

[70] Liotta LA, Rao CV, Barsky SH. Tumour invasion and the extracellular matrix. Lab Invest. 1983; **49**: 636-649.

[71] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009; **119**: 1420-1428.

[72] Guarino M. Epithelial-mesenchymal transition and tumor invasion. Int J Biochem Cell Biol. 2007; **39**: 2153-2160.

[73] Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. Cell Res. 2009; **19**: 156-172.

[74] Maruyama H, Kawamura H, Takemoto N, Komatsu Y, Aburaya M, Hosoya E. Effect of Juzentaihoto on phagocytes. Jpn J Inflammation. 1988; **8**: 461-465.

[75] Ohnishi Y, Fujii H, Hayakawa Y, et al. Oral Administration of a Kampo (Japanese Herbal) Medicine *Juzentaihoto* Inhibits Liver Metastasis of Colon 26-L5 Carcinoma Cells. Jpn J Cancer Res. 1998; **89**: 206–213.

[76] Ohnishi Y, Yamaura T, Tauchi K, et al. Expression of the anti-metastatic effect induced by Juzentaihoto is based on the content of Shimotsu-to constituents. Biol Pharm Bull. 1998; **7**: 761-765.

[77] Saiki I. A Kampo Medicine "*Juzentaihoto*"--Prevention of Malignant Progression and Metastasis of Tumor Cells and the Mechanism of Action. Biol Pharm Bull. 2000; **23**: 677-688.

[78] Ogasawara M, Murata J, Ayukawa K, Saiki I. Differential effect of intestinal neuropeptides on invasion and migration of colon carcinoma cells in vitro. Cancer Lett. 1997; **119**: 125-130.

[79] Esatbeyoglu T, Jaschok-Kentner B, Wray V, Winterhalter P. Structure elucidation of procyanidin oligomers by low-temperature 1H NMR spectroscopy. J Agric Food Chem. 2011; **59**: 62-69.

[80] Kamei T, Kumano H, Iwata K, Nariai Y, Matsumoto T. The effect of a traditional Chinese prescription for a case of lung carcinoma. J Altern Complement Med. 2000; **6**: 557-579.

[81] Nakagawa T, Tashiro I, Fujimoto M, et al. Keishibukuryogan reduces renal injury in the early stage of renal failure in the remnant kidney model. Evid Based Complement Alternat Med. 2011: 914249.

[82] Amagaya S. Keihi. Kampo and the newest treatment. 2006; 15: 62-63.

[83] Ka H, Park HJ, Jung HJ, et al. Cinnamaldehyde induces apoptosis by

ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. Cancer Lett. 2003; **196**: 143-152.

[84] Nakano N, Nishiyama C, Tokura T, et al. Procyanidin C1 from apple extract inhibits FcεRI-mediated mast cell activation. Int Arch Allergy Immunol. 2008; **147**: 213–221.

[85] Terra X, Palozza P, Fernandez-Larrea J, et al.: Procyanidin dimer B1 and trimer C1 impair inflammatory response signalling in human monocytes. Free Radic Res. 2011; **45**: 611-619.

[86] Singh T, Katiyar SK. Green tea catechins reduce invasive potential of human melanoma cells by targeting COX-2, PGE2 receptors and epithelial-to-mesenchymal transition. PLoS One. 2011; **6**: e25224.

[87] ShibaChen PN, Chu SC, Kuo WH, Chou MY, Lin JK, Hsieh YS. Epigallocatechin-3 gallate inhibits invasion, epithelial-mesenchymal transition, and tumor growth in oral cancer cells. J Agric Food Chem. 2011; **59**: 3836-3844.

18. Acknowledgement

I would like to express my deep appreciation and gratitude to Professor Dr. Ikuo Saiki for his supervision, continuous encouragement and kind support. I deeply appreciate to my supervisor, Assistant Professor Dr. Satoru Yokoyama who has provided a great basis, knowledge and idea for my research. I am also deeply grateful Associate Professor Dr. Yoshihiro Hayakawa for his shrewd comment, suggestion and discussion.

I would like to thank Professor Dr. Hiroaki Sakurai, Department of Cancer Cell Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, for his curious suggestion and long-term attention to my research.

I would like to special thanks to Shu Etsu and all the great members in the Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, for their kindness, academic advice, extensive discussion and support throughout my PhD course.

Finally, I am grateful to the Grant-in-Aid support for JSPS fellows (DC2) from Japan Society for the Promotion of Science during my PhD course.