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キーワード：がん間質線維芽細胞, 間葉系幹細胞/前駆細胞, 血管外膜線維芽細胞, 分化

【目　的】

がん組織には、がん細胞のみならず線維芽細胞・血管・炎症細胞からなるがん間質がある。肺がん・膵臓がん・乳がんなどのがん種では、がん間質ががん組織内の大部分を占める。近年、がんと間質細胞が作り出すがん微小環境はがん発生・進展に深く関わっていることが明らかとなってきた。これらのがんにおけるがん治療を考えると、がん細胞のみに着目するのではなくがん間質も含めたがん組織全体を把握することは必須である。がん間質を構成する細胞の中で、血管についての知見は多く、血管が栄養供給源としてがん組織の進展に寄与するという報告もある。しかしながら、がん間質には筋線維芽細胞が多くみられるという知見はあるが、その線維芽細胞の生物学的特徴を詳細に調べた研究は少ない。また、がん間質に含まれる線維芽細胞の由来も定かではない。

ヒトのがん病理組織切片では血管外膜の線維芽細胞が、がん間質線維芽細胞に連続的に移行している組織像がしばしばみられることより、血管外膜細胞はがん間質に成り得る細胞集団の一つである可能性が高い(Figure 1)。本研究ではがん間質線維芽細胞の起源の一つと考えられるヒト血管外膜細胞の生物学的特徴を明らかにするとともに、がん細胞との相互作用について検討することを目的とする。

【結　果】

ヒト肺動脈血管外膜線維芽細胞の特徴

1) 初期培養したヒト肺動脈血管外膜由来細胞は、*in vitro* で線維芽細胞類似の細胞形態であり、CD29・CD44・CD105陽性、CD3・CD14・CD20・CD34・CD45・CD68・CD117・CD133陰性であった。2) *in vitro* migration assayでは、がん細胞の培養上清に対し遊走能を示した。3) 又、8種類のサイトカインでの検討ではPDGF-bbに最も高い遊走能を示した。4) 肺動脈血管外膜線維芽細胞は、脂肪細胞、骨芽細胞、筋線維芽細胞への分化誘導が可能であった。このことから、正常の肺組織由来線維芽細胞は持たないが、血管外膜線維芽細胞は間葉系幹細胞/前駆細胞を有することがわかった。

血管外膜線維芽細胞とがん細胞の相互作用(mouse xenograft model)

1) ヒト血管外膜線維芽細胞をヒト肺がん細胞株A549と共にマウス皮下に移植した結果、A549単独移植群と比較して、腫瘍体積を約3倍増加させることが確認できた。これは、間葉系幹細胞

を有さない正常の肺組織由来線維芽細胞を A549 と移植した群に比べても腫瘍体積は大きかった(Figure 2)。2)マウス皮下に移植後２週間の、移植腫瘍中の増殖細胞を免疫染色により評価した。その結果、血管外膜線維芽細胞と共に移植した移植腫瘍中の増殖率は A549 単独群に比べて有意に高いことがわかった(Figure 3)。

【考察・今後の展望】

以上より、ヒト肺動脈血管外膜線維芽細胞はがん組織内に積極的に動員され、腫瘍増殖に寄与している可能性が示唆された。又、ヒト肺動脈血管外膜線維芽細胞には間葉系幹細胞/前駆細胞が含まれていることが判明した。正常の肺組織由来線維芽細胞と比較しても血管外膜線維芽細胞と共にマウス皮下に移植した群の腫瘍体積が大きくなったことより、我々は間葉系幹細胞が腫瘍増殖に寄与しているのではないかと考えている。

今後、どの様な相互作用を介して血管外膜線維芽細胞が腫瘍体積の増加に寄与しているかの検討を進めていきたい。更に、当研究室においてリンパ管マーカーである podoplanin が肺がん患者の間質で陽性率が高い程予後不良であること、及び肺がん細胞付近の血管外膜に podoplanin 陽性が含まれていることが分かった。このことより、新たな展望として、血管外膜線維芽細胞の podoplanin 陽性細胞と陰性細胞を単離しそれぞれががん細胞と共にマウス皮下に移植した際に腫瘍体積にどのように影響を与えるかも検討したい。

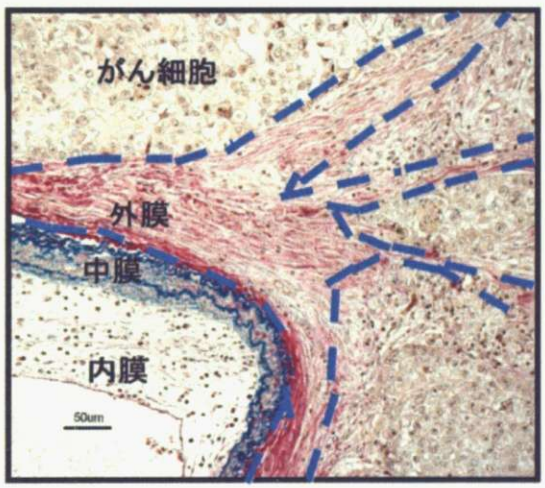

Figure1. 血管外膜ががん間質に連続的に移行している
（ヒト肺がん組織切片）

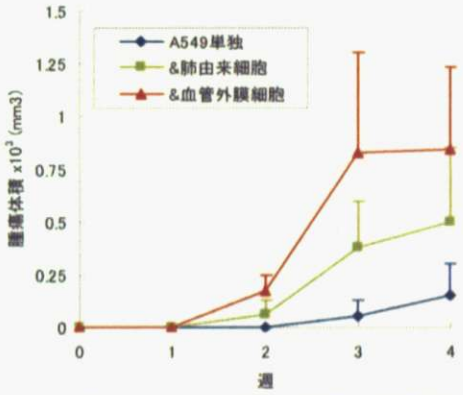

Figure2. マウス皮下の腫瘍体積

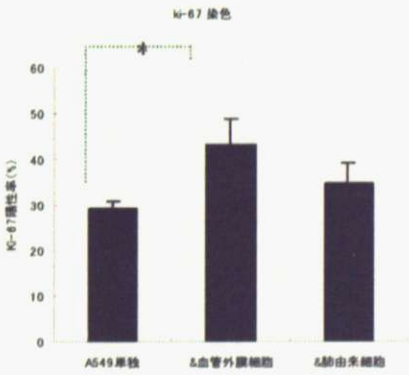

Figure3. ２週目腫瘍の増殖率

発表論文

Human vascular adventitial fibroblasts contain mesenchymal stem/progenitor cells

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Biochemical and Biophysical Research Communications, in press

Abstruct

Microenvironment of cancer cells and stromal cells such as fibroblasts, blood vessels, and inflammatory cells are engaged with cancer development. Fibroblasts in stroma are known to show desmoplastic reaction, however the origins or further biological details remain unclear. In human pathological specimen, vascular adventitial fibroblasts continuously penetrating into cancer stroma are often seen. This study is to characterize the biological features of vascular adventitial fibroblasts, one of a possible origin of stromal fibroblasts, and also to investigate the cross interaction with cancer cells. 1) The primarily cultured human vascular adventitia showed fibroblast-like shape and were CD29, CD44, CD105 positive, and CD3, CD14, CD20, CD34, CD45, CD68, CD117, CD133 negative. 2) Human vascular adventitial fibroblasts (hVAFs) migrate to cancer cell cultured medium and different cytokines, especially PDGF-bb. 3) hVAFs had ability to differentiate to adiopocyte, osteoblast, and myofibroblast. 4) Tumor volume of mouse xenograft of A549 injected with hVAFs increased threefold compared to A549 alone, and was larger than A549 injected with human lung fibroblasts (hLFs) also. The growth rate of A549 injected with hVAFs at week two was significantly higher than A549 injected alone. These results indicate that hVAFs contain mesenchymal stem/progenitor cells and has

potency to increase tumor volume.

Introduction

Cancer microenvironment

Cancer refers to any one of a large number of diseases characterized by the development of abnormal cells that divide uncontrollably and have the ability to infiltrate and destroy normal body tissue. In many types of cancer, such as breast cancer, pancreatic cancer, and lug cancer, cancer tissue is composed of different kinds of cells forming microenvironment [1]. The cells in the cancer microenvironment include stromal fibroblasts, infiltrating immune cells, the blood and lymphatic vascular networks, and the extracellular matrix (Fig. 1A, 1B, 1C). It has been hypothesized that these components are functionally organized to promote survival of cancer cells in the host and generate a favorable microenvironment for cancer cells in both primary and metastatic sites [2; 3; 4] (Fig. 1C).

Over a century ago, Paget suggested the cancer microenvironment as the 'seed and soil hypothesis'. This emphasized the tumor microenvironment and tumor-host cross talk in organ-specific cancer metastasis and the 'seed-soil' compatibility contributing to the establishment of metastasis and colonization of cancer cells. However, molecular features of the 'soil' are less well understood

than those of the 'seed', and understanding the 'soil' is now an important issue in the cancer research and therapy. Both cancer cells and their surrounding environment need to be fully characterized in order to understand how cancer grows in the body, and both need to be considered when developing new interventions to fight the disease.

Vascular adventitial fibroblasts and Differentiation

Arterial walls of vessels are heterogenous three-layered structures composed of an intima, media, and adventitia (Fig. 1D & 1E). The vascular adventitia is the outermost connective tissue of the vessel, which contains fibroblasts. Each layer exhibits specific histological, biochemical, and functional characteristics, and each contributes in unique ways to maintaining vascular homeostasis and to regulating the vascular response to stress or injury. Endothelial cells and smooth muscle cells (SMCs), the principal cellular constituents of the intima and media, have received much attention from vascular biologists, whereas the adventitial fibroblasts have been largely overlooked. However, an increasing volume of experimental data indicates that the adventitial compartment of blood vessels is a critical regulator of vessel wall

function in health and disease. Animal models have demonstrated that the vascular adventitia contribute to the pathology of a variety of diseases [5], such as atherosclerosis [6; 7; 8], vascular restenosis [9; 10], and vascular fibrosis induced by radiation [11] by changing its functions, having a migratory myofibroblast phenotype. Adult mouse aorta has been reported to possess a cell population that can be induced to differentiate into osteoblasts and adipocytes [12]. We therefore hypothesized that human vascular adventitia possesses mesenchymal differentiation ability.

In human pathological specimen, histological picture of vascular adventitial fibroblasts continuously penetrating into cancer stroma are often seen (Fig. 1D). Furthermore, pathological paper has reported that vessels in tumor stroma lack vascular adventitial fibroblasts in gastric carcinomas, indicating the possibility that vascular adventitial fibroblastic cells may be associated with tumor stroma formation [13]. This supports the idea that the vascular adventitia may be one of the possible origins of tumor stroma.

Mesenchymal stem cell and cancer

Mesenchymal cells are primordial cells of mesodermal origin forming the connective tissue throughout the body. Mesenchymal cells would therefore give rise to fibroblasts, osteoblasts, chondrocytes, smooth muscle cells, and endothelial cells, as well as stromal cells with hematopoietic support (Fig. 1F).

Mesenchymal stem/progenitor cells (MPCs) are described as cells that are capable of differentiating into at least one mesenchymal lineage, such as an adipocyte lineage, osteoblast lineage, or myofibroblast lineage [14]. MPCs have a spindle-shaped, fibroblast-like morphology in their undifferentiated stage. Recent studies have showed that human MPCs having a fibroblast-like shape are stored in various tissues and organs, such as bone marrow [15; 16; 17], adipose tissue [18], skin [19], and umbilical cord blood [20; 21].

In the investigation of the origins of the invasive and metastatic phenotypes of cancer cells, current models depict these phenotypes as traits that are acquired through exposure of epithelial cancer cells to paracrine signals that they receive from mesenchymal cell types within the cancer-associated stroma [22]. Although several evidence demonstrate the contributions of stromal cells to primary tumor growth [23], direct experimental demonstration of

the influence of these various cells on the metastatic abilities of cancer cells has been difficult to obtain. This is due to the complexity of the mesenchymal cell types that are recruited into the stroma. Recent reports proposed the model that the bone-marrow-derived mesenchymal stem cell is a cell type that is recruited into the stroma developing tumors [24; 25]. But still, the heterogeneity of the cancer stromal fibroblasts with unclear origin remains together with necessity to determine whether MSCs could supply contextual signals that serve to promote cancer expansion.

The aim of this study is to characterize the cell population, human vascular adventitial fibroblasts, which is the possible origin of the cancer stromal fibroblasts including its potency for mesenchymal differentiation ability. And to investigate the cross interaction of human vascular adventitial fibroblasts and cancer cells.

Material and Method

Vascular adventitia isolation and adventitial fibroblast cell culture

Human pulmonary arteries and lung specimens were obtained from the surgically resected lungs of lung cancer patients. The adventitia of the pulmonary artery was carefully removed from the media and intima. The adventitia was then cut into pieces and plated on a culture dish containing mesenchymal stem cell medium (Toyobo, Tokyo, Japan). The lung tissue was also cut into small pieces and plated on a culture dish containing mesenchymal stem cell medium. The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. The tissue was then removed and cultured for two more days. When the cells reached 80% confluence they were harvested with 0.25% trypsin and 1 mmol/L EDTA and replated at a density of 1 $\times 10^4$ cells/cm^2. All specimens were collected after the subjects gave their written informed consent, approved by the Institutional Review Board of the National Cancer Center.

Characterization of vascular adventitial fibroblasts (VAFs)

Immunohistochemical staining

Immunohistochemical staining was performed with DAKO EnVision + System-HRP (Dako, Glostrup, Denmark). Cultured cells were fixed in 10% formaldehyde for 15 minutes, and then blocked for 30 minutes with 5% milk in PBS. Cells were incubated with primary antibodies at room temperature for one hour. The antibodies used were polyclonal anti-rabbit collagen type 1 (Calbiochem, San Diego, CA) at a working dilution of 1:25 and polyclonal anti-rabbit vimentin (Santa Cruz, Santa Cruz, CA) at a working dilution of 1:50.

Flow cytometry analysis

Cultured hVAFs were characterized by flow cytometry with FACSCalibur cytometer (Beckton Dickinson, San Jose, CA) and a minimum of 10,000 events counted with Cell Quest software. The hVAFs used in this analysis were from passages two to five. The conjugated antibodies used were CD117-PE, CD68-FITC (DAKO A/S, Glostrup, Denmark), CD105-PE, CD44-PE, CD45-PE, CD14-FITC (eBioscience, San Diego, CA), and CD29-PE (Chemicon, Temecula, CA). The non-conjugated antibodies used were CD3, CD34 (DAKO A/S,

Denmark), CD20 (eBioscience, San Diego, CA), and CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were trypsinized, centrifuged, and incubated with each antibody for 15 minutes on ice in the dark. Excess antibody was removed by washing the cells with PBS (containing 3% FBS and 0.05% Na_2N). Polyclonal rabbit anti-mouse immunoglobulins/FITC (Dakocytomation, Glostrup, Denmark) as a secondary antibody for non-conjugated antibodies was added and incubation was continued for another 15 minutes on ice in the dark. The cells were rinsed with PBS to remove excess antibody, and a FACS scan was performed using Cell Quest software (Beckton Dickinson Labware, Franklin Lakes, NJ).

Chemoattractant assay

In vitro migration assay was performed using a 24-well plate with 8.0 microns pore size cell culture inserts (Beckton Dickinson Labware, Franklin Lakes, NJ). 2×10^4 cells of hVAFs were plated in the culture insert with either supernatant or recombinant chemokines in the lower plate. After six hours of incubation in 37C, culture inserts were fixed with 10% paraformaldehyde buffer for 20 minutes at room temperature followed by rinse with PBS and staining with

Meyer's hematoxylin for 30 minutes at room temperature. The number of cells were counted in nine fields of $2.4x10^3 \mu m^2$ through a X20 microscope objective lens.

Adipogenic induction and Oil Red O staining

hVAFs and human lung fibroblasts (hLFs) were plated at a density of 2.1 $\times 10^4$ cells/cm^2 and cultured until semi-confluence. The medium was then changed to adipogenic induction medium (Cambrex Bio Science Walkersville, Walkersville, MD), and the cells were cultured for three more days, at which time the medium was changed to adipogenic maintenance medium (Cambrex Bio Science Walkesville, Walkersville, MD) for two days. Three complete cycles of induction/maintenance medium stimulated optimal adipogenic differentiation, forming adipocytes. To confirm their identity, cells were fixed with 10% paraformaldehyde buffer for 15 minutes at room temperature and incubated with 60% isopropanol for one minute. The cells were then stained with oil red O (Sigma-Aldrich, Saint Louis, MO) solution (three volumes of 0.3% of oil red O in isopropanol plus two volumes of distilled water) for 15 minutes at 37 C, 5% CO_2 and counterstained with Meyer's hematoxylin for five minutes at room

temperature. Control cells were assayed in the same manner, but stem cell culture medium was used instead of adipogenic induction/maintenance medium.

Osteogenic induction and von Kossa / Alkaline phosphatase (ALP) staining

Osteogenesis was induced by plating hVAFs and hLFs at a density of 3.1×10^3 cells/cm^2. One day after plating, the medium was replaced with fresh osteogenic induction medium (Cambrex Bio Science Walkesville, Walkersville, MD). The medium was changed every three to four days for three weeks, and calcium deposition was detected by von Kossa's method as a marker for osteogenic differentiation. Cells were fixed with 10% paraformaldehyde buffer for 20 minutes, and then stained with 5% silver sulfate for two hours at room temperature. After washing the cells with both 5% sodium thiosulfate and distilled water for three minutes, they were counterstained with nuclear fast red solution (Sigma-Aldrich, Saint Louis, MO). Alkaline phosphatase was detected as another marker for osteogenic differentiation by using an alkaline phosphatase staining kit (Muto Chemical, Tokyo, Japan) according to the manufacturer's instructions. Briefly, cells were fixed with fixative solution for five seconds at –4C, and then stained by incubation with Fast Blue RR Salt

solution at 37C for two hours. Excess stain was removed with water, and the cells were counterstained with 1% safranin. Control cells were subjected to the same assay with stem cell culture medium instead of osteogenic induction medium. Alkaline phosphatase staining was quantified by counting positive cells in five $2.4x10^3 \mu m^2$ fields through a X20 microscope objective lens and calculating the percentage of cells stained.

Myogenic induction

To assess the myogenic differentiation ability of hVAFs and hLFs, we added transforming growth factor (TGF)-beta1 (R&D Systems, Minneapolis, MN) to mesenchymal stem cell medium to a final concentration of 5 ng/ml or platelet-derived growth factor (PDGF)-BB (R&D Systems, Minneapolis, MN) to a final concentration of 20 ng/ml, cultured the cells for five days. The medium was replaced every other day with medium containing newly added TGF-beta1 or PDGF-BB. Cells were lysed and Western blotting was performed for smooth muscle actin and calponin expression as markers for myogenic differentiation. Control cells were assayed in the same manner using mesenchymal stem cell medium without recombinant proteins.

Western blot analysis

Western blot analysis was performed as follows. Cells were lysed in whole-cell extraction buffer (20 mM Hepes-NaOH, 0.5% NP-40, 15% Glyserol) containing Complete, a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Proteins were separated on 12% SDS-polyacrylamide gel and transferred to an Immobilon-P, PVDF (polyvinylidene fluoride) membrane (MILLIPORE, Billerica, MA). Blots were saturated with blocking buffer (5% skim milk in TBS-T) for one hour at room temperature and then incubated overnight at 4C with anti-human mouse monoclonal alpha-smooth muscle actin antibody (Sigma, Sain Louis, MO), mouse monoclonal calponin antibody (Novocastra, Newcastle, UK), or polyclonal goat actin antibody (Santa Cruz, Santa Cruz, CA). After washing in TBS-T, membranes were incubated for one hour at room temperature with HRP-Rabbit Anti-mouse IgG or HRP-rabbit Anti-goat IgG (Zymed, San Francisco, CA). ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) were used to develop high-performance chemiluminescence film (GE Healthcare, Buckinghamshire, UK).

Mouse xenograft

3.5×10^6 cells of A549 human lung adenocarcinoma cells alone, or with either 3.5×10^6 cells of hVAFs or hLFs was grown as xenograft tumors in SCID mice. Tumor growth was determined by caliper measurement of the length, width, and height. Tumor volume was calculated as the product of scaling factor 0.52, tumor length, width, and height. Tumor size was measured every week until week four.

Growth rate of the xenograft tissue

Xenograft tumors were stained for Ki-67 (MIB-1) using a mouse monoclonal antibody to human Ki-67 (Dakocytomation, Glostrup, Denmark). The growth rate was calculated by counting the positive cells out of at least 1,000 cells.

Determination of Microvessel Density

Xenograft tumors were stained for Von willebrand factor eight using a mouse monoclonal antibody to human Von willebrand factor eight (DAKO Corporation, Carpinteria, CA). The microvessel density was determined by

averaging the second to fourth highest vascularity (hot spots). The number of microvessels was counted at x200.

Results

Isolation and culture of human pulmonary artery adventitial fibroblasts

We isolated human pulmonary artery adventitia by peeling away from arterial SMCs (Fig. 2A & 2B) and cultured until adherent cells grew out (Fig. 2C). The cultured human vascular adventitial fibroblasts (hVAFs) from pulmonary arteries were spindle-shaped and similar in appearance to human lung fibroblasts (hLFs) (Fig. 2C). The doubling time of both hVAFs and hLFs cell populations was 1.5 days. Immunohistochemistry showed that both cell populations were positive for vimentin and collagen type 1, which are fibroblast markers (Fig. 2D).

As shown in Table 1, the number of passages of both the hVAFs and hLFs was independent of age and sex. It was not affected by the patients' stage either (data not shown). The average number of passage for hVAFs was around 11 and as for hLFs, it was around 10. The morphology of the hVAFs and hLFs did not change until the final two or three passages. When both cell populations were about to reach senescence, the cells increased in size.

Immunophenotyping

Flow cytometry analysis showed that both the cultured hVAFs and hLFs were negative for the leukocyte marker CD45, the hematopoietic stem cell markers CD117, CD133, and CD34, the endothelial cell marker CD34, the B-cell marker CD20, the T-cell marker CD3, the macrophage marker CD68, and the monocyte/granulocyte marker CD14 (Fig. 2E). The hVAFs and hLFs reacted with antibodies to beta-1 integrin (CD29), cell adhesion receptor (CD44), and endoglin (CD105) (Fig. 2E). These results suggested that hVAFs and hLFs have similar morphological and immunophenotypical characteristics in culture.

Migration assay (Chemoattractant)

hVAFs migrated toward various types of supernatant from cancer cell lines in a concentration dependant manner (Fig. 3A). As for chemoattractant assay for seven major cytokines known for migration, hVAFs showed most motility to PDGF-bb. It also migrated toward TGF-beta, IGF-2, and basic-FGF in the concentration dependent manner (Fig. 3B).

Adipogenic differentiation

After the three complete rotations of culture with adipogenic induction medium and adipogenic maintenance medium, oil red O staining was performed. hVAFs showed optimal adipocyte formation during the last rotation of change of medium. The adipocytes formed by induced hVAFs were large and frequent (Fig. 4A). The hLFs formed smaller lipid droplets than the hVAFs, and their differentiation ability was very low (Fig. 4A).

Osteogenic differentiation

The morphological changes in hVAFs during osteogenic induction were first noted around ten days after plating, when the cells began to change from spindle-shaped to cuboidal. After the morphological change, marked calcium deposition was observed under the microscope, and it was later confirmed by von Kossa staining (Fig. 4B). Non-induced hVAFs were barely positive for alkaline phosphatase staining and the von Kossa staining. Brownish calcium deposits were spread evenly across the plate of induced hVAFs (Fig. 4B). The induced hVAFs that stained positive for alkaline phophatase stained more deeply purple than the induced hLFs (Fig. 4B). The alkaline phosphatase staining of

the induced hVAFs was more intense than the staining of the differentiation-induced hLFs (Fig. 4B & 4C). We also performed an adipogenic and osteogenic induction assay of the hVAFs cultured in alpha-MEM instead of MF-medium. The hVAFs were induced to differentiate to both lineages, but their differentiation was weaker than that of the hVAFs cultured in MF-medium (data not shown).

Myogenic differentiation

TGF-beta1 and PDGF-BB were added to the culture medium for five days to induce myogenic differentiation of the hVAFs and hLFs and myogenic induction was assessed by Western blot of alpha-smooth muscle actin (SMA) and calponin expression. hVAFs showed an increase in both SMA and calponin expression after five days of induction with TGF-beta1, but not with PDGF-BB (Fig. 4D). hLFs, on the other hand showed an increase in SMA and calponin in response to induction with both TGF-beta1 and PDGF-BB.

Mouse xenograft model

We found that hVAFs accelerated the growth of A549 tumors in SCID mouse xenograft model. At week three the tumor volume of A549 injected with hVAF increased threefold compared to A549 alone (Fig. 5A). A549 injected with hLFs also formed larger tumor than the tumor with A549 alone, but even though it was not significantly different, the tumor size were smaller than the one injected with hVAFs in average. By Ki-67 staining of tumor sections, the growth rate of A549 cells of hVAFs co-injection tumor was significantly higher than that of A549 single injection at week 2 (Fig. 5B & 5D). Microvessel density did not show difference between tumors at week two or four (Fig. 5C & 5E).

Discussion

The origin of the cancer stromal fibroblasts and what it does for cancer progression has always been a question. As shown in figure 1D, vascular adventitia penetrating into cancer stroma is often seen in pathological specimen. We therefore thought that vascular adventitita would be one of the origins of the cancer stromal fibroblasts. In this study, we found that the hVAFs accelerated tumor growth in *in vivo* model. A549 with hVAFs and A549 without hVAFs showed significant difference from week three. Ki-67 staining revealed that at week two, the growth rate of A549 in tumor injected with hVAFs were more than ten percent higher than that of A549 injected alone. This suggests that hVAFs interacts with A549 or provides some kind of niche for A549 to accelerate its tumor growth. Interestingly, even though A549 injected with hLFs used as control in this study did not differ significantly with tumor volume of A549 with hVAFs, but average size of tumor was always smaller than tumor with hVAFs in all specimen used. We hypothesized that this difference came from distinction of whether the cells possess MPCs or not.

An important characteristic of MPCs is that they migrate to sites of tissue injury primarily as a result of the production of inflammatory mediators produced

during tissue damage and remodeling [26]. As tumors are thought as wounds that never heal, recent studies report that circulating MPCs are recruited to tumor stroma [27]. We performed the differentiation assay of cancer stromal fibroblasts and compared its differentiation ability with hVAFs and hLFs to see how much MPCs does stromal fibroblasts possess. The data revealed that cancer stromal fibroblasts showed some differentiation ability, less than hVAFs and more than hLFs. This supports the fact that MPCs including hVAFs may be recruited to stromal fibroblasts.

The microvascular density so far, does not differ between the tumors. But further investigation is needed to find if there are any relationships between where hVAFs exist within the tumor and where microvessel density is high. To figure this question out, we are now planning to perform in vivo mouse xenograft model of GFP-labeled hVAFs and hLFs done by lentivirus transfection co-injected with A549.

Migration assay revealed the hVAFs' ability to migrate toward supernatants of various cancer cell lines as well as several cytokines. From seven cytokines, hVAFs showed motility to PDGF-bb, TGF-beta, IGF-2, and basic-FGF in concentration dependant manner revealing the possession of

receptors for these cytokines. However, although PDGF-bb was the major motility factor for the hVAFs within seven cytokines, it is not yet verified that PDGF-bb expressed from each cell lines stimulated the hVAFs to migrate. Rather, Real Time-PCR revealed that the mRNA level of PDGF from each cell lines did not match the migration level of the hVAFs (data not shown). It is likely that several-conjugated amount of migratory cytokines expressed from each cell lines stimulates hVAFs for migration.

This is the first study to show that human adventitial fibroblasts have the ability to differentiate into adipocytes, osteoblasts, and myofibroblasts. hVAFs exhibited myogenic induction in response to TGF-beta1 and showed high expression of calponin and alpha-smooth muscle actin. Calponin expression is known to be restricted to smooth muscle cells and is a marker of a differentiated contractile phenotype of developing smooth muscle. Thus, this finding may also mean that hVAFs have the ability to differentiate into smooth muscle cells (SMCs), which would be consistent with a previous report indicating that murine VAFs have the capacity to differentiate into SMCs [7]. In this study PDGF-BB did not induce myofibroblast differentiation by hVAFs. Since hVAFs migrated toward PDGF-BB in a concentration-dependant manner in the chemoattractant

assay, the hVAFs possess a signal transduction pathway via the PDGF-BB receptor (data not shown). Though the hVAFs have PDGF-BB receptor, it did not differentiate by PDGF-BB induction. This may mean that the signal pathway that resides in the hVAFs and the hLFs to myofibroblasts differs.

Although the adipogenic differentiation ability of our hLFs was poorer than that of the hVAFs, the induced hLFs formed small lipid droplets in some areas. Osteogenic induction revealed that alkaline phosphatase staining of the hLFs was indeed weaker than that of the hVAFs, but it was still positive compared to the control hLFs. This finding supports the previous study suggesting that the MPCs reside in various parts of the body but that their differentiation ability varies [28]. In addition to the differentiation ability related to the source of MPCs, we suspect that their differentiation ability varies with the culture medium. This was suggested by the fact that the hVAFs had lower differentiation ability in response to both adipogenic and osteogenic induction medium when cultured in alpha-MEM before the induction assay. Based on all of the above findings taken together, mesenchymal differentiation potential is thought to depend on both cell origin and the culture-condition.

It is not yet clear whether the hVAFs contains multipotent cells or is a

mixture of committed progenitors with restricted single-potential. Cloning of hVAFs at the single cell level will clarify this point. We challenged to perform clone analyses by diluting the cells to single cell in 96 well plates. However we could not obtain enough cells to perform differentiation assay due to cell viability. To solve this problem, it may be necessary to use immortalized hVAFs without eliminating their differentiation ability [29].

The vascular adventitia is composed of a variety of cells, including SMCs, capillary cells, neuronal cells, and fibroblasts. So there are possibilities that cultured vascular adventitia contaminated smooth muscle cells and/or blood-borne fibroblasts [30]. However, we think that the hVAFs in the current study were derived from adventitia fibroblasts for the following reasons. First, it was possible to rule out contamination by smooth muscle cells based on cell morphology. The typical 'hill and valley' growth pattern of vascular smooth muscle cells was not seen in the cultured hVAFs. Moreover, the smooth muscle cells expressed both SMA and calponin in the absence of TGF-beta1 and PDGF-BB, whereas the hVAFs expressed SMA to some extent in the absence of induction (Fig. 3D). Another possible contaminant is blood-borne fibroblasts. When we cultured blood-borne fibroblasts, they reached

senescence before passage eight (data not shown), whereas the hVAFs that we cultured stopped proliferating by at least after passage nine, which makes contamination by blood-borne fibroblasts unlikely.

MPCs are considered as cells with potential therapeutic applications. They are currently being used in a clinical trial as a treatment for myocardial infarction [31]. A previous study found that MPCs from human bone-marrow improved the heart function of damaged hearts made by coronary artery bypass graft surgery in a rat model [32]. The fact that vascular adventitia contain MPCs can be applied in this case. Autologous grafts, such as of the internal thoracic artery, are used for coronary artery bypass grafting. Since it is speculated that the grafts contain MPCs in their adventitia, implantation of grafts or grafts whose outer layer is coated with MPCs from adventitia may accelerate recovery from the heart injury, if maintained under suitable conditions before grafting. Because hVAFs can be easily maintained and have a fairly long life span after culture, hVAFs can be considered to be another source of MPCs for the treatment of clinical conditions such as myocardial damage.

As mentioned before, since most of the antibodies showed similar pattern between hVAFs and hLFs (Fig. 2D &2E) and markers for MPCs has yet

been determined, it is difficult to discriminate between these two cell types. In some pathological specimen, stromal fibroblasts show positive for lymphatic endothelial marker, podoplanin. Podoplanin is a noble lymphatic endothelial marker, which also reacts with an oncofetal antigen. It is a mucin-type sialoglycoprotein, which is known as a platelet aggregation-inducing factor that is up regulated in a number of human cancers and has been implicated in tumor progression (Fig. 6E). As shown in figure 6A and 6B, some vascular adventitia revealed positive for podoplanin when it is near cancer, but not in normal sites. Furthermore, recent study has significantly proven that lung cancer patients having podoplanin positive stromal fibroblasts have a poor prognosis compared to patients with podoplanin negative stromal environment (Kawase et al. submitting). These data suggests that the hVAFs may be positive for podoplanin antibody, and that it may be a unique protein that discriminates with hLFs. Further investigation concerned with podoplanin is planned for improved understanding of interaction of hVAFs and cancer cells.

Future Plans

Material and Method

FACS sorting for aggrus/podoplanin marker

Cultured hVAFs and hLFs were sorted with BD FACSAria Cell Sorter (Beckton Dickinson, San Jose, CA) and a minimum of 10,000 events counted with BD FACSDiva Software for analysis. Staining of the cells was done with same methods as flow cytometry analysis written above using podoplanin (abcam, Tokyo, Japan) and polyclonal rabbit anti-mouse immunoglobulins/FITC (Dakocytomation, Glostrup, Denmark) as a secondary antibody.

Results and Discussion so far

FACS sorting of hVAFs for aggrus/podoplanin

From flow cytometry analysis we found that both hVAFs and hLFs possessed podoplanin positive cells (Fig. 6C). The rate of podoplanin positive cells within each specimen derived cell population varied, but hVAFs contained from 20% to 50% where hLFs contained from 7% to 10%. When cells were cultured after sorting, podoplanin positive cells seemed to adhere more than negative cells for both hVAFs and hLFs (Fig. 6D). This suggests the possibility

that podoplanin expression may be linked to cell adhesion molecule. Furthermore, podoplanin positive cells showed wider phenotype, which may be indicating the activation of fibroblasts. Further investigation is needed to discuss this matter, and we are planning to investigate whether or not podoplanin positive cells would interact with cancer cells to form some kind of niche to accelerate tumor formation in any ways.

Acknowledgement

We thank Hiroko Hashimoto and Mai Okumoto for technical support and Satoshi Fuijii, Motohiro Kojima, Takashi Ito, Naho Atsumi, Masaru Sanada and Motoko Suzaki for helpful advice and experimental support. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare; a Grant for Scientific Research Expenses for Health Labour and Welfare Programs; the Foundation for the Promotion of Cancer Research, 3rd- Term Comprehensive 10-Year Strategy for Cancer Control.

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Figure Legends

Fig. 1. Introduction of cancer microenvironment, vascular construction, and differentiation compartment of mesenchymal stem cell

(A) Specimen of lung cancer patient showing heterogeneity of cancer microenvironment. (B) Cancer cells in tumor covered in black revealing cancer cells account for small amount of volume in some cancer tissue. (C) Model of tumor-host invasion field. (Liotta et al. *Nature* 411, 375-379) (D) Pathological specimen of human lung cancer. Vascular adventitia is continuously penetrating into cancer stroma. (E) Model of aortic vessel. (F) Model of differentiation compartment of mesenchymal stem cell. (James E. Dennis and Pierre Charbord *Stem Cell* 2002;20;205-214)

Fig. 2. Characteristics of cultured human vascular adventitial fibroblasts (hVAFs) and human lung fibroblasts (hLFs)

(A) Section of vessel before desquamation of adventitia. (B) Section of vessel after desquamation. (C) Morphology of hVAFs and hLFs. Cultured hVAFs and hLFs were spindle-shaped and grew in a disorganized fashion. (D) Immunohistochemistry of hVAFs and hLFs. Vimentin on the left, collagen

type-1 on the right. Control panel in the upper left corner. (E) Flow cytometry analysis of hVAF and hLF immunophenotypes. The immunophenotype of hVAFs is shown at the top.

Fig. 3. Migration assay of hVAFs toward supernatants of cancer cell lines and seven cytokines.

(A) Migration assay toward supernatants of eight cancer cell lines. Number of cells migrated were counted from nine fields. (B) Migration assay toward seven types of cytokines. Number of cells migrated were counted from nine fields.

Fig. 4. Adipogenic, osteogenic, and myogenic differentiation of hVAFs and hLFs

(A) Adipogenic induction of hVAFs and hLFs. Cells were stained with oil red O. (B) Osteogenic induction of hVAFs and hLFs. Cells were stained with alkaline phosphatase and by the von Kossa assay. (C) Quantitative analysis of alkaline phosphatase staining. (D) Myogenic induction of hVAFs and hLFs. Western blotting for expression of alpha-smooth muscle actin and calponin.

Fig. 5. Mouse xenograft model.

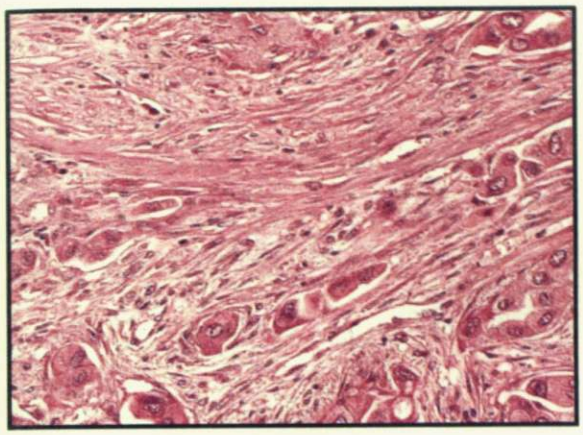
(A) Tumor volume measurements of 3.5×10^6 cells of A549 alone A549 with either 3.5×10^6 cells of hVAFs or same number of hLFs injected subcutaneously into SCID mice. Asterisk, $P<0.01$ using t-test. (B) Growth rate of the xenograft tissue. The growth rate was calculated by counting the positive cells out of at least 1,000 cells. Asterisk, $P<0.01$ using t-test. (C) Determination of Microvessel Density. The microvessel density was determined by averaging the second to fourth highest vascularity (hot spots). The number of microvessels was counted at x200. (D) Immunohistochemical staining of Ki-67. (E) Immunohistochemical staining of von Willebrand factor eight.

Fig. 6. Expression of podoplanin in hVAFs and hLFs.

(A) Human lung cancer specimen showing positive staining for podoplanin at vascular adventitia where cancer cells exist around (top picture). Same specimen's vascular adventitia negative for podoplanin where cancer cells are not around. Lymphatic vessel is stained positive for podoplanin (bottom picture). (B) Close view of box in (A) where the vascular adventitia is stained positive for podoplanin. (C) Flow cytometry analysis of podoplanin.

Histogram of hVAFs on the top. (D) Cultured hVAFs and hLFs after sorted for podoplanin. (E) Model of podoplanin. Green circles indicate glycosylation. PLAG domain stands for platelet aggregation domain.

A



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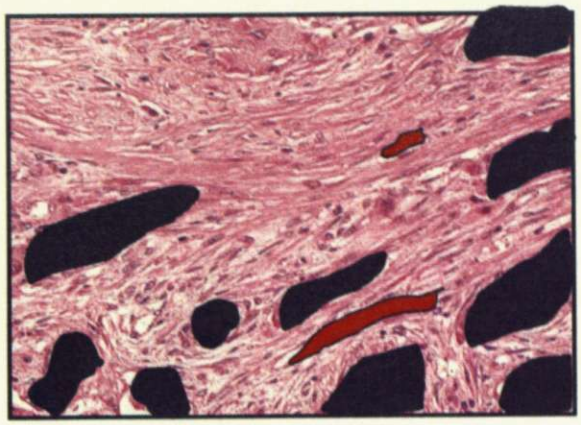

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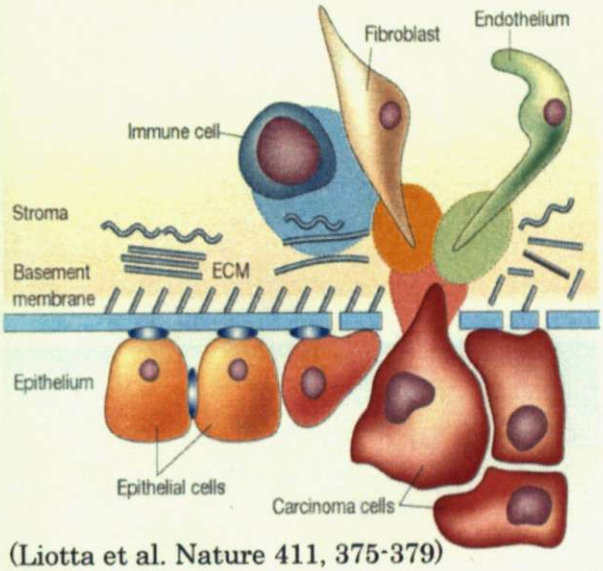
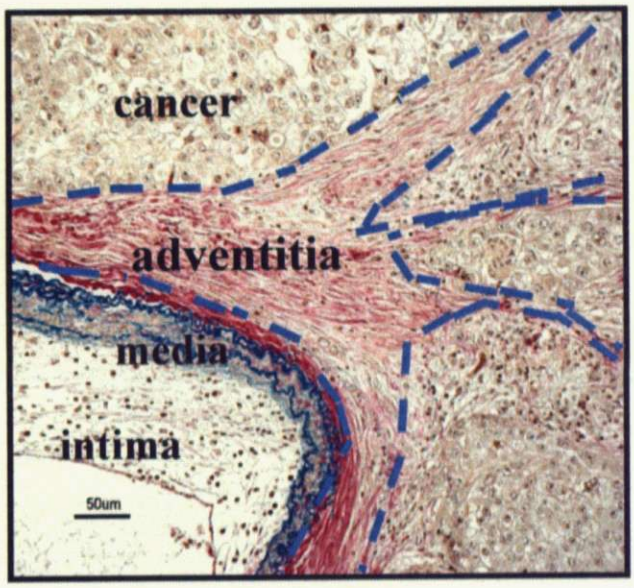


(Liotta et al. Nature 411, 375-379)

D



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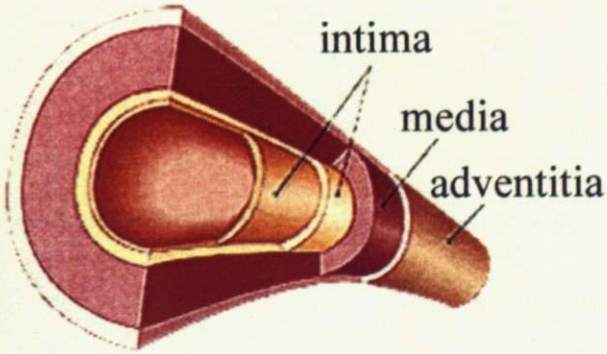

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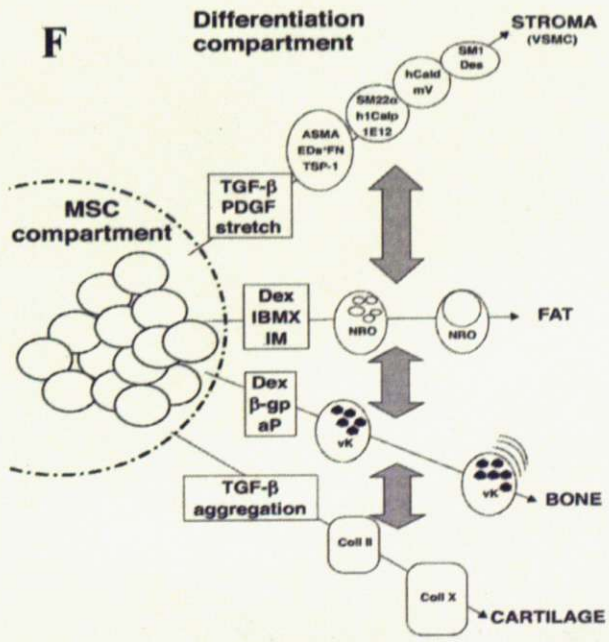


Figure 1

(James E. Dennis and Pierre Charbord *Stem Cell* 2002;20:205-214)

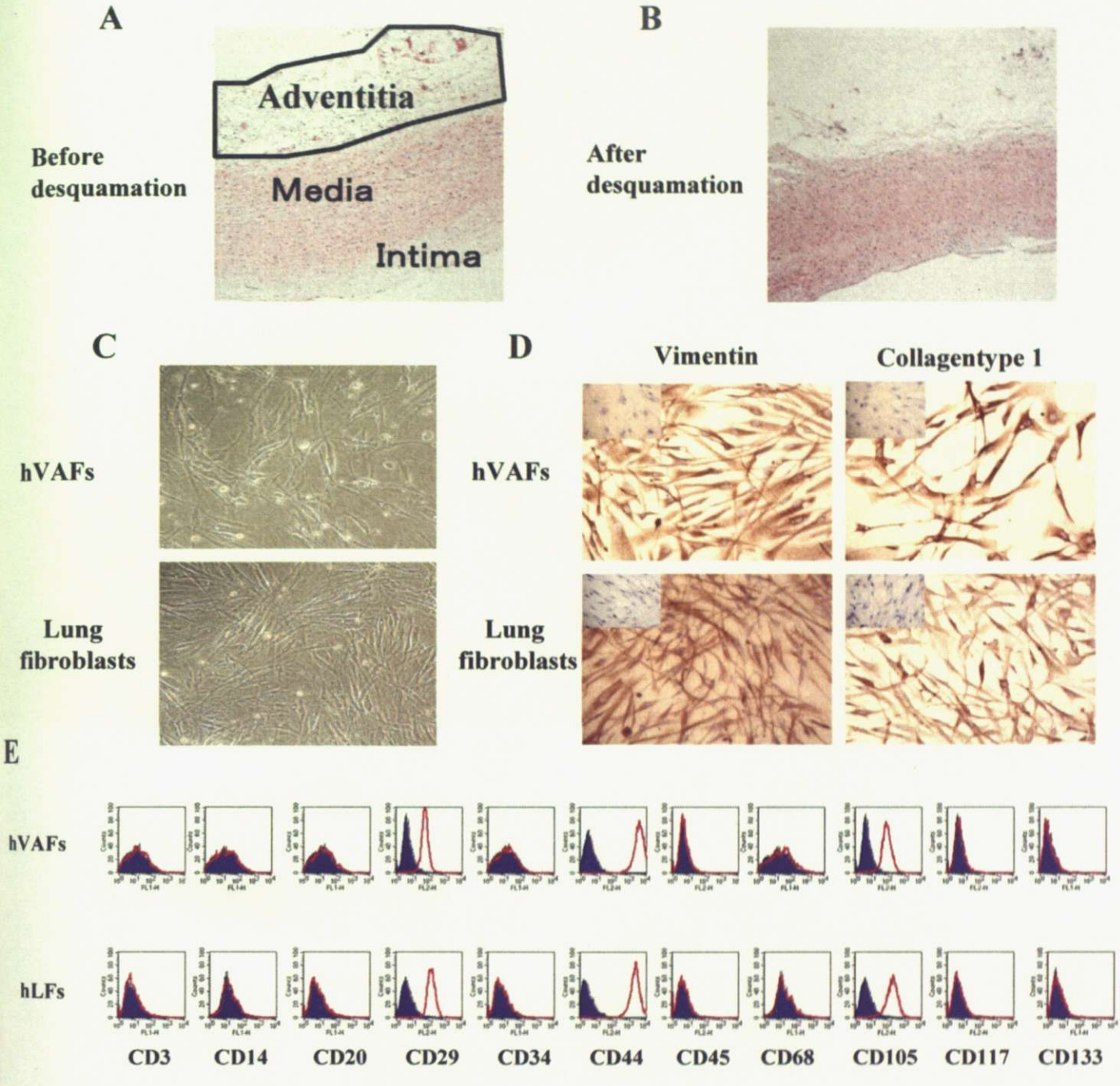

A
Before desquamation
Adventitia
Media
Intima
B
After desquamation
C
hVAFs
Lung fibroblasts
D
Vimentin
Collagentype 1
hVAFs
Lung fibroblasts
E
hVAFs
hLFs
CD3
CD14
CD20
CD29
CD34
CD44
CD45
CD68
CD105
CD117
CD133

Figure 2

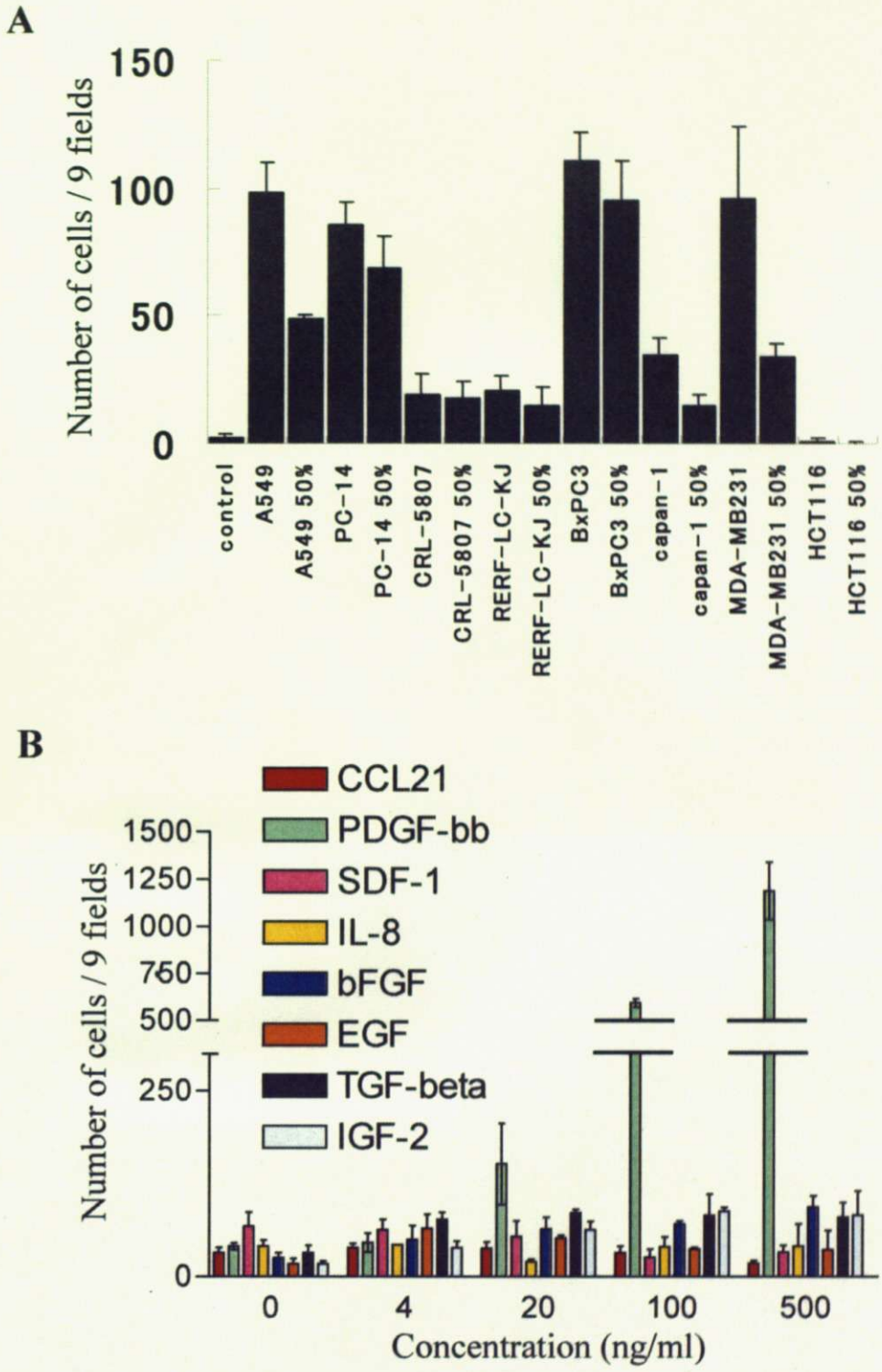
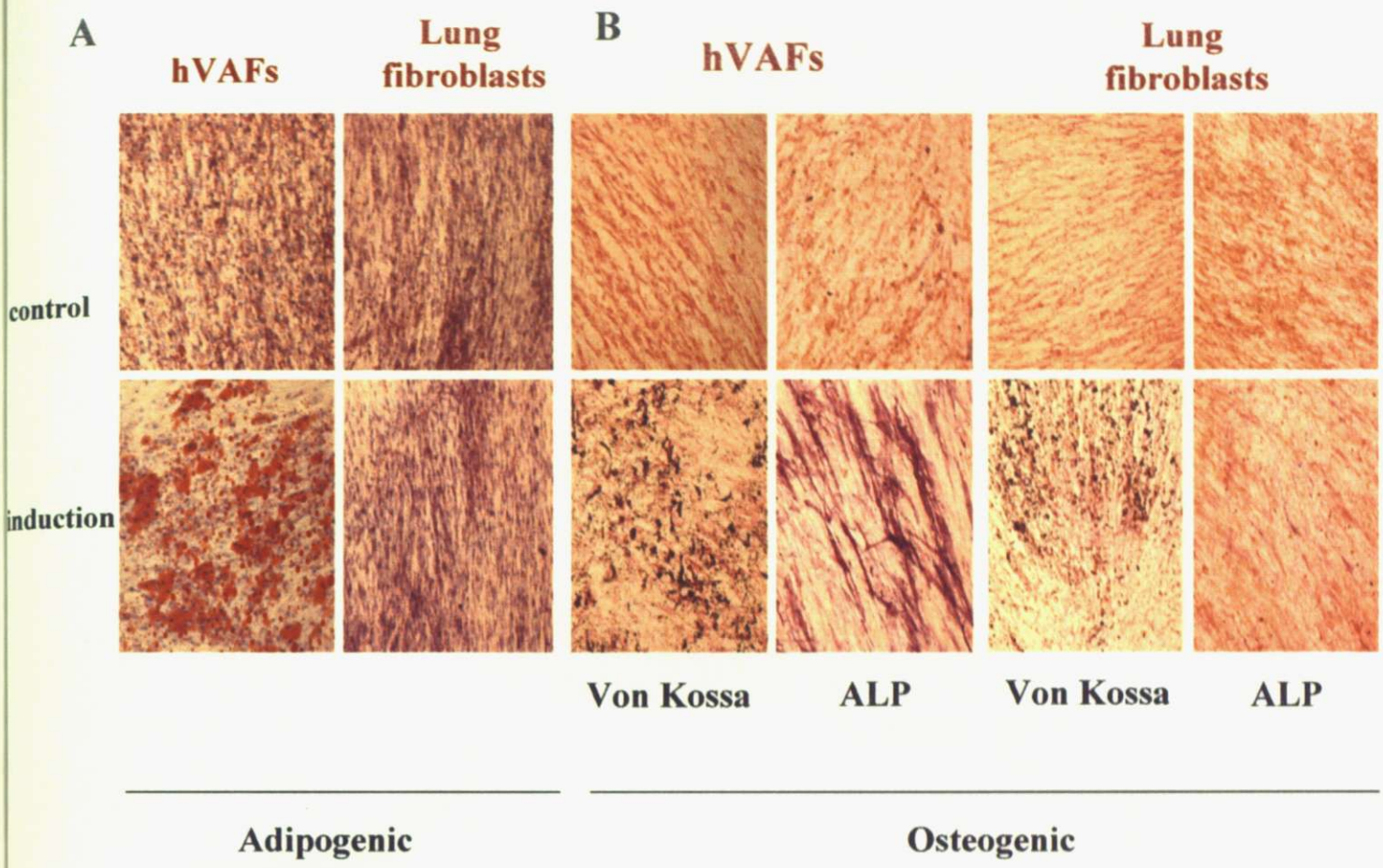
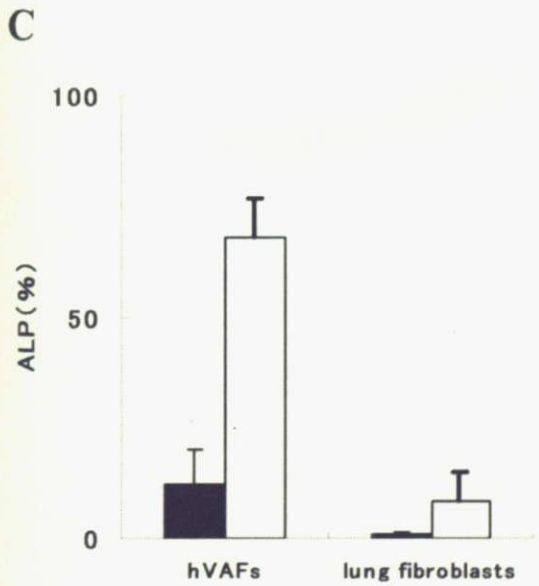

A
Number of cells / 9 fields
150
100
50
0
control
A549
A549 50%
PC-14
PC-14 50%
CRL-5807
CRL-5807 50%
RERF-LC-KJ
RERF-LC-KJ 50%
BxPC3
BxPC3 50%
capan-1
capan-1 50%
MDA-MB231
MDA-MB231 50%
HCT116
HCT116 50%
B
Number of cells / 9 fields
1500
1250
1000
750
500
250
0
CCL21
PDGF-bb
SDF-1
IL-8
bFGF
EGF
TGF-beta
IGF-2
0
4
20
100
500
Concentration (ng/ml)

Figure 3


A
hVAFs
Lung fibroblasts
B
hVAFs
Lung fibroblasts
control
induction
Von Kossa
ALP
Von Kossa
ALP
Adipogenic
Osteogenic


C
100
50
0
ALP(%)
hVAFs
lung fibroblasts

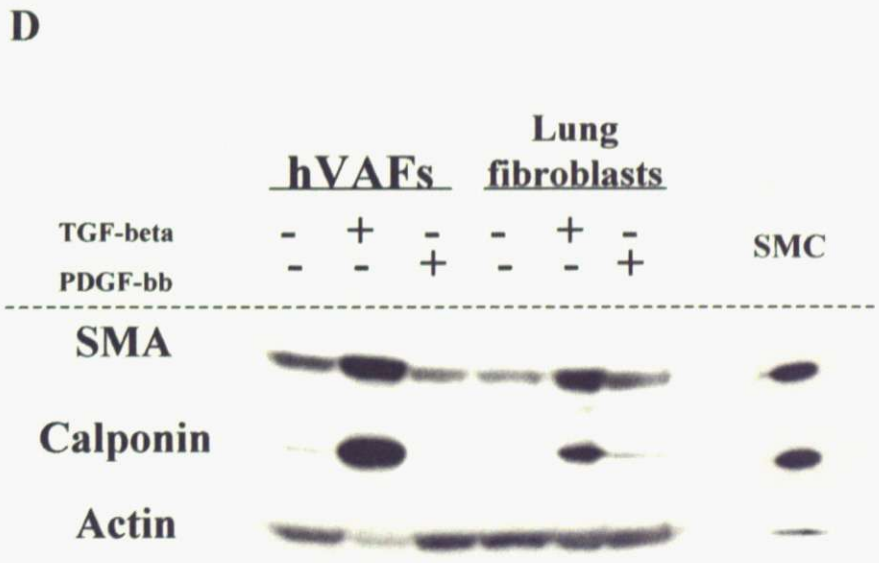

D
hVAFs
Lung fibroblasts
TGF-beta
PDGF-bb
SMC
SMA
Calponin
Actin

Figure 4

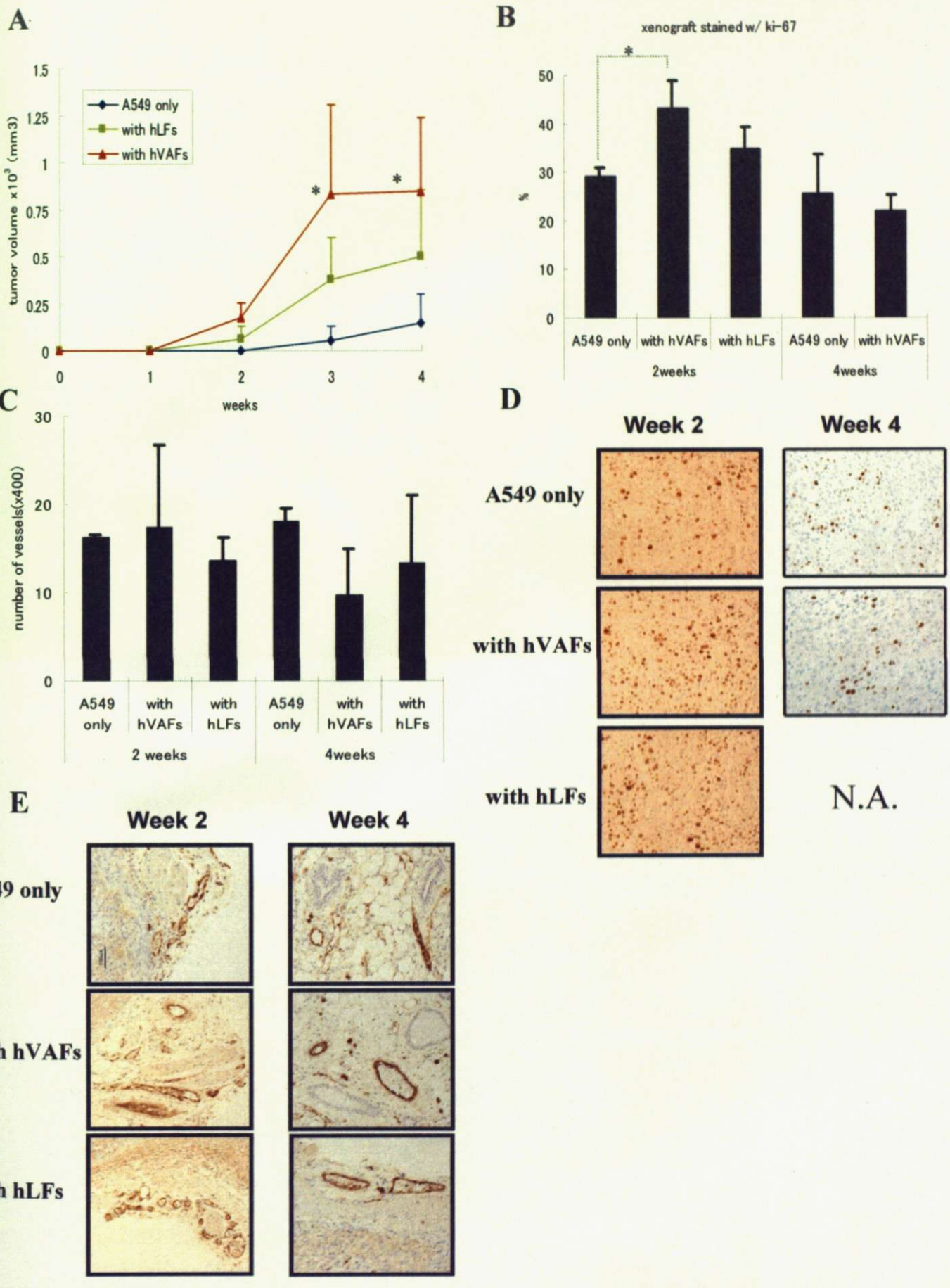

A
tumor volume x10³ (mm3)
1.5
1.25
1
0.75
0.5
0.25
0
A549 only
with hLFs
with hVAFs
*
*
0
1
2
3
4
weeks
B
xenograft stained w/ ki-67
*
%
50
40
30
20
10
0
A549 only
with hVAFs
with hLFs
A549 only
with hVAFs
2weeks
4weeks
C
number of vessels(x400)
30
20
10
0
A549 only
with hVAFs
with hLFs
A549 only
with hVAFs
with hLFs
2 weeks
4weeks
D
Week 2
Week 4
A549 only
with hVAFs
with hLFs
N.A.
E
Week 2
Week 4
A549 only
with hVAFs
with hLFs

Figure 5

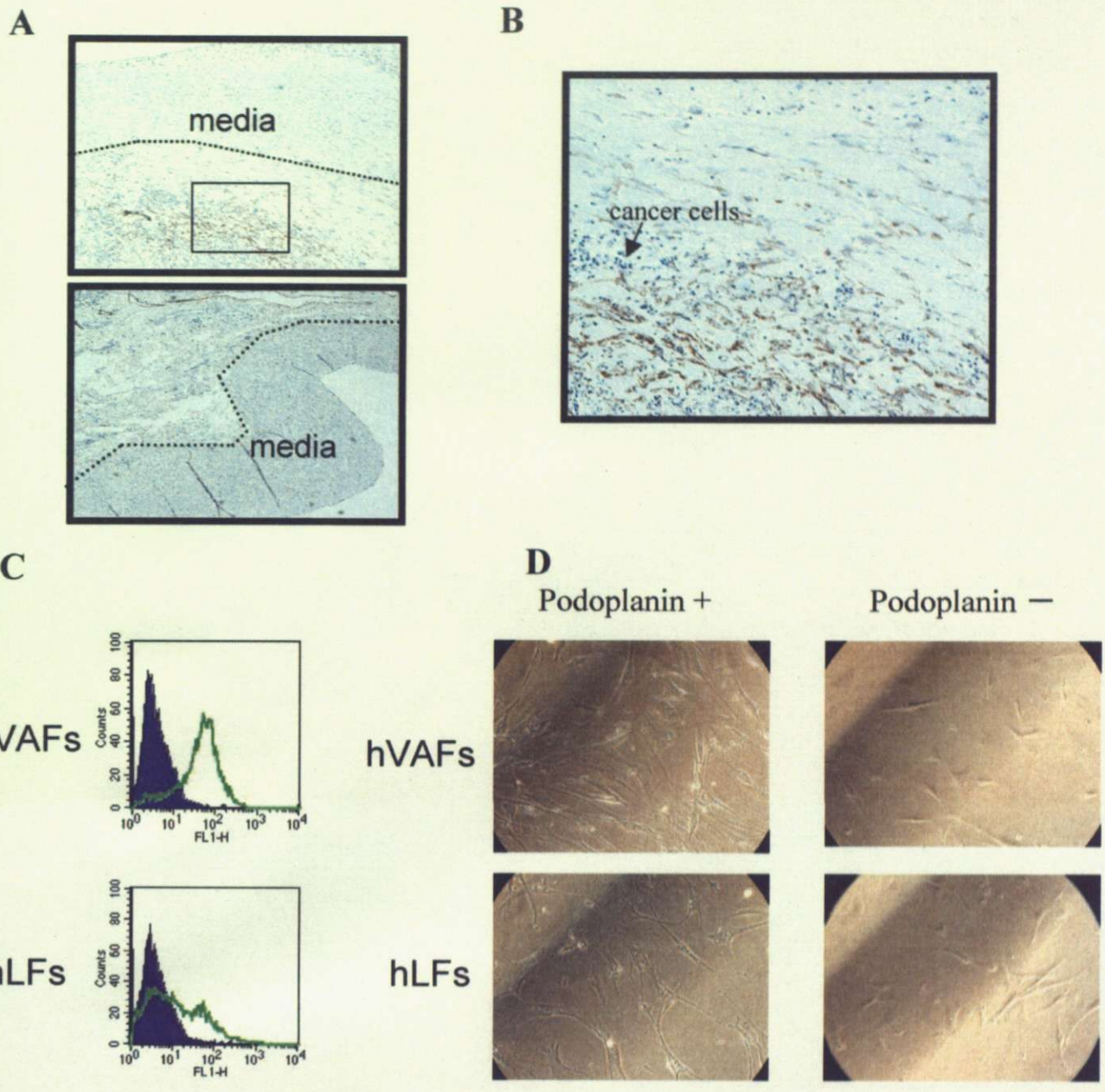

A
media
media
B
cancer cells
C
hVAFs
Counts
FL1-H
hLFs
Counts
FL1-H
D
Podoplanin +
Podoplanin −
hVAFs
hLFs
E
N Terminal
PLAG domain
T52
C Terminal

Figure 6

Table 1. Number of passage per individual

case	age	sex	hVAFs	lung fibroblasts
1	79	male	9	11
2	71	male	12	6
3	78	male	10	8
4	60	male	10	15
5	69	female	9	9
6	65	male	15	N.A.
7	74	male	13	N.A.
8	77	male	11	N.A.
9	53	male	11	N.A.
10	56	female	11	N.A.

Number of passages of hVAFs and hLFs from corresponding patients

Table 1