Establishment of Highly Metastatic Cell Line (Lu10) from Murine Mammary Carcinoma Cell Line MCH66 and Biological Characteristics of Lu10

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Objective: Mouse mammary cancer cell line MCH66 shows invasion-independent metastasis. To elucidate this metastatic mechanism, the biological characteristics putatively related to metastasis were analyzed using several cell lines with different metastatic abilities derived from MCH66.
Methods: Metastatic capacity, invasive activity, growth property, and mRNA expressions of factors associated with endothelial cell proliferation were comparatively analyzed in MCH66 and its sublines.
Results: Lu10 subline exhibited higher metastatic potential to the lungs and lymph nodes (100%) than MCH66 or Lu1 subline (0/5, 0/5 each). The growth rate was almost identical between Lu10 and MCH66, and Lu10 revealed weaker invasive activity *in vitro* than MCH66. In Lu10 tumors in mice, well-developed sinusoidal blood vessels and dilated lymphatics were noted compared with in Lu1 tumors. Accordingly, Lu10 showed higher expression of vascular endothelial growth factor (VEGF)-C, -D, platelet-derived growth factor (PDGF)-B and pleiotrophin than Lu1, while the expression of other growth factors such as VEGF-A, midkine, angiogenin, hepatocyte growth factor, PDGF-A, and basic fibroblast growth factor remained unchanged between Lu1 and Lu10.
Conclusion: These data indicate that high invasiveness and rapid growth are not required for this metastatic process, and some angiogenic mediators are involved in blood-borne and lymphatic metastasis.

Key words: invasion-independent metastasis, MCH66, Lu10-subline, biological characteristics, VEGF-C, VEGF-D

INTRODUCTION

It is generally accepted that cancer metastases are multistep processes including detachment from the primary site, stromal invasion and migration, vascular invasion, survival in the blood stream, extravasation and proliferation at a remote metastatic site [1–4]. However, we have found a unique metastatic pathway, which needs no active vascular invasion in mouse mammary carcinoma cell line MCH 66 and this is designated as invasion-independent metastasis [5, 6]. In this model, MCH 66 metastasizes to the lung from the subcutaneous tumor formed at the site of injection via blood vessels in which tumor cell nests are wrapped by vascular endothelial cells and dropped into dilated lumina of tumor blood vessels. Tumor cell nests grow in the blood vessels during transportation and the cancer cell nests are trapped in pulmonary small vessels. Then, tumor cell nests further increase in size at the site of lodgment, fulfill extravasation and invade pulmonary parenchyma [6]. In addition, we have observed the same metastatic pathway in human carcinoma, such as renal cell carcinoma, hepatocellular carcinoma and follicular thyroid carcinoma [7]. As specific morphological features, dilated sinusoid-like tumor blood vessels in or around the tumor have been noted in an animal model and in human cancers [6, 7]. The precise mechanisms of cancer metastasis in this manner and the biological peculiarities of cancer cells, however, have not been elucidated in humans or even in the animal model [6, 7]. Therefore, we attempted to establish several cell lines with various capacities for metastasis from MCH 66 and obtained highly metastatic cell lines, Lu10, Lu5, and a non-metastatic cell line, Lu1. Using these cell lines, we analyzed the biological characteristics and expression patterns of mRNAs for a number of vascular endothelial cell growth factors, since unique and close relationships between tumor cell nests and vascular endothelial cells have been observed in invasion-independent metastasis.

MATERIALS AND METHODS

1. Cell culture of MCH66 and its sublines

From a spontaneous mammary tumor of a C3H mouse, MCH66 was established and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-bio, Kerrville, TX, USA) at 37°C in a humidified 5% CO_2 atmosphere. The cell line initially revealed metastatic potential when 1×10^7 cells were inoculated into the fat pads of C3H mice. Thereafter, however, MCH66 showed weak metastatic ability.

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2. Establishment of cell lines with various metastatic potential

MCH66 cells, 1×10^7, suspended in 200 μl serum-free DMEM were subcutaneously inoculated into the abdominal wall of mice. After a few weeks, metastatic lung colonies were formed in selected mice. Coarsely isolated cells from the lung tumors were re-cultured to establish a cell line, Lu1. Repeating this *in vivo-in vitro* selection at ten cycles, we established ten cell lines, from Lu1 to Lu10.

3. Implantation and confirmation of metastatic potential

Three sublines (Lu1, Lu5 and Lu10) among Lu1 to Lu10 were selected according to differences in selection cycles. These sublines and MCH66 were subcutaneously inoculated into the abdominal wall of mice. Inoculum number was as above. After 8 weeks, mice were sacrificed and the tumors at inoculated sites were removed and weighed. Both lungs were removed and metastatic lung colonies were counted grossly. Other metastatic sites, if present, were evaluated macroscopically.

4. Determination of population doubling time

Cells, 1×10^4, were seeded onto 12-well-tissue-culture plates [Becton Dickinson (BD), Franklin Lakes, NJ, USA] and the number of cells per well was determined every 24 hrs for 4 successive days. Growth curves were depicted and doubling times were estimated from the growth curves.

5. Invasion assay

Matrigel (BD) was dissolved in phosphate-buffered saline (PBS) at 0.25 g/L and 50 μl of this solution was placed onto each insert bottom (Cell culture insert; BD). After drying this insert overnight at room temperature, 700 μl DMEM containing 1% fetal bovine serum (FBS) and mouse fibronectin (at 10 μg/ml) (BD) was added to each well of 12-well tissue-culture plates. Cells (2×10^5) suspended in 200 μl DMEM containing 1% FBS were plated above the Matrigel. Eight hours later, the inserts were removed from the wells and the upper side of the insert was wiped off. To fix the cells invading the Matrigel and through the Millipore membrane, the insert was floated in methanol for 5 min. These cells were stained by floating the inserts in hematoxylin solution for 5 min. The insert bottom was cut off, fixed on slide glass and the number of cells was counted under a light microscope.

6. Assessment of sinusoidal vessel area

Morphometry of sinusoidal vessels was assessed on histological sections at low power magnification (× 40). The areas of sinusoidal vessels and tumor parenchyma exclusive of necrosis were measured per field using a computerized image analyzer (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). The proportion of the total vascular area to that of non-necrotic tumor tissue was used as an index of vascularity for each tumor.

7. Immunohistochemistry

Immunohistochemical studies were performed on paraffin-embedded sections using an indirect immunoperoxidase method. After deparaffinization of sections, the slides were soaked in citrate buffer and microwaved at 100ºC for 10 min for antigen retrieval. Endogenous peroxidase was quenched by incubation with 0.3% hydrogen peroxide in methanol for 15 min. Each slide was reacted with monoclonal antibody LYVE (Lymphatic vessel endothelial receptor)-1 (MBL, Nagoya, Japan) diluted 1:100 as the primary antibody, and then with HISTOFINE simple stain mouse MAX-PO (RAT) (Nichirei Bioscience, Tokyo, Japan). Polyclonal primary antibody Prox-1 (AngioBio Co., Del Mar, CA, USA) diluted 1 : 5000 was applied to the slides, followed by HISTOFINE SAB-PO (Nichirei Bioscience). These reagent kits were used according to the manufacturers' instructions. These sections were counterstained for nuclei with hematoxylin.

8. RT-polymerase chain reaction (PCR)

First, total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Superscript III (Invitrogen) with oligo (dT) was used to generate full-length cDNA from total RNA.

Then, we semi-quantitatively analyzed the expression of a number of vascular endothelial cell growth factors using RT-PCR. The factors included pleiotrophin [8–10], midkine [11, 12], VEGF-A [13], VEGF-C [14, 15], VEGF-D [16, 17], angiogenin [18, 19], platelet-derived growth factor-A [20, 21], PDGF-B [22–24], basic fibroblast growth factor (bFGF) [25, 26] and hepatocyte growth factor [27, 28]. Primer sequences are shown in Table 1. The thermal cycle was 1 min at 95ºC for denaturing; 1 min at 55ºC for PDGF-A, hepatocyte growth factor, angiogenin and pleiotrophin, 56ºC for VEGF-D, 58ºC for β-actin, VEGF-A and VEGF-C, 60ºC for basic fibroblast growth factor, PDGF-B and midkine for annealing; 1 min at 68ºC for extension. This cycle was repeated as shown in Fig. 7.

9. Statistical analysis

All results are expressed as the mean ± standard errors. Statistical comparisons were made using Student's t-test. Differences were considered to be significant at $p < 0.05$.

RESULTS

1. Growth properties of cell lines

Growth curves shown in Fig. 1 revealed an almost similar growth rate between MCH66 and Lu10, and the growth speed of both cell lines was slower than that of Lu1 or Lu5 (statistically not significant). Doubling times of MCH66, Lu1, 5 and 10 were 21.6, 12.6, 14.4 and 18.6 hrs, respectively (statistically not significant).

2. Morphology

Morphology of each cell line observed by phase-contrast microscopy was almost the same and showed epithelial, polygonal configuration with prominent nuclei. Cells grew adherently on the culture dish. The histology of tumors formed at the inoculated sites of MCH66, Lu1, 5 and 10 was almost similar (Fig. 2). Tumors disclosed confined, solid growth with central necrosis. In and around the tumor, sinusoidal ves-

Table 1

Primer name	Sense	Anti-sense
β-actin	5'- GGGTCAGAAGGACTCCTATG-3'	5'-TCGGTCAGGATCTTCATGAG-3
pleiotrophin	5'-GCAATATCAGCAGCAACGTAGAAA-3'	5'-TGGCGTCTTTTAATCCAGCATC-3'
midkine	5'-AGTGTTCGGAGTGGACCTGGGG-3'	5'-TCCGTTCAGGCTCCAGGCGA-3'
vascular endothelial growth factor (VEGF)	5'-CGAGACCCTGGTGGACATCT-3'	5'-CACCGCCTCGGCTTGTCAC-3'
VEGF-C	5'-CAACAAGGAGCTGGATGAAG-3'	5'-GGCCTTTTCCAATACGATGG-3';
VEGF-D	5'-ACCAACACATTCTTCAAGCC-3'	5'-GCGATCTTCATCAAACGTCA-3'
angiogenin	5'-GTTGGAAGAGATGGCGATAAGC-3'	5'-GACTGACTCTTAATGGCTTTTGAGAC-3';
platelet-derived growth factor (PDGF)-A	5'-CGGTCATTTACGAGATACCT-3'	5'-TTTTGTTTTCGCTCTCTGTG-3'
PDGF-B	5'-GCGACCACTCCATCCGCTCCTTTG-3'	5'-CCGAGCTTGAGGCGTCTTGGCT-3'
basic fibroblast growth factor	5'-AGCGGCATCACCTCGCTTCC-3'	5'-TGGA AGAAACAGTATGGCCTTCTGTCC-3'
hepatocyte growth factor	5'-TACAGGGGAACCAGCAATACCA-3'	5'-TCAAACTAACCATCCACCCTACTGT-3'

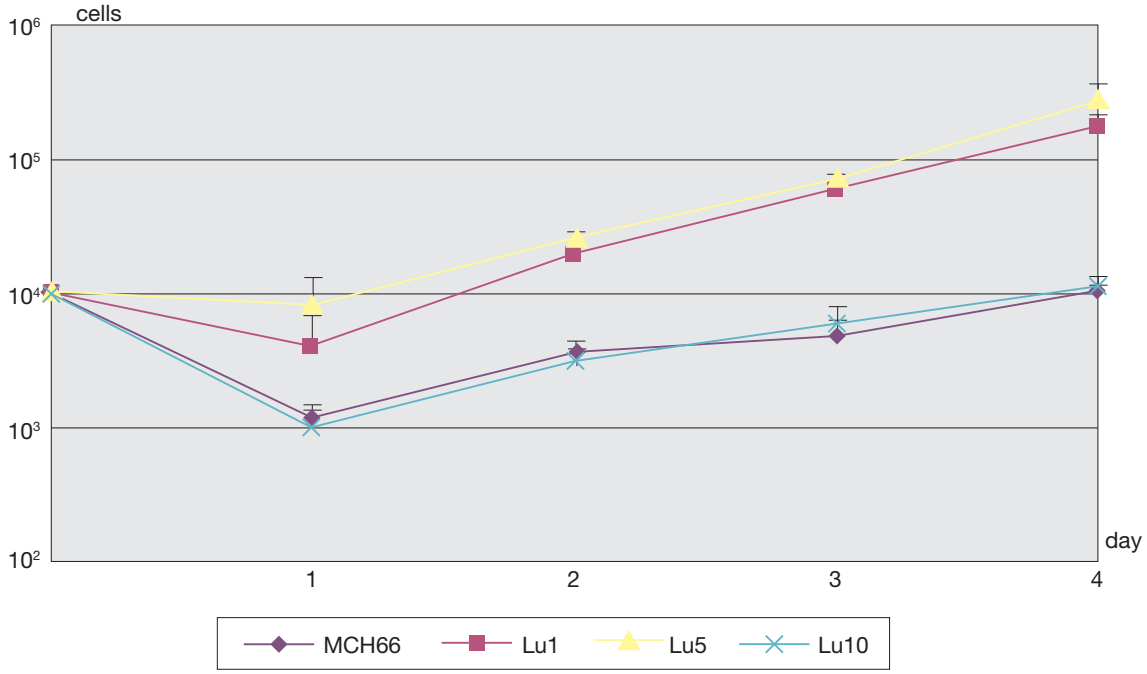


Fig. 1 Growth curves of each cell line.
Lu1 and 5 show more rapid growth than Lu10 and MCH66, but there is no difference in growth rate between Lu10 and MCH66 or between Lu1 and 5.

sel formation was seen, and this tendency was more marked in Lu10 than Lu1 (Fig. 2). These channels seemed to be composed of blood vessels containing red blood cells in their lumina. In these sinusoidal vessels, tumor cell nests surrounded by endothelial cells were being torn off (Fig. 3A, arrow) or floating in the lumen (Fig. 3B, arrow).

3. Metastatic potential

The weight of subcutaneous tumors and the metastatic status from subcutaneous tumor are shown in Table 2. The tumors of MCH66, Lu1, 5 and 10 weighed 8.8 ± 2.5 g, 4.6 ± 0.8 g, 2.5 ± 1.0 g and 8.4 ± 0.9 g, respectively (statistically not significant). The numbers of metastatic lung colonies of these cell lines were 0, 0, 13 ± 5.5 and 17 ± 7.3, respectively. Statistically, significant differences in metastatic capability were seen between MCH66 and Lu5, between MCH66 and Lu10, between Lu1 and 5, and between Lu1 and 10 ($p < 0.05$), respectively. Axillary lymph node metastases were also observed in Lu5 (1/5, 20%) and 10 (4/4, 100%) but not in Lu1. No metastasis was seen in other lymph nodes.

4. *In vitro* invasion assay

Cell counts by the invasion assay in MCH66, Lu1, 5 and 10 were 82.3 ± 8.0, 332.6 ± 171.5, 181.3 ± 61.8 and 245 ± 79.9, respectively (Fig. 4). Statistically, no

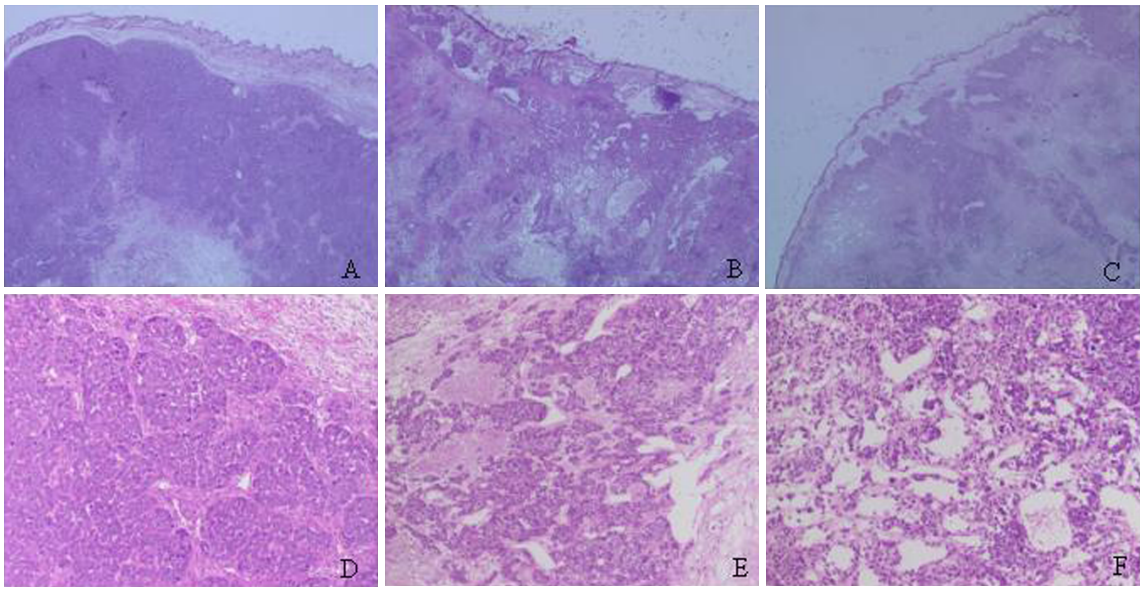


Fig. 2 Histology of tumors formed at the inoculation site.
Each tumor shows a solid growth pattern at low magnification (A: Lu1, B: Lu5, C: Lu10) and is composed of cohesive polygonal tumor cells with prominent nuclei. Central coagulation necrosis of the tumor is often seen (A, B, C) and in Lu5 (E) and Lu10 (F), development of sinusoidal vessels in or around the tumor is noticed (hematoxylin-eosin staining, same magnification in Figs A, B and C, and Figs D, E and F).

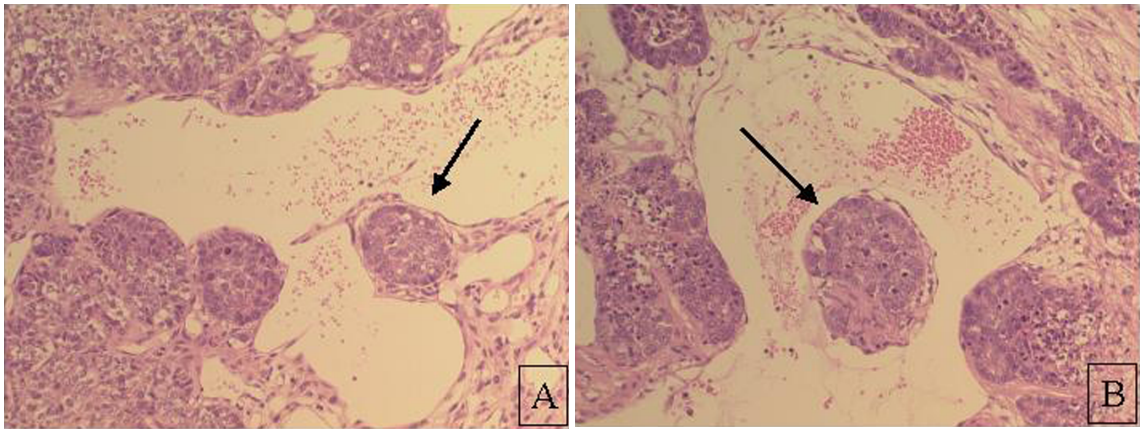


Fig. 3 Tumor cell nest surrounded by endothelial cells in the Lu10 tumor.
A. Tumor cell nests wrapped by vascular endothelial cells are seen and one is on the point of falling into the lumen (arrow). B. An endothelialized tumor cell nest is floating in the vascular lumen (arrow).

Table 2 Frequency of metastasis in each cell line

	weight (g)[*1]	lung metastasis[*2]	lymph node metastasis
MCH66	8.8 ± 2.5	0/5(0)	0/5
Lu1	4.6 ± 0.8	0/5(0)	0/5
Lu5	2.5 ± 1.0	5/5(13 ± 5.5)	1/5
Lu10	8.4 ± 0.9	4/4(17 ± 7.3)	4/4

*1: weight of subcutaneous tumor (mean ± S. D.)
*2: figures in parentheses show number of colonies (mean ± S. D.)

significant difference was seen among these results.

5. Assessment of sinusoidal vessel area

Areas of sinusoidal vessels in Lu1, 5 and 10 tumors were 7.62 ± 0.20, 20.42 ± 1.75 and 11.96 ± 0.55 % (Fig. 5). A statistically significant difference was seen between Lu1 tumor and Lu5 tumor ($p < 0.05$). There was no significant difference between Lu1 and Lu10

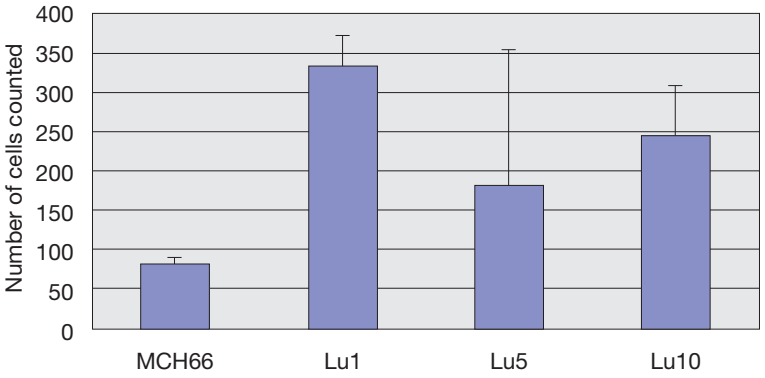


Fig. 4 Invasion assay of each cell line.
There is no significant difference in invasive activity among these four cell lines, but Lu1 shows rather high invasiveness.

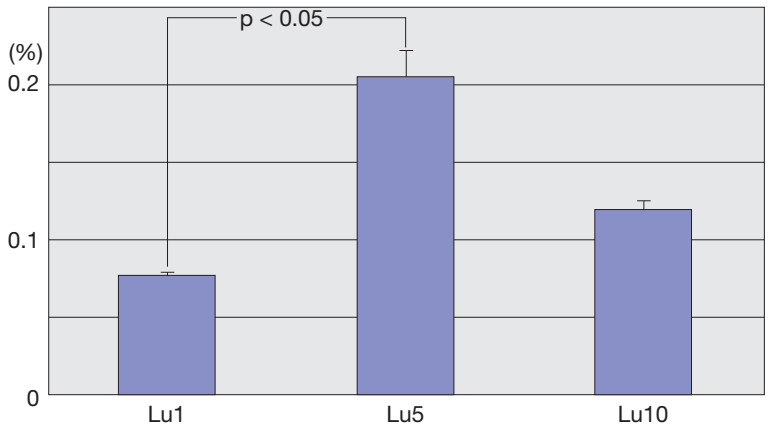


Fig. 5 Assessment of sinusoidal vessel area
The Lu5 tumor exhibits a significantly broader area of sinusoidal vessels compared with that of Lu1 tumor. There is no significant difference in sinusoidal vessel area between Lu1 and 10 tumors, and between Lu5 and 10 tumors. However, areas of sinusoidal vessels of Lu5 and 10 tumors are broader than that of Lu1 tumor.

tumors or between Lu5 and Lu10 tumors.

6. Immunohistochemistry

LYVE-1 labeled the cytoplasm of lymphatic endothelial cells, while Prox-1 marked nuclei of endothelial cells. In the Lu10 tumor, tumor cell nests wrapped up in lymphatic endothelium were easily found (Fig. 6A and B), whereas no tumor cell nests covered with lymphatic endothelium were detected in MCH66, Lu1 and Lu5 tumors.

7. RT-PCR assay for vascular endothelial cell proliferative factors

As shown in Fig. 7, Pleiotrophin mRNA was selectively detected in Lu10. And mRNAs for VEGF-C, -D and PDGF-B were seemed to be showing strong signals in Lu10 compared with those in Lu1. In addition, VEGF-C and -D mRNAs were seemed to be showing gradually increasing signals from Lu1 to Lu10, whereas VEGF-A (189, 165, 121) mRNA seemed to be unchanged. No marked change was observed in the expression of other mRNAs for PDGF-A, angiogenin, HGF, bFGF and midkine.

DISCUSSION

In the present study, we established 10 sublines from MCH66 by the method of *in vivo-in vitro* selection and analyzed the biological characteristics of three cell lines, Lu1, 5 and 10, by comparing with those of MCH66. These cell lines were chosen as the representative cycle, Lu1 (first cycle), Lu5 (five cycles) and Lu10 (ten cycles). Lu5 and 10 disclosed high metastatic ability to the lungs through an invasion-independent pathway, whereas MCH66 and Lu1 showed no lung metastasis. There were no distinctive differences in growth patterns and population doubling times among these four cell lines. According to an *in vitro* invasion assay, the four cell lines showed almost similar pattern, rather low invasiveness. These results are contrary to the general assumption that high metastatic cells are armed with high invasive capacity, active migratory activity and rather high proliferative potential [29–33]. For invasion-independent metastasis, therefore, these abilities that seemed to be needed for obtaining active metastatic capacity do not seem to be required for cancer cells.

In vitro morphology and the histology of *in vivo* tumors revealed no marked differences among these four cell lines. However, numerous dilated vascular channels were noticed in and around Lu5 and Lu10 tumors formed at the inoculation site in mice. In contrast, dilated sinusoidal blood vessels were rarely found in Lu1 and MCH66 tumors. These observations are similar to a previous report, as Sugino *et al.* referred to the relation between the invasion-independent pathway and the proliferation of dilated sinusoidal vessels [6]. In this research, moreover, areas of sinusoidal vessels showed a significant difference between Lu1 and 5 tumors, and areas of sinusoidal vessels in Lu5 and 10 tumors were broader than those in Lu1 tumor. From these, the development and extension of sinusoidal vessels seem to be essential events for invasion-independent metastasis, although the factors responsible for the extension, endothelial wrapping of tumor cell nests and cohesiveness between tumor cells and endothelial cells remain to be elucidated.

From results of RT-PCR assay, Pleiotrophin mRNA was selectively detected in Lu10. And signals of

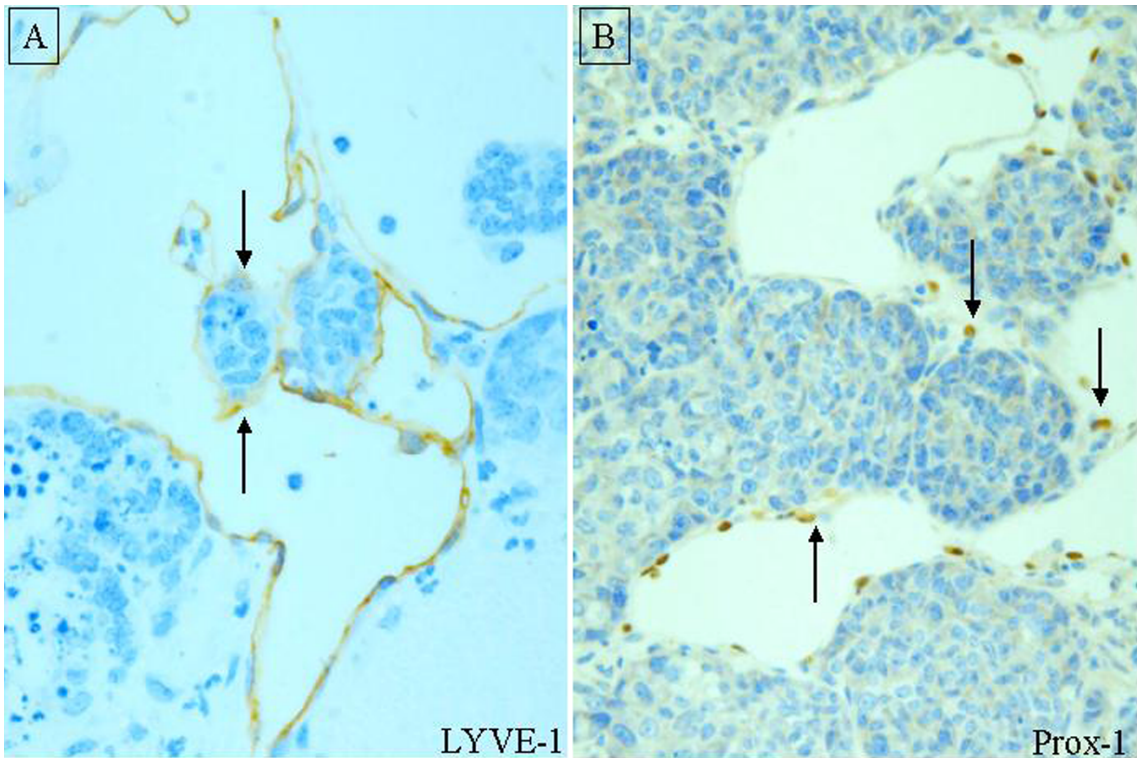
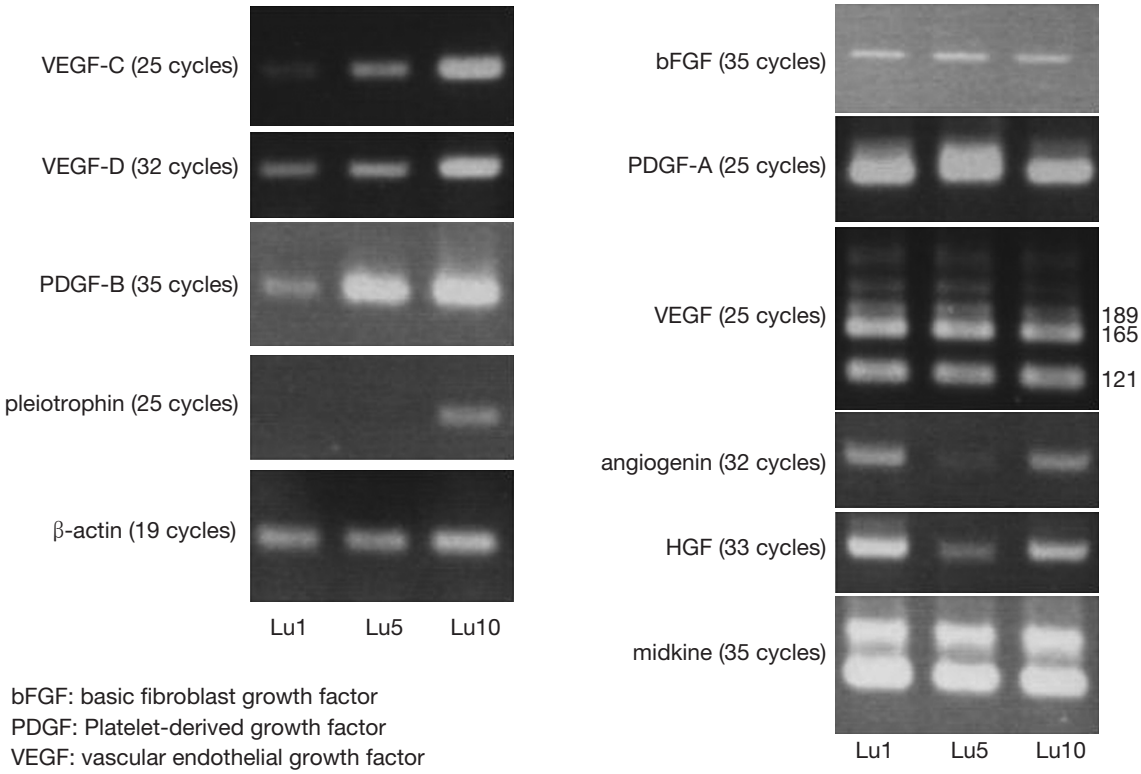


Fig. 6 Immunohistochemistry by LYVE-1 (A) and Prox-1 (B) in the Lu10 tumor
Both antibodies against lymphatic endothelial cells label endothelial cells in dilated vessels (arrows) and flat cells on tumor cell nests in dilated vessels (arrows, serial section). LYVE-1 immunoreactivity is readily recognizable rather than Prox-1 immunoreactivity.



bFGF: basic fibroblast growth factor
PDGF: Platelet-derived growth factor
VEGF: vascular endothelial growth factor

Fig. 7 mRNA expressions of various growth factors concerned with vascular endothelial cell growth.
Pleiotrophin mRNA is selectively detected in Lu10. mRNAs for VEGF-C, -D, PDGF-B are seemed to show strong signals in Lu10 compared with those in Lu1. In addition, VEGF-C and -D mRNAs are seemed to reveal gradually increasing signals from Lu1 to Lu10. The other mRNAs of VEGF-A, PDGF-A, angiogenin, HGF, bFGF and midkine exhibit no marked change in expression among these sublines.

PDGF-B, VEGF-C and -D were seemed to be stronger in Lu10 in comparison with that in Lu1, although VEGF-A mRNA remained constantly expressed in the three cell lines. In addition, Lu10 seemed to be showing gradually increasing signal of VEGF-C and -D mRNAs from Lu1, Lu5 to Lu10.

PDGF-B is a potential promotor of endothelial growth [23, 24] but pleiotrophin has diverse activities, including nervous tissue development [34, 35], spermatogenesis [36], angiogenesis [9, 10] and maintenance of the cell growth of several human cancers[8–10]. However there is no apparent evidence or literature until now, we want to propose the hypothesis that PDGF-B and pleiotrophin, possibly in combination with VEGF-A, might be involved in blood channel induction in this invasion-independent metastatic model, and pleiotrophin might also support tumor cell survival. On the other hand, VEGF-C and -D bind to VEGF receptor 3 and participate in lymphangiogenesis [14–17, 37–40]. By immunohistochemistry, tumor cell nests were wrapped up in lymphatic endothelial cells. This finding showed lymphangiogenesis and lymphatic endothelial proliferation in the Lu10 tumor. This suggests that Lu10 tumor metastasizes to lymph nodes in an invasion-independent manner, and these two molecules, VEGF-C and -D, seem to enhance lymph node metastasis in Lu10.

The results of this study provide new insight into understanding invasion-independent metastasis to the lungs and lymph nodes.

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