

# Creating a tumor-resistant microenvironment

## Cell-mediated delivery of TNF $\alpha$ completely prevents breast cancer tumor formation in vivo

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**Keywords:** tumor necrosis factor (TNF), cancer prevention, cellular therapy, fibroblast mediated delivery, mitochondrial dysfunction, breast cancer, tumor growth, tumor cell engraftment, autophagy, apoptosis

Here, we provide the necessary proof of concept, that it is possible to metabolically create a non-permissive or “hostile” stromal microenvironment, which actively prevents tumor engraftment in vivo. We developed a novel genetically engineered fibroblast cell line that completely prevents tumor formation in mice, with a 100% protection rate. No host side effects were apparent. This could represent a viable cellular strategy for preventing and treating a variety of human cancers. More specifically, we examined the autocrine and paracrine effects of the cellular delivery of TNF $\alpha$  on breast cancer tumor growth and cancer metabolism. For this purpose, we recombinantly overexpressed TNF $\alpha$  in human breast cancer cells (MDA-MB-231) or human immortalized fibroblasts (hTERT-BJ1). Our results directly show that TNF $\alpha$  functions as a potent tumor suppressor. Remarkably, TNF $\alpha$ -expressing breast cancer cells were viable, without any significant increases in their basal apoptotic rate. However, after 4 weeks post-implantation, TNF $\alpha$ -expressing breast cancer cells failed to form any tumors in xenografted mice (0 tumors/10 injections), ultimately conferring 100% protection against tumorigenesis. Similarly, TNF $\alpha$ -overexpressing fibroblasts were also viable, without any increases in apoptosis. Significantly, complete tumor suppression was obtained by co-injecting TNF $\alpha$  expressing stromal fibroblasts with human breast cancer cells, indicating that paracrine cell-mediated delivery of TNF $\alpha$  can also prevent tumor engraftment and growth (0 tumors/10 injections). Mechanistically, TNF $\alpha$  induced autophagy and mitochondrial dysfunction in both epithelial cancer cells and stromal fibroblasts, preventing energy transfer from the tumor microenvironment, likely “starving” the cancer cells to death. In addition, via qRT-PCR analysis of MDA-MB-231 cells, we observed that TNF $\alpha$  mediated the upregulation of gene transcripts associated with inflammation and senescence [IL-1- $\beta$ , IL-6, IL-8, MCP-1, COX-2, p21(WAF1/CIP1)] and downregulated known tumor-promoting genes (collagen VI and MMP2). Recombinant overexpression of TNF $\alpha$  receptor(s) in MDA-MB-231 cells also significantly reduced tumor growth, but was not as effective as the TNF $\alpha$  ligand itself in preventing tumor growth. Thus, we propose that stromal cell-mediated delivery of TNF $\alpha$  to human tumors [using transfected fibroblasts or mesenchymal stem cells (hMSCs)] may be a novel and effective strategy for the prevention and treatment of human cancers.

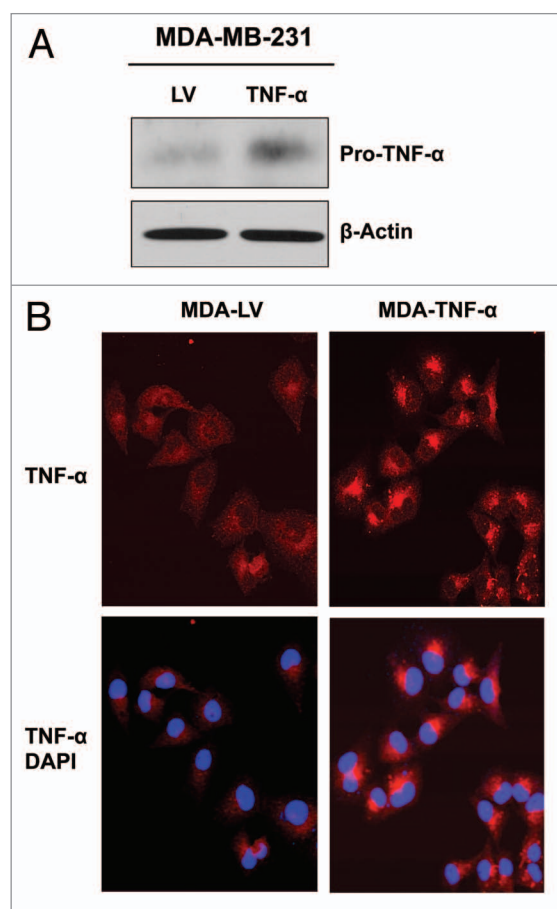
### Introduction

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is an inflammatory cytokine that is key part of the innate immune response, in both infectious disease and cancer(s).<sup>1,2</sup> It has also been implicated in the pathogenesis of chronic inflammatory diseases (rheumatoid arthritis, inflammatory bowel disease, psoriasis, refractory asthma), as well as Alzheimer disease.<sup>3,4</sup> TNF $\alpha$  is able to induce acute inflammatory events [fever (pyrogen) and sepsis (via IL-1 and IL-6)], as well as chronic inflammation.<sup>5,6</sup> It behaves as a catabolic cytokine

that drives ROS production, oxidative stress, autophagy and mitochondrial dysfunction, as well as programmed cell death (apoptosis).<sup>7-12</sup> In accordance with these metabolic findings, for many years TNF $\alpha$  was also known as the hormone cachexin or cachectin, a mediator of tumor-induced cachexia (“wasting”), which results in negative energy balance.<sup>7-12</sup> TNF $\alpha$  is named for its ability to kill tumor cells, but this activity has not yet been successfully exploited for anticancer therapy.

Here, we assessed the compartment-specific functional effects of the cellular expression of TNF $\alpha$  on tumor engraftment and

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Submitted: 12/15/12; Accepted: 12/20/12  
<http://dx.doi.org/10.4161/cc.23370>



**Figure 1.** Stable TNF $\alpha$  overexpression in human epithelial breast cancer cells. MDA-MB-231 human breast cancer cells were transduced with a TNF $\alpha$  lentiviral vector or the LV empty vector control, and selected with puromycin to generate stable cell lines. Then, transduced cells were subjected to immunoblotting and immunofluorescence analysis to validate TNF $\alpha$  overexpression. (A) Immunoblotting shows a band at ~24 kDa, corresponding to the pro-TNF $\alpha$  precursor form.  $\beta$ -actin is shown as a control for equal protein loading. (B) Immunofluorescence with anti-TNF $\alpha$  antibodies demonstrates TNF $\alpha$  overexpression in cancer cells. Red, TNF $\alpha$  specific staining; blue, DAPI nuclear staining.

cancer metabolism. Remarkably, cellular expression of TNF $\alpha$  in epithelial cancer cells (MDA-MB-231) was not cytotoxic *in vitro*, but completely prevented tumor growth *in vivo*. Similarly, cellular expression of TNF $\alpha$  in stromal fibroblasts also completely prevented tumor formation *in vivo*, via paracrine mechanism(s). We propose that the tumor suppressor effects of TNF $\alpha$  are due to its ability to interrupt symbiotic metabolic coupling between epithelial cancer cells and their host stromal microenvironment. Thus, cellular delivery of TNF $\alpha$  to human tumors *in vivo* may represent a viable new strategy for inducing primary tumor regression and preventing metastatic cancer progression.

As such, TNF $\alpha$  expressing stromal cells [either fibroblasts or mesenchymal stem cells (MSCs)] may represent the missing “magic bullets” that we need for successful targeted anticancer therapies. In fact, TNF $\alpha$  pre-treatment of MSCs increases their ability to “home” or accumulate within tumors *in vivo*.<sup>13</sup>

Finally, our results also suggest that successful tumor-host engraftment requires metabolic co-operation or symbiosis with the host, as normally occurs during a parasite-host infection. Thus, we should consider treating cancer as an “infectious disease,” with “antibiotics” that prevent or interrupt metabolic co-operation. Our current findings with the cellular delivery of TNF $\alpha$  provide the required proof-of-principal that this simple “antibiotic” or “metabolic uncoupling” strategy can effectively prevent tumor growth *in vivo*.

## Results

**MDA-MB-231 cancer cells overexpressing TNF $\alpha$  display increased autophagy and decreased mitochondrial activity, without any changes in apoptosis.** MDA-MB-231 cancer cells were transduced with a TNF $\alpha$  lentiviral vector or an LV empty vector control. After selection, stable cell lines were subjected to immunoblotting and immunofluorescence analysis to validate the TNF $\alpha$  overexpression. **Figure 1A and B** show, respectively, the results of this analysis, demonstrating successful TNF $\alpha$  overexpression.

We first assessed if TNF $\alpha$  overexpression promotes the apoptosis of MDA-MB-231 cells by immunoblotting with specific antibodies against apoptotic markers. **Figure 2A** shows that caspase-3 is significantly upregulated and activated in MDA-MB-231 cells overexpressing TNF $\alpha$  relative to control cells. However, MDA-MB-231 cells overexpressing TNF $\alpha$  display the downregulation of the pro-apoptotic marker Bax, and this is consistent with the upregulation of the anti-apoptotic molecule BCL-2. These results suggest that TNF $\alpha$  induces the upregulation of certain components of the apoptosis machinery and may prime cancer cells toward apoptosis.

To directly evaluate steady-state apoptosis levels, MDA-MB-231 cells overexpressing TNF $\alpha$  or control cells were analyzed by flow cytometry using Annexin-V and PI probes. **Figure 2B** shows that MDA-MB-231 cells overexpressing TNF $\alpha$  did not show any significant differences in apoptotic rates relative to the empty vector control cells. To independently validate these results, MDA-MB-231 cells overexpressing TNF $\alpha$  or control cells were subjected to an MTT assay. **Figure 2C** demonstrates that MDA-MB-231 cells overexpressing TNF $\alpha$  show no differences in cell viability/proliferation relative to control cells. These results indicate that TNF $\alpha$  does not induce non-specific cytotoxicity in MDA-MB-231 cells.

We next assessed if TNF $\alpha$  overexpression promotes autophagy in MDA-MB-231 cells using immunoblotting with specific antibodies directed against autophagic markers. **Figure 3A** shows that several autophagy markers (Beclin-1, BNIP3 and Lamp-1) are upregulated in TNF $\alpha$  overexpressing MDA-MB-231 cells relative to control cells. Since the mitophagy marker BNIP3 is upregulated in TNF $\alpha$ -overexpressing MDA-MB-231 cancer cells, we postulated that mitochondrial activity might also be decreased. To this end, we investigated the status of OXPHOS complexes (I–V) by immunoblotting. **Figure 3** shows the downregulation of the key subunits of the OXPHOS complexes (namely components III and V) in MDA-MB-231 cells overexpressing TNF $\alpha$ ,

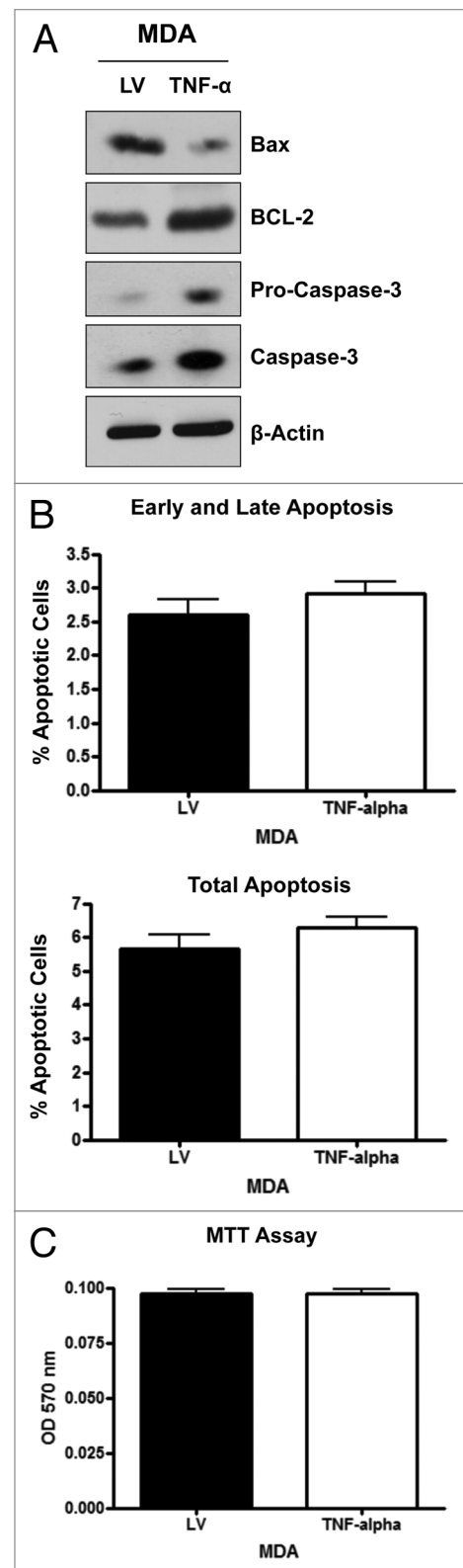
**Figure 2.** MDA-MB-231 cells overexpressing TNF $\alpha$  show the upregulation of apoptotic machinery, without a steady-state induction of increased apoptosis. **(A)** MDA-MB-231 cells overexpressing TNF $\alpha$  or the LV empty vector were subjected to immunoblotting with specific antibodies directed against apoptotic markers. Note that caspase-3 is significantly upregulated and activated in TNF $\alpha$ -expressing MDA-MB-231 cells, while Bax is downregulated consistent with BCL-2 upregulation.  $\beta$ -actin is shown as a control for equal loading. **(B)** Apoptosis was directly measured with Annexin-V and PI probes by flow cytometry (FACS analysis). MDA-MB-231 cells overexpressing TNF $\alpha$  did not show any significant increases in apoptotic rates relative to the empty vector control. Early and late apoptosis, represents Annexin-V-positive/PI-negative cells; Total apoptosis, represents Annexin-V-positive and/or PI-positive cells. **(C)** MTT assay demonstrates that MDA-MB-231 cells overexpressing TNF $\alpha$  show no differences in cell viability/proliferation, relative to empty vector control cells. These results indicate that TNF $\alpha$  does not increase the steady-state apoptotic rate in epithelial cancer cells.

relative to LV control cells, providing an indication of impaired mitochondrial activity.

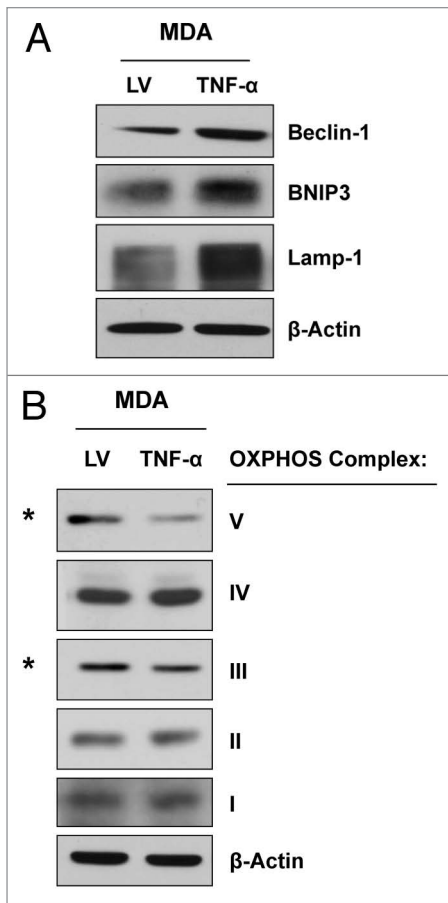
**TNF $\alpha$  overexpression in epithelial cancer cells completely abolishes breast cancer tumor growth in vivo.** To evaluate the effects of TNF $\alpha$  overexpression in MDA-MB-231 cells on tumor growth in vivo, we employed a murine xenograft model. To this end, MDA-MB-231 cells carrying TNF $\alpha$  or the LV empty control vector were injected into the flanks of nude mice, and tumors were measured using calipers for a period of 30 d. **Figure 4A–C** shows the tumor growth curve as well as the tumor mass and volume of the dissected tumors, clearly demonstrating that MDA-MB-231 cells overexpressing TNF $\alpha$  did not develop tumors in any of the injected mice (0/10; 0 tumors out of 10 injections). However, MDA-MB-231 vector alone control cells formed robust tumor xenografts, as expected (10/10; 10 tumors out of 10 injections). These results demonstrate that TNF $\alpha$  overexpression completely inhibits the in vivo growth of MDA-MB-231 cells.

**TNF $\alpha$  overexpression in fibroblasts induces autophagy and decreases mitochondrial activity, without causing apoptosis.** To evaluate if the effects of TNF $\alpha$  are cell type-specific, we overexpressed TNF $\alpha$  in the hTERT-BJ1 fibroblast cell line. LV empty vector control fibroblasts were generated in parallel. Immunoblotting (**Fig. 5A**) and immunofluorescence analysis (**Fig. 5B**) validated the overexpression of TNF $\alpha$  in hTERT-BJ1 cells. We next checked if fibroblasts overexpressing TNF $\alpha$  display increased levels of apoptosis by immunoblotting with antibodies against apoptotic markers. **Figure 6A** shows no changes in the levels of caspase-3, Bax and BCL-2. To independently assess apoptotic rates, hTERT-BJ1 cells overexpressing TNF $\alpha$  or LV control cells were analyzed by flow cytometry (FACS) using Annexin-V and PI probes. **Figure 6B** demonstrates that hTERT-BJ1 cells overexpressing TNF $\alpha$  did not show any significant differences in their apoptotic rates relative to the empty vector controls. These results indicate that TNF $\alpha$  does not induce apoptosis in non-transformed fibroblasts.

We next evaluated the activation status of the autophagy program by immunoblotting with specific antibodies directed against autophagic markers. **Figure 6C** shows that TNF $\alpha$  overexpression induces autophagy in hTERT-BJ1 cells, as judged by elevated expression and activation of cathepsin B, Lamp-1 and LC3B.



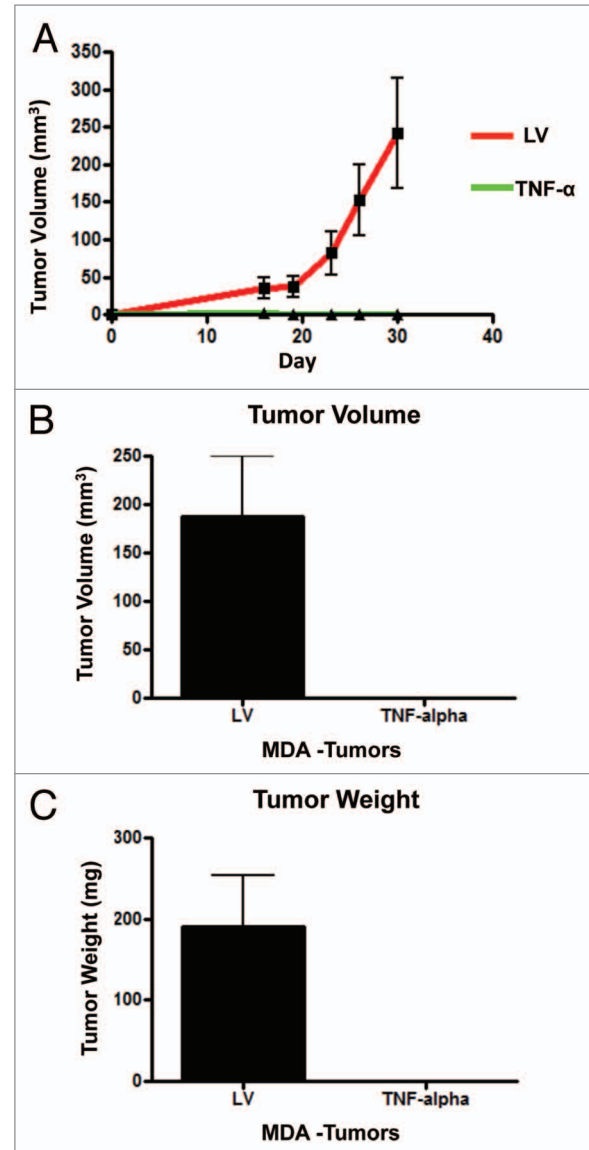
Finally, we asked if TNF $\alpha$  overexpression in hTERT-BJ1 cells results in decreased mitochondrial activity, similarly to what we observed in MDA-MB-231 cells. Immunoblot analysis shows that TNF $\alpha$  overexpression in hTERT-BJ1 cells induces the downregulation of key subunits of the OXPHOS complexes



**Figure 3.** MDA-MB-231 cells overexpressing TNF $\alpha$  display the induction of autophagy and a reduction in mitochondrial OXPHOS. MDA-MB-231 cells overexpressing TNF $\alpha$  or LV empty vector were subjected to immunoblotting with specific antibodies directed against autophagic markers and OXPHOS complex components. (A) Note that several autophagy markers (Beclin-1, BNIP3 and Lamp-1) are upregulated in TNF $\alpha$ -overexpressing MDA-MB-231 cells relative to control cells. (B) MDA-MB-231 cells overexpressing TNF $\alpha$  display the downregulation of the OXPHOS complexes III and V, relative to control cells.  $\beta$ -actin is shown as an equal loading control.

(namely subunits III and V; Fig. 6D), providing an indication of decreased mitochondrial respiration.

**Fibroblasts overexpressing TNF $\alpha$  completely prevent breast cancer tumor growth in vivo.** To evaluate if fibroblasts overexpressing TNF $\alpha$  inhibit breast cancer tumor growth in vivo, hTERT-BJ1 cells (carrying TNF $\alpha$  or LV empty vector) were co-injected with MDA-MB-231 cells into the flanks of nude mice. Tumor growth was monitored for over a 4-week period post-implantation. The tumor growth curves show that MDA-MB-231 cells co-injected with TNF $\alpha$ -overexpressing fibroblasts did not form any tumors (Fig. 7A). Measurements of the weight and volume (Fig. 7A and B) of tumors shows that TNF $\alpha$ -overexpressing fibroblasts completely prevented the growth of co-injected MDA-MB-231 cells, relative to control cells (0/10; 0 tumors out of 10 injections). These results indicate that fibroblast-mediated delivery of TNF $\alpha$  to the tumor site may be an effective and feasible strategy for tumor inhibition and regression.



**Figure 4.** TNF $\alpha$  overexpression in cancer cells completely abolishes tumor growth in vivo. MDA-MB-231 cells (carrying TNF $\alpha$  or the LV empty vector control) were injected into the flanks of nude mice. Tumors growth was serially followed using calipers for 30 d post-injection. (A) Note that the growth curves clearly show that MDA-MB-231 cells overexpressing TNF $\alpha$  did not develop any tumors. After mice were sacrificed, tumor volume (B) and weight (C) were measured, demonstrating no growth of TNF $\alpha$ -MDA-MB-231 cells in all xenografted mice (0/10; 0 tumors grew from 10 injections). Thus, expression of TNF $\alpha$  confers 100% protection against tumor formation.

MDA-MB-231 cancer cells overexpressing TNF $\alpha$  receptors show the upregulation of apoptotic markers in vitro, and show significantly reduced tumor growth in vivo. To further evaluate the cell-autonomous tumor inhibition properties of TNF $\alpha$ , MDA-MB-231 cells were transduced with vectors encoding TNF $\alpha$  receptors (TNFR-1A and TNFR-1B) or a LV empty vector. Cells were first subjected to immunoblotting with antibodies directed against apoptotic markers. Figure 8 shows that MDA-MB-231 cells overexpressing TNFR-1A and TNFR-1B

display the upregulation and activation of caspase-3 relative to empty vector control cells. Conversely, the levels of the apoptotic activator Bax were slightly decreased.

We next employed a tumor xenograft model to establish the behavior of MDA-MB-231 cells expressing TNFR-1A and TNFR-1B *in vivo*. To this end, MDA-MB-231 cancer cells overexpressing TNFR-1A or TNFR-1B or LV empty vector were injected into the flanks of mice, and tumor growth was monitored for 30 d. The tumor growth curves (Fig. 9A), tumor volume (Fig. 9B) and tumor weight (Fig. 9C) all directly show that MDA-MB-231 cells overexpressing TNFR-1A and TNFR-1B display significantly reduced tumor growth rates, relative to control cells. However, recombinant expression of TNFR-1A had the most robust tumor suppressor activity.

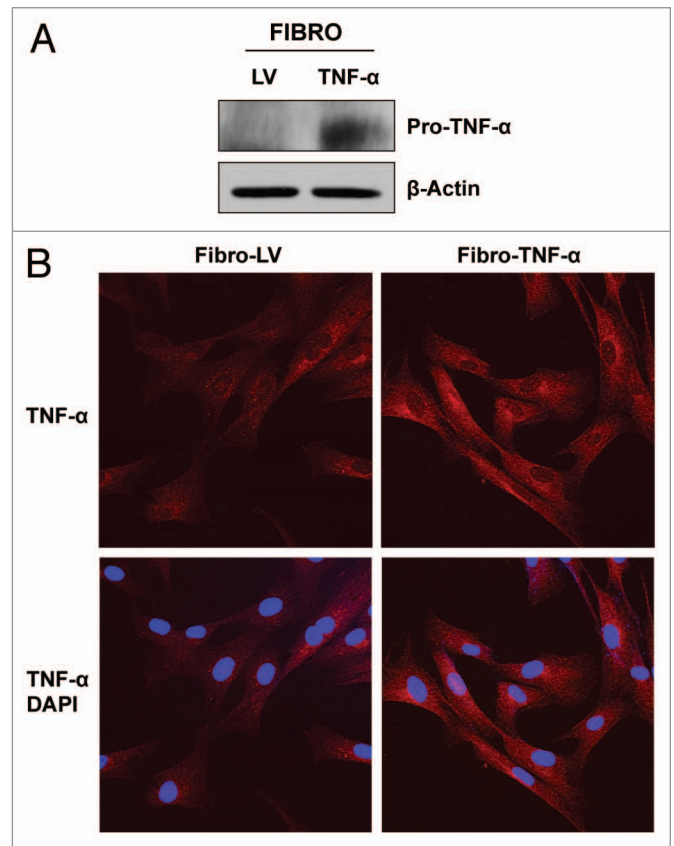
To verify if TNFR-1A and TNFR-1B expression is maintained in the tumor xenografts, tumor xenograft sections derived from TNFR-1A, TNFR-1B or LV-control MDA-MB-231 cells were subjected to immunohistochemistry with anti-TNFR-1A or anti-TNFR-1B antibodies. Figure 10A and B shows that the expression of TNFR-1A and TNFR-1B (brown staining) is preserved in the epithelial compartments of the tumor xenografts.

MDA-MB-231 cells overexpressing TNF $\alpha$  show the transcriptional elevation of genes functionally associated with cellular inflammation and senescence and decreased expression of genes associated with extracellular matrix remodeling. To further understand the mechanism(s) by which TNF $\alpha$  suppresses tumor growth, MDA-MB-231 cells overexpressing TNF $\alpha$  or vector alone control cells were analyzed by quantitative real time PCR (RT-PCR) for markers of inflammation, and extracellular matrix remodeling. Figure 11A shows that TNF $\alpha$  induces the upregulation of several transcripts associated with the inflammatory response, including IL-1- $\beta$ , IL-8, IL-6, MCP-1 and COX-2. TNF $\alpha$  was also found greatly upregulated, as expected. In addition, several markers associated with extracellular matrix remodeling, such as collagen VI and MMP2 were selectively downregulated in TNF $\alpha$ -expressing cancer cells, relative to controls. Previous studies have shown that collagen VI deposition favors tumor growth.<sup>14</sup> Similarly, it is well known that increased MMP2 levels are associated with tumor aggressiveness.<sup>15</sup>

Interestingly, TNF $\alpha$ -expressing cancer cells display the downregulation of a marker of hypoxia, HIF-2, relative to controls (Fig. 11C). We have previously shown that increased HIF-2 $\alpha$  expression in MDA-MB-231 cancer cells promotes tumor growth.<sup>16</sup> Finally, a marker of senescence, p21 (WAF1/CIP1) was found to be elevated in TNF $\alpha$ -expressing MDA-MB-231 cancer cells, relative to controls (Fig. 11C). We have previously shown increased p21 expression in MDA-MB-231 cancer cells inhibits tumor growth, by inducing senescence and autophagy.<sup>17</sup>

## Discussion

Here, we determined the specific effects of the stable recombinant expression of TNF $\alpha$  in human breast cancer cells (MDA-MB-231) and human immortalized fibroblasts (hTERT-BJ1 cells). Interestingly, stable expression was achieved via lentiviral transduction, but did not lead to any significant increases in

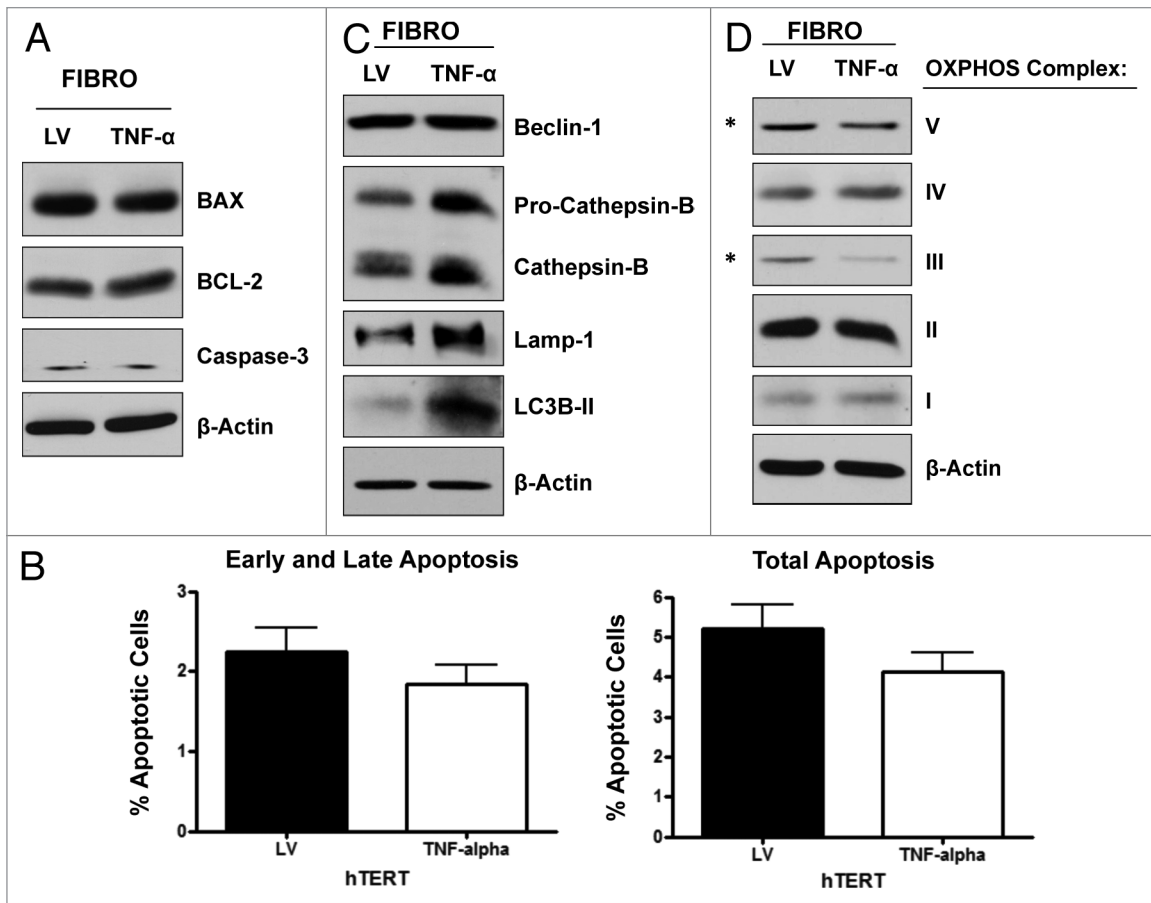


**Figure 5.** Stable TNF $\alpha$  overexpression in immortalized human fibroblasts. hTERT-BJ1 fibroblasts were transduced with a TNF $\alpha$  expressing vector or LV empty vector control. Then, the resulting transduced fibroblasts were subjected to immunoblotting or immunofluorescence analysis to validate TNF $\alpha$  overexpression. (A) Immunoblotting shows a band at ~24 kDa, corresponding to pro-TNF $\alpha$ , the precursor form.  $\beta$ -actin demonstrates equal protein loading. (B) Immunofluorescence with TNF $\alpha$  specific antibodies clearly demonstrates TNF $\alpha$  expression (see white arrows) in hTERT-BJ1 cells. Red, TNF $\alpha$  specific staining; blue, DAPI nuclear staining.

basal apoptotic rates, indicating that the cells remained viable. As expected, TNF $\alpha$  expression resulted in the induction of multiple autophagy markers as well as the onset of mitochondrial dysfunction, as evidenced by a loss of expression of specific OXPHOS components, especially Complex V, the mitochondrial ATP synthase.

Most importantly, MDA-MB-231 cells expressing TNF $\alpha$  failed to form any tumors *in vivo*, resulting in 100% protection against tumorigenesis. These results suggest that TNF $\alpha$  induces metabolic dysfunction in cancer cells, preventing their ability to form viable tumors *in vivo*. These findings are also consistent with the idea that autophagy and mitochondrial dysfunction in cancer cells has a potent tumor-suppressor effect.<sup>18-25</sup>

Similarly, the tumor suppressor effects of TNF $\alpha$  could be achieved by co-injecting TNF $\alpha$ -expressing human fibroblasts with human breast cancer cells. As such, we propose that generalized metabolic dysfunction in both tumor cells and their surrounding microenvironment will prevent the onset of metabolic symbiosis, effectively “starving” the cancer cells to death<sup>18-25</sup> (Fig. 12).



**Figure 6.** TNF $\alpha$  overexpression induces autophagy and deregulates mitochondrial activity in fibroblasts, without inducing apoptosis. **(A)** Immunoblotting shows that fibroblasts overexpressing TNF $\alpha$  have similar levels of the apoptotic markers (caspase-3, Bax and BCL-2), as control cells. **(B)** Apoptosis was measured with Annexin-V and PI probes by flow cytometry (FACS analysis). hTERT-BJ1 fibroblasts overexpressing TNF $\alpha$  did not show any significant increases in apoptosis, relative to empty vector controls. Early and late apoptosis, represents Annexin-V-positive/PI-negative cells; total apoptosis, represents Annexin-V-positive and/or PI-positive cells. **(C)** TNF $\alpha$  overexpression in hTERT-BJ1 cells induces autophagy, as assessed by the elevated expression and/or activation of cathepsin B, Lamp-1 and LC3B (LC3B-II is the cleaved active form). **(D)** Immunoblotting shows that TNF $\alpha$  overexpression in hTERT-BJ1 cells induces the downregulation of key subunits of the OXPHOS complex, namely components III and V.  $\beta$ -actin indicates equal protein loading.

This has important therapeutic implications, because mesenchymal stem cells (MSCs) and fibroblasts are known to migrate or “home” to human tumors *in vivo*, where they contribute significantly to the mass of the tumor microenvironment. So, fibroblasts or MSCs transfected with TNF $\alpha$  could be used for the direct cellular delivery of this inflammatory cytokine *in vivo*, thereby promoting tumor regression.

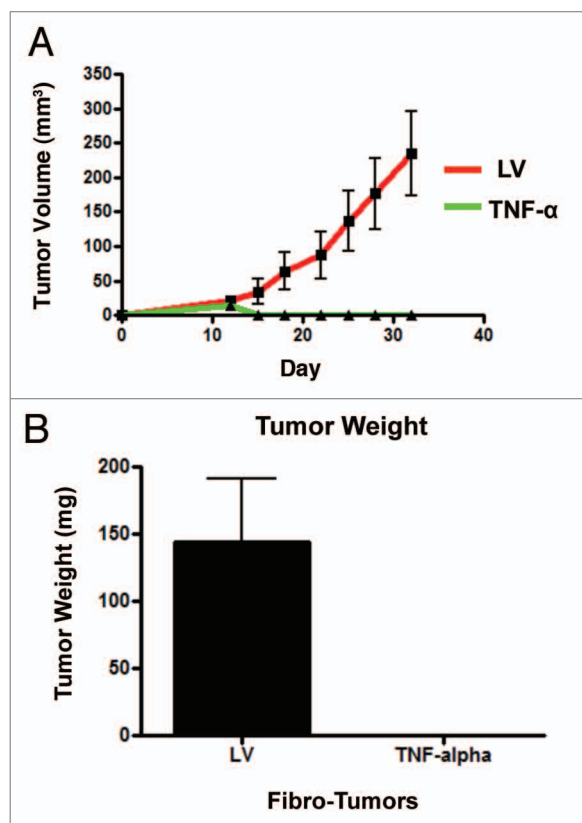
While this manuscript was being finalized for publication, several other papers were published suggesting that TNF $\alpha$  has potent tumor suppressor activity. For example, Munich et al., showed that dendritic cell exosomes, which contain TNF $\alpha$ , can kill melanoma (B16), squamous cell carcinoma (KLN205) and colon carcinoma cells (MC38).<sup>26</sup> In addition, skeletal muscle myoblasts, which naturally secrete TNF $\alpha$ , were effective in preventing the tumor growth and metastasis of prostate cancer cells.<sup>27</sup> Finally, Prockop and colleagues most recently showed that pre-treatment of hMSCs with TNF $\alpha$  can be used to inhibit the progression of lung metastatic foci *in vivo*.<sup>28</sup> However, none of these papers directly demonstrated that recombinant expression

and cellular delivery of TNF $\alpha$  was indeed sufficient to prevent tumor growth *in vivo*.

Interestingly, pre-treatment of hMSCs with TNF $\alpha$  increases their ability to home to and accumulate within tumors, and this phenomenon is dependent upon NF $\kappa$ B activation.<sup>13</sup> As such, cellular delivery of TNF $\alpha$  via the injection of non-tumorigenic cells (human fibroblasts or hMSCs) could be an attractive new approach for anticancer therapy.

## Materials and Methods

**Materials.** Antibodies were purchased from the following commercial sources: Beclin-1 (NBP1-00085, Novus), Lamp-1 (E-5, sc-17768, Santa Cruz Biotech), BNIP3 (ab10433, Abcam), Cathepsin B (FL-339, sc-13985, Santa Cruz Biotech), OXPHOS (MS601, Mitosciences),  $\beta$ -actin (A5441, Sigma), caspase-3 (9662, Cell Signaling), Bax (06-499, Millipore), TNF $\alpha$  (ab9739, Abcam), TNFR-1A (ab107860, Abcam), TNFR-1B (ab15563, Abcam), BCL-2 (ab7973, Abcam). Other reagents were purchased

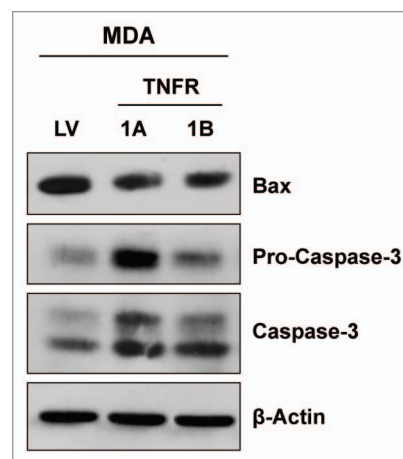


**Figure 7.** Fibroblasts harboring TNF $\alpha$  completely prevent breast cancer tumor growth in vivo. MDA-MB-231 cells were co-injected with hTERT-BJ1 cells (carrying TNF $\alpha$  or the LV empty vector control) into the flanks of nude mice. Tumors growth was serially measured using calipers for 31 d. (A) Note that the growth curves clearly show that MDA-MB-231 cells co-injected with TNF $\alpha$ -overexpressing fibroblasts did not develop any tumors. After 31 d, the mice were sacrificed. Measurements of tumor weight (B) show that TNF $\alpha$ -overexpressing fibroblasts completely prevented tumor growth of co-injected MDA-MB-231 cells, relative to control cells.

as follows: DAPI nuclear stain (Sigma), anti-fade mounting reagent (S2828, Invitrogen), biotinylated anti-rabbit antibody (BA-1000, Vector), normal goat serum (S-1000, Vector), streptavidin-HRP (K0690, Dako), Avidin (AB972H-A, Biocare Medical), Biotin (AB972H-B, Biocare Medical), DAB (K3468, Dako).

**Cell cultures.** MDA-MB-231 cells (a human triple negative breast cancer cell line) and hTERT-BJ1 cells (an hTERT-immortalized fibroblast cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum in a 37°C humidified atmosphere containing 5% CO<sub>2</sub>.

**Cell line derivation.** Lentiviral plasmids (from GeneCopoeia) were as follows: EX-NEG-Lv105 (empty vector), TNF $\alpha$  (EX-A0276-Lv105), TNFR-1A (EX-Z2785-Lv105), TNFR-1B (EX-A0254-Lv105). Lentiviral particles were generated by transfecting the GeneCopoeia 293Ta packaging cells with lentiviral plasmids, using the Lenti-Pac HIV Expression Packing Kit (GeneCopoeia), according to manufacturer's recommendations. Cells were transduced with lentiviral particles in the presence of 5  $\mu$ g/ml Polybrene (Santa Cruz Biotech). Transduced cells



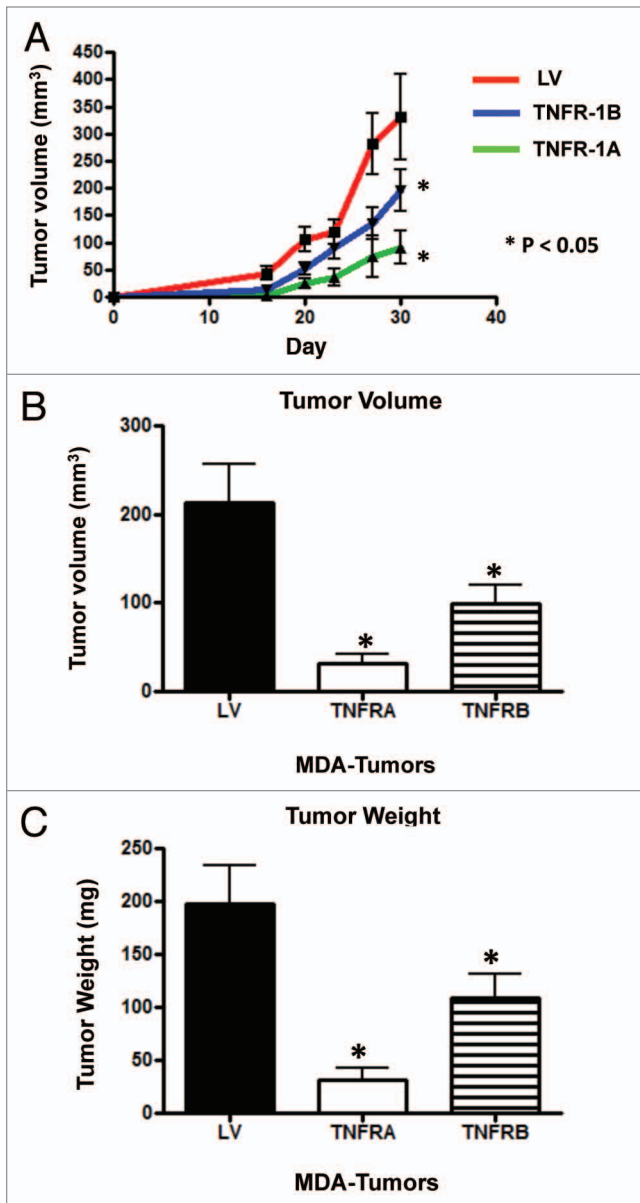
**Figure 8.** Overexpression of TNF $\alpha$  receptors induces the upregulation of apoptotic markers in cancer cells. MDA-MB-231 cells were transduced with TNF $\alpha$  receptor (TNFR-1A and TNFR-1B) vectors or an LV empty vector control and subjected to immunoblotting with apoptotic markers. Note the upregulation and activation of caspase-3 in MDA-MB-231 harboring TNFR-1A and TNFR-1B, relative to empty vector control cells.  $\beta$ -actin is shown as a control for equal protein loading.

were selected with 2  $\mu$ g/ml (MDA-MB-231 cells) or 1.5  $\mu$ g/ml (hTERT-BJ1 cells) puromycin.

**Immunoblotting.** Protein lysates were obtained by cell scraping into lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100 and 60 mM n-octylglucoside) containing protease and phosphatase inhibitors. Samples were incubated on a rotating platform at 4°C, and then centrifuged at 12,000 g for 10 min at 4°C to remove insoluble debris. Protein concentrations were determined using the BCA reagent (Pierce). Samples were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. All subsequent wash buffers contained 10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20, which was supplemented with 5% nonfat dry milk for the blocking solution and 1% BSA for the antibody diluent. Horseradish peroxidase-conjugated secondary antibodies were used to visualize bound primary antibodies with an ECL detection kit (Pierce).

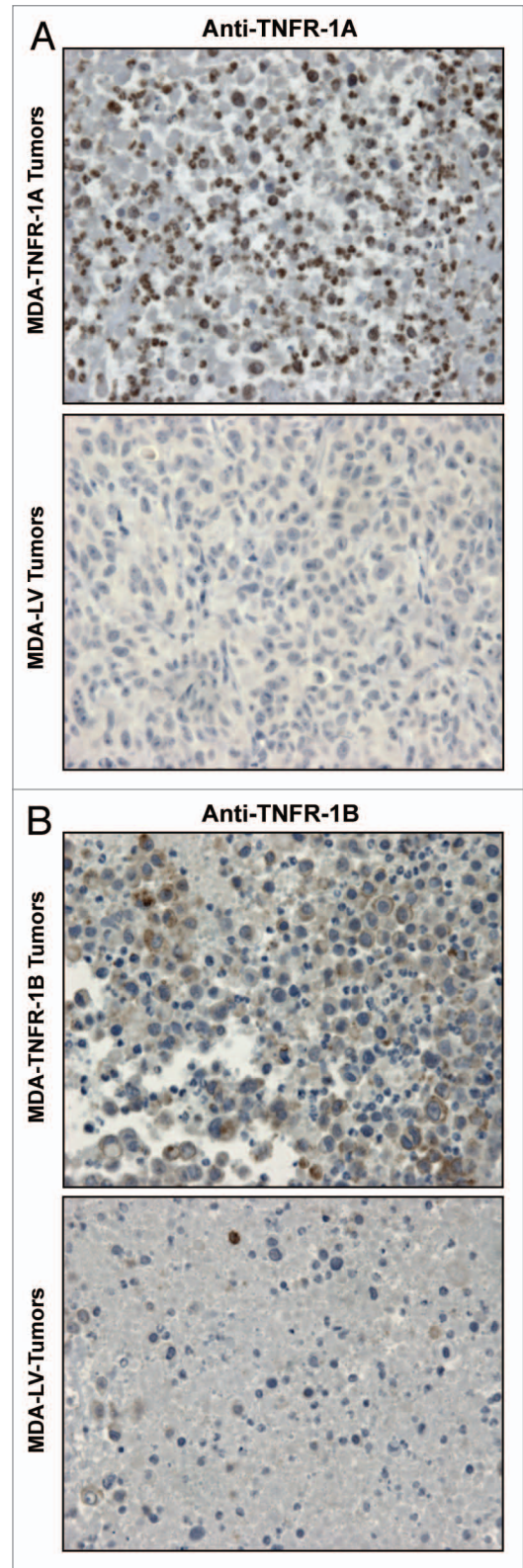
**Immunofluorescence.** Cells were plated onto coverslips in 12-well plates for 48 h in complete media. Then, cells were rinsed with PBS containing 0.1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (PBS/CM) and fixed with 2% paraformaldehyde in PBS/CM for 30 min. After fixation, cells were washed three times with PBS/CM and permeabilized with IF buffer (PBS/CM with 0.1% Triton-X100 and 0.2% BSA) for 10 min. Then, fixed cells were quenched with 50 mM NH<sub>4</sub>Cl in PBS/CM for 10 min, rinsed and incubated with anti-TNF $\alpha$  antibodies for 24 h at 4°C. Cells were washed with IF buffer and incubated with fluorescent secondary antibodies (Molecular Probes) for 30 min. After washing, cells were incubated with DAPI nuclear stain, rinsed and mounted with ProLong Gold anti-fade reagent (Molecular Probes). Images were acquired with a Zeiss LSM510 Meta confocal microscope system and analyzed with Zeiss LSM Browser.

**Apoptosis assay by Annexin V and PI staining (flow cytometry).** Cells were seeded in a 12-well plate (50,000 cells/well).



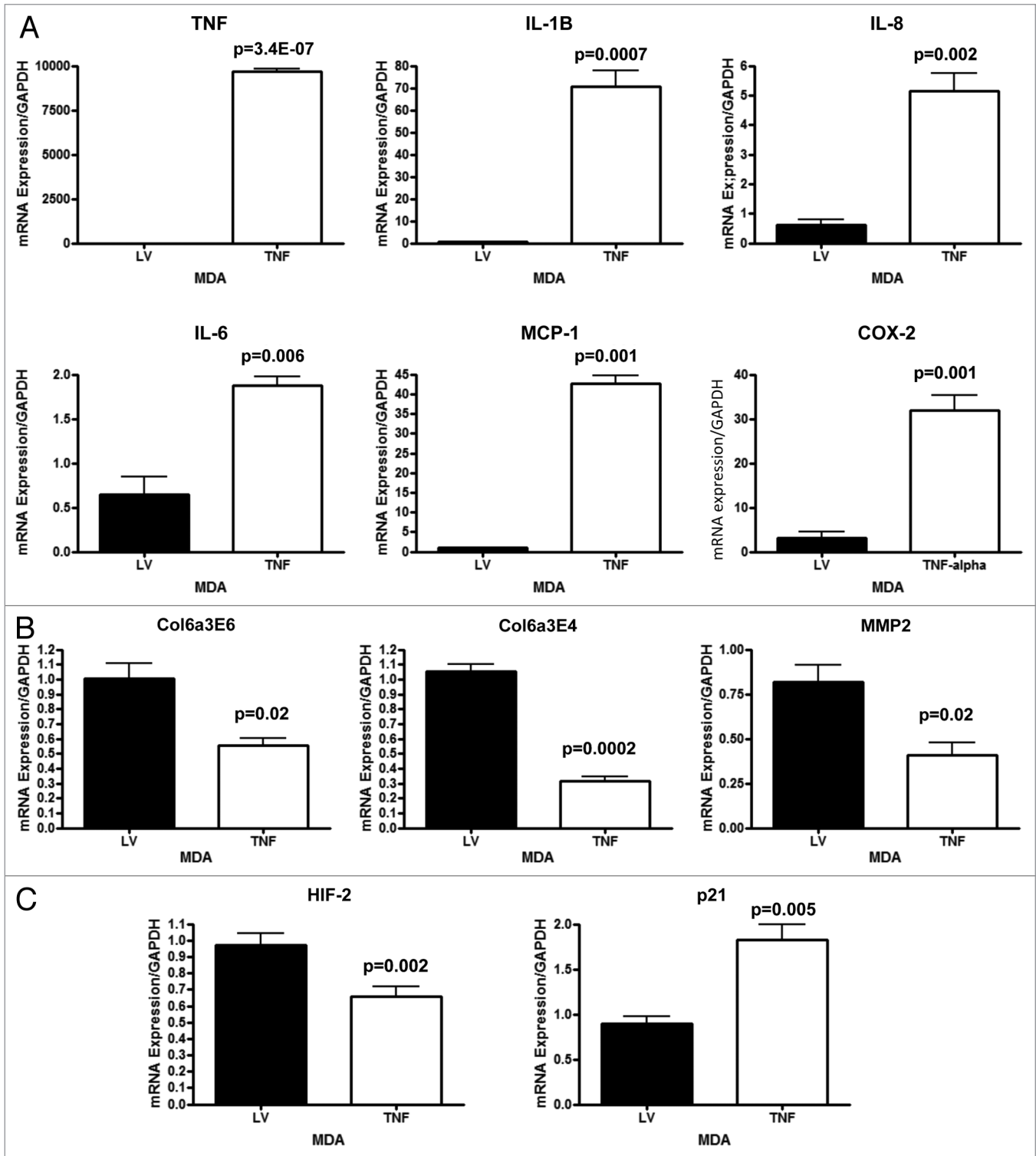
**Figure 9.** Cancer cells overexpressing TNFR-1A or TNFR-1B show significant tumor growth inhibition in vivo. MDA-MB-231 cells (carrying TNFR-1A, TNFR-1B or the LV empty vector control) were injected into the flanks of nude mice. Tumors were measured with calipers for 30 d. Note that the serial growth curve (A), final tumor volume (B) and final tumor weight (C) all clearly show that TNFR-1A and TNFR-1B transduced MDA-MB-231 cells display significantly reduced tumor growth relative to control cells.

The day after, the medium was collected into a 15-ml tube, and cells were rinsed with PBS and trypsinized (100  $\mu$ l of 0.05% trypsin-EDTA per well). Cells were placed into the same tube as the collected medium. Cells were spin down at 1,000 rpm for 5 min and resuspended in 500  $\mu$ l of 1 $\times$  Annexin binding buffer (556454, BD Biosciences), with 4  $\mu$ l of Annexin V-APC conjugate (550474, BD Biosciences) and 0.5  $\mu$ l propidium iodide (PI) (71-04-00, KPL). Samples were incubated in the dark for 5–15 min, and then analyzed by flow cytometry under the

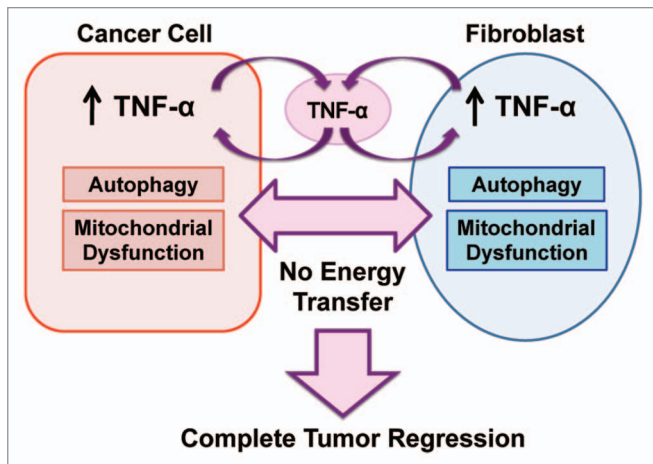


**Figure 10.** TNFR-1A and TNFR-1B overexpression in MDA-MB-231 cell tumor xenografts. TNFR-1A and TNFR-1B overexpression in MDA-MB-231 cells was validated on the tumor xenografts derived from TNFR-1A, TNFR-1B or LV-control MDA-MB-231 cells. Sections were subjected to immunohistochemistry with anti-TNFR-1A (A) or anti-TNFR-1B (B) antibodies. The positive-staining (brown color) represents TNFR-1A or TNFR-1B overexpression. Original magnification, 60 $\times$ .





**Figure 11.** MDA-MB-231 cancer cells overexpressing TNF $\alpha$  display elevated transcripts associated with inflammation and senescence and decreased transcripts associated with extracellular matrix remodeling. MDA-MB-231 cells overexpressing TNF $\alpha$  or vector alone were analyzed by quantitative real time PCR (qRT-PCR) for markers of inflammation, senescence and extracellular matrix remodeling. (A) Note that TNF $\alpha$  induces the upregulation of several transcripts associated with an inflammatory response, including IL-1 $\beta$ , IL-8, IL-6, MCP-1 and COX-2. TNF $\alpha$  was also found to be elevated, as expected. (B) In addition, several markers associated with extracellular matrix remodeling, including collagen VI and MMP2, were selectively down-regulated in TNF $\alpha$ -expressing cancer cells, relative to controls. (C) Finally, a marker of hypoxia, HIF2 $\alpha$ , was decreased and a marker of senescence, p21 (Cip1/Waf1) was found to be elevated in TNF $\alpha$ -expressing cancer cells, relative to controls. We have previously shown that increased HIF2 $\alpha$  expression in MDA-MB-231 cancer cells promotes tumor growth, and that increased p21 expression in MDA-MB-231 cancer cells inhibits tumor growth.<sup>16,17</sup>



**Figure 12.** Proposed metabolic mechanism(s) underlying the potent tumor suppressor effects of TNF $\alpha$  in the tumor microenvironment. TNF $\alpha$  can be released either by tumor cells or cancer associated fibroblasts (CAFs). Then, TNF $\alpha$  induces autophagy both in the fibroblast cell compartment and in the epithelial cancer cell compartment. In addition, TNF $\alpha$  induces mitochondrial dysfunction in both compartments. The resulting lack of energy transfer between the two metabolic compartments (epithelia vs. stroma) promotes complete tumor regression and/or inhibits tumor growth.

following parameters: voltage settings-FSC (250), SSC (245), APC (500), PE Texas Red (PI, 400).

**MTT assay.** The MTT assay (M5655, Sigma) was performed according to manufacturer's recommendations. Cells were seeded in 48-well plate (nine wells for each cell line) and incubated at 37°C for 48–72 h. Then, the MTT solution (5 mg/ml in phenol-free medium) was added to each well to equal one-tenth of the original culture volume, and incubated for 3–4 h. At the end of the incubation period, the medium was removed and the converted dye was solubilized with 200  $\mu$ L of DMSO. Absorbance was measured in a plate reader at a wavelength of 570 nm.

**Pre-clinical animal studies.** Tumor cells (MDA-MB-231;  $1 \times 10^6$  cells) were injected alone or co-injected with hTERT-BJ1 fibroblasts ( $3 \times 10^5$  cells) in 100  $\mu$ L of sterile PBS into the flanks of athymic NCr nude mice (NCRNU; Taconic Farms; 6–8 weeks of age). Mice were sacrificed at 4–5 weeks post-injection; tumors were excised to determine their weight and size, using calipers. Tumor volumes were calculated using the formula  $V = (x^2 y)/2$ , where V is the tumor volume, x is the length of the short axis, and y is the length of the long axis.

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**Immunohistochemistry.** Formalin-fixed paraffin embedded sections (6  $\mu$ m) were deparaffinized by heating for 30 min at 55°C, followed by xylene incubation and rehydration to water. Antigen retrieval was performed with citrate buffer (pH 6.0) using a pressure cooker. Endogenous peroxidase was blocked with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 15 min, followed by avidin/biotin blocking. Then, slides were blocked with 10% goat serum for 60 min and incubated overnight with anti-TNFR-1A (1:200) or anti-TNFR-1B (1:100) at 4°C. Slides were then incubated with biotinylated secondary antibodies (1:500) for 30 min at room temperature, followed by streptavidin-HRP incubation for 30 min at room temperature. Bound antibodies were visualized with DAB solution. Slides were counterstained with hematoxylin and mounted.

**Real-time PCR.** RNA extraction was performed using Trizol-Phenol-Isopropanol. cDNA was prepared using the First-Strand cDNA Synthesis Kit for Real-Time PCR (75780 50 RX) from Affymetrix. All primers were from Invitrogen (Taqman Gene Expression Assay, GEx, 4331182), TaqMan Gene Expression Assay consist of a pair of unlabeled PCR primers and a TaqMan probe with a FAM for all genes and VIC for the GAPDH house-keeping gene (20 $\times$ ). Primers were diluted 1:20 in 2 $\times$  master mix [Hot Start-IT Master Mix (2 $\times$ , usb 75766)]. Q-PCR was performed by mixing 1:1 the primer master mix with the cDNA and run under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

**Statistical analysis.** Statistical significance was examined using the Student t-test. Values of  $p < 0.05$  were considered significant.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

F.S. was the recipient of a Young Investigator Award from the Breast Cancer Alliance. U.E.M. was supported by a Young Investigator Award from the Margaret Q. Landenberger Research Foundation. Funds were also contributed by the Margaret Q. Landenberger Research Foundation (to M.P.L.).

This work was also supported, in part, by a Centre grant in Manchester from Breakthrough Breast Cancer in the UK and an Advanced ERC Grant from the European Research Council.

Also, these developments were made possible through the resources of Thomas Jefferson University.

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