# The Role of Fas/FasL in the Metastatic Potential of Osteosarcoma and Targeting this Pathway for the Treatment of Osteosarcoma Lung Metastases

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**Abstract** Pulmonary metastases remain the main cause of death in patients with Osteosarcoma (OS). In order to identify new targets for treatment, our laboratory has focused on understanding the biological properties of the tumor microenvironment that contribute to or interfere with metastasis. Dysfunction of the Fas/FasL signaling pathway has been implicated in tumor development, and progression. Here we describe the status of Fas expression in murine nonmetastatic K7 and metastatic K7M2 cells and human nonmetastatic SAOS and LM2 and metastatic LM6 OS cells. We demonstrated that Fas expression correlates *inversely* with metastatic potential. Pulmonary metastases from patients were uniformly $Fas^-$ supporting the importance of Fas expression to the metastatic potential. Since FasL is constitutively expressed in the lung, our data suggests that $Fas^+$ tumor cells undergo apoptosis and are cleared from the lung. By contrast, $Fas^-$ tumor cells evade this host defense mechanism and form lung metastases. We confirmed these findings by blocking the Fas pathway using Fas Associated Death Domain Dominant-Negative (FDN). $Fas^+$ cells transfected with FDN were not sensitive to FasL, showed delayed clearance and formed lung metastases. $Fas^+$ cells were also able to form lung metastases in FasL-deficient mice. Using our mouse model systems, we demonstrated that aerosol treatment with liposomal 9-Nitrocamptothecin and Gemcitabine (chemotherapeutic agents known to upregulate Fas expression) increased Fas expression and induced tumor regression in wild type mice. Lung metastases in FasL deficient mice did not respond to the treatment.

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We conclude that Fas is an early defense mechanism responsible for clearing invading $Fas^+$ tumor cells from the lung. $Fas^-$ cells or cells with a nonfunctional Fas pathway evade this defense mechanism and form lung metastases. Therapy that induces Fas expression may therefore be effective in patients with established OS lung metastases. Aerosol delivery of these agents is an ideal way to target treatment to the lung.

## Introduction

The lung is the most common site of metastatic spread in patients with osteosarcoma (OS). While combination chemotherapy and surgery has resulted in a disease-free survival rate of 60–65%, this cure rate has not changed for over 20 years.[1–9] Pulmonary metastases remain the major cause of death in these patients. Our laboratory has therefore focused on understanding the biologic properties in the tumor microenvironment that support and contribute to OS cell growth in the lung with the goal of identifying new targets for therapy. Altering the tumor microenvironment may be a reasonable therapeutic approach for the treatment of OS as metastases are usually limited to the lung and are the leading cause of death.

Fas and its ligand FasL are cell surface receptors which belong to the TNF receptor family. Interaction of the Fas receptor on cells with FasL results in ligand-mediated cell death.[10–13] Two different apoptosis-signaling pathways have been identified. The type I pathway involves Caspase 8 with subsequent activation of Caspase 3. The type II or mitochondrial pathway involves Caspase 9 (Fig. 1). While Fas is constitutively expressed on T cells, B cells and in numerous tissues, the constitutive expression of FasL is limited to the testes, small intestine, anterior chamber of the eye, and the lung.[14,15] Fas/FasL induced cell death is a critical regulator of immune homeostasis and is required for the maintenance of peripheral tolerance.[10] Deletion of activated T cells and inflammatory cells at the end of an immune response is mediated by this pathway. Constitutive expression of FasL in tissues, therefore, creates an immunotolerant microenvironment as B cells and activated T cells, which express Fas, are eliminated upon entering the organ. Constitutive expression of FasL prevents a massive immune response which can damage these organs. Indeed, herpes eye infections in FasL-deficient mice resulted in large immune cell infiltration into the anterior chamber of the eye resulting in blindness. By contrast wild-type mice showed a short controlled immune response, clearing the infection without sequelae.[14,16–19] Fas-mediated cell death has also recently been implicated as a regulator of tumor development, out-growth and progression. Downregulation of Fas or impaired Fas signaling have been correlated with tumor progression.[10,12,20–22]

The organ microenvironment can influence the success or failure of metastatic cells to survive and grow at distant sites. As OS metastasizes almost exclusively to the lung and lung epithelium constitutively expresses FasL, we investigated whether the expression of Fas on OS cells correlated with their metastatic potential. For these investigations, we used two different OS mouse models. The first is a human OS mouse model[23] where parental SAOS cells were injected i.v. into mice, a lung metastasis harvested and those cells reinjected i.v. (LM2 subline). This process was repeated five additional times to create the very metastatic LM6 and LM7 sublines.

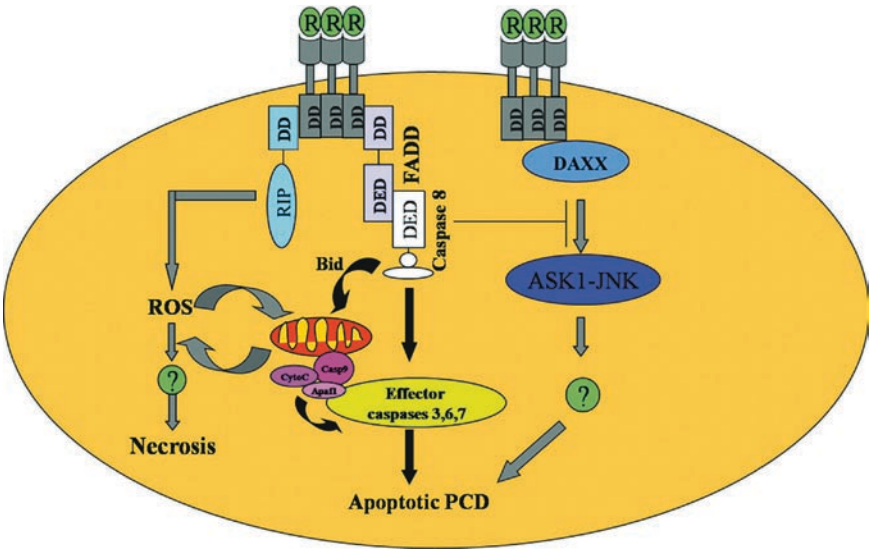


**Fig. 1** Apoptosis signaling pathways triggered by interaction of Fas receptor on cells with FasL. The Type I pathway, involves Caspase 8 with subsequent activation of Caspase 3 and apoptosis. The Type II or mitochondrial pathway involves Caspase 9

Specific characteristics of the parental and LM2–LM7 cell lines are depicted in Table 1. The second is the K7M2 mouse OS model created in a similar fashion. The parental K7 cells are poorly metastatic compared with the K7M2 variant.[24,25]

## Role of Fas in the Metastatic Potential of OS Cells in the Lung

As FasL is constitutively expressed on lung epithelium, our hypothesis was that tumor cells expressing the Fas receptor with a functional Fas signaling pathway will be eliminated by the engagement of the FasL expressed in the lung. These $Fas^+$ cells would therefore be unable to form lung metastases. $Fas^-$ OS cells, by contrast, would escape this host defense mechanism and form lung metastases (Fig. 2). Indeed, the poorly metastatic parental SAOS cells expressed high levels of Fas and Fas cell surface protein while the metastatic sublines LM6 and LM7 showed low to no Fas expression (Fig. 3a, b). Similarly, the metastatic K7M2 cells showed a lower intensity of cell surface Fas compared with the nonmetastatic K7 cells (Fig. 3c). LM6, LM7 and K7M2 lung nodules were $Fas^-$ by immunohistochemistry,[26–28] as were lung nodules from patients with OS.[29] Furthermore, transfection of the Fas gene into LM7 cells inhibited their ability to form lung metastases following i.v. administration while control transfection had no effect on metastatic potential.[26,30] These data support our hypothesis that Fas expression correlates inversely with the ability of OS cells to form lung metastases.

If Fas-mediated cell death is responsible for clearing OS cells from the lung and inhibiting tumor growth in this organ, $Fas^+$ cells with a blocked Fas signaling pathway

**Table 1** Metastatic characteristics of the SAOS parental and LM sub lines. No lung metastases were seen 17 weeks following the i.v. injection of SAOS parental or LM2 cells. LM3–LM7 sub lines all form lung metastases. LM7 is the most metastatic subline

| | | Lung metastases[b] | | | |
|---|---|---|---|---|---|
| Cell line | Doubling time (h)[a] | Time of sacrifice (weeks) | Incidence[c] | Median no (range) | Diameter (mm) |
| SAOS parental | 45.7 ± 3.3 | 17 | 0 | 0 | 0 |
| LM2 | 43.6 ± 4.2 | 17 | 0 | 0 | 0 |
| LM3 | 44.1 ± 2.6 | 17 | 2/5 | 0 (0–1) | 0.5–1.0 |
| LM4 | 40.0 ± 0.9 | 17 | 3/4 | 9 (0–100) | 0.5–2.0 |
| LM5 | 37.2 ± 3.8 | 17 | 4/4 | 88 (7–>200) | 0.5–5.0 |
| LM6 | 34.9 ± 1.4 | 12 | 9/9 | 92 (30–>200) | 0.5–5.6 |
| LM7 | 26.8 ± 1.3 | 10 | 12/12 | 100 (30–>200) | 0.5–7.0 |

[a]SAOS parental or LM cells ($3 \times 10^3$) were plated and incubated at 37°C for 24, 48, 72 and 96 h. The cells were labeled with [$^3$H]-thymidine during the last 24 h of incubation. Doubling time was calculated using the following formula: time × log 2/log ($n/n_0$) where $n_0$ is the cpm of cells incubated for 24 h and $n$ is the cpm of cells incubated for 48, 72 and 96 h. It was expressed as the average of three independent experiments.

[b]Nude mice were injected with $1 \times 10^6$ of the indicated cells. Mice injected with SAOS, LM1, LM2, LM3, LM4 or LM5 were sacrificed 17 weeks later. Mice injected with LM6 and LM7 cells were sacrificed earlier because of signs of distress. The lungs were removed, fixed and tumor nodules were counted and measured.

[c]Number of tumor-positive mice/number of inoculated mice.

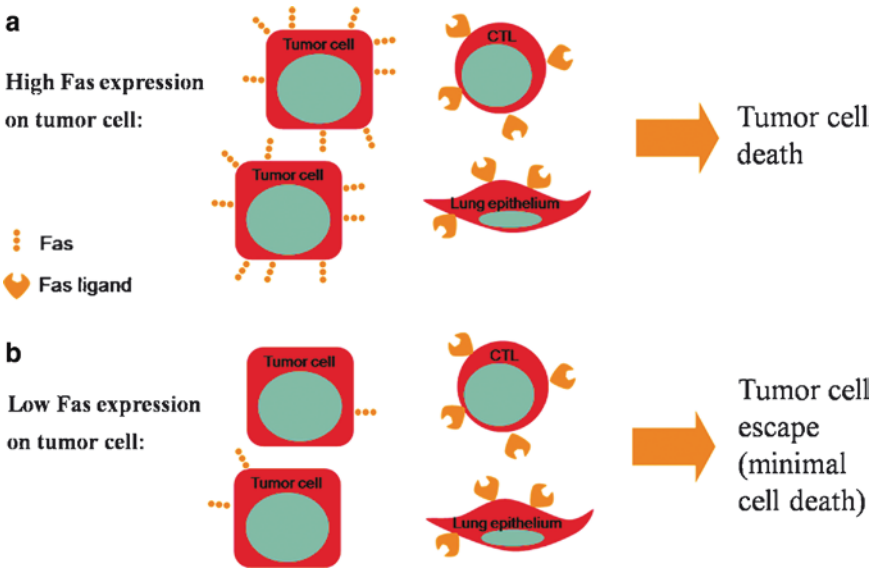


**Fig. 2** Fas expression correlates inversely with the metastatic potential of OS cells to the lung. (**a**) Fas$^+$ tumor cells enter the lung and undergo apoptosis triggered by FasL constitutively expressed by lung endothelium. (**b**) Tumor cells with low or no Fas expression evade this host defense mechanism

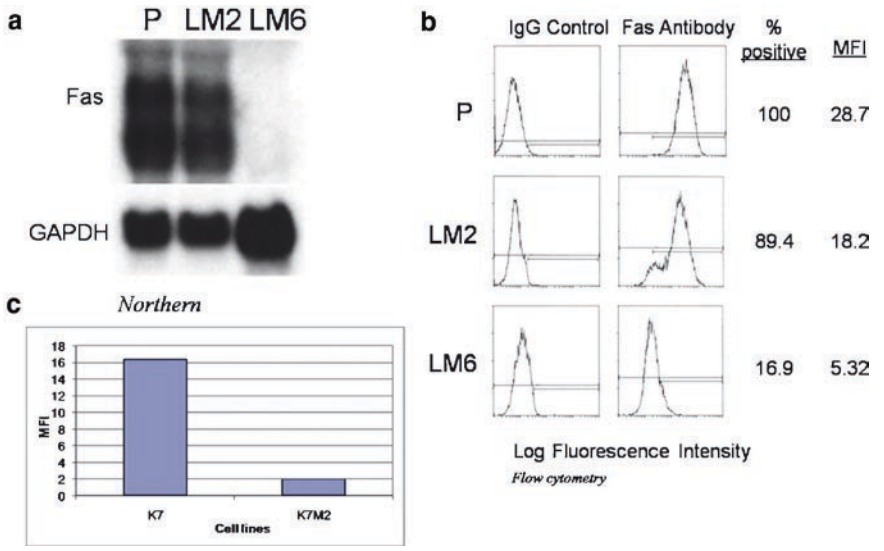


**Fig. 3** Fas expression correlates inversely with the metastatic potential of human and murine OS cells. (**a**) Northern blot analyses shows high Fas expression in poorly metastatic parental SAOS-2 and LM2 cells and no Fas expression in the metastatic LM6 cells. (**b**) Flow cytometry confirms higher cell surface Fas protein expression in parental SAOS-2 and LM2 cells compared with LM6 cells. (**c**) Higher Fas expression in poorly metastatic parental K7 cells compared with the metastatic K7M2 cells

will not be susceptible to this clearance mechanism and should form lung metastases when injected intravenously. To test this hypothesis, we inhibited the Fas signaling pathway in Fas$^+$ nonmetastatic K7 OS cells by transfecting these cells with Fas associated death-domain dominant negative (FDN). FDN blocks apoptosis in both type I and type II cells by inhibiting Caspase 8 at the DISC complex (Fig. 4). K7/FDN cells were not sensitive to FasL-induced cell death. Fas receptor expression in these cells was unaffected. We demonstrated that K7/FDN cells were retained in the lung compared to control-transfected K7/neo cells.[31] Two days after i.v. injection, there were five times the number of K7/FDN cells in the lung compared with the control-transfected cells. K7/FDN cells formed numerous large pulmonary metastases while the lungs from mice injected with K7/neo cells were clear (Fig. 5). The K7/FDN tumors were Fas$^+$ by immunohistochemistry with some Fas$^-$ cells as well.[31] The important finding here is that blocking the Fas signaling pathway resulted in retention of Fas$^+$ cells in the lung and the subsequent development of Fas$^+$ tumor nodules.

The absence of FasL in the tumor microenvironment should also allow Fas$^+$ OS cells to form lung metastases (Fig. 6). To address this question, Fas$^+$ nonmetastatic K7 cells were injected i.v. into FasL-deficient mice. All of the mice developed lung metastases.[31] The immunohistochemistry analysis of these nodules revealed both Fas$^+$ and Fas$^-$ cells.[31]

The K7M2 subline contains both Fas$^+$ and Fas$^-$ cells. However, the lung nodules formed following i.v. or intrabone injection into wild-type Balb/c mice are all Fas$^-$. If constitutive FasL in the lung is responsible for clearing Fas$^+$ cells, then K7M2

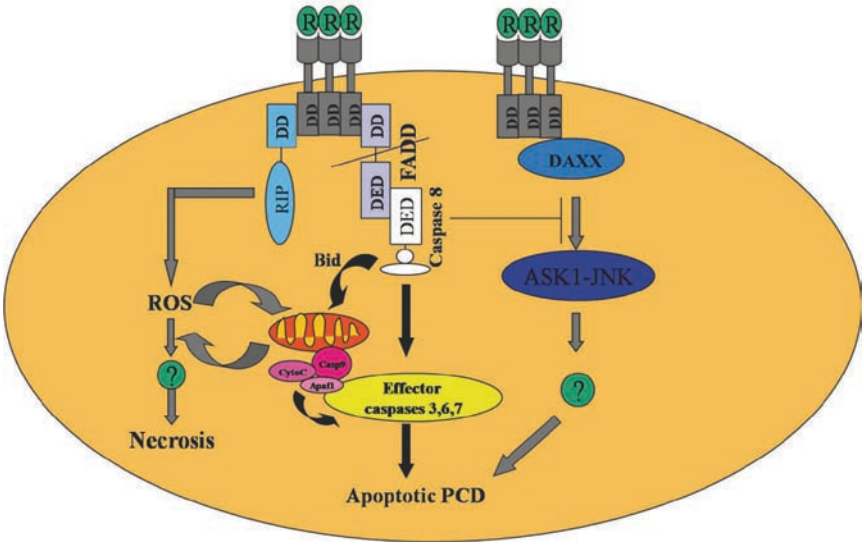


**Fig. 4** Blocking the Fas signaling pathway with Fas Associated Death Domain Dominant-Negative (FDN) to inhibit FasL-induced cell death. FDN blocks apoptosis in both type I and type II cells by inhibiting C8 at the DISC complex

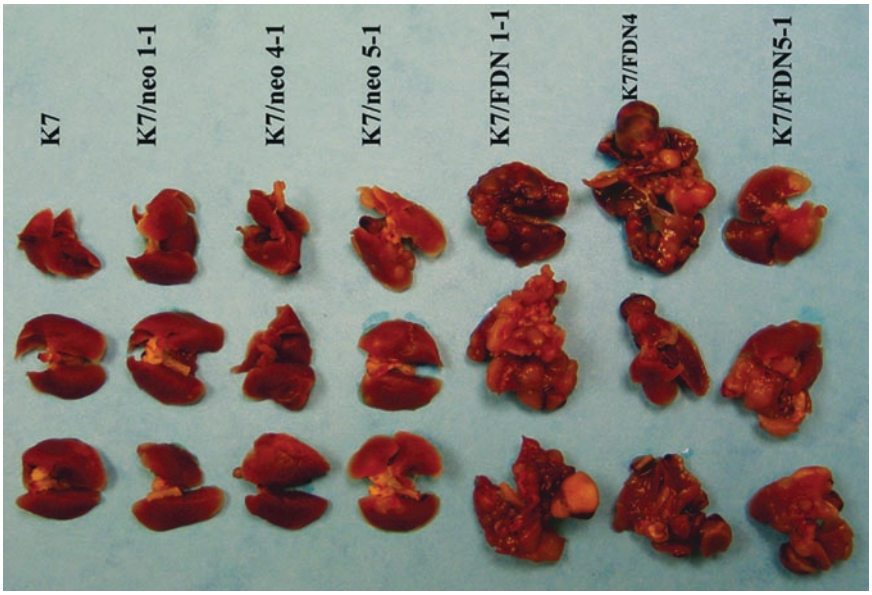


**Fig. 5** Blocking the Fas signaling pathway alters the metastatic potential of Fas$^+$ K7 cells. K7 cells were transfected with FDN or control vector (neo) and injected i.v. into mice. The mice were sacrificed 4 weeks later and lung metastases were quantified. K7/FDN cells induced numerous large pulmonary metastases compared with K7/neo and K7 cells

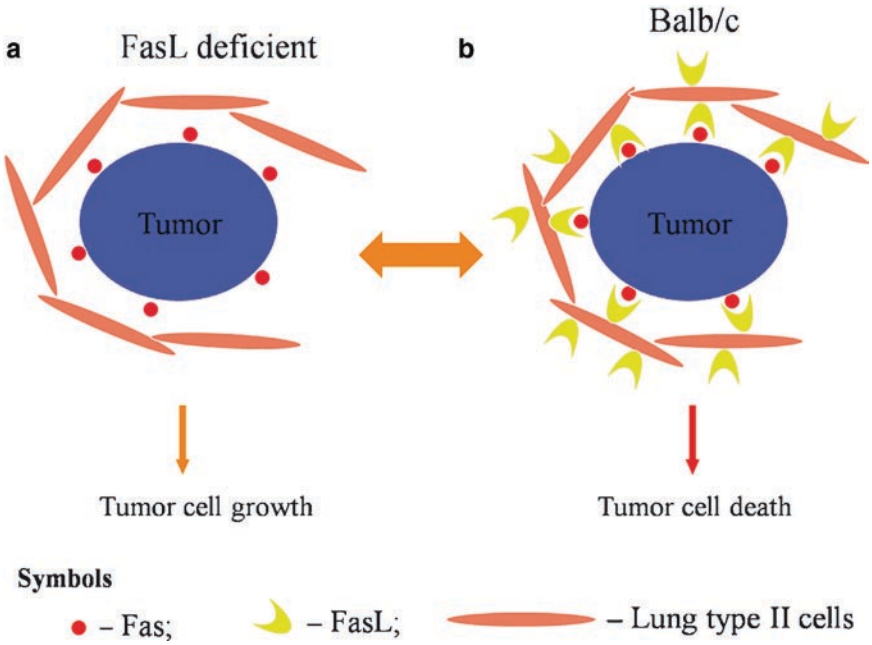


**Fig. 6** (**a**) Absence of FasL in the lung microenvironment allows Fas$^+$ cells to survive and grow. (**b**) By contrast, constitutive FasL in the lung of wild-type Balb/c mice binds to the cell surface Fas activating the Fas pathway which leads to apoptosis

cells injected into FasL-deficient mice should form heterogeneous lung metastases comprised of both Fas$^+$ and Fas$^-$ cells as the Fas$^+$ will not be eliminated (Fig. 6). Indeed, we demonstrated this phenomenon,[28] (Fig. 7). K7M2 nodules in FasL deficient mice contained areas of Fas$^+$ as well as Fas$^-$ cells within the same lung (Fig. 7b). By contrast, wild-type BALB/c mice injected with K7M2 cells developed only Fas$^-$ lung nodules (Fig. 7a). Taken together, these data confirm our hypothesis that FasL is responsible for eliminating the Fas$^+$ OS cells once they enter the lung. These data were the first to demonstrate that the expression of Fas and the presence of a functional Fas signaling pathway contributes to the ability of OS cells to form lung metastases. We were also the first to demonstrate that the pulmonary microenvironment plays a critical role in the metastatic potential of OS cells.

## Therapeutic Effect of Aerosol Therapy on Established OS Lung Metastases

Having demonstrated that Fas expression is a critical determinant for OS cell growth in the lung, we next determined whether upregulating Fas expression in established Fas$^-$ OS lung nodules would result in tumor regression. Our hypothesis

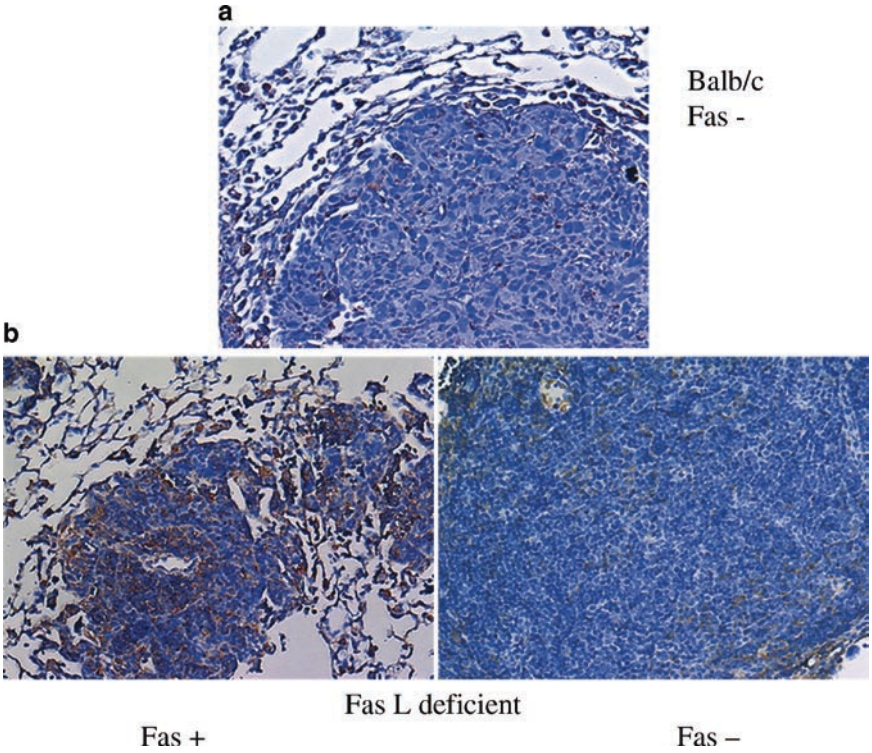


**Fig. 7** Representative picture of Fas expression in K7M2 OS lung nodules in Balb/c and FasL deficient mice. K7M2 cells were injected i.v. into Balb/c and FasL deficient mice. The mice were sacrificed 2 weeks later, lungs were resected and stained for Fas expression. (**a**) K7M2 OS lung metastases from Balb/c mice were $Fas^-$. (**b**) K7M2 OS lung metastases from FasL deficient mice showed heterogeneous Fas expression with areas of $Fas^+$ and $Fas^-$ cells within the same lung

was that agents that stimulate the reexpression of Fas in $Fas^-$ lung nodules would result in tumor cell apoptosis induced by the FasL-expressing lung cells (Fig. 8). We demonstrated that both gemcitabine and liposomal 9-nitrocamptothecin (L-9NC) increased Fas expression in LM7 and K7M2 cells in vitro.[27,28,32] For in vivo analysis of efficacy, we elected to deliver these chemotherapy agents via the aerosol route. Aerosol technology has several advantages over systemic therapy. The agent is delivered directly to the organ where the tumor is growing avoiding dilution in the bloodstream. Aerosol administration avoids the first pass metabolic degradation in the liver and GI tract. This allows the achievement of high pulmonary drug concentrations with minimal systemic exposure resulting in decreased or minimal systemic toxicity. Finally, the drug is uniformly distributed throughout the lung. As OS metastasizes almost exclusively to the lung, aerosol therapy makes sense and is appealing. We demonstrated that the administration of aerosol L-9NC, initiated 8 weeks following tumor cell injection, or aerosol gemcitabine initiated 3 days after tumor cell injection, induced Fas expression in

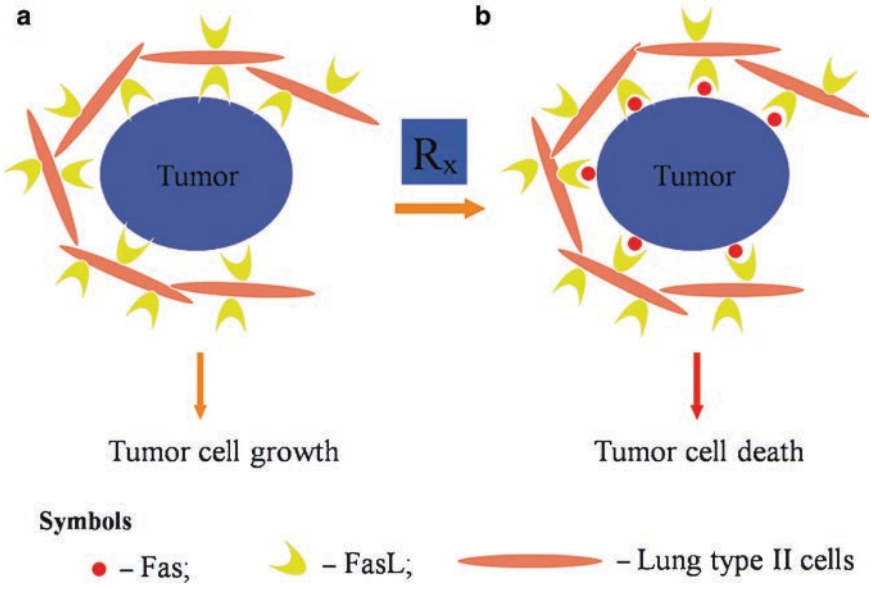


**Fig. 8** Therapy induced expression of Fas on Fas⁻ tumor cells. (**a**) Fas⁻ tumor cells are not eliminated from the lung. (**b**) Treatment of Fas⁻ lung metastases with agents that stimulate the reexpression of Fas will result in tumor cell apoptosis induced by the FasL expressing lung cells

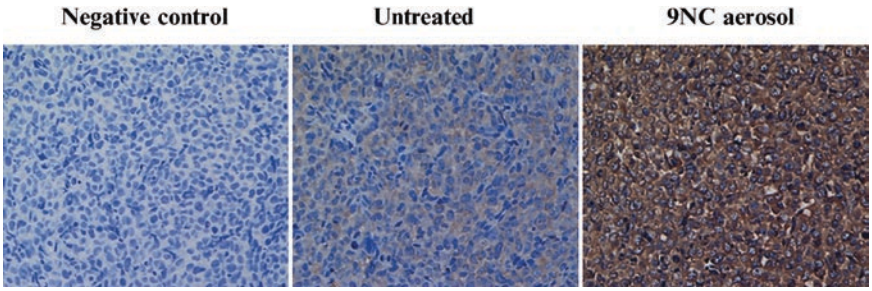


**Fig. 9** Fas expression in LM7 lung metastases following aerosol liposome-9NC. Nude mice with established pulmonary metastases were treated with aerosol liposome-9NC daily for 6 weeks. The mice were sacrificed, the lungs were sectioned and evaluated by IHC for Fas expression. Brown staining represents positive Fas expression. Untreated pulmonary metastases were Fas⁻ whereas those treated with aerosol L-9NC were Fas⁺

established OS lung nodules (Fig. 9), tumor cell apoptosis (Fig. 10), and tumor regression.[27,28,32] To confirm that the effect of aerosol chemotherapy was mediated in part by the constitutive FasL in the lung, the in vivo aerosol therapy studies were repeated in FasL-deficient mice. No therapeutic affect was seen when FasL deficient mice were treated with aerosol Gemcitabine.[28] Although aerosol Gemcitabine induced Fas expression in the pulmonary nodules, these nodules continued to proliferate in size and number.[28] These data indicate that targeting the

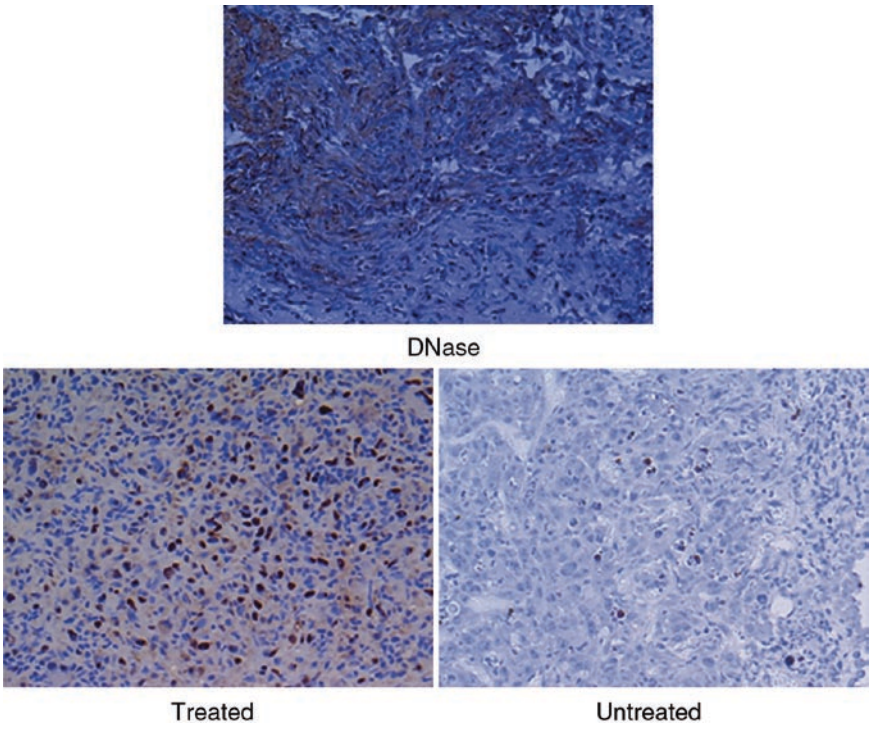


**Fig. 10** Apoptosis of K7M2 OS lung metastases after treatment with aerosol Gemcitabine. Balb/c mice with established pulmonary metastases were treated with aerosol Gemcitabine and sacrificed after 2 weeks. Sections were analyzed for TUNEL as a marker of apoptosis. Brown staining represents apoptosis. Increased apoptosis was observed in the pulmonary metastases from Gemcitabine treated mice compared with those from control untreated mice

Fas pathway is a therapeutic opportunity for treating patients with established OS lung metastases and that the efficacy of aerosol chemotherapy may be closely linked to the microenvironment in the lung.

## Summary

The metastatic process is complicated, involving multiple steps and factors that contribute to the ability of cancer cells to degrade the extracellular matrix in the primary tumor site, escape into the circulation, travel through the bloodstream from the local site to a distant organ, survive in the new organ microenvironment, and finally to initiate new vasculature to bring the needed oxygen and nutrients to support tumor growth in the new environment. The microenvironment itself can be a key factor in either permitting or inhibiting tumor cell survival and growth.

We have demonstrated that $Fas^+$ OS cells are rapidly cleared from the lung while $Fas^-$ cells remain. OS lung nodules are uniformly $Fas^-$. Inhibiting the Fas signaling pathway interferes with the clearance of $Fas^+$ OS cells resulting in the formation of $Fas^+$ lung metastases. Similarly, the lack of FasL in the host microenvironment allowed $Fas^+$ nonmetastatic cells to induce pulmonary metastases.[28,31]

We were the first to demonstrate that the Fas pathway plays a critical role in the metastatic potential of OS cells and that the lung microenvironment can influence treatment efficacy of OS lung metastases. Based on our data, we hypothesize that Fas is an early defense mechanism responsible for clearing invading $Fas^+$ tumor cells from the lung. $Fas^-$ cells or cells with a blocked or nonfunctional Fas pathway can evade FasL-induced cell death and go on to form lung metastases. Our data also suggest that inducing the expression of Fas can result in tumor regression, which is mediated by the FasL lung microenvironment. Identifying agents that enhance Fas expression in lung metastases or restore Fas signaling pathway activity may have therapeutic potential for patients with established unresponsive lung metastases. Our data also suggest that delivery of these agents by the aerosol route should be considered.

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