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Review

An attempt to isolate genes responsible for spontaneous and experimental metastasis in the mouse model

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Summary. Cancer develops and progresses as genetic alterations occur subsequently. Onset process of cancer has become well understood in some types of cancer, such as colorectal cancers. In this process, responsible alterations were identified in numbers of oncogenes such as k-ras, and tumor suppressor genes such as p53, as Vogelstein proposed earlier in the multistage carcinogenesis theory. In contrast, our understanding remains short to draw such an adequate diagram for the process during which cancer becomes more malignant, i.e., metastatic. To examine the molecular basis for this progression step, mouse metastasis models have been established where tumor cell lines are inoculated into mice and metastasize to specific organs. The model using B16 melanoma cells is one of the most developed. BL6 subline, one of the most metastatic, was obtained from F10 subline simply through six rounds of *in vitro* selection. Nonetheless, BL6 cells metastasize lungs much more heavily than F10 cells when injected subcutaneously. The difference in gene expression between the two sublines is considered rather small but relevant for spontaneous metastasis. We began our research by elaborating a method for the construction of subtracted cDNA libraries, and made it applicable to BL6 and F10 cells. As a result, we were able to isolate a couple of genes that were expressed differently between the two sublines. As might be expected, each of the genes appeared to play a role more or less in distinct aspects of spontaneous metastasis of B16 melanoma cells. Moreover, similar roles were expected for the genes in the process by which human melanoma cells metastasize.

Key words: B16 melanoma, PP2A, Connexin, Nop5/Sik family, Subtracted cDNA library

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Introduction

Cancer is a genetic disease resulting from an accumulation of genetic changes in various regulatory genes. A multistep genetic model of tumorigenesis has been proposed for human neoplasms, especially in colon carcinomas (Vogelstein et al., 1988). However, such a genetic model is not clearly understood in the acquisition of metastatic ability (Liotta, 1992). Fidler devised an excellent metastasis model of mouse melanoma and established B16 mouse melanoma sublines with different metastasis potentials and behaviors (Poste and Fidler, 1980). A recent advanced method in molecular genetics has been applied to reveal genetic changes accumulated during the process for the acquisition of metastatic ability.

Among B16 mouse melanoma cells, we analyzed F10 and BL6 cells. They are nearly identical except for their metastatic behavior (Poste et al., 1980). BL6 cells were obtained through six rounds of *in vitro* selection of F10 cells. Both cell types colonize the lungs when injected into the tail vein (experimental metastasis assay). In contrast, when injected subcutaneously into the footpad (spontaneous metastasis assay), BL6 cells are much more metastatic than F10 cells (Poste et al., 1980). Since the spontaneous metastasis assay reflects the *in vivo* sequence of metastatic events, it appears that BL6 cells may possess the full complement of factors essential for metastasis, all or some of which may be missing from F10 cells. Alternatively, F10 cells, but not BL6 cells, may express a factor that suppresses metastasis. Genes encoding such factors are likely to be differentially expressed between the two cell lines.

To know the difference in gene expression between F10 and BL6 cells, we used an improved method for constructing subtracted cDNA libraries (Ito et al., 1998, 1999; Kobori et al., 1998). This method was designed to identify genes that are expressed differently between two types of cells. Its usefulness has been demonstrated by our previous studies, in which we intended to clone transcriptional target genes for mi transcription factor (MITF) (Ito et al., 1998, 1999). The subtraction method

was improved so elaborately that we were able to detect a subtle difference in gene expression caused by a mutation in the MITF gene. We applied the same method to F10 and BL6 cells, and yielded a set of genes that were transcriptionally upregulated in BL6 cells. Here we review a role of these genes in metastasis, and also refer to other researchers' studies on experimental metastasis of B16 melanoma sublines.

Isolation of cDNA clones transcriptionally upregulated in BL6 cells

The method for constructing a subtracted BL6 plasmid cDNA library was described previously (Ito et al., 1998). Briefly, total RNA was extracted from BL6 cells. Poly $(A)^+$ RNA was purified and the BL6 plasmid cDNA library was constructed. To prepare single-stranded DNA, the plasmid DNA was introduced into *E coli* cells. Transformed cells were infected with helper phage and single-stranded DNA was purified. To prepare RNA drivers, total RNA was extracted from F10 cells. Poly $(A)^+$ RNA was purified and labeled by photobiotin. The single-stranded DNA was hybridized with biotinylated RNA. After hybridization, streptoavidin was added. The mixture was incubated and avidin-biotin complex was discarded by centrifuge. After repeating the subtraction process, the recovered single-stranded DNA was converted to double-stranded plasmid DNA. The plasmid DNA was introduced into *E coli* cells.

After the subtraction process had been repeated four times, we rescued approximately 1,500 clones. One clone after another was subjected to Northern blot analysis and eleven clones have been confirmed to be upregulated in BL6 cells so far (Fig. 1). Sequencing identified the clones. Seven clones were connexin 26 (Cx26), protein phosphatase 2A (PP2A) B56g subunit, Sik-similar protein (Sik-SP), phakinin (a lens-specific intermediate filament protein), B-myc (a member of the myc gene family, assumed to function as an inhibitor of cellular proliferation), archain (a clathrin-associated protein, possessing a possible role in vesicle structure or trafficking) and acetyl-CoA transporter (a membrane protein required for the formation of O-acetylated ganglioside). Four clones of the rest were clone 23, 438, 785 and 929, and had no significant homology to known genes. Clone 23 and 929 were later designated as MLZE (Melanoma-derived Leucine Zipper, Extra-nuclear factor) and TIB929 (Transcript induced in BL6 cells) (a new member of GTPase superfamily, shortly characterized in Nakaji et al., 1999), respectively. None of the clones isolated were previously reported to be involved in metastasis. We further studied connexin 26, PP2A B56g, Sik-SP and MLZE for their roles in metastasis.

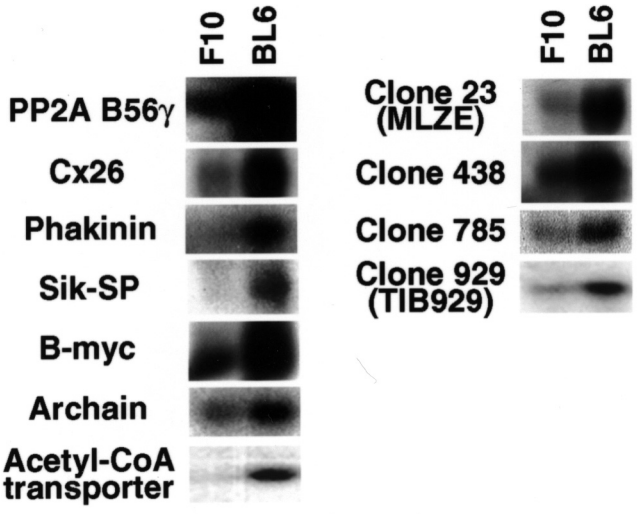

Fig. 1. A list of genes expressed more abundantly in BL6 cells than in F10 cells. By using subtracted cDNA library, we isolated eleven clones upregulated in BL6 cells. Seven clones were identical to connexin 26 (Cx26), protein phosphatase 2A (PP2A) B56γ subunit, Sik-similar protein (Sik-SP), phakinin, B-myc, archain and acetyl-CoA transporter. Four clones of the rest, clones 23, 438, 785 and 929, had no significant homology to known genes. Clone 23 and 929 were later designated as MLZE (Melanoma-derived Leucine Zipper, Extra-nuclear factor) and TIB929 (Transcript induced in BL6 cells), respectively.

Cx 26

Cx26 is one of the genes whose mRNA expression levels are most different between F10 and BL6 cells (Ito et al., 2000b). To our surprise, the expression level for Cx26 in BL6 cells was as high as that in the liver tissue, one of the tissues that express Cx26 most abundantly. Connexins are a family of at least 12 proteins that make up the intercellular channels of gap junctions (Beyer et al., 1990). Gap junction hemichannels, called connexons, regulate passage of ions and low-molecular-weight molecules between cells (Charles et al., 1992). Most cells or tissues express multiple Cx genes and show functional coupling via homologous gap junctions (Bennett et al., 1991). Various lines of evidence support the involvement of homologous gap junctional intercellular communication (GJIC) in carcinogenesis (Eghbali et al., 1991; Zhu et al., 1992; Grossman, 1994; Mesnil et al., 1995; Hirshi et al., 1996). For instance, homologous GJIC is believed to decrease as hepatocytes and keratinocytes acquire potential to proliferate autonomously (Sakamoto et al., 1992; Kamibayashi et al., 1995). On the other hand, the role of connexins in the acquisition of more malignant phenotypes, such as metastasis, remains controversial (Nicolson et al., 1988, 1990; Hamada et al., 1988; Ren et al., 1990).

At the beginning of our study, we examined whether BL6 cells were GJIC-proficient, since there was no mutation in the coding sequence of the Cx26 mRNA expressed abundantly in BL6 cells. We performed a dye-transfer assay to examine whether homologous GJIC ability increased in BL6 cells. Unexpectedly, under *in vitro* culture conditions, neither F10 nor BL6 cells showed any homologous GJIC ability by themselves.

Then, we devised a coculture system to assess the heterologous GJIC (Fig. 2). In this system, an opened vein segment was placed at the bottom of a culture dish and the dye-labeled melanoma cells were seeded onto it. BL6 cells could transfer dye into endothelial cells (dye-coupling) but F10 cells could not. By the transfection with Cx26, F10 cells acquired a similar dye-coupling activity. Conversely, transfection with dominant-negative forms of Cx26 abrogated the dye-coupling proficiency of BL6 cells. These results indicated that Cx26 mediated not homologous but heterologous GJIC between melanoma cells and endothelial cells.

We next examined whether such a difference in GJIC ability might be involved in spontaneous metastasis of F10 and BL6 cells. Dye-coupling-proficient F10 clones, obtained by the transfection with wild-type Cx26, were as spontaneously metastatic as BL6 cells. On the contrary, dye-coupling-deficient BL6 cells, obtained by the transfection with dominant negative forms of Cx26, significantly reduced their spontaneously metastatic potential. Although there were no direct data that show how Cx26 might exert its effects *in vivo*, the results above suggested an involvement of heterologous gap junctions between melanoma and endothelial cells in spontaneous metastasis. Recently, heterologous functional gap junctions were found to form between glioma cells and surrounding astrocytes, resulting in the phenotypic transformation of the astrocytes (Zhang et al., 1999). Both glioma and melanoma cells appear able to communicate directly with distinct types of normal host cells. Taken together, we propose that such communication might contribute to the susceptibility of the host to tumor invasion.

Interestingly, Cx26 seems to have a dual function in tumor cells. One is to promote carcinogenesis through abrogating GJIC. The other is to advance malignant progression through acquiring GJIC. We further examined the expression of Cx26 in human melanocytic lesions by immunohistochemistry to evaluate the clinical relevance of Cx26 in the natural course of melanoma carcinogenesis and malignant progression. Cx26 expression was low in melanocytes, nevus cells and melanoma cells that were still resident in the epidermal basal layer. In contrast, melanoma cells markedly increased Cx26 expression after detachment from the basal layer or in the process of invading the dermis. Upon these observations, one possibility should be pointed out: that once tumor cells have acquired autonomous growth potential, higher expression of Cx26 might promote invasion and metastasis. Kamibayashi et al. reported decrease of Cx26 expression in the carcinogenesis from papilloma to squamous cell carcinoma (Kamibayashi et al., 1995). In addition, they demonstrated that Cx26 expression increased in the cancer cells metastasizing into lymph nodes, consistent to our scenario.

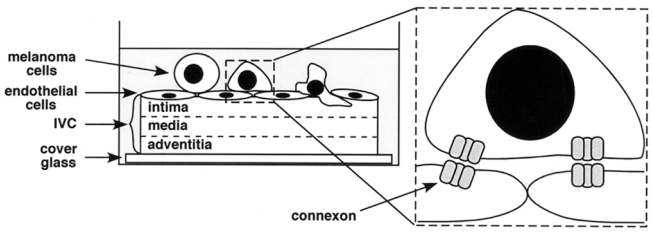

Fig. 2. A coculture system developed to examine the heterologous gap junctional intercellular communication (GJIC) between melanoma cells and endothelial cells. A segment of mouse inferior vena cava (IVC) was opened and fixed upon a culture dish filled with growth medium. Melanoma cells labeled with fluorescent dye were seeded onto endothelial cells. 4 hours later, the cocultured tissue was fixed and observed through a microscope equipped with epifluorescence. The activity of the GJIC was measured as the number of the melanoma cells that had transferred fluorescent dye to endothelial cells.

PP2A B56γ subunit

As shown in Fig. 1, PP2A B56γ was most upregulated in BL6 cells (Ito et al., 2000a). PP2A is an intracellular serine/threoine protein phosphatase that regulates a variety of cellular processes, including signal transduction, cell cycle progression and development (Shenolikar, 1994; Wera and and Hemmings, 1995). PP2A holoenzymes consist of catalytic (C) and structural (A) subunits associated with a variable regulatory (B) subunit (Usui et al., 1988). The B regulatory subunit modulates enzyme activity, substrate specificity and subcellular localization. To date, three unrelated families of the PP2A regulatory subunits have been identified, denoted PR55 (or B), B56 (B'), and PR72 (B''). B56γ subunit is a member of the B56 regulatory subunit and includes three alternative splicing isoforms, B56γ1, B56γ2 and B56γ3.

Genomic analysis of PP2A B56γ locus in BL6 cells revealed that one allele was rearranged by insertion of retrotransposon, intracisternal type A particle (IAP). Consequently, a chimeric mRNA was expressed in BL6 cells that consists of a part of the IAP sequence followed by the N-terminally truncated B56γ1 mRNA. Its expression level was much higher than that for B56γ1 probably due to the strong promoter activity of long terminal repeat sequence present in the IAP. The chimeric mRNA contained a shorter open reading frame starting from the second methionine codon of B56γ1. We found that the chimeric message was indeed translated in BL6 cells, and termed this truncated protein Δγ1.

To know the effect of Δγ1 on PP2A, we used okadaic acid (OA), which inhibits PP2A activity completely at a low concentration. Our earlier experiments characterized Δγ1 as a general inhibitor for PP2A, as Δγ1 produced effects similar to those of OA treatment. OA has been shown to render living cells more motile and rounded with reduced cytoskeletal organization. Similarly, the NIH3T3 cell clone expressing Δγ1 (3T3Δγ1) showed a greater migratory ability than 3T3 cells. However, the effect of Δγ1 on cell morphology was the opposite to that of OA. OA treatment rendered NIH3T3 cells round in shape and

poor in actin stress fiber, while 3T3Δγ1 cells were quite flat, well spread out, and rich in actin stress network.

Increased cell migration and well-developed cytoskeleton observed in $3T3^{\Delta\gamma1}$ cells are a characteristic of several types of highly invasive tumor cells (Verschueren et al., 1994; Malliri et al., 1998). We examined whether Δγ1 increases metastatic potentials of the F10 cells. The competence for lung metastasis was not affected by transfection with Δγ1. In contrast, F10 cells transfected with Δγ1 showed a higher incidence of metastasis to lymph nodes than did F10 cells. Δγ1 appeared to be involved in the metastasis of melanoma cells probably through its effect on actin-based cytoskeleton.

We wished to seek for the target substrate of PP2A whose dephosphorylation or phosphorylation affects cytoskeleton. Since accelerated spreading of $3T3^{\Delta\gamma1}$ cells on fibronectin suggested that the cells could form focal adhesions (FA) more efficiently, we paid attention to paxillin, an intracellular component of FA (Fig. 3). Paxillin is a multidomain-containing adaptor molecule that provides binding sites for various proteins at the FA. Paxillin was localized efficiently to nascent FA at the early stage of $3T3^{\Delta\gamma1}$ cell-contact to fibronectin. B56γ1 and Δγ1 was localized in the cytoplasm, and a part of both isoforms co-localized with paxillin at FA. We then examined the levels of phosphorylation in paxillin. Paxillin phosphorylation was constitutively enhanced in 3T3Δγ1 cells compared to 3T3 cells, especially on the serine residue. An *in vitro* phosphatase assay confirmed the direct inhibitory effect of Δγ1 on the dephosphorylation of paxillin by the PP2A holoenzyme containing B56γ1. Δγ1 appeared to prevent the B56γ1-containing holoenzyme from dephosphorylating paxillin, resulting in constitutive hyperphosphorylation of the molecule (Fig. 3). Recent reports indicate that serine and threonine phosphorylation of the paxillin LIM domains is required for paxillin to recruit to nascent FA (Brown et al., 1998). In Δγ1-expressing tumor cells, paxillin might recruit into FA at accelerated rates in response to integrin-mediated signals because paxillin is constitutively phosphorylated.

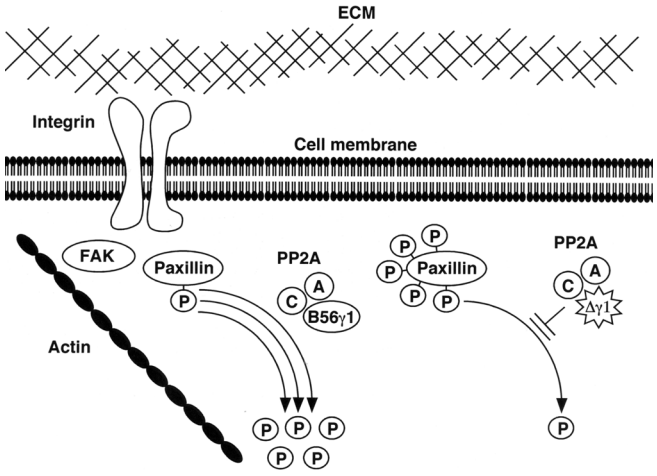

Fig. 3. A role for the B56γ1-containing PP2A holoenzyme and its truncated isoform Δγ1 in the focal adhesion. Integrin-mediated focal adhesions (FA) serve as a bridge between extracellular and intracellular events (Burridge and Chrzanowska-Wodnicka, 1996). Extracellular domains of integrins adhere to extracellular matrix (ECM). The cytoplasmic domains of integrins are linked to cytoskeletal actin, probably through some cytoplasmic structural molecules such as vinculin and talin. In addition, FA consists of regulatory molecules, including focal adhesion tyrosine kinase (FAK) and paxillin. FAK is one of the kinases that phosphorylates paxillin. However, the mechanism for the reversal of phosphorylation of paxillin has not been understood. We showed for the first time that PP2A was one of the phosphatases that dephosphorylated paxillin. Δγ1 appeared to prevent the PP2A holoenzyme from dephosphorylating paxillin.

We found only one mutation in one isoform of PP2A. The mutation resulted in the production of Δγ1, and Δγ1 was potent for targeting of PP2A to paxillin. Consequently, Δγ1 could produce inhibitory effects on the PP2A heterotrimer and cause distinct cellular events such as adhesion, spreading, migration and metastasis, all of which are characteristic features of malignant cells.

Sik-SP

Hypertrophy of the nucleolus is one of the cytological features of cancer cells (Busch and Smetana, 1970) and is closely related to the rapidity of cancer cell proliferation (Derenzini et al., 1998, 2000). The nucleolus is a structural and functional unit in which ribosomal RNA (rRNA) and ribosome synthesis occur (Olson et al., 2000) (Fig. 4). Therefore, hypertrophy of the nucleolus is regarded as a state in which rRNA and ribosome synthesis has increased. Recently, molecular basis for the nucleolar hypertrophy has become gradually known. In cancer cells possessing larger nucleoli and a shorter cell-doubling time, several nucleolar proteins are expressed more abundantly, including the RNA polymerase I upstream-binding factor (Derenzini et al., 1998), topoisomerase I (Derenzini et al., 1998), fibrillarin (Derenzini et al., 1998), nucleolin (Olson, 1990), and protein B23 (Olson, 1990). These proteins augmented the activity of rRNA processing and synthesis (Derenzini et al., 1998; Olson, 1990). In addition to tumor growth rate, nucleolar size can predict the metastatic behaviors of a tumor. In cutaneous (Gambini et al., 1992; Barzilai et al., 1998) and uveal (McLean et al., 1997; Bechrakis et al., 2000) melanomas, the mean nucleolar area in patients who died of metastasis was significantly larger than the mean in patients who did not develop metastasis. The increase in nucleolar size is believed to reflect the increase in nuclear and nucleolar activity during the process of malignant progression from poorly to highly metastatic melanoma cells (Gambini et al., 1992; McLean et al., 1997; Barzilai et al., 1998). However, the genetic alterations that might be implicated in the changes in nucleolar size and function during such processes have not been studied intensively.

From the subtracted BL6 cDNA library, we isolated Sik-SP gene (Nakamoto et al., 2001), which is a member of the Nop5/Sik family that consists of highly conserved nucleolar proteins (Vorbruggen et al., 2000). The Nop5/Sik family members bind box C/D small nucleolar ribonucleoproteins and play a pivotal role in rRNA processing necessary for ribosome assembly (Maxwell and Fournier, 1995; Tollervey, 1996) (Fig. 4). In yeast, at least two members, Nop56 and Nop58, are present (Gautier et al., 1997). Deficiency in either causes yeast to undergo growth arrest because of defects in protein synthesis (Gautier et al., 1997; Wu et al., 1998). In mammalian cells, the presence of the Nop5/Sik family members has already been confirmed, including Sik-SP (Lyman et al., 1999; Newman et al., 2000; Yang et al., 2000). However, the function of Sik-SP has not been well characterized.

We assumed that Sik-SP might have a similar nucleolar function to that of yeast Nop5/Sik family members. Consistently, GFP-tagged Sik-SP showed a well-concentrated localization within the nucleolus. To evaluate whether Sik-SP is involved in ribosome biogenesis, two parameters were measured: magnitude of ribosomal RNA synthesis per nucleus and magnitude of protein production from the same amount of mRNA of an exogenous luciferase gene (Fig. 5). Sik-SP increased both values and, in addition, nucleolar size. Therefore, Sik-SP seemed to promote ribosome biogenesis in the nucleolus.

Previous reports indicated a clear relationship between nucleolar size and cell proliferation rate (Derenzini et al., 1998, 2000). Although the nucleolar size of BL6 cells (11.4 mm^2) was larger than that of F10 cells (7.86 mm^2), there was no difference in the cell-doubling time between F10 (12.9 h) and BL6 (13.1 h) cells. In addition, transfection of Sik-SP did not significantly shorten the cell-doubling time of F10 cells. However, we found significant effects of Sik-SP on cell growth recovery after serum-starved culture condition. Cells were serum-starved for 3 days, and then serum was added to the culture. In this culture condition, BL6 cells and Sik-SP-transfected F10 cells readily entered a logarithmic growth phase within 24 hours upon the serum addition. But F10 cells and vector-control F10 cells required one more day to do so. Sik-SP seemed to confer on melanoma cells a greater ability to grow in response to serum stimulation through keeping the nucleolar function high.

This function of Sik-SP might help cancer cells grow promptly in response to environmental changes, such as tumor angiogenesis, which release cells from stress conditions, such as hypoxia, low pH and nutrient deprivation. This speculation was supported by the study on clinical cases. We examined gene expression for human Nop58 (hNop58), a human member of the Nop5/Sik family, in human melanoma lesions, and found that it closely linked to metastasis and nucleolar size.

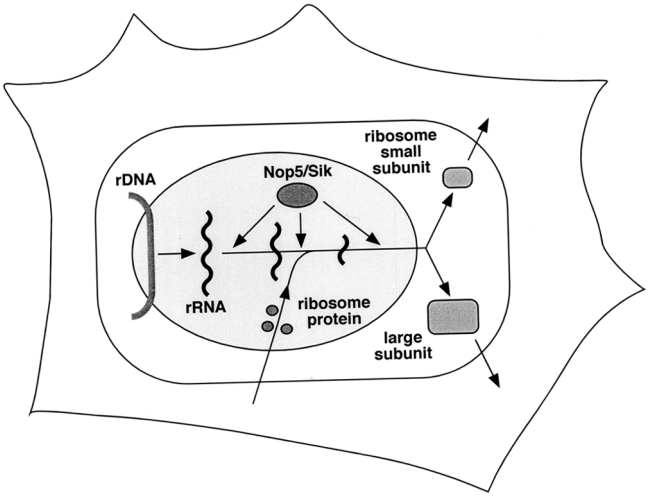

Fig. 4. Involvement of Nop5/Sik family proteins in ribosome synthesis. In the nucleolus, ribosomal RNA (rRNA) is transcribed from ribosomal DNA (rDNA) and proceeds to processing steps including excision, nucleotide modification and assembly with ribosomal proteins. After processing, the mature ribosome subunits (small subunit and large subunit) are exported to the cytoplasm. Nop5/Sik family proteins play an essential role in the rRNA processing.

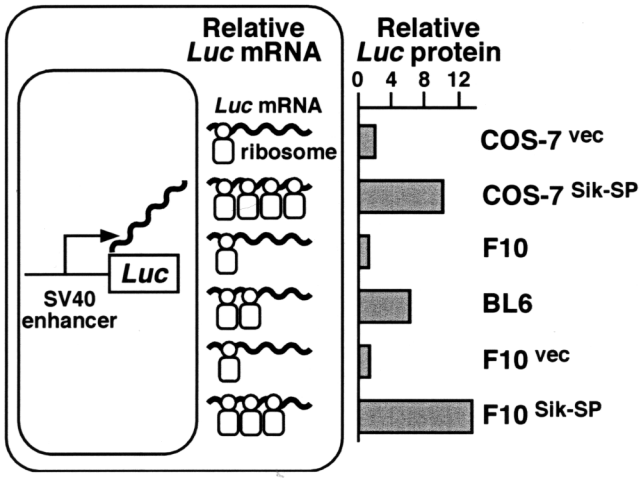

Fig. 5. Translational rather than transcriptional upregulation of an exogenous gene in Sik-SP-expressing cells. COS-7, F10 and BL6 cells were transfected with a plasmid vector that expresses the Renilla luciferase (Luc) gene under the control of the simian virus 40 enhancer/early promoter. In some cases, COS-7 and F10 cells were simultaneously transfected with an empty vector or a vector expressing Sik-SP. Twenty-four hours later, the cells were examined for Luc gene dosage (Luc plasmid DNA), Luc gene expression (Luc mRNA) and Luc activity (Luc protein). The values for Luc mRNA and Luc protein were normalized against the value for the Luc plasmid DNA (relative Luc mRNA and relative Luc protein). Relative Luc mRNA was comparable among all types of cells. However, relative Luc protein was significantly higher in COS-$7^{Sik\text{-}SP}$, BL6 and $F10^{Sik\text{-}SP}$ cells than in COS-7^{vec}, F10 and $F10^{vec}$ cells. The Luc protein production seemed to increase in its translational, but not transcriptional, level through increased amounts of ribosome in Sik-SP-expressing cells.

MLZE

MLZE is a novel gene and another example whose mRNA expression levels are most different between F10 and BL6 cells (Watabe et al., 2001). The predicted amino acid sequence of human MLZE (hMLZE) contains one leucine zipper structure (Alber, 1992) and two potential nuclear localizing signals (NLS) (Dingwall and Laskey, 1991). This feature suggests that MLZE plays a role of transcription factor in the nucleus. Consistently, we observed nuclear localizing activity of GFP-tagged NLS sequence in hMLZE. However, the function of MLZE is not clear.

To assess the clinical relevance of hMLZE, we examined by immunohistochemistry hMLZE expression in clinical cases of malignant melanoma. Based upon Clark invasion level (Clark et al., 1969), patients were divided into two groups; Clark levels I/II melanoma group and Clark levels III/IV/V melanoma group. Melanoma of the former group shows low metastatic behavior and corresponds to radial growth phase (RGP) melanoma (Clark et al., 1989). Melanoma of the latter group shows highly metastatic behavior and corresponds to vertical growth phase (VGP) melanoma (Clark et al., 1989). The ratio of the number of patients positive versus negative for MLZE was significantly larger in VGP melanoma group (6/11) than in RGP melanoma group (2/15).

hMLZE gene was mapped to 8q24.1-2, which contains the c-myc gene and is often amplified in malignant melanoma (Bastian et al., 1998). c-myc is one of the genes whose expression has been examined extensively in malignant melanoma (Lazaris et al., 1995; Bergman et al., 1997). We examined the expression of c-myc in the same samples by immunohistochemistry with c-myc antibody. The ratio of the number of patients positive versus negative for c-myc was larger in the the VGP melanoma group (4/11) than in the RGP melanoma group (3/15), but the difference between the two groups was not significant.

We also examined immunoreactivity for hMLZE and c-myc at each anatomic level of the melanoma spread; epidermis level, papillary dermis level, reticular dermis level and subcutaneous fat level. None of the hMLZE-positive cases in the VGP melanoma group showed a decrease in immunoreactivity to hMLZE within the lower invasive component of the tumor. Moreover, the strength of hMLZE staining increased at the lower invasive component of the tumor in two cases. In contrast, c-myc-staining substantially decreased at the lower invasive component of the tumor.

Although the function of hMLZE remains unknown, chromosomal localization and expression profiles of hMLZE suggested an involvement of MLZE in melanoma progression. Several markers, such as HMB45 (Gown et al., 1986) and c-myc (Bergman et al., 1997), are used to help diagnosis of malignant melanomas. Anti-HMB45 antibody often fails to detect the lower invasive component of melanoma. In fact, the strength of c-myc staining decreased substantially in the lower invasive component in one case. In contrast, we did not find the strength of hMLZE-staining to decrease within the invasive descent of the melanoma spread. On the contrary, immunoreactivity for hMLZE increased remarkably as melanoma cells spread deeper into the dermis in two cases. These findings suggested not only that MLZE could be a useful marker to indicate the deepest site of melanoma spread, but also that MLZE plays a possible role in melanoma progression.

Genes involved in experimental metastasis

Besides subtracted cDNA library, microarrays have become an alternative method to compare the gene expression pattern of two or more than two types of cells. This technique provides researchers with expression profiles of ten thousand genes at once. Edwin A. Clark and his colleagues used this method to examine the difference in gene expression among B16 cells with different potentials for experimental metastasis (Clark et al., 2000). By repeating experimental metastasis assay, they selected highly metastatic clones A375 M1 and M2 cells and B16 F1, F2, and F3 cells from human A375 P and mouse B16 F0 cells, respectively. RNAs extracted from each type of cell were used to prepare the labeled cRNAs, and hybridized to oligonucleotide microarrays. Clark et al. found that 32 genes showed consistently enhanced expression in pulmonary metastases produced by the highly metastatic clones. They divided the isolated genes into several categories. One category is made up of the genes that regulate the actin-based cytoskeleton, including RhoC and thymosin ß4. Another category is made up of the genes that encode extracellular matrix or that regulate their assembly, including fibronectin, collagen Iα2 and collagen IIIα1.

Among the genes isolated, further examination was done on RhoC. RhoC is a member of the Rho GTPase family that can regulate many cellular functions, most notably cytoskeletal organization in response to extracellular factors (Van Aelst and D'Souza-Schorey, 1997). Overexpression of RhoC in poorly metastatic A375P cells markedly enhanced metastasis in experimental metastasis assay. Conversely, inhibition of RhoC in highly metastatic A375M cells markedly suppressed the generation of metastasis in experimental metastasis assay. Rho activity seemed necessary and sufficient for metastasis in these melanoma cells. Migratory and invasive ability of melanoma cells were also measured using 8 mm-pore-size filters and matrigel-coated chambers, respectively. Metastatic A375M cells were more migratory and more invasive than the poorly metastatic A375P cells. Furthermore, RhoC could enhance the migratory and invasive capacity of the A375P cells, whereas inhibition of Rho decreased motility and invasion of the A375M cells, indicating that RhoC may be involved in metastasis by controlling cytoskeletal events essential for motility.

Concluding remarks

There is an apparent difference when F10 cells are compared with BL6 cells, and when B16-F0 cells are compared with F1, F2, and F3 cells, with respect to their metastatic behavior in mice. To obtain a clue for the molecular basis of the metastatic ability, others and ourselves began by identifying the difference in gene expression between the sublines. Such an approach seems reasonable because cancers progress by way of accumulation of genetic alterations. Subtraction and microarray are the two representatives of methods aiming to discover the difference in gene expression. The microarray method seems less laborious than the subtraction method because the former does not require the construction of cDNA libraries and the sequencing of each library clone. However, the subtraction method sometimes provides researchers with a surprise; in the standard microarray method genes of interest are selected from the pools of the reported cDNA sequence fragments, whereas the subtracted cDNA library contains considerable numbers of clones carrying unknown genes. In fact, we isolated several unknown clones in the subtracted cDNA library, such as MLZE and TIB929. Studies on the unknown genes may often be difficult to carry out, but lead us to a new field of metastasis research.

Particular genes that regulate cytoskeleton and extracellular matrix became known to play pivotal roles in metastasis (Hall, 1991; Suwa et al., 1998; Maniotis et al., 1999). Consistently, Clark et al. isolated a couple of such clones as a gene upregulated in F1, 2 and 3 cells when compared to F0 cells, and showed the responsibility of RhoC in experimental metastasis of those cells. This function appeared to result from the direct effects of RhoC on actin-based cytoskeletal organization. On the other hand, we found a mutation in the PP2A B56γ subunit gene in BL6 cells, but not in F10 cells. Functional analysis revealed that a truncated isoform arising from the mutation could enhance phosphorylation levels of paxillin, a molecule making up focal adhesions, and therefore result in development of actin-based cytoskeleton. In addition, we isolated Cx26 as a gene markedly upregulated in BL6 cells, and showed its responsible role in spontaneous metastasis of F10 and BL6 cells. Although Cx26 itself does not function as an adhesion molecule, Cx26-mediated interaction between melanoma and endothelial cells could be a trigger for cytoskeletal alteration in either type of cells. Comparative analyses on B16 sublines reminded us again the importance of studies on cytoskeletal systems of metastatic tumor cells.

In spite of every effort of researchers, it remains to be resolved what alterations in gene expression are essential for the acquisition of spontaneously metastatic ability by BL6 cells. Our studies showed that upregulation of Cx26 might be a candidate for such alterations. However, our studies also suggested that the Cx26 upregulation did not solely explain the entire *in vivo* events of spontaneous metastasis. Alternatively, spontaneous metastasis of BL6 cells could be attributable not to a single gene alteration, but to its combination. In fact, we isolated more than 10 clones as a gene upregulated in BL6 cells. As summarized here, each of the genes was found to have a role more or less in melanoma progression. It is likely that alterations occurred in numbers of genes during the repeated *in vitro* selection from F10 to BL6 cells. Even though Cx26 and RhoC may play a significant role, it seems that only a part of the alterations has been revealed so far. The fact that BL6 cells, but not F10 cells, are readily able to achieve spontaneous metastasis remains reproducible even at this time when thirty years have passed since the two sublines were established by Dr. Fidler. The answer we seek for must be hiding behind BL6 cells. With this conviction in mind, BL6 cells will continue to be the best partner for our metastasis researches in the future.

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