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Differential Expression of Transforming Growth Factor-β1 Gene in 3LL Metastatic Variants[1]

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ABSTRACT

***In vitro* and *in vivo* metastatic variants derived from Lewis lung carcinoma (3LL) were examined for the level of the expression of several growth-regulated genes, oncogenes, and transforming growth factor (TGF) genes. To determine whether the proliferative advantage of metastatic cells is due to an increased growth fraction of the cell population or to a deregulated expression of some growth-regulated genes, the mRNA levels of the S-phase-specific H3 histone gene were compared with that of some cell cycle-related genes (vimentin, calcyclin, c-*myc*, and *p53*) and oncogenes (Ki-*ras*, Ha-*ras*, c-*sis*, c-*src*, c-*fes*, and c-*erb*). In addition, to evaluate whether an autocrine pattern of cell proliferation is responsible for the proliferative advantage of metastatic cells, the level of the expression of TGF genes (α and β1) was studied.**

Northern blot analysis demonstrated that in 3LL metastatic variants the expression of TGF-α as well as the expression of all growth-regulated genes and oncogenes studied are similar. Only the TGF-β1 gene is expressed at higher levels in highly metastatic 3LL variants maintained either *in vitro* or *in vivo*. Data suggest that the proliferative advantage of 3LL metastatic cells is not due to a deregulated expression of some growth-regulated genes and oncogenes, but more likely is acquired through the expression of genes which might interfere with the ability of the tumor cells to escape hostile microenvironmental conditions.

INTRODUCTION

Only a few cells within a primary tumor possess the ability to survive the sequence of hostile events which occur during metastasis formation. In fact, the majority of the tumor cells are destroyed by microenvironmental conditions and by the host response, but the overgrowth of tumoral subpopulations derived from these few cells is responsible for the majority of cancer deaths. It has been suggested that progenitor cells in the primary tumors possess a selective genetic advantage which enables them to metastasize. In an attempt to identify such progenitor cells, clonal subpopulations from a single tumor have been derived (1), and major research efforts have been directed toward the identification of genes which may be specifically involved in the metastatic process (2, 3). Since the growth of malignant cells depends on their ability to adapt to hostile microenvironmental conditions, and since the proliferation of tumor cells is controlled by various hormones and growth factors, it has been suggested that quantitative and qualitative differences in the requirements of tumor cells for different growth factors could represent the mechanism through which tumor cells escape a negative control which would prevent cell division (4, 5). The identification of TGFs[3] as positive and negative autocrine growth factors (6–8) has suggested that the malignant phenotype could arise from an excessive production of positive growth factors and/or from the failure of the tumor cells to respond to specific negative growth factors. A functional interaction between growth factors and oncogene expression has been reported, while changes in the expression of oncogenes have attracted considerable attention and have been proposed as determinants of tumor progression (9).

In the attempt to identify genes which might be responsible for the higher metastatic capacity of clonal subpopulations derived from 3LL (10), we have compared the level of the expression of several growth-regulated genes, oncogenes, and TGF genes in metastatic variants maintained either *in vitro* or *in vivo*. Since it has been previously reported that some oncogenes and growth-regulated genes (c-*myc*, c-*fos*, *p53*, and c-*ras*) are expressed in a cell-cycle-dependent manner (11), to exclude the possibility that an increased expression of these genes simply reflects an increased growth fraction of the cell populations, we have compared the expression of growth-regulated genes to that of H3 histone gene, whose expression is specific for the S phase of the cell cycle (12).

Results reported here demonstrate that 3LL metastatic variants express similar levels of all growth-regulated genes and oncogenes studied; only the TGF-β1 gene was found to be expressed at higher levels in highly metastasizing 3LL cells.

MATERIALS AND METHODS

Tumor Lines and Metastasis Formation. *In vitro* variants C87 and BC215 were derived from 3LL and were maintained in culture according to previously reported methods (13). Cell suspensions of cultured cells, harvested by trypsinization from log-phase cultures, were injected into syngeneic mice (C57Bl/6) to analyze *in vivo* growth. *In vivo* tumor lines M1O87 and BM21548 and the OL of 3LL were passaged in 2–3-month-old male C57Bl/6 mice. Each mouse received an injection in the leg muscle of O.1 ml of a suspension containing 2.5×10^5 viable cells. Lung metastases were evaluated 21 days after tumor implant. The metastatic behavior of 3LL in *in vitro* clones and *in vivo* sublines evaluated as artificial and spontaneous lung colonization capacity is summarized in Table 1.

RNA Extraction and Hybridization. Total RNA was extracted by the guanidinium thiocyanate method (14) from 3LL cells, grown under the indicated conditions. The cells were harvested from log-phase cultures (3 days after seeding). The tumors were excised 10 days postimplant, and lung metastases were isolated 21 days postimplant. The days at which the tumors or the metastases were excised correspond to the log phase in their growth curves. Polyadenylated$^+$ RNA (4 μg/lane) was fractionated on formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized following the standard procedure (15). ^{32}P nick-translated, gel-purified fragments of DNA probes (2×10^9 cpm/μg) were used for hybridization. The size of the transcripts was determined in relation to 18S and 28S markers. Quantitation of the levels of RNA was carried out directly on the autoradiograms of the blots, using a soft-laser densitometer.

Plasmids and DNA Probes. The probes used were the following: pFO422, carrying the human histone H3 gene (16); c-*myc* 3′, a *Cla*I-*Eco*RI fragment from human genomic DNA (17); hp4F1/vimentin and hp2A9/calcyclin, human cDNA clones (18); pHFβA-1, composed of a cDNA *Bam*HI insert encoding the entire β-actin protein (19); the insert of SW11-1, containing Ki-*ras*2 cDNA (20); H-1, containing a 5.5-

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[3] The abbreviations used are: TGF, transforming growth factor; 3LL, Lewis lung carcinoma; cDNA, complementary DNA; OL, original tumor line.

Table 1 *Metastatic properties of 3LL variants*

	Spontaneous lung metastases[a]	Artificial lung metastases[b]
In vivo lines		
OL	35 ± 4	20 ± 2
M1087	110 ± 8	75 ± 15
BM21548	16 ± 3	12 ± 4
In vitro clones		
C87	20 ± 9.5	18 ± 5
BC215	4 ± 1.5	3 ± 1.2

[a] Lung colonies evaluated 24 days after i.m. implantation of 2×10^5 cells. Means ± SE.
[b] Lung nodules evaluated 19 days after i.v. injection of 2×10^5 cells. Means ± SE.

kilobase *Eco*RI fragment from Ha-*ras* oncogene (21); pHSPp53, containing a complete murine p53 cDNA (22); v-*erb*B cDNA, a *Bam*HI (0.5-kilobase) fragment inserted into pBR322; and *p26*, containing a region of the human c-*fes* gene. All of these plasmids were the gift of Prof. R. Baserga (Temple University, Philadelphia, PA). hpBSTGF-β1, containing a 2.2-kilobase cDNA coding for the human transforming growth factor β1 (23), was kindly provided by Dr. R. De Martin (European Molecular Biology Laboratory, Heidelberg, Germany). phTGF1-10-925, containing a 0.925-kilobase cDNA coding for the human transforming growth factor α, was kindly provided by Prof. Giulia Colletta (Department of Experimental Medicine, University of Rome). The v-*sis* 0.98-kilobase fragment and v-*src* 0.8-kilobase fragment were purchased from Oncor (Gaithersburg, MD).

RESULTS

Low- and high-metastatic 3LL variants were so designated according to their ability to form spontaneous and artificial metastases in the lung following transplantation into syngeneic C57Bl/6 mice (see Table 1). In Fig. 1 we compare the level of the expression of G1-related genes (calcyclin, vimentin, c-*myc*, and *p-53*) to the level of the S-phase gene, histone H3, in 3LL cells. To obtain quantitatively comparable data, the same RNA blots were normalized for the expression of the β-actin gene. The RNA was extracted from *in vitro* (C87, high metastatic; and BC215, low metastatic) and *in vivo* (M1O87, high metastatic; and BM21548, low metastatic) 3LL variants and from the OL of 3LL. Fig. 1 demonstrates that the level of expression of different genes (c-*myc*, vimentin, calcyclin, *p53*, and *H3*) is the same in all metastatic variants whether maintained *in vitro* or *in vivo*. The hybridization patterns observed for the *erb*-B and Ki-*ras* probes reported in Fig. 2 correspond to those previously described (24, 25) and demonstrate that the two oncogenes are equally expressed in low- and high-metastatic 3LL variants. The level of expression of the Ha-*ras* gene is extremely low in all clones and sublines of 3LL, whereas the mRNA for c-*fes*, v-*src*, and v-*sis* oncogenes was not detectable in either the *in vivo* or *in vitro* variants under the conditions used (data not shown).

In Fig. 3 we report the expression of the TGF-α and β1 genes in *in vitro* and *in vivo* variants. By comparing the level of the expression of TGF genes with that of the β-actin gene, it appears that the expression of TGF-β1 is higher in highly metastasizing 3LL cells maintained either *in vitro* (C87 clone) or *in vivo* (M1087 subline), whereas the level of TGF-α is similar in all 3LL variants. To further verify that an association exists between the expression of the TGF-β1 gene and the metastatic capacity of 3LL cells, the TGF-α and β1 mRNAs were quantitated in metastatic lesions of 3LL metastatic variants. In this view, in Fig. 3 the expression of TGF genes in primary tumors is compared with that of the corresponding metastatic lesions. The figure shows that only the TGF-β1 expression is changed in metastases of the low-metastatic variant (BM21548). Because of the reported heterogeneity of tumor cells in metastatic lesions and because of the variability in the total number of lung nodules in different mice, the TGF-β1 expression in primary tumors and in the corresponding metastases has been evaluated several times. Consistently, a higher level of the TGF-β1 mRNA has been observed in metastatic nodules of the low-metastatic variant (BM21548) with respect to corresponding primary tumors.

DISCUSSION

Metastatic variants derived from Lewis lung carcinoma have been used to correlate the expression of several growth-regu-

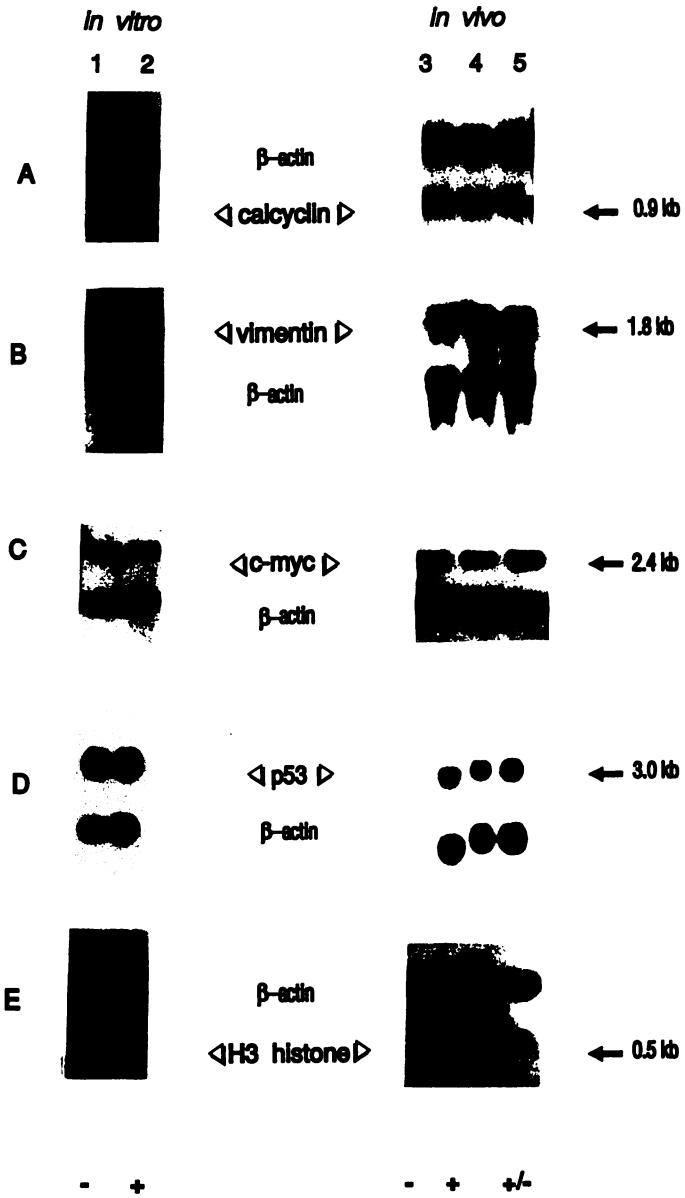

Fig. 1. Calcyclin (*A*), vimentin (*B*), c-*myc* (*C*), p53 (*D*), and H3 histone (*E*) expression on *in vitro* and *in vivo* 3LL variants and in the primary tumors of the OL of 3LL. *Lane 1*, BC215 (low metastatic); *Lane 2*, C87 (high metastatic); *Lane 3*, M1087 (high metastatic) primary tumor; *Lane 4*, BM21548 (low metastatic) primary tumor; *Lane 5*, OL (intermediate metastatic) primary tumor. Total RNA (20 μg/lane; *E*, H3 histone) and polyadenylated$^+$ RNA (4 μg/lane; *A–D*) were electrophoresed on agarose/formaldehyde gel and blotted onto nitrocellulose. Hybridization was carried out with purified nick-translated inserts, as described in "Materials and Methods." As a control the β-actin gene expression is reported (approximately 2 kilobases).

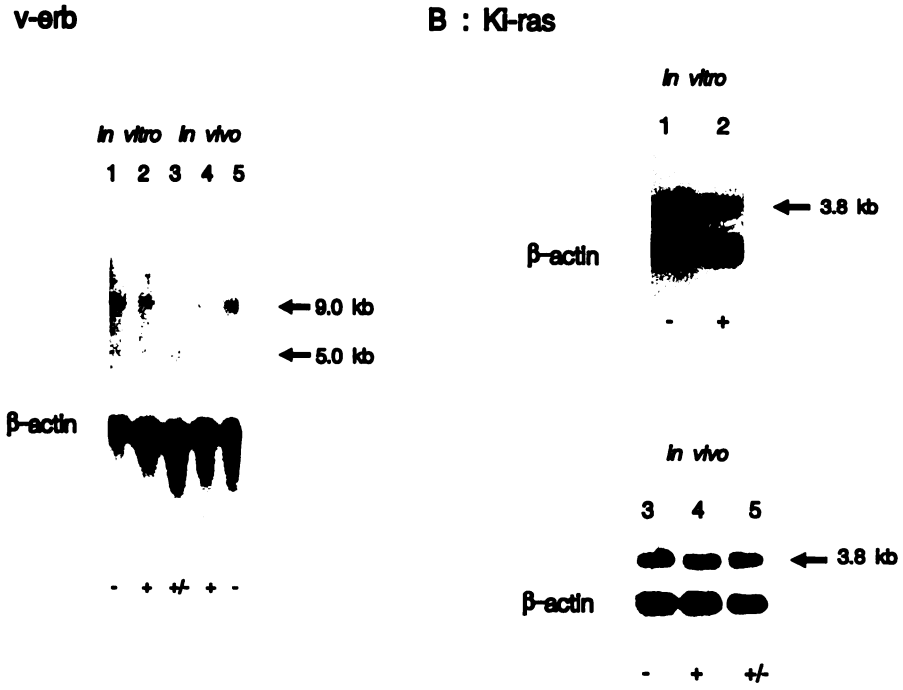

Fig. 2. v-*erb*-b (*A*) and Ki-*ras* (*B*) expression on *in vitro* and *in vivo* 3LL variants and in the primary tumors of the OL of 3LL. *A: Lane 1*, BC215 (low metastatic); *Lane 2*, C87 (high metastatic); *Lane 3*, OL (intermediate metastatic) primary tumor; *Lane 4*, M1087 (high metastatic) primary tumor; *Lane 5*, BM21548 (low metastatic) primary tumor. *B: Lane 1*, BC215 (low metastatic); *Lane 2*, C87 (high metastatic); *Lane 3*, BM21548 (low metastatic) primary tumor; *Lane 4*, M1087 (high metastatic) primary tumor; *Lane 5* OL (intermediate metastatic) primary tumor. Polyadenylated$^+$ RNA (4 μg/lane) was electrophoresed, blotted, and hybridized as described in Fig. 1.

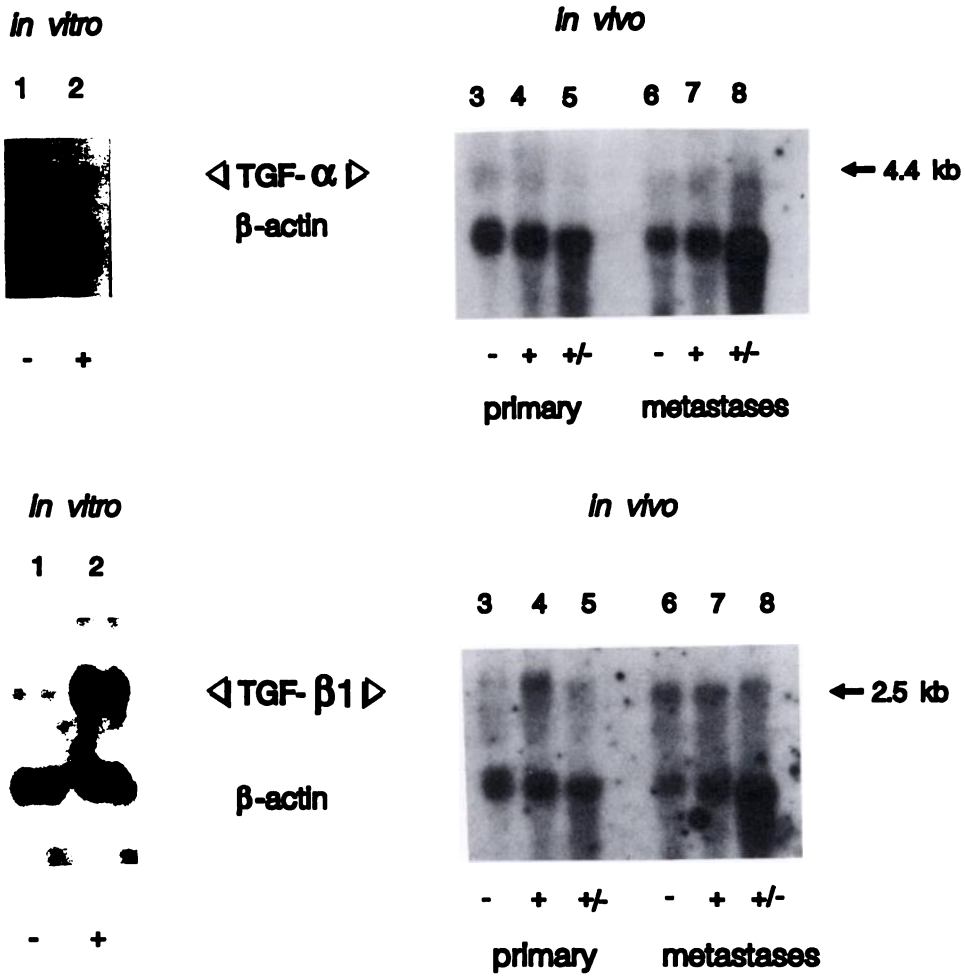

Fig. 3. TGF-α and TGF-β1 expression on *in vitro* and *in vivo* 3LL variants and in the OL of 3LL. mRNA was extracted from cultured cells (BC215 and C87) and from the primary tumors and the metastases of different lines (BM21548, M1087, and OL). *Lanes 1*, BC215 (low metastatic); *Lanes 2*, C87 (high metastatic); *Lanes 3*, BM21548 (low metastatic) primary tumor; *Lanes 4*, M1087 (high metastatic) primary tumor; *Lanes 5*, OL (intermediate metastatic) primary tumor; *Lanes 6*, BM21548 (low metastatic) metastases; *Lanes 7*, M1O87 (high metastatic) metastases; *Lanes 8*, OL (intermediate metastatic) metastases. Polyadenylated$^+$ mRNA (4 μg/lane) was electrophoresed, blotted, and hybridized as described in Fig. 1.

lated genes, oncogenes, and TGF genes with the metastatic phenotype. Since we have previously reported that 3LL variants show different abilities to proliferate in response to growth factors and exhibit a higher autocrine capacity (26), we have attempted to determine whether a deregulated expression of a number of growth-regulated genes, oncogenes, or TGFs (α and β1) could account for the reported proliferative advantage. To evaluate whether specific differences in the expression of

growth-regulated genes are due to a proliferative advantage acquired by a tumoral subpopulation, or whether they simply reflect the conditions of growth in culture, *in vitro* and *in vivo* tumor lines have been used. To exclude the possibility that an overexpression of some oncogenes and growth-regulated genes could reflect an increased growth fraction of the cell populations (11), we have compared their expression to that of the H3 histone gene, which is an S-phase-specific gene (13).

The results indicate that the H3 histone and the growth-regulated genes studied (*p53*, vimentin, calcyclin, and c-*myc*) are expressed at the same level in low- and high-metastatic variants either *in vivo* or *in vitro*, therefore demonstrating that the growth fraction of the different cell populations is similar. The mRNAs for *sis*, *src*, and *fes* oncogenes were not detectable in the conditions employed, whereas *erb*-b, Ki-*ras*, and Ha-*ras* oncogenes were equally expressed in *in vitro* cells and *in vivo* tumors. By contrast, the TGF-β1 gene is clearly expressed at higher levels in highly metastatic variants, whereas TGF-α is unvaried. Since we report an increased expression of the TGF-β1 gene in highly metastatic variants maintained either *in vitro* or *in vivo*, the results indicate that TGF-β1 expression is independent of culture conditions and suggest that such a growth factor is constitutively overexpressed in 3LL metastatic cells. The fact that in the metastatic lesions of the low-metastatic variant the level of TGF-β1 mRNA approaches the level observed in the primary and secondary tumors of the highly metastatic line further supports the view that the TGF-β1 gene is highly expressed in 3LL cells which metastasize to the lung.

Taken together these results indicate that the overgrowth of tumoral subpopulations which possess the ability to metastasize might be independent of the increased growth fraction and the overexpression of several oncogenes. The data suggest, rather, that in 3LL tumors, an autocrine pattern of cell proliferation may contribute to the ability of the cells to metastasize. The fact that a higher expression of the TGF-β1 gene in these epithelial cells does not influence the expression of the growth-regulated genes and oncogenes here studied does not rule out the possibility that other proliferation-associated genes (27) are affected. Moreover, the reported reduced nutritional requirements for cell proliferation, the complete serum independence in anchorage-independent growth, and the higher autocrine capacity of metastatic 3LL cells (26), coupled with the constitutively higher expression of the TGF-β1 gene here described, are in good agreement with data reported by other authors (28). Since TGF-β1 may influence metastatic cell growth via complex pathways which do not necessarily involve mitogenesis (29), further studies have to be undertaken to determine the mechanism(s) by which TGF-β1 may affect metastatic phenotype.

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