

Fas-Negative Osteosarcoma Tumor Cells Are Selected during Metastasis to the Lungs: The Role of the Fas Pathway in the Metastatic Process of Osteosarcoma

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Abstract

Low expression of Fas by different tumors including osteosarcoma, correlates with poor prognosis. We found that osteosarcoma lung metastases from patients expressed negligible amounts of Fas, but primary tumors often expressed high Fas levels. The reason for this discrepancy is unknown. We hypothesized that because FasL is constitutively expressed in the lungs, Fas-positive (Fas⁺) tumor cells entering the lungs would bind with FasL and die from Fas-induced apoptosis, resulting in the "selection" of Fas-negative (Fas⁻) cells, which would eventually form metastases. To test this hypothesis, we injected K7 osteosarcoma cells, which express functional Fas *in vitro*, into mice and confirmed that its bone tumors were Fas⁺, but lung metastases were Fas⁻. Next, to inhibit Fas signaling without affecting Fas expression, we transfected these cells with a FADD-dominant negative (FDN) plasmid and developed K7/FDN cells. Metastases formed by K7/FDN cells contained Fas⁺ tumor cells. Moreover, K7/FDN cells were retained in the lungs longer and formed more lung metastases than K7 cells. In addition, the incidence of lung metastases in FasL-deficient mice injected with K7 cells was higher than that in wild-type mice. Metastases from FasL-deficient mice but not from wild-type mice contained Fas⁺ tumor cells. Based on that, we conclude that Fas⁻ osteosarcoma cells are selected during lung metastases formation and that inhibition of Fas signaling in tumors or lack of FasL in the host environment allows the proliferation of Fas⁺ osteosarcoma cells in the lungs and promotes metastases growth. Therefore, Fas may be considered as a new therapeutic target for osteosarcoma treatment. (Mol Cancer Res 2007;5(10):991–9)

Received 1/5/07; revised 6/8/07; accepted 6/14/07.

Grant support: National Cancer Institute grant R01 CA42992 (E.S. Kleinerman) and Legends of Friendswood and Amschwand Sarcoma Cancer Foundation Awards (N.V. Koshkina). Animal experiments were supported in part by the NIH Core grant CA16672.

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doi:10.1158/1541-7786.MCR-07-0007

Introduction

Metastasis from primary tumors to other organs is a major cause of death from cancer: according to one estimate, ~70% of patients with cancer have microscopic metastases at the initial diagnosis of cancer (1). Early diagnosis and early initiation of treatment are linked with favorable outcome in nearly all forms of cancer. Therefore, a better understanding of the earliest steps in the metastatic process is critical for designing effective targeted therapies for metastatic disease.

A common site of metastasis in many types of solid tumors, including osteosarcoma, is the lung. Osteosarcoma that metastasize to the lung carry a poor prognosis because metastatic disease is generally resistant to salvage chemotherapy (2). In our previous studies of the mechanisms by which osteosarcoma metastases progress to the lungs, we examined tumor tissues from patients with osteosarcoma and found that most osteosarcoma lung metastases expressed negligible levels of Fas receptor (3), but that it was expressed at various levels in primary bone tumors.³ Studies with the LM7 osteosarcoma animal model revealed that the metastatic potential of osteosarcoma cells inversely correlated with Fas expression (4). These findings suggest that Fas signaling is implicated in the metastatic progression of osteosarcoma.

The Fas receptor and its ligand (FasL) belong to the tumor necrosis factor death receptor superfamily and participate in regulating tumorigenesis in several types of primary malignancies and metastases (5-7). Our previous work indicated that chemotherapeutic agents can up-regulate Fas expression in osteosarcoma lung, whereas untreated lung metastases remain negative for Fas (8). These results indicate that the *fas* gene is present in osteosarcoma cells that formed pulmonary metastases, but why it would not be expressed in the untreated lung metastases remains unknown. Two explanations for these findings are possible. First, Fas expression in metastatic lesions could be suppressed by some aspect of the pulmonary environment (e.g., the lungs may produce factors that inhibit Fas expression by tumors). Alternatively, Fas-negative (Fas⁻) tumor cells could be somehow selected during the process of metastasis formation and growth in the lungs. Identification of which mechanism is involved in the loss of Fas expression in metastasis has important clinical implications because it would

³ Unpublished data.

allow us to identify targets either in cancer cells or in the lung environment, which could be used to restore the Fas signaling activity and presumably to increase Fas-mediated apoptosis in tumors.

Lung epithelial cells are one of few cell types in humans that constitutively express FasL (9, 10). Because lungs are continuously exposed to environmental antigens, they have a residential population of activated immune cells that also express FasL (10, 11). On the basis of these findings, we hypothesized that tumor cells possessing the Fas receptor and a functional Fas pathway are most likely to be eliminated by engagement with the FasL expressed in the lungs, leading to natural selection of Fas⁻ tumor cells in the pulmonary microenvironment, where they would survive and form metastases. To test this hypothesis, we inhibited the Fas signaling in K7 osteosarcoma cells while leaving the Fas receptor unaffected and injected these cells into mice. The examination of lung metastasis in these mice revealed the presence of Fas-positive (Fas⁺) tumor cells in the pulmonary metastases, indicating that Fas had not been suppressed in the lungs of mice. Further analysis confirmed that the presence of Fas⁺ tumor cells with corrupted Fas signaling can contribute to the retention and growth of those tumor cells in the lungs. The presence of Fas⁺ tumor cells was also observed in the osteosarcoma lung metastases from FasL-deficient mice. These findings lead us to conclude that inhibition of Fas signaling in tumors or lack of FasL in the host environment, allows the proliferation of Fas⁺ osteosarcoma cells in the lungs, which could promote the growth of metastases in the lungs. These findings indicate, for the first time, that in the presence of the functional Fas pathway, Fas⁺ tumor cells will bind to FasL expressed in the lungs and die from Fas-induced apoptosis, leaving only Fas⁻ tumor cells to be “selected” for growth in the pulmonary environment.

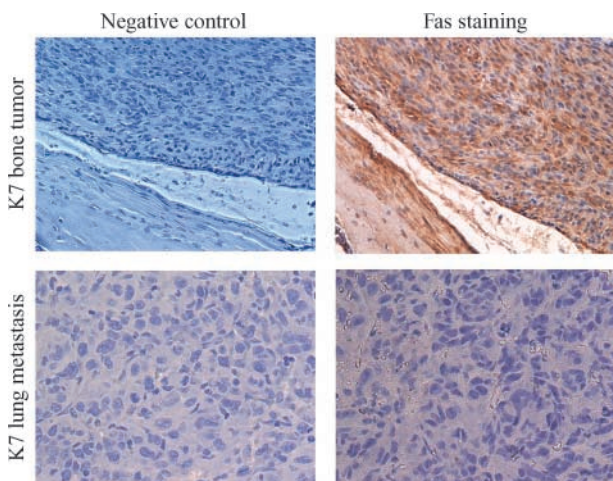


FIGURE 1. Fas receptor expression in primary bone tumor and in lung metastases formed by K7 cells. Mice were injected either intratibially (for bone tumor assessment) or intravenously (for lung metastasis assessment) with K7 parental cells, and tumor tissues were sectioned and stained with anti-mouse Fas antibody. Negative controls were prepared by omitting the primary antibody. The presence of Fas receptors is visible in the bone tumor specimen but not in the lung tissue section (brown). $N = 5$ mice/group; experiments were repeated thrice.

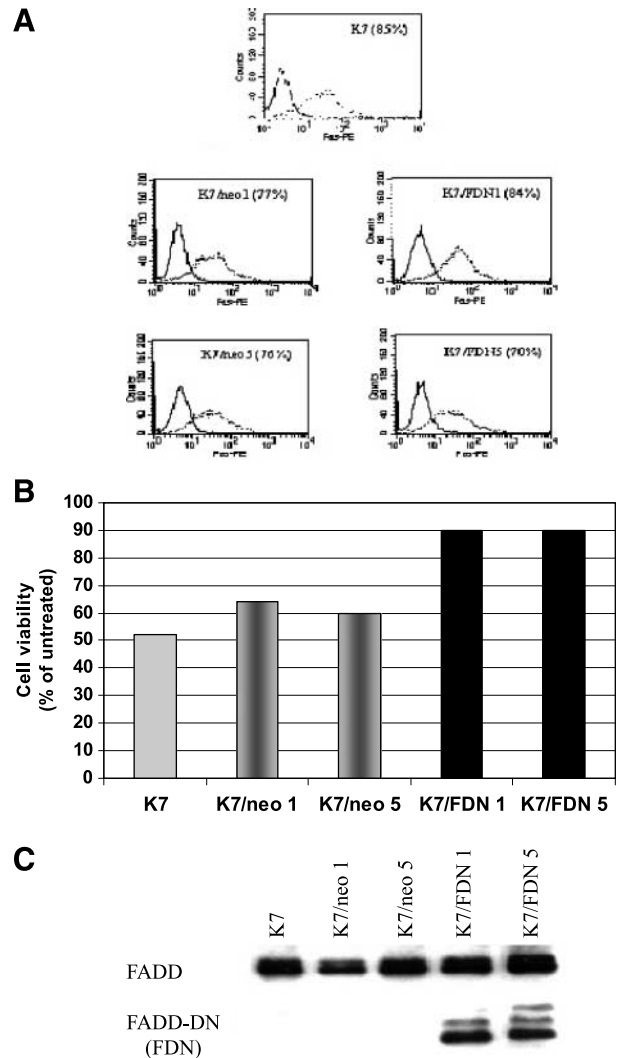


FIGURE 2. *In vitro* characterization of K7 mouse osteosarcoma cells and their transfectants. **A.** Fas expression in parental K7 cells and selected clones of cells transfected with neomycin vector (K7/neo) or with a FADD-dominant negative construct (K7/FDN). Cells were labeled with phycoerythrin-conjugated anti-mouse Fas antibody and analyzed by flow cytometry for Fas expression. Percentages refer to the portion of Fas⁺ cells. Transfected cells showed similar levels of Fas as parental cells. **B.** Cytotoxic effect of FasL on K7, K7/neo, and K7/FDN cells. Cells were exposed to 100 ng/mL of soluble FasL (the IC₅₀ value) for 24 h and assessed for viability by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay. Transfection of K7 cells with the FDN plasmid inhibited Fas-induced apoptosis. **C.** Expression of FADD protein and its dominant-negative form by K7, K7/neo, and K7/FDN clones. Western blots show that all cells expressed full-length FADD protein but only the K7/FDN cells, transfected with a dominant-negative form of FADD, expressed its truncated form (FDN). Each experiment was repeated thrice.

Results

Fas⁻ Osteosarcoma Cells Are Selected in the Pulmonary Environment during the Formation of Metastases

Our hypothesis is that Fas⁺ osteosarcoma cells with a functional Fas pathway are eliminated from the lungs through their interaction with the FasL, which is constitutively expressed in the lungs and residential activated immune cells. Therefore, Fas⁺ tumor cells in which the Fas receptor is

expressed, but is not functional owing to inhibition of downstream signaling, would be expected to evade this innate host defense mechanism and form lung metastases. In other words, we should be able to detect Fas⁺ tumor cells in lung metastasis. If an environmental suppression mechanism is responsible for the lack of Fas in lung metastases, then the Fas receptor will be suppressed independently of the Fas pathway activity.

For these experiments, we opted to use K7 osteosarcoma cells. These cells form rapidly growing tumors after injection into the tibia bone of mice, but rarely form lung metastases in mice (12). Immunohistochemical staining of primary bone tumors that formed after intratibial injection of K7 cells into mice showed that the bone tumors remained Fas⁺, but the lung metastases that formed after intravenous injection of K7 cells were Fas⁻ (Fig. 1). *In vitro*, 85% of K7 cells expressed high levels of Fas (Fig. 2A) and died after treatment with soluble FasL (Fig. 2B).

Next, to inhibit the downstream Fas signaling without affecting the Fas receptor, we transfected K7 cells with a FADD-dominant negative (FDN) plasmid or with a control neomycin-resistant (neo) plasmid. The expression of FDN protein in selected clones was confirmed by Western blot

analysis (Fig. 2C). Fas expression levels in K7/FDN and K7/neo clones were not significantly different from that in parental K7 cells (Fig. 2A), but only K7/FDN cells were resistant to FasL treatment *in vitro* (Fig. 2B, $P < 0.05$ when compared with K7 or K7/neo cells), indicating that the Fas pathway in K7/FDN cells was inhibited downstream of the receptor. Western blot analysis of other proteins that can mediate Fas-mediated apoptosis resistance (FAP, cFLIP, and Bcl-2) showed that their levels of expression were not different between K7, K7/neo, and K7/FDN clones (data not shown). As expected, the lung metastases formed after intravenous injection of K7 or K7/neo cells in syngeneic mice were Fas⁻, but the K7/FDN lung metastases included both Fas⁺ and Fas⁻ cells (Fig. 3A), pointing out that Fas⁺ tumor cells in which the Fas pathway was inhibited could survive in the lungs. The proliferation rates of tumor cells in metastases formed by K7, K7/neo, and K7/FDN cells was similar according to immunohistochemical staining with proliferating cell nuclear antigen antibody (Fig. 3B). Together, these findings suggest that blocking the Fas pathway interfered with the elimination of Fas⁺ tumor cells in the lungs, thereby allowing them to survive and form lung metastases.

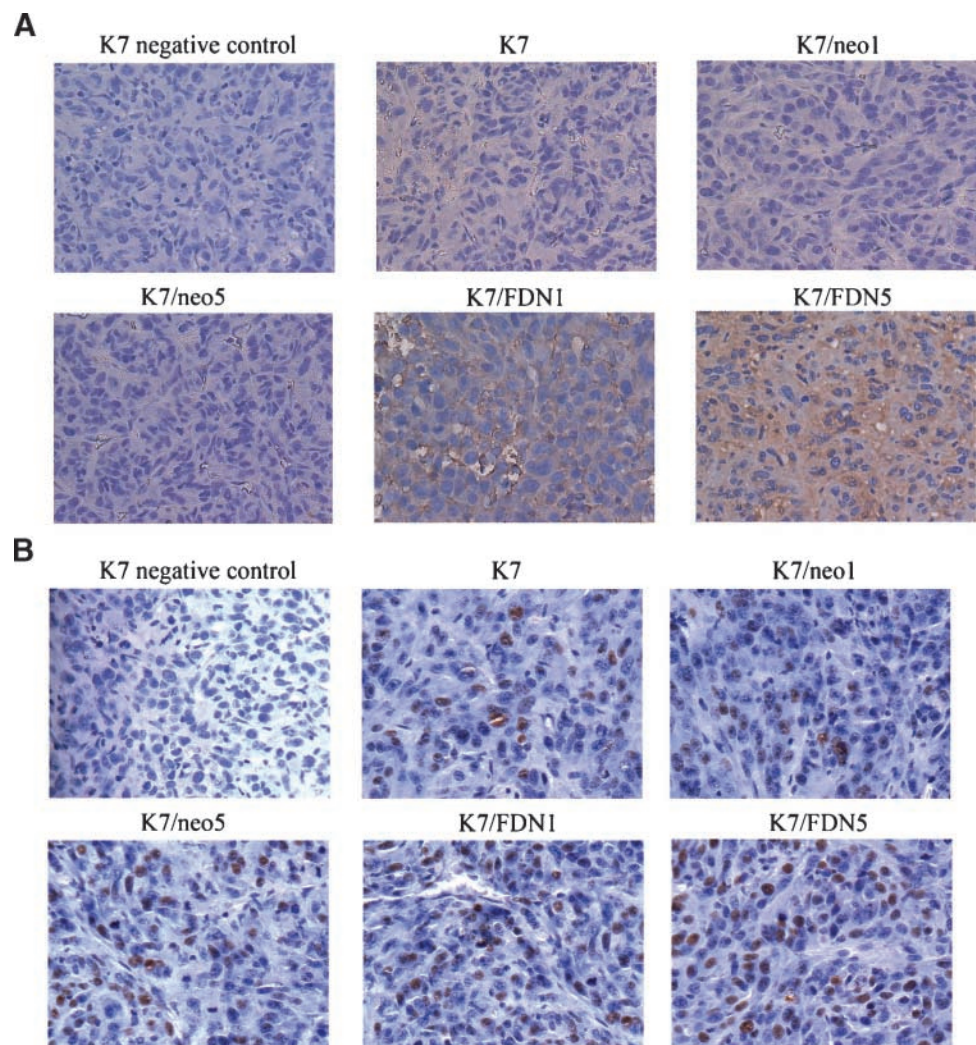


FIGURE 3. Immunohistochemical staining of metastases formed by K7, K7/neo, and K7/FDN osteosarcoma cells for Fas receptor expression and proliferation. Negative controls were prepared by omitting the primary antibodies. **A.** Lungs from mice injected with K7, K7/neo, or K7/FDN cells were immunohistochemically stained with anti-mouse Fas antibody. The presence of Fas receptors is visible only in the lung metastases from K7/FDN mice but not from K7 or K7/neo (brown). **B.** K7, K7/neo, and K7/FDN lung metastases were stained for proliferation markers with anti-proliferating cell nuclear antigen antibody. Dividing cells showed no differences between tumors (dark brown). $N = 5$ mice/group; experiments were repeated twice.

TABLE 1. Retention of K7 Osteosarcoma Cells and Their Transfectants in the Mouse Lungs after Intravenous Injection

Cells	Percentage of cells retained in the lungs after injection (%)			
	1 (h)	6 (h)	24 (h)	48 (h)
K7*	100	34.4 ± 8.9	4.4 ± 3.1	0.6 ± 0.3
K7/neo*	100	25.6 ± 9.0	1.9 ± 0.4	0.6 ± 0.4
K7/FDN*	100	39.0 ± 6.0	10.0 ± 2.3	6.8 ± 2.3

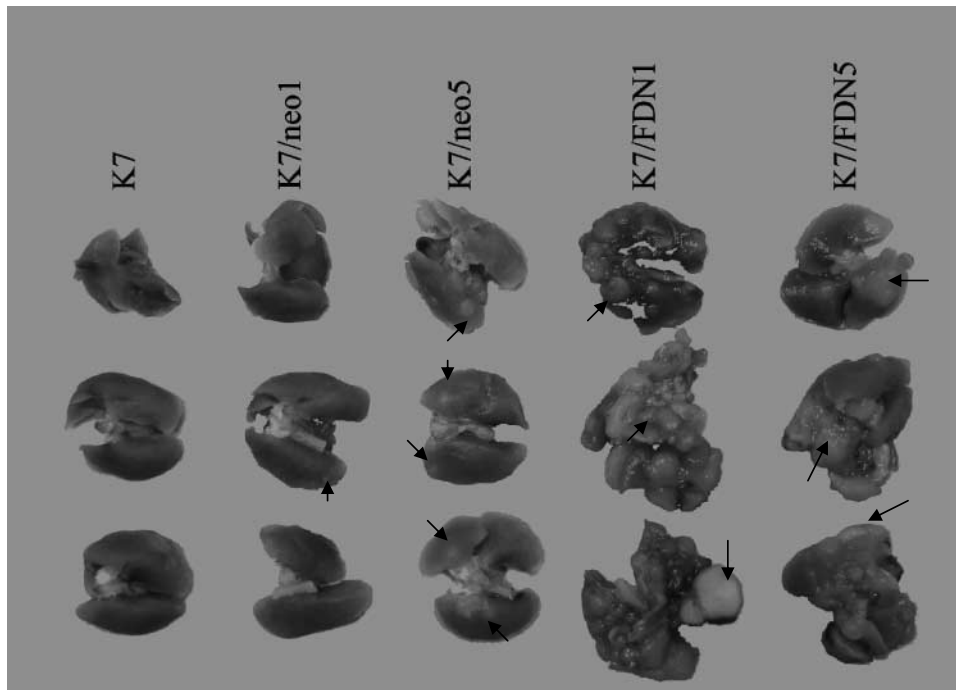
*N = 5 mice/group were used; experiments were repeated twice.

Inhibition of the Fas Pathway Increases the Number of Osteosarcoma Cells Retained in the Lungs

If our hypothesis is correct, then blocking the Fas pathway should increase the number of tumor cells retained in the lungs,

because Fas⁺ cells could contribute to the growth of metastasis. To address this question, we used a single-cell fluorescent imaging system that allowed the quantification of injected cells in the lung (13). When fluorescently labeled K7, K7/neo, or K7/FDN cells were intravenously injected into mice, more K7/FDN cells were retained in the lungs than K7 and K7/neo cells; moreover, at 24 h after injection, the number of K7/FDN cells in the lungs was ~5 times higher than the number of K7 or K7/neo cells, and by 48 h, that number had increased to 12 times higher (Table 1). No difference in retention was observed between K7 and K7/neo cells. These findings indicate that inhibition of the Fas pathway could facilitate the formation of metastases by retaining additional osteosarcoma cells in the lungs, whereas intact Fas signaling could suppress the formation of metastases by reducing or eliminating the retention of tumor cells that arrived in the lungs.

A



B

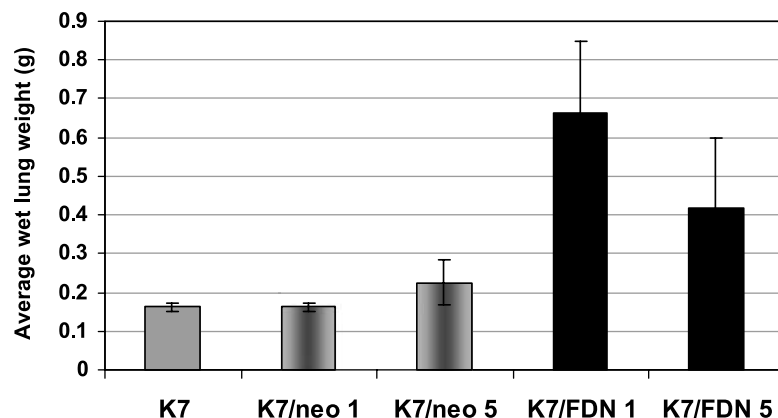


FIGURE 4. Inhibition of Fas signaling in K7 cells by transfection with the FDN plasmid increases the metastatic potential of these cells in mouse lungs. Untransfected parental K7 cells, K7/neo, or K7/FDN cell clones were injected by tail vein into BALB/c mice. Average net wet-lung weight (**A**) and representative lungs (**B**) from mice injected with K7 cells, K7/neo, or K7/FDN clones. Arrows, visible lung metastases. N = 5 mice/group; experiments were repeated twice.

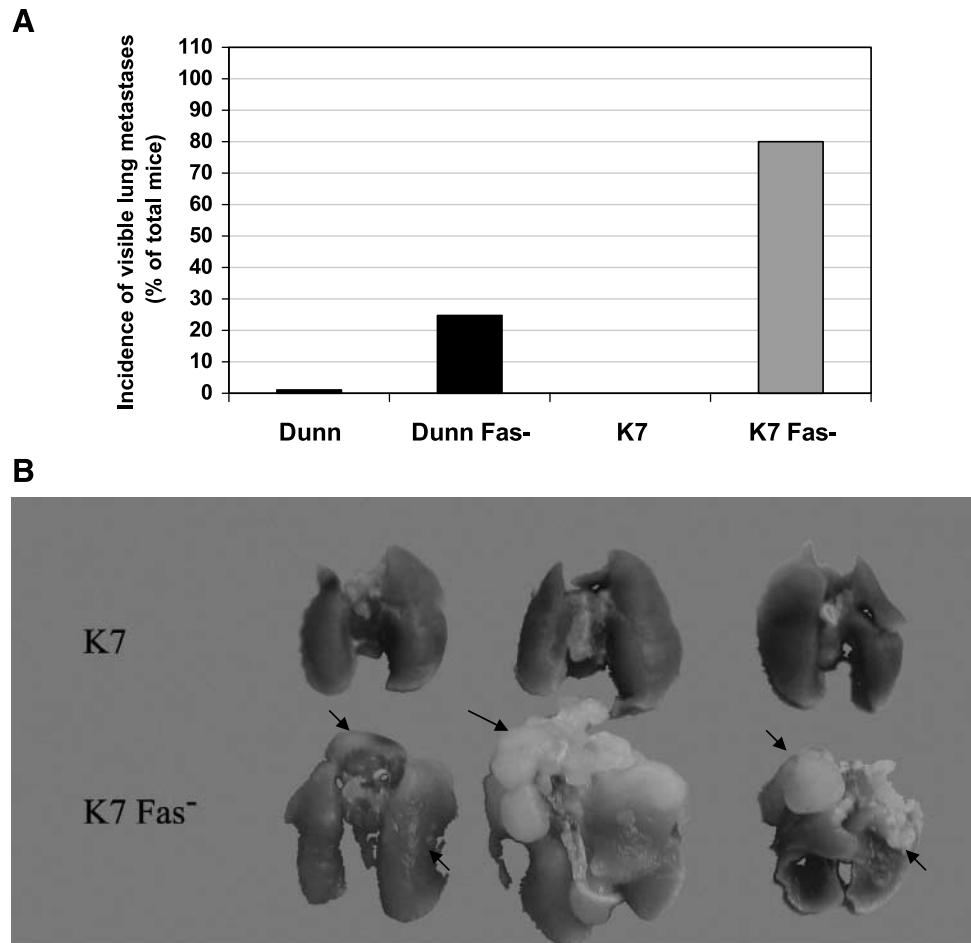


FIGURE 5. Fas⁻ mouse osteosarcoma cells promote the growth of tumor metastases in the lungs. **A.** Dunn or K7 cells were labeled with phycoerythrin-conjugated anti-mouse Fas antibody or phycoerythrin-conjugated IgG₁ isotype-matched control antibody, sorted, and the Dunn or Dunn Fas⁻ cells were injected subcutaneously into syngeneic C3H mice. Similarly, K7 and K7 Fas⁻ cells were injected intravenously into BALB/c mice. The presence of visible lung metastases in mice was evaluated at 4 wk (for Dunn cells) or at 5 wk (for K7 cells) after tumor cell inoculation. **B.** Representative lungs from mice injected with parental K7 or K7 Fas⁻ cells. Arrows, visible metastases. *N* = 5 mice/group; experiments were repeated twice.

Inhibition of the Fas Pathway Promotes Osteosarcoma Lung Metastasis

To further confirm that Fas⁺ cells with a dysfunctional Fas pathway could contribute to the growth of osteosarcoma lung metastases, we injected parental K7 cells, which rarely form metastases (12), K7/neo cells, or K7/FDN cells intravenously in mice. As expected, K7 and K7/neo cells, which contained mostly Fas⁺ cells and had a functional Fas pathway, produced few lung metastases (Fig. 4A). By contrast, K7/FDN cells, which express an inactive Fas receptor, formed lung metastases so large that they more than doubled the weight of the mouse lungs (Fig. 4B, *P* < 0.05). In conjunction with our discovery of Fas⁺ osteosarcoma cells in metastases formed by K7/FDN cells (Fig. 3), these findings showed that dysfunction of the Fas signaling pathway enabled the survival and growth of Fas⁺ osteosarcoma cells in the lungs and significantly promoted the growth of lung metastases in mice.

Fas⁻ Osteosarcoma Cells Are More Likely to Form Lung Metastases than Parental Cells Which Contain a Mixed Population of Fas⁻ and Fas⁺ Cells

If inhibition of the Fas signaling promotes the formation of osteosarcoma metastases in the lungs, then substitution of Fas⁺ cells with a functional Fas pathway for Fas⁻ cells in tumor cell

populations should increase the growth of metastases. To test this assumption, we used two murine osteosarcoma lung metastasis models, K7 and Dunn. Dunn cells occasionally form spontaneous lung metastases after subcutaneous injection in C3H mice. No visible lung metastases were seen in injected mice on day 29, and only 60% of the mice had microscopic metastatic lung disease (Fig. 5A). However, when only the Fas⁻ population of Dunn cells (Dunn Fas⁻ cells) were injected into C3H mice in the same amounts as the parental cells (which contained both Fas⁺ and Fas⁻ cells), 25% of the mice had visible metastases and 100% of mice had microscopic disease (Fig. 5A).

Similarly, parental K7 cells formed no visible lung metastases, whereas K7 Fas⁻ cells formed visible lung metastases in 75% of mice 5 weeks after intravenous injection (Fig. 5B). Collectively, these findings indicated that enrichment of tumor cells with the Fas⁻ population enhanced the ability of osteosarcoma cells to form lung metastases.

Lack of FasL in the Host Environment Increases the Metastatic Potential of Osteosarcoma Cells In vivo

In this experiment, we used another approach to show that the lungs do not suppress Fas expression in osteosarcoma tumor cells. If the host lungs or activated immune cells do not express

functional FasL, then the Fas receptor on tumor cells will not be able to bind appropriately with the ligand and thus Fas⁺ tumor cells will be able to survive in the lungs. In other words, we should be able to detect Fas⁺ osteosarcoma cells in lung metastases and these cells may contribute to the growth of metastases. To address this question, we intravenously injected FasL-deficient mice with Fas-expressing parental K7 osteosarcoma cells. The incidence of micrometastases was 60% in the wild-type mice and 100% in the FasL-deficient mice. Immunohistochemical staining of lung tissues revealed the presence of both Fas⁺ and Fas⁻ cells in tumors from FasL-deficient mice, but tumors from the wild-type mice were Fas⁻ (Fig. 6). We conclude that the lack of FasL in the host environment enabled the survival and growth of Fas⁺ osteosarcoma cells in lung metastases and contributed to the promotion of osteosarcoma metastasis in the lungs.

Discussion

Fas-induced apoptosis has an important role in tumorigenesis and in therapeutic cell destruction. In numerous clinical studies, lack of Fas receptor expression in primary tumors correlated with poor prognosis (14-17). However, its role in metastatic progression is not completely understood. The down-regulation or mutation of the apoptosis-inducing Fas receptor has been proposed as a mechanism by which cancer cells can avoid destruction by immune cells which express membrane-bound FasL (6, 7). Activated immune cells are not the only source of FasL in the human body. Constitutive FasL expression has been detected in the epithelial cells of the lungs (18) and few other organs (9-11, 19, 20). Relevant to this finding, we and others have reported that Fas-mediated apoptosis can be lost during metastatic spread of different malignancies to the lungs in animal models (4, 5, 7, 21). Until now, the mechanism responsible for this loss has been unknown.

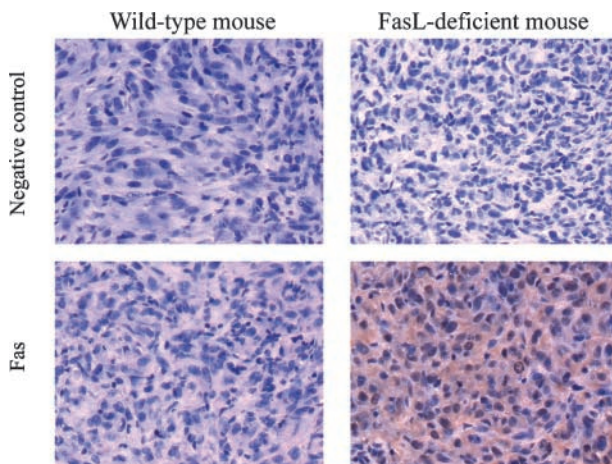


FIGURE 6. Fas receptor expression in lung metastases formed by K7 cells in normal and FasL-deficient mice. Mice were injected intravenously with K7 cells, and 5 wk later, the mice were killed and the lungs were removed and processed for immunohistochemical staining with anti-mouse Fas antibody. Negative controls were prepared by omitting the primary antibodies. The presence of Fas receptor is visible in the lung tissue from FasL-deficient mice but not in the wild-type mice (brown). *N* = 5 mice/group; experiments were repeated twice.

In this study, we showed that injecting K7 mouse osteosarcoma cells, most of which expressed functional Fas receptor *in vitro*, into the tibias of mice caused the formation of primary bone tumors and those bone tumors were Fas⁺. Intravenous injection of these cells led to the formation of lung metastases that were Fas⁻. Also, spontaneous micrometastases that episodically formed in the lungs of mice after intratibial injection of K7 cells were negative for Fas (data not shown). To identify the mechanism responsible for the absence of Fas in lung metastases, we inhibited downstream Fas signaling in these cells without affecting the integrity of Fas receptor and expression of other apoptosis-inhibitory molecules, such as Bcl-2, FLIP, and FAP (data not shown) by creating K7/FDN cells. Pulmonary metastases formed by K7/FDN cells after intravenous injection contained many Fas⁺ tumor cells, indicating that Fas receptor in tumor cells is not suppressed by factors in the lung environment; if that was not the case, the Fas receptor would be inhibited regardless of the functionality of the Fas pathway. Rather, selection of Fas⁻ tumor cells takes place during the metastatic process of osteosarcoma in the lungs, which allowed Fas⁺ cells with inhibited Fas signaling to survive in this environment. We realize that using intravenous injection of tumor cells in our animal model did not allow us to test metastasis from the bone tumor per se, but it did allow us to address the question of the ability of osteosarcoma cells circulating in the blood to form lung metastases because tumor cells' access to the distant organs of the body is predominantly through the hematogenous circulation. We found that the number of tumor cells with inhibited Fas pathway (K7/FDN cells) retained in the lungs after intravenous injection was significantly higher than the number of cells with functional Fas signaling (K7 or K7/neo cells). Assuming that apoptosis induced by death receptors takes place quite rapidly (for K7 cells, 50% cell death was detected 24 h after treatment with FasL; Fig. 2B), we would expect to start observing this difference 24 h after the *in vivo* injection of tumor cells. In fact, we observed no difference between the number of K7/FDN, K7/neo, and K7 cells in the lungs 6 h after intravenous injection of these cells, but by 24 h, ~5 times as many K7/FDN cells were detected in the lungs as were parental K7 cells or K7/neo cells, and this number further increased to 10 times at 48 h. After that time, only 0.5% of injected K7 and K7/neo cells were detected in the lungs. One could speculate that during this period, Fas⁺ tumor cells with a functional Fas pathway died from apoptosis induced through binding between Fas on tumor cells and FasL, expressed either by lung epithelial cells or by pulmonary residential activated immune cells. Tumor cells that remained in the lungs at 48 h most likely had dysfunctional Fas pathways or did not express Fas. Logically, then, one could assume that tumor cells which remain in the lungs for extended periods can survive and form lung metastases.

The possible contribution of tumor cells with an inhibited Fas pathway in the growth of lung metastases was further studied by comparing the metastatic potential of K7/FDN cells with that of the control K7 or K7/neo cells. The number and size of lung metastases formed by K7/FDN cells were significantly higher than those formed by K7 or K7/neo cells. This finding could be explained by the fact that a Fas⁺

population of K7/FDN tumor cells was able to survive and remain in the lungs (Fig. 1; Table 1) and eventually contribute to the growth of osteosarcoma metastases in the lungs. This was further confirmed by the finding that the proliferation rates of K7, K7/neo, and K7/FDN metastatic cells did not differ *in vivo* (Fig. 3B). This indicates that an increased number of metastases formed by K7/FDN cells emerged for the reason that more K7/FDN-metastasized cells could survive in the lungs due to the Fas pathway inhibition in them, not because these cells divide more quickly.

We realize that inhibition of FADD may affect other pathways regulated by tumor necrosis factor death receptors, which themselves may have a role in tumorigenesis and metastases formation (22, 23). The use of other techniques such as small interfering RNA may allow more specific down-regulation of protein expression, but in most cases, they cannot completely inhibit protein expression. To bypass the problem with lack of FDN specificity, we specifically inhibited the Fas signaling in two different osteosarcoma tumor cell lines, Dunn and K7, by using flow cytometry to select tumor cells that lack Fas receptor on their cell surfaces. As expected, injection of parental K7 and Dunn cells, which presumably consisted of both Fas⁺ and Fas⁻ cells, showed low metastatic activity (12, 24). The injection of a similar number of only Fas⁻ cells significantly increased the metastatic growth of these cells in the lungs. Notably, the Dunn osteosarcoma cells in this experiment were injected subcutaneously and the growth rate of primary tumors which formed after inoculation of Fas⁻ Dunn cells were similar to those of the parental Dunn cells ($P > 0.14$; data not shown). The difference was reflected only in their spontaneous formation of metastases in the lungs. This observation indicates that the status of the Fas pathway in primary tumors can have no effect on their growth, perhaps because of the absence of FasL in the subcutaneous environment of the wild-type mice, but that Fas status affects tumor growth in a FasL-expressing environment, such as the lungs. This supposition, regarding subcutaneous tumor growth, correlates with the findings of Schroter et al., who injected immortalized fibroblast NIH3T3 cells in which Fas expression had been restored by subcutaneous transfection with *fas* plasmid into normal and FasL-deficient mice, and found that tumors grew similarly in both sets of mice (25). Those investigators concluded that host-derived FasL was not crucially involved in tumor suppression and suggested that FasL produced by the tumor cells had a critical role. However, this conclusion could be countered by the fact FasL is not expressed in the subcutaneous tissues of the wild-type mice, and therefore, the only source of FasL at that site could be activated immune cells. Because no difference was noted in tumor growth between normal and FasL-deficient mice in their study, the amount of FasL from this source at this site was probably not sufficient to induce apoptosis in Fas⁺ tumor cells. In our studies, we showed that FasL in the host environment was important for the formation of metastases in the lungs, which constitutively express FasL. When we intravenously injected K7 cells into wild-type or FasL-deficient mice, the lack of FasL in the tissues or in the activated immune cells of FasL-deficient mice was associated with the formation of more visible lung metastases than in the wild-type animals. Similar

observations were described by Owen-Schaub et al. (5), who also observed the increased metastatic potential of murine melanoma cells in the lungs of FasL-deficient mice but not in the lungs of normal mice. Later, they identified FasL in the bone marrow-derived cells as having an essential role in the suppression of melanoma lung metastasis growth by using a chimeric bone marrow transplant animal model (26). These findings indicate that FasL is present in various sources in the pulmonary environment and that any of these sources might have a role in the growth of lung metastasis.

Although our findings agree with those of numerous reports on the role of Fas in tumorigenesis, they differ from those of Liu et al., who found that changes in Fas expression were characteristic of, but not solely responsible for, enhanced metastatic growth in the lungs (21). This difference could be explained by their use of different approaches to disrupt the Fas pathway in tumor cells or their use of different types of tumor cells. Perhaps, in their models, the single attenuation of Fas signaling was not sufficient to promote metastasis and other alterations were needed to generate a more aggressive metastatic phenotype. In fact, this group later identified a large set of differentially expressed genes in primary tumor cells compared with their *in vivo*-selected highly metastatic subline (27).

Currently, more evidence is emerging that the role of the Fas signaling may vary in different tumor types, revealing previously unknown pro-oncogenic activities of the Fas pathway in tumorigenesis (28-32). For example, Lee et al. showed that overexpression of functional Fas receptors in two different types of murine tumor cells had opposite effects: in renal cell carcinoma, this change delayed tumor growth and reduced the potential of tumor cells to metastasize to the lungs (7), but in Lewis lung carcinoma, the change induced tumor progression *in vivo* (29). Findings from Mitsiades et al. also illustrated a proliferative rather than an apoptotic role for Fas in thyroid carcinoma cell lines and provide some evidence that Fas expression was associated with more aggressive thyroid cancers (32). In analyzing differences between this type of malignancy and others in which cells undergo apoptosis after treatment with anti-Fas agonistic antibodies, they found that binding of the Fas receptor with those antibodies *in vitro* induced the recruitment of the Fas-inhibitory molecule c-FLIP, and consequently, promoted signal transduction events (e.g., phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinases and activation of nuclear factor κ B and activator protein-1 transcription factors). The ability of FLIP to stimulate these events and thus promote nonapoptotic signaling pathways has been previously noted in some normal and malignant cells (29, 32-34). All of these findings indicate that we cannot generalize the role of the Fas pathway, as it may vary among tumor types and sites of tumor growth.

In summary, we showed here that Fas⁻ osteosarcoma cells are selected during the formation of metastases in the lungs. Inhibition of Fas signaling in tumors, or the lack of FasL in the host environment, enabled the proliferation of Fas⁺ osteosarcoma cells in the lungs and promoted lung metastases growth in two autochthonous murine osteosarcoma models (K7 and Dunn). We conclude that the Fas pathway has an important role in osteosarcoma lung metastasis and should be considered as a potential therapeutic target for the treatment of osteosarcoma.

This pathway could be targeted through the identification of agents that enhance Fas expression in lung metastases or restore Fas pathway activity. Such agents could be used in the future to treat patients with osteosarcoma lung metastases and those at high risk for metastasis to the lungs.

Materials and Methods

Cell Lines and Reagents

The mouse osteosarcoma K7 cell line was obtained from Dr. Louis C. Gerstenfeld (Children's Hospital, Boston, MA; ref. 12). Dunn murine osteosarcoma cells were provided by Dr. Akira Myoui (University of Osaka, Osaka, Japan; ref. 24). All cell lines were maintained in complete DMEM (Whittaker Bioproducts, Inc.) supplemented with 10% heat-inactivated bovine serum (Intergen).

Plasmids and Establishment of Stable Clones

The plasmid constructs for mouse FDN and a control plasmid (neo) were a gift from Dr. Astar Winoto (University of California, Berkeley, CA; ref. 35). Stable clones expressing FDN or neo proteins were established in K7 cells by transfection using nonviral FuGENE 6 Transfection Reagent (Roche Applied Biosciences) and were further selected and maintained in 1.0 mg/mL of hygromycin-containing DMEM medium.

Animal Models

All wild-type mice were purchased from the National Cancer Institute. FasL-deficient mice, BALB/c strain (Cpt.C3-Tnfsf6^{slid}), were purchased from Jackson Laboratory. For the K7 experiments, nontransfected K7 cells, K7 cells stably transfected with FDN plasmid (K7/FDN clones) or with control plasmid (K7/neo clones) were injected (1×10^6 cells/injection) via the tail vein into wild-type BALB/c mice or FasL-deficient mice. For the induction of osteosarcoma bone tumor growth, 2×10^5 of K7 cells were injected into the tibia of BALB/c mice. For the Dunn cell experiments, 5×10^6 Dunn cells were injected subcutaneously into syngeneic C3H mice. At the end of each experiment (at 4-5 weeks after tumor inoculation), animal lungs were weighed and subjected to gross examination for the presence of visible metastases or histologic analyses for the presence of micrometastases and Fas expression. Experiments were conducted with five mice per group and each experiment was repeated at least twice. All animal experiments were approved by The University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Western Blot Analysis

Cells to be subjected to analysis were lysed with proteinase inhibitor cocktail (Roche Applied Diagnostics) in radioimmunoprecipitation assay buffer. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories). Lysates containing 50 μ g of protein were boiled for 5 min before being loaded onto an 18% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham Biosciences). Specific protein bands were detected with rabbit polyclonal anti-FADD antibody (Upstate Cell Signaling Solutions), polyclonal anti-FAP anti-Bcl-2 antibodies (R&D Systems, Inc.), anti-FLIP (Santa Cruz Biotechnology, Inc.)

using the enhanced chemiluminescence Western blotting analysis system (Amersham Biosciences) according to the manufacturer's instructions.

Flow Cytometric Analysis and Cell Sorting

Fas receptor expression in cells was detected by flow cytometry as described previously (8). Briefly, osteosarcoma cells were resuspended in fluorescence-activated cell sorting buffer (2% FCS and 0.1% of sodium azide solution in PBS) and incubated either with fluorescently labeled antibodies (1 mg/mL of phycoerythrin-conjugated monoclonal anti-Fas antibodies or isotype-matched, phycoerythrin-conjugated control IgG₁; BD Biosciences). Samples were analyzed with a FACScan system (Becton Dickinson).

Fas⁻ and Fas⁺ cell populations were sorted after being labeled with phycoerythrin-conjugated anti-Fas antibody with a FACSAria cell sorting system (Becton Dickinson). Parental cells were labeled with isotype-matched control phycoerythrin-labeled IgG₁ antibody, processed through the sorter, and used as control cells. Cell concentration and viability were determined immediately after flow sorting with trypan blue reagent, which stains dead cells. Cell viability after sorting was >90%.

Immunohistochemical Staining

Tissue staining for Fas was done as described previously (8). Briefly, deparaffinized and rehydrated 5- μ m tissue sections were blocked with 3% H₂O₂. Nonspecific binding was blocked with 10% normal horse and 1% normal goat serum solutions in PBS. Primary polyclonal rabbit anti-mouse Fas antibody (Santa Cruz Biotechnology) was applied overnight at 4°C. Secondary antibody goat anti-rabbit IgG antibody labeled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) was then applied for 1 h and the slides were developed with 3,3'-diaminobenzidine as a substrate and lightly stained with hematoxylin. Negative controls were prepared by omitting the primary antibodies.

For proliferation status of metastases, mouse anti-proliferating cell nuclear antigen monoclonal antibody, clone PC10 (Dako) was used. The procedure used was similar to the Fas staining method, except that the secondary antibody labeled with horseradish peroxidase was rat anti-mouse IgG_{2a} (Harlan Bioproducts for Science, Inc.).

Cytotoxicity Assay

The sensitivity of osteosarcoma cells to soluble FasL (Alexis Biochemicals) was determined by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay as described previously (8). Briefly, 3,000 cells/well were grown in 96-well plates and treated with or without 100 ng/mL of soluble FasL for 24 h; wells without cells served as negative controls. 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide reagent was added to each well at a concentration of 0.08 mg/mL for 2 to 4 h. Cells were lysed with 0.1 mL of DMSO. Cytotoxicity was quantified by using a microtiter plate reader at 570 nm.

Ex vivo Imaging of Tumor Cell Retention in the Lungs

Retention of tumor cells in the lungs after intravenous injection was assessed as described previously (13). Briefly,

cells were fluorescently labeled with 5 $\mu\text{mol/L}$ of 5-chloromethylfluorescein (Molecular Probes) according to the manufacturer's recommendations. BALB/c mice (five mice per cell line per time point) were injected via tail vein with 0.5×10^6 tumor cells and then euthanized by CO_2 inhalation at 1, 6, 24, and 48 h later. Collected lungs were inflated by the injection of 0.75 mL of PBS into the trachea and dissected free for *ex vivo* imaging by inverted fluorescent videomicroscopy (Leica DM IRB, Leica Microsystems) at $100\times$ magnification. Ten fluorescent images from each lung were randomly selected for analysis with OpenLab Software to define and count fluorescent chloromethylfluorescein tumor cell events at 10 pixels or larger. The total number of events per mouse lung, in five mice per cell line per time point, were summarized as means; the percentage of survival of metastatic cells was defined by normalizing the mean number of the cells present at 6, 24, and 48 h, with the mean number present at 1 h for each cell line.

Statistical Analyses

Statistical analysis in the lung metastasis studies was determined using an unpaired, two-tailed Student's *t* tests, with $P < 0.05$ considered statistically significant.

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