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Transformation of Epithelial Madin-Darby Canine Kidney Cells with p60$^{v\text{-}src}$ Induces Expression of Membrane-Type 1 Matrix Metalloproteinase and Invasiveness[1]

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ABSTRACT

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is expressed both in carcinoma cells and in surrounding stromal fibroblasts. MT1-MMP localizes to the surface of tumor cells and is thought to play an important role in tumor invasion. To analyze the mechanism of MT1-MMP gene expression in epithelial tumor cells, the dog kidney epithelial cell line Madin-Darby canine kidney (MDCK) was transformed by oncogenes, including v-*src*, and expression of MT1-MMP was examined. Transformation of MDCK cells with v-*src* resulted in loss of cell-to-cell contacts and morphological change. Expression of MT1-MMP in v-*src*-transformed cells was identified by Northern and Western blotting. Gelatin zymography analysis showed that progelatinase A in the culture medium was processed from latent to activated form by MDCK cells transformed with v-*src*. The MDCK cells transformed by v-*src* were tumorigenic in the subcutis (ectopic) and kidney (orthotopic) of nude mice and spontaneously metastasized to the lung after orthotopic implantation. These results suggest that MT1-MMP induced by v-*src* transformation may promote invasiveness of transformed cells.

INTRODUCTION

MMPs[3] are overexpressed in various human malignancies (1). However, because MMPs, including gelatinase A and 72-kDa type IV collagenase, are secreted as an inactive zymogen, activation is another key regulatory step for MMP function *in vivo*. Molecular events in tumors appear conducive to MMP activation. Activated gelatinase A was specifically observed in tumor tissues, suggesting the localization of progelatinase A activator in tumor tissues (2–4). We have identified MT1-MMP as an activator of progelatinase A expressed on the surface of tumor cells (5). MT1-MMP functions not only as an activator but also as the receptor for gelatinase A on the cell surface (6).

Expression of MT1-MMP mRNA was identified both in carcinoma cells and in stromal fibroblasts (5, 7–11), whereas progelatinase A mRNA is synthesized in stromal cells (11–14). During mouse embryogenesis MT1-MMP is co-expressed with progelatinase A specifically in mesenchymal cells (15). Thus, expression of MT1-MMP mRNA in tumor epithelial cells is unique and should contribute to invasion.

Oncogenic transformation of kidney epithelial cell line MDCK with v-*src* reportedly down-regulates cell-to-cell adhesions and stimulates invasion into collagen gels (16). It is suggested that interference with epithelial cell adhesion can up-regulate mesenchymal gene programs (17). In this study we examined expression of MT1-MMP in MDCK cells transformed with v-*src* and the possible role of MT1-MMP for invasion was discussed.

MATERIALS AND METHODS

Cell Lines. MDCK cells were obtained from Japanese Cancer Research Resources Bank and cultured in DMEM supplemented with 5% FCS. Transformed cells were obtained by transfecting expression plasmids for v-*src*, Ha-*ras*, or c-*fos* into MDCK cells with a selectable *neo*^{r} gene (neomycin phosphotransferase gene of *Escherichia coli*) using the calcium phosphate method (18). Transfected MDCK cells were cultured in the presence of 800 μg/ml G418, and the resistant cells were cloned.

Northern Analysis of MT1-MMP and v-*src* mRNA Levels. Total RNA was extracted from the cells by the guanidine isothiocyanate method. Fifteen micrograms of total RNA were electrophoresed in a 1% agarose gel with 2.2 M formaldehyde. RNA was then transfered to a nylon membrane and hybridized with ^{32}P-labeled human MT1-MMP and v-*src* cDNA probes. Hybridization signals were examined using a BAS 1000 Bioimage Analyzer (Fuji Film, Tokyo, Japan). The amount of rRNA in the gel was visualized by ethidium bromide staining.

MT1-MMP Protein Levels. Cells were solubilized with SDS-PAGE sample buffer with the aid of sonication and electrophoresed on 10% SDS-PAGE minigels, transblotted to a nitrocellulose filter, and blotted with anti-MT1-MMP monoclonal Ab 114-1F2 (5).

Tyrosine Kinase Assay. Cells were solubilized in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1% NP-40, and pp60^{src} was immunoprecipitated with mouse anti-*src* Ab. A kinase reaction was performed with γ-^{32}P-labeled ATP, and phosphorylated proteins were resolved by 10% SDS-PAGE and visualized by BAS 1000.

Gelatin Zymography. Samples were mixed with SDS sample buffer in the absence of a reducing agent, and SDS-PAGE gels (10%) copolymerized with 1 mg/ml gelatin were used for electrophoresis to resolve the latent and activated species of gelatinase A. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h to remove the SDS and then incubated for 24 h at 37°C in reaction buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3). The gels were stained with 0.25% Coomassie brilliant blue R250.

Subcutaneous and Renal Subcapsule Injection. Tumorigenicity of transformed MDCK cells was assayed by s.c. inoculation of 1×10^6 cells into athymic nude mice. Renal subcapsule injection of cells was performed as described previously (19). Briefly, transformed cells (1×10^6) were injected in a volume of 0.05 ml PBS. Visible bulla formation between the renal parenchyma and capsule was the criterion for a successful injection. Three weeks after inoculation, the mice were killed, and the kidneys and lungs were removed and processed for histology. The sections were stained with H&E.

RESULTS

Expression of MT1-MMP in MDCK Cells Transformed with the v-*src* Gene. Four clones of MDCK cells transfected with the v-*src* gene were selected. Parental (MDCK) and MDCK cells transfected with control plasmid (neo) exhibited the characteristic cobblestone morphology (Fig.1). Three v-*src* clones (src1, src2, and src3) partially lost cell-to-cell contacts and acquired a rather spindle-shape morphology. Another v-*src* clone (src4) acquired a round morphology and lost cell-to-cell contacts. Anchorage-dependent cell growth rate

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[3] The abbreviations used are: MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; Ab, antibody.

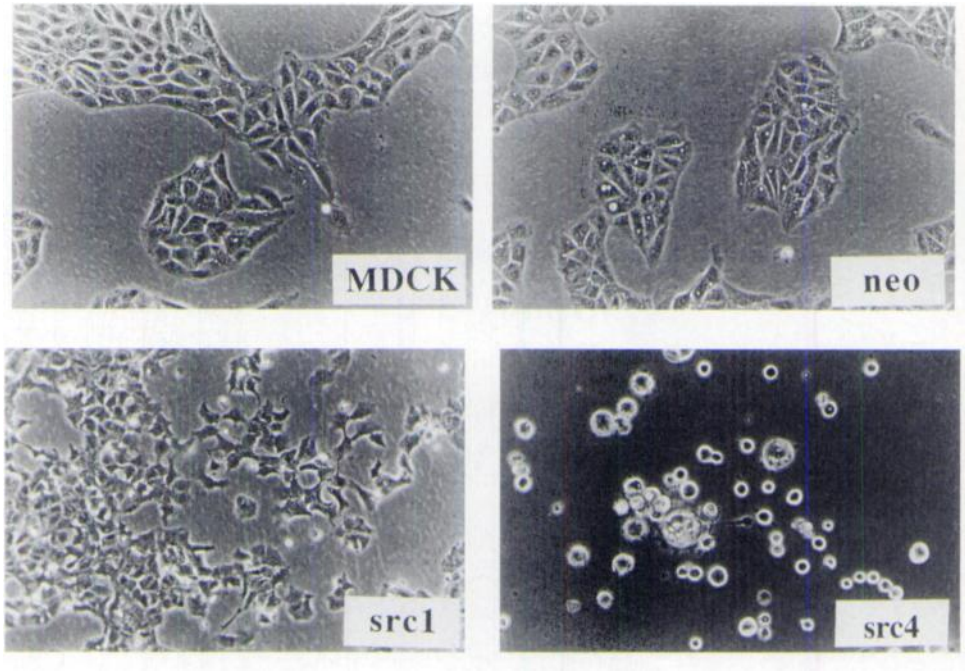

Fig. 1. Morphological changes of MDCK cells induced by transformation with v-src. A subconfluent culture of MDCK cells (*MDCK*), MDCK cells transfected with a control plasmid (*neo*), and two clones of MDCK cells transformed by v-*src* gene (*src1* and *src4*, respectively) are shown.

was not altered among v-*src*-transformed clones, neo, and parental MDCK cells (data not shown). Expression of v-*src* and MT1-MMP genes was examined by Northern hybridization (Fig. 2). The v-*src* clone that exhibited the most drastic morphological change (src4) synthesized the highest level of v-*src* mRNA. src1, src2, and src3, which showed a partial morphological change, expressed a lower level of v-*src* mRNA. Parental and control MDCK cells (MDCK and neo) did not synthesize detectable levels of v-*src* mRNA. Two clones of MDCK cells transfected with the v-*src* gene (src1 and src4) were also examined for v-src protein ($pp60^{src}$) expression by measuring tyrosine kinase activity of immunoprecipitated $pp60^{src}$ (Fig. 3). Autophosphorylation of $p60^{src}$ was seen with phosphorylated IgG heavy chain most intensely in v-*src* MDCK clone src4, which showed the highest v-*src* mRNA expression and the most drastic morphological change.

MT1-MMP mRNA was not detected in either parental MDCK cells or cells transfected with control plasmid (Fig. 2, *top panel*). The v-*src* MDCK clone that expressed an undetectable level of v-src mRNA (src1) did not synthesize a detectable level of mRNA transcript for MT1-MMP; however, src4 (highest v-*src* mRNA expression) showed high-level MT1-MMP mRNA expression. src2 and src3, which synthesized intermediate levels of v-*src* mRNA, expressed MT1-MMP mRNA in proportion to the v-*src* mRNA level. Expression of mRNA for other membrane-type MMPs, MT2-MMP and MT3-MMP was not detected with either parental or transformed cells (data not shown). MT1-MMP protein synthesis by MDCK, neo, src1, and src4 was examined by Western blotting using a monoclonal Ab specific for MT1-MMP. Immunoblotting analysis demonstrated synthesis of 63-kDa MT1-MMP protein in v-*src* clone src4 but not in other cells (Fig. 4). MT1-MMP mRNA expression was not detected in MDCK cells transfected with Ha-*ras* and c-*fos* genes by either Northern or Western analysis (data not shown).

Gelatin Zymography. Gelatin zymography was performed to monitor the synthesis and activation of gelatinase A by MDCK cells transformed with v-*src* (Fig. 5). MDCK cells and v-*src* transformants produced a low level of progelatinase A into culture supernatant. Thus, culture medium containing FCS, which contained progelatinase A, was used to examine the processing of progelatinase A (Fig. 5, *Lane culture medium*). Progelatinase A of 66 kDa derived from FCS was not processed by parental, control MDCK cells and a low v-*src*

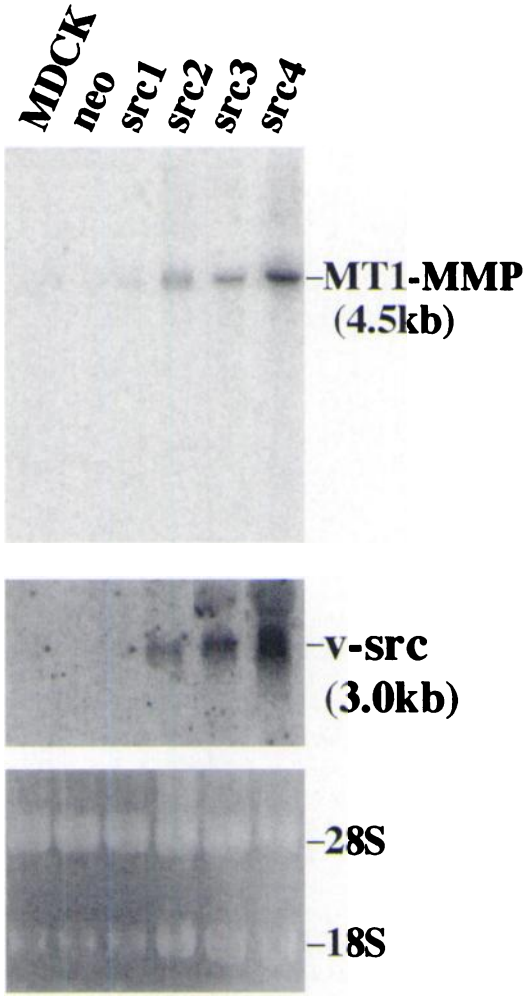

Fig. 2. Northern hybridization analysis. Total RNA (15 μg) from MDCK cells (*Lane MDCK*), MDCK cells transfected with control plasmid (*Lane neo*), and four clones of v-*src*-transformed cells (*Lanes src1, src2, src3,* and *src4*, respectively) was electrophoresed in a gel, stained with ethidium bromide (*bottom*), and then examined by Northern blotting probed with ^{32}P-labeled human MT1-MMP (*top*) or v-src (*middle*) cDNA fragments.

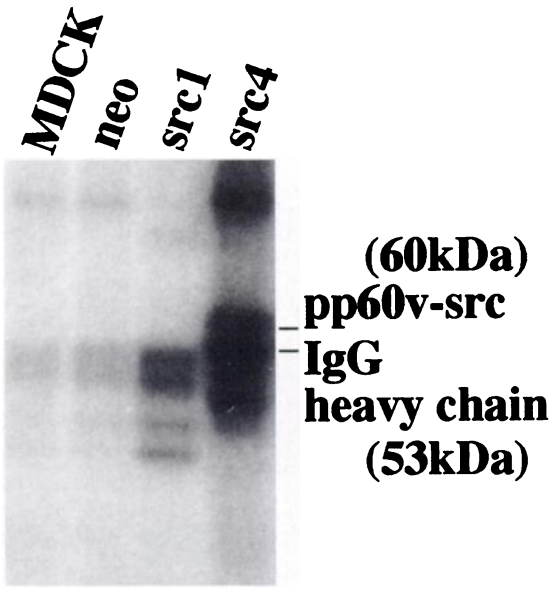

Fig. 3. *In vitro* tyrosine autophosphorylation. MDCK cells (*Lane MDCK*), MDCK cells transfected with control plasmid (*Lane neo*), and two clones of v-src MDCK cells (*Lanes src1* and *src4*, respectively) were immunoprecipitated with a monoclonal Ab against Src protein, and a kinase reaction with γ-^{32}P-ATP was performed, followed by SDS-PAGE and autoradiography. The 53-kDa IgG heavy chain and pp60$^{v\text{-}src}$ are indicated.

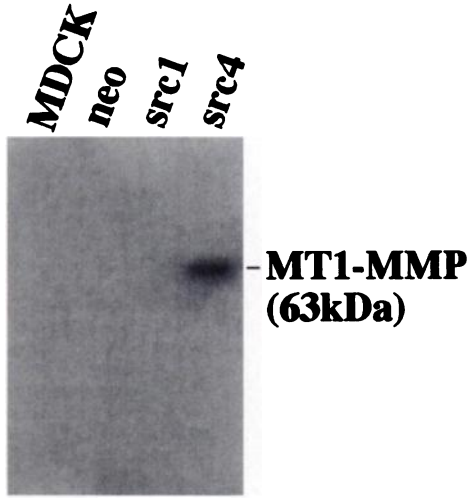

Fig. 4. Western analysis for MT1-MMP expression. MDCK cells (*Lane MDCK*), MDCK cells transfected with control plasmid (*Lane neo*), and two clones of v-*src*-transformed cells (*Lanes src1* and *src4*, respectively) were solubilized and subjected to SDS-PAGE. After blotting to a nitrocellulose filter, MT1-MMP protein was detected with a monoclonal Ab against MT1-MMP.

clone, src1 (Fig. 5, *Lanes MDCK, neo*, and *src1*, respectively). The activated gelatinase A species of 62 kDa was detected in culture medium from v-*src* high-producer clone src4 (Fig. 5, *Lane src4*). The activation of progelatinase A by src2 and src3 clones was faint (data not shown). Progelatinase B expression was not enhaced but rather decreased by v-*src* transformation, and progelatinase B was not processed by either control or v-*src*-transformed cells.

Tumorigenicity and Production of Metastasis by MDCK Cells Transformed by the v-*src* Gene. MDCK cells mock transformed (neo) and transformed with v-*src* (clone src4; 1.0×10^6 cells per mouse) were implanted into ectopic (s.c.) and orthotopic sites (renal subcapsule) of athymic nude mice. Mock-transformed MDCK cells (neo) did not grow in either subcutis or kidney of nude mice; however, v-*src*-transformed MDCK cells (clone src4) formed tumors in both organs (Table 1 and Fig. 6). Transformed MDCK cells implanted into kidney not only produced tumors but also metastasized to lung with high incidence. Histology showed invasive tumor growth in the kidney and the s.c. tissue. Synthesis of activated gelatinase A in tumors formed in mice trasplanted with v-src MDCK cells was confirmed by gelatin zymography (data not shown).

DISCUSSION

MT1-MMP is coexpressed with its substrate progelatinase A in mesenchymal cells during mouse embryogenesis (15). In human tumor tissues, MT1-MMP is overexpressed both in cancer cells and in stromal fibroblasts (5, 7–11), whereas progelatinase A is synthesized in fibroblasts (11–14). The expression in cancer cells should be associated with the invasive growth (5, 20, 21), and that in fibroblasts may be related to the tissue-remodeling process caused by invasive growth of cancer cells. Although MT1-MMP expressed on the surface of tumor cells is thought to play an important role in tumor invasion,

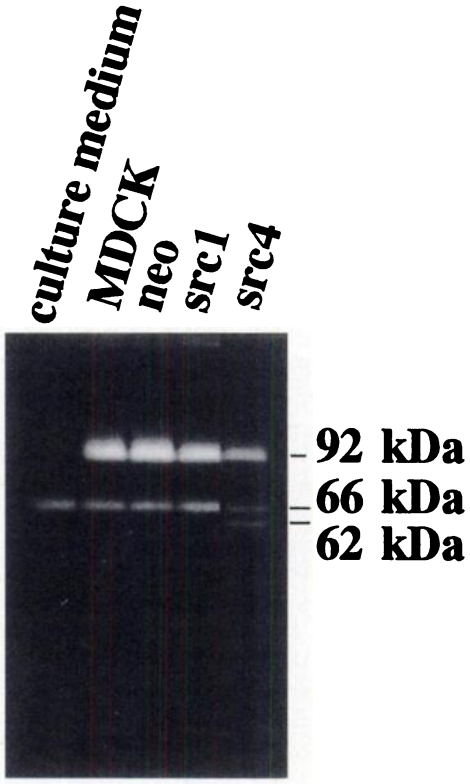

Fig. 5. Gelatin zymography. An aliquot of culture medium (*Lane culture medium*) and culture supernatant from MDCK cells (*Lane MDCK*), MDCK cells transfected with control plasmid (*Lane neo*), and two clones of v-*src*-transformed cells (*Lanes src1* and *src2*, respectively) were subjected to gelatin zymography analysis.

Table 1 *Tumorigenicity and metastasis in nude mice by s.c. and renal subcapsular injection of v-src-transformed MDCK cells*

Cell type[a]	s.c., tumor incidence[b] in subcutis	Renal subcapsular	
		Tumor incidence[b] in kidney	Tumor incidence[c] in lung
neo	0/5	0/5	0/5
src4	5/5	5/5	4/5

[a] v-*src*-transformed MDCK cells were injected at 1×10^6 cells into hypoderm or renal subcapsule of athymic nude mouse. Tumor formation was monitored at 3 weeks after inoculation.

[b] Number of mice with a tumor per total number of mice receiving injections.

[c] Number of mice with macroscopic and microscopic metastasis of lungs per total number of mice receiving injections into renal subcapsule.

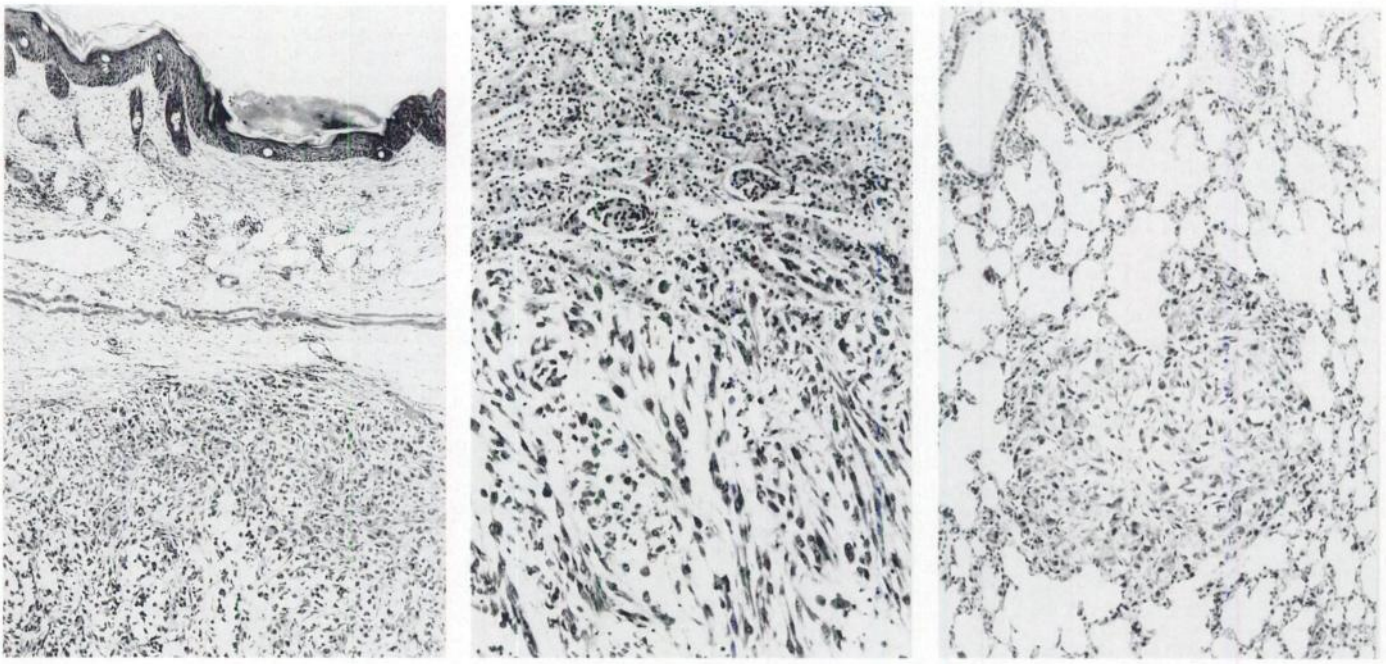

Fig. 6. Histology of v-src MDCK cells growing in nude mice. *Left*, subcutaneous tumor 3 weeks after implantation (H&E; magnification, ×80). *Middle*, v-*src* MDCK tumor in the kidney of nude mice (H&E; magnification, ×160). *Right*, v-*src* MDCK tumor metastasized to the lung (H&E; magnification, ×160).

the mechanism of gene expression in epithelial tumor cells remains to be clarified.

The present study demonstrates that transformation of kidney epithelial cell line MDCK by v-*src* induced expression of the MT1-MMP gene and the loss of cell-to-cell contacts. The effects of v-*src* on intercellular adhesion are to counteract junctional assembly through phosphorylation of the E-cadherin-catenin complex (16). MDCK cells transformed by v-*src* were invasive and metastatic when implanted orthotopically into nude mice. Previously we have shown that expression of MT1-MMP in tumor cells enhances pulmonary metastasis in an experimental metastasis assay (20). Thus, MT1-MMP expressed in v-*src* MDCK cells is thought to be associated with invasion *in vivo* along with the loss of cell-to-cell adhesions.

MT1-MMP was originally identified as an activator of progelatinase A and was recently revealed to degrade components of extracellular matrix such as type I–III collagen and fibronectin (22). Which activity of MT1-MMP contributes to the degradation of extracellular matrix still remains to be solved.

Enhancement of MT1-MMP mRNA synthesis by effectors such as concanavalin A has been reported; however, the induction was not dramatic (23–25). Furthermore, induction by concanavalin A and 12-*O*-tetradecanoylphorbol-13-acetate was not always at a transcriptional level (23). Transformation of MDCK with the v-*src* gene is the first case in which MT1-MMP mRNA was induced from undetectable to high level. However, such a difference in MT1-MMP expression is seen between epithelial-like breast cancer cell lines, which lack MT1-MMP, and the more invasive, mesenchymal-like breast cancer cell lines, which express significant levels of MT1-MMP (24). Down-regulation of epithelial cell-to-cell adhesions may cause mesechymal gene induction (17), and thus the transformed MDCK cells express MT1-MMP; however, gelatinase A expression was not induced in transformed MDCK cells. Selective induction of the MT1-MMP gene but not gelatinase A is similar to that in human tumor cells *in vivo* (11). Thus, MDCK cells transformed by v-*src* will constitute a useful system to study the regulatory mechanism of MT1-MMP gene expression in human tumor cells.

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