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A Radiation-induced Murine Ovarian Granulosa Cell Tumor Line: Introduction of v-*ras* Gene Potentiates a High Metastatic Ability

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A non-metastatic epithelial tumor cell line, OV3121, was established from ovarian granulosa cell tumor in B6C3F1 mouse irradiated with ^{60}Co-gamma rays. OV3121 cells showed an epithelial morphology and grew in monolayer with a population doubling time of 28–30 h. The production of estradiol and the expression of cytokeratin confirmed the epithelial origin of the line. No pulmonary metastasis was observed from solid tumors after subcutaneous (s.c.) injection or after intravenous (i.v.) injection of a clonal subline, OV3121-1 cells. We examined the experimental metastasis of individual clones of OV3121-1 cells, containing various introduced viral oncogenes: v-Ha-*ras*, v-Ki-*ras*, v-*fms*, v-*mos*, v-*raf*, v-*src*, v-*sis*, v-*fos* and v-*myc*. Among them, only OV3121-1 cells with v-Ha-MuSV or v-Ki-MuSV produced lung colonies at high frequencies. In a more detailed analysis, the v-Ha-*ras* transfectants OV-ras4 and OV-ras7 were found to form colonies in various organs by metastasis from tumors after s.c. injection, as well as lung colonies after i.v. injection. Moderately metastatic OV-ras7 cells showed high gelatinolytic activity at 72 kDa (MMP-2) and 92 kDa (MMP-9) as compared with the parental OV3121-1 and OV-Neo control cells by zymographic analysis. However, more metastatic OV-ras4 cells produced progressively weaker bands of 72 kDa gelatinolytic activity. No gross alterations in the expression of MMP-1, MMP-3, TIMP-1 and TIMP-2 transcripts were detected in these cell lines. These results suggest that this ovarian granulosa cell tumor line may provide a useful system for understanding the mechanisms by which oncogenes influence the occurrence of metastasis.

Key words: Metastasis — Oncogene — Ovarian granulosa cell tumor — Steroidogenesis — v-Ha-*ras*

Metastatic spread is the most important form of tumor progression because it is the most life-threatening aspect of the disease. Metastasis is a multistep process in which tumor cells become detached from the primary tumor, enter the circulation, are arrested at a distant site, exit the bloodstream, and grow.[1] During tumor progression many changes may occur genotypically and phenotypically, including increases in gene dosage, expression of oncogenes and tumor suppressor genes, and genetic instability. To elucidate the role of oncogenes in the metastatic process, one approach is to introduce an oncogene into a non-metastatic cell line and to monitor the metastatic ability of the oncogene-bearing cells in appropriate hosts. Indeed, many reports have indicated that activation of *ras* oncogenes plays a role in tumor progression.[2–7] We have reported that NIH3T3 cells transformed by the oncogenes *mos, raf, src, fes* and *fms* formed experimental metastases when injected i.v. into nude mice and these genes were more efficient at metastatic conversion than mutant *ras* gene.[8] Other studies have also suggested a role for various oncogenes in malignant tumor progression.[9] Although most human cancers are epithelial in origin, fibroblastic cells have most commonly been used for these studies. Therefore, it seemed desirable to use non-metastatic or weakly metastatic epithelial tumor cells as recipient cells for the introduction of various oncogenes using a syngeneic mouse system. For this purpose, we have made many attempts to establish cell lines from primary or transplantable tumors with non-metastatic behavior.

In this paper, we report the successful establishment of a non-metastatic epithelial tumor cell line (OV3121) derived from an ovarian granulosa cell tumor in B6C3F1 mouse irradiated with ^{60}Co-gamma rays. This line has been analyzed with respect to the growth properties, cellular morphology, steroidogenesis, tumorigenicity and metastatic behavior in syngeneic mice. We also report that gene transfer of various oncogenes resulted in the

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recovery of cell clones with metastatic potential at a frequency significantly above that observed following control gene transfer.

MATERIALS AND METHODS

Mice Six- to 8-week-old (C57BL/6N×C3H/He)F1 (B6C3F1) female mice were purchased from Charles River Japan, Inc., Atsugi.

Establishment and culture of mouse tumor line An ovarian granulosa cell tumor developed in a B6C3F1 mouse which was given ^{60}Co gamma-irradiation.[10] The primary tumor, designated as 6HF1 3121, was transplanted s.c. on the back of a syngenic mouse. A cell line was established by s.c. back transplantation of the primary tumor for the second transplant generation, then tumor cells were removed for *in vitro* cultivation. This ovarian granulosa cell tumor cell line was designated as OV3121. Subclones were obtained after two cycles of a metal cylinder isolation of colonies and subsequent colony formation in soft agar.

The medium was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 m*M* L-glutamine, 100 μg/ml of streptomycin, 100 IU/ml of penicillin, and 8% heat-inactivated fetal calf serum (FCS) (Hazleton Res. Products, St. Lenexa, KA). The cells proved to be mycoplasma-free as tested by DNA staining (Hoechst 33258).

Transforming retroviruses and infection Defective transforming viruses were rescued from non-producer cell lines[11] after infection with high multiplicities of ecotropic murine leukemia virus (MuLV) stocks.[12] Techniques for assaying murine leukemia and sarcoma viruses and infectious center assays to determine the proportion of cells in a population containing leukemia and sarcoma viruses have been described previously.[13, 14] pM-MuLV (myc)-transformed NIH3T3 cells (v-*myc* virus producer)[15] were kindly provided by Dr. H. Fan (University of California, Irvine, CA).

Transfection of the v-Ha-*ras* gene The plasmid pBW 1160, containing the v-Ha-*ras* gene and the aminoglycoside phosphotransfer gene conferring resistance to the neomycin analogue, G418, under control of a Moloney murine leukemic virus (Mo-MuLV) LTR was kindly provided by Dr. D. R. Rowly (National Cancer Institute, NIH, Bethesda, MD). The pBW1160 plasmid DNA was introduced into 6×10^6 OV3121-1 cells by electroporation using an Electric Cell Borer (Rikoukagaku Lab., Tokyo) by applying 2200 V five times. Transfected cells were selected with 800 μg/ml of G418 (Gibco Lab., Grand Island, NY). Individual colonies were obtained after two cycles of metal cylinder isolation.

Tumorigenicity and experimental metastasis assay The tumorigenicity of the cell lines was tested by s.c. injection of $1–5\times10^6$ cells suspended in 0.2 ml of phosphate-buffered saline (PBS) into 6- or 8-week-old B6C3F1 mice. Using calipers, tumor growth was measured weekly in terms of the diameters of the tumor. Mice were killed 150 days after cell inoculation or when moribund, and the tissues were examined for metastasis in various organs and processed for histology.

For the experimental metastasis assay, cells were harvested by a brief exposure to 0.25% trypsin in 0.02% EDTA, washed twice in serum-free DMEM and resuspended to the desired concentration. Viabiliy was greater than 95% as assessed by Trypan blue exclusion. To test the metastatic potential, aliquots of 1×10^4 to 1×10^6 cells in 0.5 ml were injected into the lateral tail vein of 8-week-old B6C3F1 mice. Eight weeks later, mice were killed and Bouin's fixative instilled directly in the trachea with a syringe. The lungs were removed, and metastases were counted under a dissecting microscope.[8]

Isolation of DNA and Southern analysis DNA was isolated from the cultured cells as described previously.[16] Cellular DNA (5 μg) was digested with restriction endonuclease *Eco*RI and processed for Southern blotting hybridization.[17] The probes used were: v-Ha-*ras* (pBS-9), v-Ki-*ras* (pHiHi-3), v-*myc* (pMC3), v-*fos* (pfos-1), v-*raf* (361 1E-H), v-*fms* (pSM3), v-*src* (pPvuIIE), v-*abl* (pV-abl) and v-*mos* (pHT10). All of these plasmids were obtained from the Japanese Cancer Research Resources Bank (Tokyo).

Isolation of RNA and Northern analysis Total cellular RNA was extracted from the cells using the acid guanidinium thiocyanate-phenol-chloroform method.[18] Electrophoresis, transfer and hybridization were carried out as described previously.[12] The relative intensity of each of the Northern bands was estimated with a Fujix Bio-Image Analyzer BAS 2000 (Fuji Photo Film Co., Ltd., Kanagawa) using phosphor imaging plates.[19] Glyceraldehyde phosphate dehydrogenase (GAPDH) probe was used as an internal control. The probes for MMP-1, -2, -3, -9, TIMP-1 and TIMP-2 were kindly provided by Dr. M. Seiki[20] (Cancer Research Institute, Kanazawa University, Kanazawa).

Isolation of protein and Western analysis The amount of oncoprotein p21 was detected by the standard Western blotting technique.[21] Total protein lysates (100 μg) prepared from equivalent numbers of cells were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Nitrocellulose papers were washed and incubated with 3% bovine serum albumin for 3 h at 37°C and then sequentially with medium containing p21 monoclonal antiserum Y13-259, for 16 h at 4°C, rabbit anti-rat IgG for 2.5 h in an ice-water bath, and 5×10^5 cpm/ml ^{125}I-labeled Protein A for 1 h in an ice-water bath. The nitrocellulose papers were air-dried and exposed to Kodak XAR film.

This antibody recognizes both the Ki- and Ha-*ras* gene products.[22] p21 monoclonal antibody Y13-259 was kindly provided by Dr. D. R. Lowy.

Zymographic analysis Cell-free supernatant from cells cultured for 48 h in the absence of serum was concentrated 20-fold with a Centricut (Kurabo, Osaka). Zymography was done as described by Koshikawa *et al.*[23] Briefly, gelatin zymography of proteinases was carried out on 7.5% polyacrylamide slab gels under nonreducing conditions. For the zymography, proteinases separated on the gels containing 1 mg/ml gelatin were renatured and then incubated in 50 m*M* Tris-HCl (pH 7.5) with $CaCl_2$ at 37°C for 24 h. The gels were stained with Coomassie Brilliant Blue R-250. The molecular weight markers used were rabbit muscle phosphorylase *b* (Mr 97,400), bovine serum albumin (Mr 66,200), hen egg

Table I. Biological Characteristics of OV3121 and Its Sublines

Cells	Doubling time[a] (h)	Cloning efficiency[b] (%)	Tumor formation[c]	Cytokeratin[d]	Production of steroid hormones[e]			
					Estradiol (pg/ml)	Estriol (ng/ml)	Pregnenolone (ng/ml)	Progesterone (ng/ml)
OV3121	30	36	5/5	+	710±45	—	—	—
OV3121-1	30	45	6/6	+	1249±161	—	—	—
OV3121-2	28	42	5/5	+	643±184	—	—	—

a) Doubling time of cells was determined as described.[24]
b) Colony formation in semi-solid agar was assayed as described.[12]
c) Viable cells (5×10^6) were injected s.c. into syngeneic B6C3F1 mice. The results are given as number of mice with tumors/number of mice given injection.
d) Expression of cytokeratin was performed using the immunohistochemical procedure as described.[25]
e) Cells were seeded at 2×10^6 cells per 10 ml of 5% FCS containing DMEM and cultured for 24 h. The medium was replaced and the supernatant was collected 72 h thereafter. Secretion of steroid hormones was tested by radioimmunoassay at Otsuka Assay Laboratory (Tokushima). Assay was done in triplicate. —: Undetectable, below the detection limit of the assay.

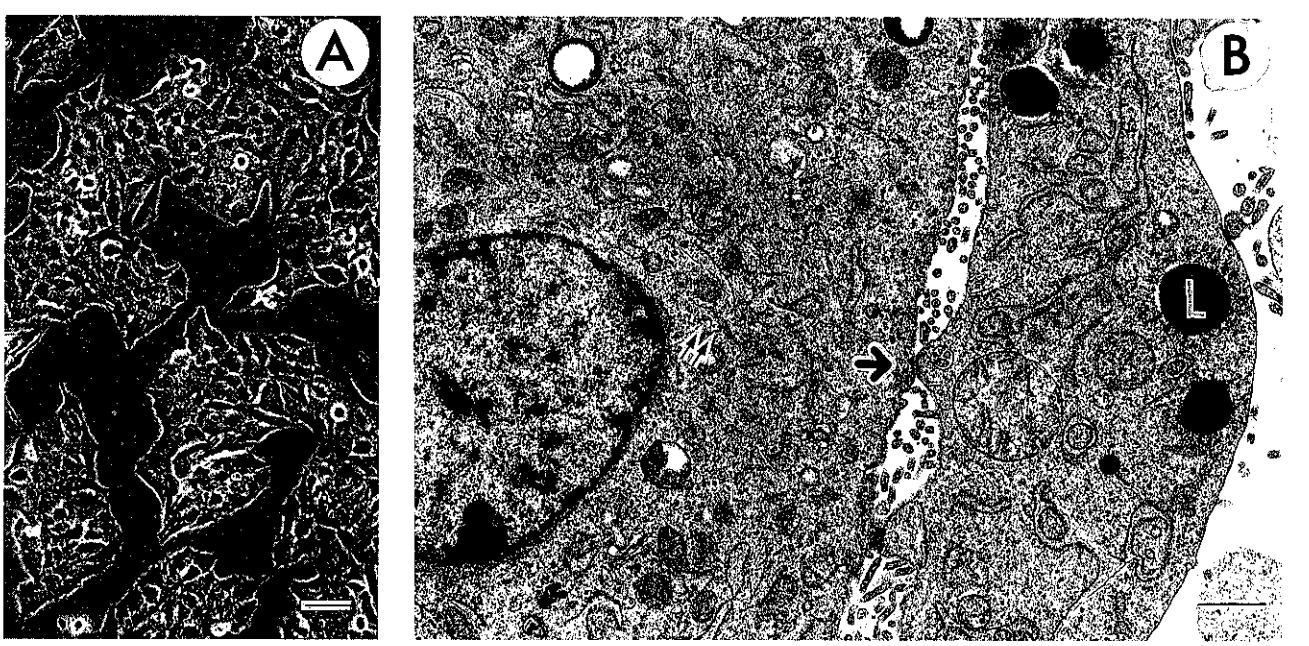

Fig. 1. (A) Phase-contrast micrograph of established OV3121-1 cells at the 20th passage. OV3121 cells showing typical cobblestone-like epithelial monolayers. Scale bar=20 μm. (B) Electron micrograph of OV3121-1 cells at the 20th passage. Intermediate junctions are present between adjacent cells (single arrow), and the cytoplasm contains tonofilaments (double arrow) and many lipid droplets (L). The electron micrograph was taken using a JEM-1200 as described previously.[16] Scale bar=1 μm.

while ovalbumin (Mr 45,000), bovine carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500), and hen egg white lysozyme (Mr 14,400).

RESULTS

Establishment and characterization of an ovarian granulosa cell tumor line After examination of six ^{60}Co-gamma ray-induced ovarian tumors for transplantation to syngenic mice, only one granulosa cell tumor was found to be tumorigenic but not metastatic. This granulosa cell tumor did not show any metastasis after 10 serial passages by subcutaneous injection into syngenic mice over 18 months.

A tumor cell line (designated OV3121) was established *in vitro* from the transplantable solid tumor at the second passage level. The parental OV3121 cells and clonal derivatives (OV3121-1 and OV3121-2) were biologically quite similar, growing *in vitro* in a monolayer with a population doubling time of 28–30 h (Table I). They formed colonies in soft agar, with a cloning efficiency of 36–45%. OV3121 and clonal cells showed typical cobblestone-like epithelial monolayers growing to confluence as polygonal cells with well-defined margins. The cells have scanty amphophilic cytoplasm, a small ovoid nucleus and small nucleolus (Fig. 1A). Ultrastructural examination demonstrated the presence of intermediate junctions or gap junctions and tonofilaments, confirming the epithelial nature of OV3121 cells (Fig. 1B). The cells contained a large number of lipid droplets, at low or high density. No secretory granules were observed.

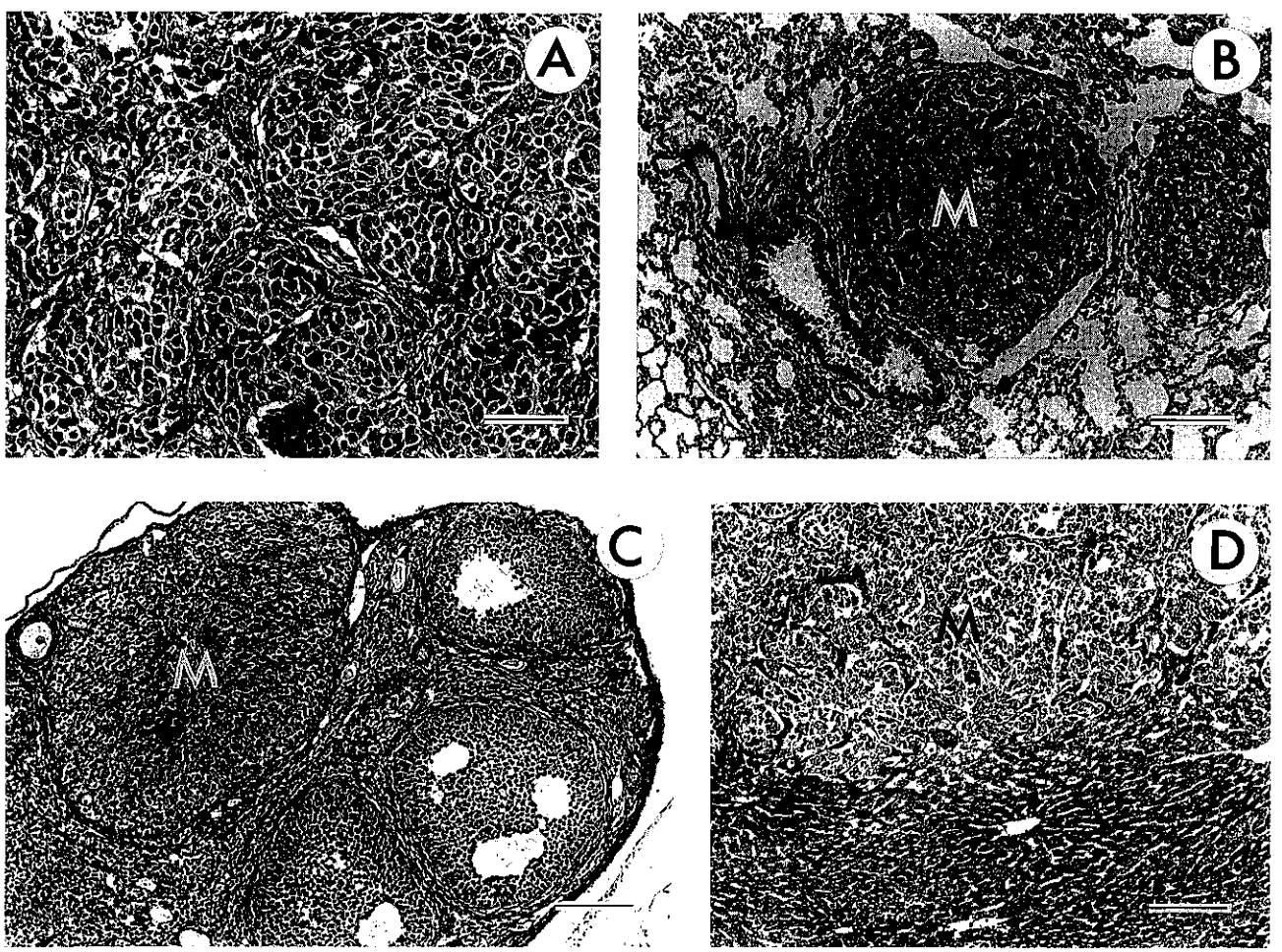

Fig. 2. (A) Histology of a tumor established by s.c. injection of OV3121-1 cells into syngeneic B6C3F1 mouse. Tumor cell growth and abundance of blood pooling are apparent. Scale bar=20 μm. (B) Histology of lung metastasis produced by v-Ki-*ras*-introduced OV3121-1 cells. (C) Histology of ovary metastasis produced by v-*sis*-introduced OV3121-1 cells. (D) Histology of liver metastasis produced by v-*mos*-introduced OV3121-1 cells. Original magnification for (A): ×200, scale bar=20 μm. Original magnification for (B) (C) and (D): ×100, scale bar=40 μm. The letter "M" overlies a metastatic deposit of tumor cells.

Table II. Non-metastatic Phenotypes of OV3121-1 Cells after Various Treatments[a)]

Treatment (dose)		Tumor formation[b)]	Spontaneous metastasis		Experimental metastasis	
			Lung	Ext. lung	Lung	Ext. lung
Passage	10	9/9	0/9	0/9	0/10	1/10 (liver)
	35	10/10	0/10	0/10	0/9	0/9
	51	7/7	0/7	0/7	0/9	1/9 (liver)
TPA	10 ng/ml	6/6	0/6	0/6	0/20	3/20 (liver)
	100 ng/ml	3/3	0/3	0/3	0/10	0/10
5-Aza-C	1.8 μg/ml	5/5	0/5	0/5	0/5	1/5 (ovary)
	2.4 μg/ml	5/5	0/5	0/5	0/5	1/5 (ovary)
MNNG	2 μg/ml	3/3	0/3	0/3	0/5	0/5
EMS	500 μg/ml	5/5	0/5	0/5	0/5	0/5
X-irradiation[c)]	8 Gy	12/12	0/12	0/12	0/10	0/10

a) Exponentially growing OV3121 cells were exposed to TPA, 5-Aza-C, MNNG or EMS for 24 h, passaged once, and inoculated.
b) Tumorigenicity and metastasis were assayed as described in "Materials and Methods." Mice were observed for 150 days. Experimental metastasis assay terminated at 56 days.
c) X-ray-irradiated cells were injected after *in vitro* cultivation for 30 days.

OV3121, OV3121-1 and OV3121-2 cells were examined for the shedding of steroid hormones in the cell-free culture supernatants (Table I). Culture fluids of these cell lines were shown to contain large amounts of estradiol (E2). However, estriol (E3), pregnenolone and progesterone were not detected after 72 h in culture.

OV3121 and clonal derivatives were tumorigenic following s.c. injection into syngeneic mice (Table I). Histopathologically, these tumors were mostly arranged as groups of tumor cells with poorly developed stromal tissue (Fig. 2A). There was an abundance of blood pooling and vascularization.

Lack of metastasis of OV3121 cells and sublines OV3121, OV3121-1 and OV3121-2 cells were tumorigenic but not metastatic following s.c. injection into syngeneic mice (Table II). When 1×10^6 OV3121-1 cells were injected into a lateral tail vein, only one out of 10 mice had one metastatic focus in the liver, and no metastatic colony could be found in the lung. Injection of 1×10^6 OV3121-1 cells into the portal vein in each of 5 mice resulted in the appearance of liver colonies with a mean number of three per mouse (range 1–5). However, less than 1×10^5 OV3121 cells injected i.v. did not form a colony in any organ (data not shown). *In vitro* subcultures for up to 51 passages did not affect the low metastatic character (Table II). Furthermore, treatment with several agents including ethylmethyl sulfonate (EMS), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), X-rays, the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and the hypomethylating agent 5-aza-2′-deoxycytidine (5-Aza-C) also failed to increase the metastatic potential (Table II).

Various viral oncogenes induce metastatic conversion of the epithelial tumor cells Previously, we reported that NIH3T3 fibroblastic cells transformed by several oncogenes, *mos, raf, src, fes* and *fms*, formed experimental metastasis and these genes were more efficient at metastatic conversion than a mutant *ras* gene.[8)] We assessed the ability of non-metastatic epithelial tumor OV3121-1 cells infected with a wide variety of oncogene-containing murine sarcoma viruses to form experimental metastasis. OV3121-1 cells were infected with viral-oncogene (v-*onc*)-containing retrovirus and passaged twice. Growth of v-*onc*-introduced cells was similar to that of the parental OV3121-1 cells. The v-*onc*-introduced OV3121-1 cells were injected i.v. into syngeneic mice for assessment of lung-colonizing ability. The v-Ha-*ras*- and v-Ki-*ras*-introduced cells produced lung colonies at high frequencies (Table IV, Fig. 2B). In contrast to the ability of these two *ras*-oncogenes to induce the metastatic phenotype, other cells with the oncogenes v-*src*, v-*fms*, v-*sis*, v-*myc*, v-*fos*, v-*raf* and v-*mos* did not form lung colonies. However, metastasis of these v-*onc*-introduced cells was observed in other organs, i.e., ovary, liver, kidney and lymph nodes (Table III, Fig. 2C and 2D). The frequency of metastasis to the ovary was much higher than that of OV3121-1 or Mo-MuLV-infected OV3121 control cells.

OV3121 sublines induced by v-Ha-*ras* gene transfection are metastatic We examined in more detail the link between v-Ha-*ras* oncogene and metastatic behavior of OV3121-1 cells. Transfectants were obtained by introducing the v-Ha-*ras* oncogene into OV3121-1 cells. Firstly, we tested overall spontaneous metastatic capacity to confirm the validity of results obtained with mass cultures. The isolation of sublines with different colony morphologies should ultimately enable us to analyze the relationship between *ras* expression and various biological properties. Two of the v-Ha-*ras*-introduced clonal

Table III. Production of Experimental Metastasis by i.v. Injection of OV3121-1 Cells into which Various Viral Oncogenes Had Been Introduced[a]

Oncogene	v-*onc* sequence[b]	Frequency and organ distribution				
		Lung	Ovary	Liver	Lymph node	Kidney
v-Ha-*ras*	+	4/5 (34±18)[d]	2/5	2/5	1/5	2/5
v-Ki-*ras*	+	3/4 (29±14)	3/4	1/4	1/4	0/4
v-*src*	+	0/4	2/4	0/4	0/4	0/4
v-*fms*	+	0/3	1/3	0/3	1/3	0/3
v-*sis*	+	0/3	2/3	0/3	0/3	0/3
v-*myc*	+	0/4	1/4	0/4	1/4	0/4
v-*fos*	+	0/5	1/5	1/5	0/5	0/5
v-*mos*	+	0/5	2/5	1/5	0/5	0/5
v-*raf*	+	0/4	2/4	0/4	0/4	0/4
Mo-MuLV	−	0/4	0/4	0/4	0/4	0/4
OV3121-1	−	0/7	0/7	1/7	0/7	0/7
LT92P[c]	−	5/5 (180±35)	1/5	1/5	5/5	0/5

a) Experimental metastasis assay terminated at 56 days.
b) Detection of v-*onc* sequence was performed using Southern blotting analysis.
c) A highly metastatic lung tumor cell clone, LT92P was used as a positive control.[26]
d) Number of metastatic nodules.

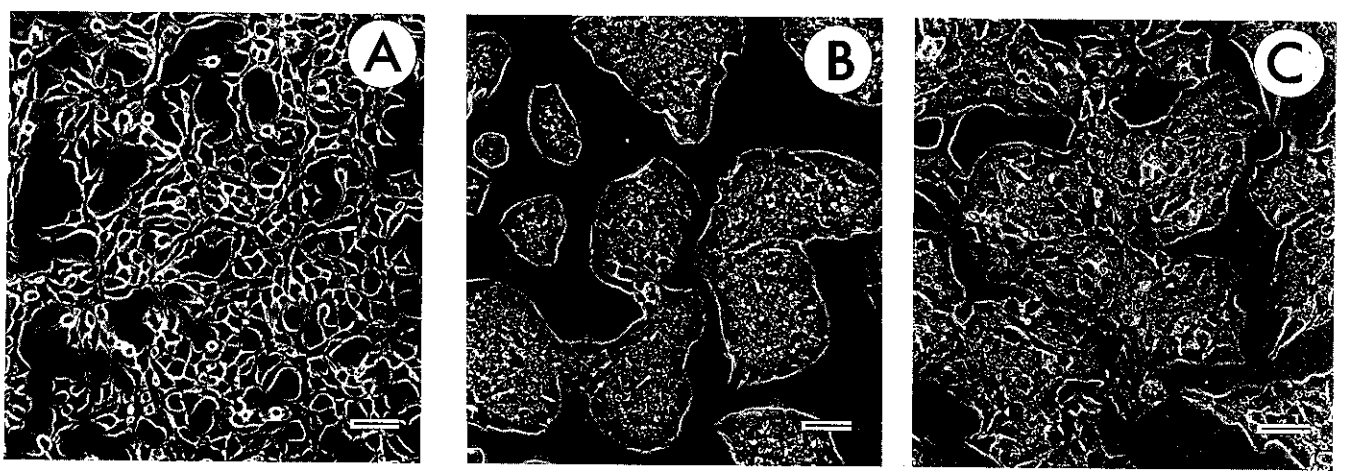

Fig. 3. Phase-contrast micrographs of v-Ha-*ras* gene transfectants. (A) OV-ras4 cells, showing predominantly spindle-shaped or more elongated cells. (B) OV-ras7 cells, showing epithelial cells packed into a more cuboidal morphology. (C) Control OV-Neo cells, showing similar morphology to the OV3121-1 cells (Fig. 1A). Scale bar=20 μm.

sublines, OV-ras4 and OV-ras7 cells, were chosen for further analysis. OV-ras4 cells formed a number of pseudopods and showed a disordered pattern and some round-shaped cells at high cell density, along with loss of intercellular contacts (Fig. 3A) as compared to OV-Neo control cells (Fig. 3C) and OV3121-1 (not shown). In contrast to the OV-ras4, OV-ras7 cells showed a compacted cobblestone-like pattern (Fig. 3B). The presence and overexpression of v-Ha-*ras* gene were confirmed by Southern and Western blot hybridization (Table IV). The growth rate of OV-ras4 cells was higher than that of the parental OV3121-1 or OV-Neo cells. However, OV-ras7 cells showed a slower growth rate than that of OV3121-1 cells.

These cells were inoculated either s.c. or i.v. into syngeneic mice to assess the spontaneous and experimental metastatic ability (Table IV). Spontaneous metastases were detected in seven of nine mice injected with OV-ras4 and three of nine injected with OV-ras7, but in none of the mice injected with either control OV-Neo or parental OV3121-1 cells. In experimental metastasis assay, OV-ras4 was highly metastatic, OV-ras7 was mod-

Table IV. Metastatic Abilities of OV3121-1 and v-Ha-*ras*-transfected Sublines

Line	v-Ha-*ras*[a]	Doubling time (h)	Growth in CDM[b]	Incidence of lung metastasis		Expression of mRNA[d]					
				Spontaneous	Experimental	MMP				TIMP	
						1	2	3	9	1	2
OV3121-1	−	30	−	0/10	0/5	−	+	−	+	−	+
OV-Neo	−	30	−	0/6	0/5	−	+	−	+	−	+
OV-ras7	+	35	−	3/9 (11±3.3)[c]	3/6 (31±15)[c]	−	+	−	++	−	+
OV-ras4	+	13	+	7/9 (18±5.8)	6/6 (>200)	−	−	−	++	−	+

a) Detection of v-Ha-*ras* sequence and elevated levels of p21 was performed using Southern blotting and Western blotting, respectively as described in "Materials and Methods."
b) CDM, chemically defined medium composed of DMEM/Ham's F12 (1:1) medium supplemented with 0.05% bovine serum albumin as described.[27]
c) Number of metastatic nodules.
d) The comparative intensity of Northern bands among parent, control and v-Ha-*ras* transfectants was determined with a Fujix Bio-Image Analyzer BAS 2000 and normalized with respect to the internal control.

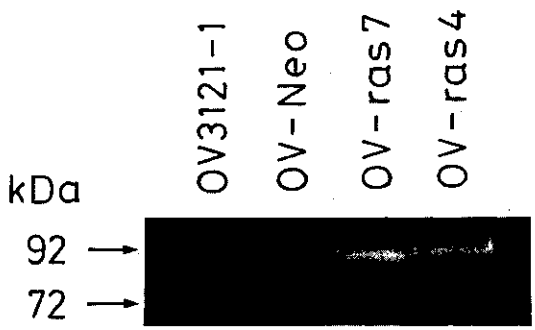

Fig. 4. Gelatin zymogram of 20-fold-concentrated serum-free culture media of OV3121-1 and v-Ha-*ras* gene transfectants. Gelatin zymography was performed as described in "Materials and Methods."

erately metastatic, and control OV-Neo was virtually non-metastatic. More than 200 colonies were observed in the lung at 68 days after i.v. injection of the OV-ras4 cells, and metastases in various organs (lymph nodes, liver, ovary and/or kidney) were found in all mice. The i.v. injection of OV-ras7 cells afforded approximately 31 colonies, mostly large. Control OV-Neo and OV3121-1 cells did not show lung colonization under the same conditions.

Production of proteolytic enzymes The aim of the next experiment was to determine whether induction of metastatic behavior by v-Ha-*ras* oncogene is associated with the expression of genes whose products are responsible for type IV collagen degradation. Therefore, we examined the mRNA expression of MMP-1, MMP-2, MMP-3, MMP-9 and their inhibitors, TIMP-1 and TIMP-2, in these 4 cells by Northern blotting (Table IV). A clear difference in mRNA expression for MMP-2 was observed between highly metastatic *ras* clone and the parental, moderately metastatic cells. OV3121-1, OV-Neo and OV-ras7 cells expressed considerable levels of mRNA for MMP-2, but OV-ras4 cells did not express it. Moreover, to confirm these results at the protein level, we tested the gelatinase activity. Gelatin zymography of 20-fold-concentrated culture media revealed that OV-ras4 cells produced a very weak band of 72 kDa (MMP-2) (Fig. 4). No evidence of activated forms of MMP-2 and MMP-9 was noted. In contrast, OV-ras7 cells produced two intense bands of gelatinolytic activity at 72 and 92 kDa (Fig. 4). Expression of MMP-1, MMP-3, TIMP-1 and TIMP-2 transcripts was not affected by the events associated with the malignant phenotype of OV3121-1 cells (Table IV).

DISCUSSION

We have established an epithelial tumor cell line, OV3121, derived from an ovarian granulosa cell tumor in B6C3F1 mouse irradiated with ^{60}Co-gamma rays. This cell line possesses a number of interesting biological properties, such as non-metastatic phenotype, secretion of estradiol and extensive angiogenesis. It has been reported that the metastatic potential of non- or low-metastatic cells can be increased by various treatments, though the magnitude of induction of metastatic phenotype differed among cells used.[28] Although some tumor cells frequently manifest phenotypic and genetic instability,[29] the non-metastatic character of OV3121-1 cells appears to be stable even after treatment of the cells with a mutagen, TPA or 5-Aza-C.

The finding in our system that v-Ha-*ras* transfection induced metastatic capability differs to some extent from other reports. For example, introduction of an activated form of c-Ha-*ras* gene into non-metastatic melanoma cells enhanced the potential for experimental metastasis

after i.v. inoculation, but not that for spontaneous metastatic ability after s.c. injection.[30] Matastatic ability of activated c-Ha-*ras*-transfected mammary adenocarcinoma cell line was detectable after s.c. injection, but not i.v. injection.[6] Our results are consistent with the data that the metastatic phenotype was acquired by transfection with the activated form of c-Ha-*ras* into immortal cells[3,7] or tumorigenic cells.[31,32] The degree of metastatic ability induced by transfection with an activated *ras* gene might depend on the origin or type of cells. OV3121-1 cell line is advantageous for the study of metastasis bacause it is completely unable to undergo spontaneous metastasis after s.c. inoculation and this phenotype is stable.

It is well known that *ras* gene expression in these cells alters the expression of a variety of genes, which are likely to contribute to induction of the metastatic phenotype.[33] Many studies have shown a correlation between production of 72 kDa (MMP-2) and 92 kDa (MMP-9) type IV collagenases/gelatinases and the metastatic potential of tumor cells.[34,35] One mechanism for the change in metastatic ability of the *ras* transfectants could be that elevated *ras* p21 expression is associated with an increase in proteolytic enzymes, including matrix metalloproteinases (MMPs), and a decrease in proteolytic inhibitors (TIMPs) of these enzymes. Garbisa *et al.*[36] have reported that conditioned media obtained from cultures of metastatic *ras*-transformed rat embryo cell lines contained high levels of a proteolytic activity capable of degrading type IV collagen, while the non-metastatic cell lines failed to produce significant levels of this activity. In this study, moderately metastatic OV-ras7 cells showed a correlation between high levels of gelatinolytic activities (72 kDa and 92 kDa) and high metastatic potential. However, surprisingly, the OV-ras4 cells with high metastatic potential were found to produce low or undetectable levels of mRNA expression of MMP-2 gene, while non-metastatic lines produced considerable levels of expression. OV-ras4 cells produced weak 72 kDa gelatinolytic activities. No correlation was found between the metastatic phenotype of the cell lines and the level of expression of two MMP (MMP-1 and MMP-3) and two TIMP (TIMP-1 and TIMP-2) transcripts. Taken together, these observations suggest that metastasis of OV-ras4 cells by v-*ras* oncogene is unrelated to enhanced MMP-2 production, but involves some other mechanism(s).

An important feature of the metastasis induced by introduction of various types of v-*onc* is organ specificity. The results of analysis of experimental metastasis of various v-*onc*-containing OV3121-1 cells can be summarized as follows: (a) *ras* oncogene induces lung metastasis: (b) other oncogenes, *src, fms, sis, myc, fos, raf* and *mos* do not induce lung colonies, but do induce metastasis to other organs (ovary, liver, kidney and lymph nodes). It is known that the organ-selective patterns of metastases are determined by factors other than anatomical location and vasculature of the primary tumor.[37] Many investigators have found that organ preference of metastasis is correlated with enhanced mitogenic responses to cytokines released from the target organs for colonization.[38-40] However, the reason for this organ preference of metastasis by OV3121-1 cells containing various introduced oncogenes is not clear. Further studies are required for delineating the mechanisms of this phenomenon.

In conclusion, OV3121 ovarian granulosa cell tumor line offers an interesting epithelial model system to understand some of the mechanisms involved in the complex metastatic process, and to evaluate various hypotheses that have been put forward.

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