Supporting Information

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SI Methods

Cell Culture. All cell lines were purchased from American Type Culture Collection unless indicated otherwise. Human cancer cell lines A549, H460, AsPC-1, Panc-2, and MDA-MB-231 and murine Lewis Lung Carcinoma (LLC) cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) FBS and maintained using standard conditions. C2C12, MCF7, mouse embryonic fibroblasts (MEFs), and MIA PaCa-2 were cultured in DMEM (Sigma-Aldrich) supplemented with 10% (vol/vol) FBS.

Microvesicle Isolation. For all experiments, microvesicles (MVs) were isolated from 250×10^6 cells cultured in serum-free medium for 48 h. Serum-free-conditioned media were then collected and harvested at $300 \times g$ for 10 min to eliminate large cells. The supernatant was then recovered, and successive centrifugations at increasing speed were performed: one at $2{,}000 \times g$ for 20 min to eliminate dead cells, then one at $10{,}000 \times g$ for 30 min to remove cell debris, and finally one ultracentrifuge at $100{,}000 \times g$ for 70 min to pellet MVs. The resulting pellet was then washed in PBS and ultracentrifuged again at the same speed. The obtained pellet was finally resuspended in 1 mL serum-free medium and used for treatment. MVs isolated from patient and mouse sera were isolated through ultracentrifugation as described earlier.

MV Treatment. C2C12 immortalized myoblasts or primary myoblasts isolated from mice were treated with MVs at indicated times. Cell numbers of myoblasts were counted on a hemocytometer.

Quantitative Real-Time PCR. Quantitative real-time PCR analysis for miRNAs was performed with the TaqMan MicroRNA assays kit (Applied Biosystems), according to the instructions of the manufacturer. Ath-miR159a and cel-miR-248 synthetic oligos were added to each sample to normalize the quantitative real-time PCR on RNAs extracted from MVs.

Nanosight. The MVs prepared from the above-mentioned cell lines were analyzed using a Nanosight NS300 (Nanosight Ltd). The MV preparations were stored at −80 °C, thawed at room temperature, and diluted in PBS solution that was free of any contaminant particles. All samples were diluted to $\sim 10^9$ particles/mL for analysis. Video capture and analysis on the NS300 was used to create size and concentration profiles for each sample.

MTS Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS)-Cell Titer 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (catalogue no. G3580), and cell viability was determined by using 96-well plate on a Spectra MAX M2 plate reader (Molecular Devices), following the manufacturer's protocol.

siRNA Transfection. For transfection of A549 cells with Exiqon Negative Control A or miRCURY, LNA inhibitor hsa-miR-21 Lipofectamine 2000 (Invitrogen) was used, following the manufacturer's instructions.

Single Fiber Isolation. Single myofibers were prepared from gastrocnemius muscles according to a previously established protocol (1). Isolated single myofibers were fixed with 2% (vol/vol) formaldehyde and further analyzed with TUNEL (Roche, fluorescein) assay and immunofluorescence staining.

Myoblast Isolation and Culture. Primary myoblasts were isolated as previously described (2) and preplated twice, using noncoated tissue culture dishes, and then cultured either on a matrigel-coated 96-well plate for MTS assay reading at indicated times or on a matrigel-coated 12-well plate for protein and RNA analysis.

Immunofluorescence and TUNEL Staining. Immunofluorescence staining and Western blotting were performed as previously described (2). TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche), following the manufacturer's protocol. Quantitation was performed from 50 myofibers per muscle per animal.

Trypan Blue Staining. Trypan blue dye was purchased from Gibco (15250-061), and staining was performed following the manufacturer's protocol. Dead cells are blue in color under the microscope and were counted using a hemocytometer.

Western Blot. Western blots were performed as previously described (2). Antibodies used and their dilutions are listed: p-JNK (Cell Signaling Technology, 1:2,000), p-c-Jun (Cell Signaling Technology, 1:1,000), p-p38 (Cell Signaling Technology, 1:2,000), and vinculin (Abcam, 1:3,000).

Mice. Cachexia in the LLC model were induced as previously described (2). $TLR7^{-/-}$ mice were obtained from Jackson Laboratory. Wild-type C57B6 male mice at the same age and weight were used as $TLR7^{+/+}$ controls. All genotypes were determined by PCR, using tail DNA. All procedures used in this study complied with federal guidelines and the institutional policies of the Ohio State University Animal Care and Use Committee.

Statistics. All quantitative data are represented as mean or mean ± SEM. Analysis was performed between different groups, using a two-tailed Student t test. Statistical significance was set at a P value of 0.05 as significant and a value of 0.01 as highly significant.

1. Keire P, Shearer A, Shefer G, Yablonka-Reuveni Z (2013) Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. *Methods Mol Biol* 946: 431–468.

2. He WA, et al. (2013) NF-κB-mediated Pax7 dysregulation in the muscle microenvironment promotes cancer cachexia. *J Clin Invest* 123(11):4821–4835.

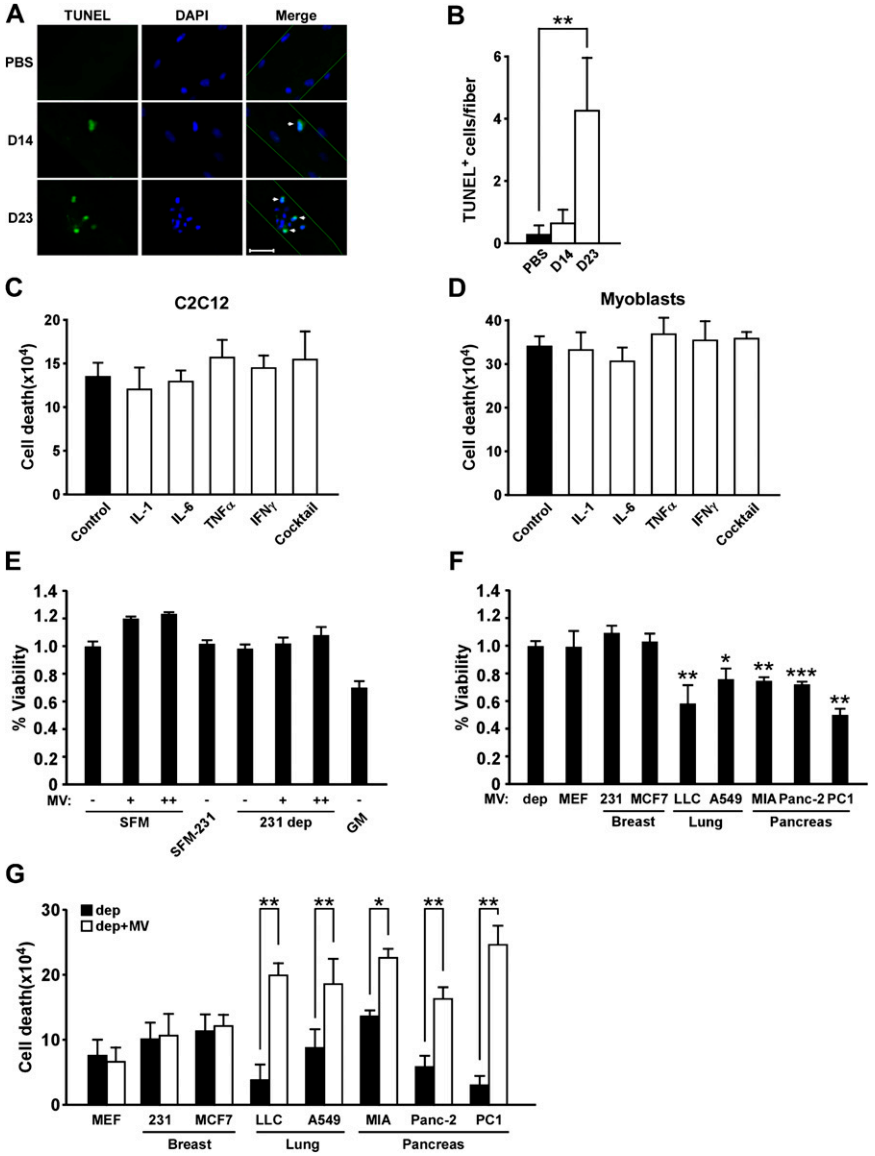

Fig. S1. MVs derived from lung cancer and pancreatic cancer, but not breast cancer, induce apoptosis on primary myoblasts. (*A*) TUNEL staining was performed on single myofibers isolated from in vivo xenograft LLC mouse models 14 and 23 d after tumor injection (D14 and D23, respectively). As negative control, myofibers derived from PBS-injected mice were used. (Scale bar, 20 μm.) (*B*) Quantitation of TUNEL$^+$ cells in *A*. (*C*) Individual cytokines or a mixture of IL-1, IL-6, TNFα, and IFNγ (all at 5 ng/mL) were used to treat C2C12 cells for 24 h, and cell death was assessed by trypan blue dye staining. As negative control, cells were treated with serum-free medium. (*D*) Same experiment as in *C* was performed on primary myoblasts. (*E*) MTS assay performed on primary myoblasts incubated for 8 h with breast cancer cell line MDA-MB-231-derived MVs, diluted either in serum-free medium (SFM) or in SFM depleted of MVs (231 dep). As control, SFM from MDA-MB-231 cells (SFM-231) and growth medium (GM) were used. "+" or "++" indicate a low or high amount of MVs being used to treat myoblasts. (*F*) MTS assay performed on C2C12 cells incubated with MVs isolated from the indicated cell lines. As control, C2C12 cells were also incubated with SFM depleted of MVs or MEF-derived MVs. (*G*) Trypan blue assay performed on C2C12 cells with different tumor-derived MVs and their corresponding MV-depleted media. Treatment with MEF-derived MVs was used as control. Treatments with MVs derived from MDA-MB-231, MIA-PaCa-2, and AsPC-1 are indicated as 231, MIA, and PC1, respectively. Results are presented as average ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

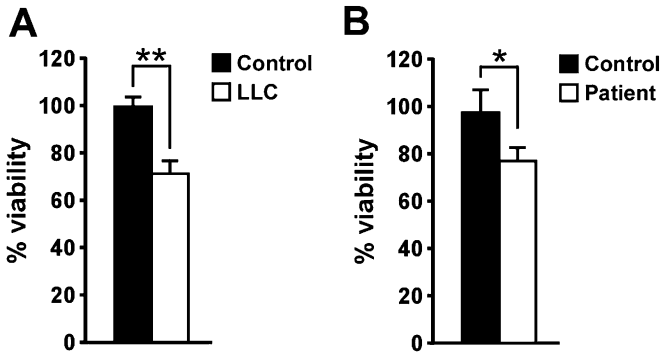

Fig. S2. MVs derived from cachectic serum impair myoblast viability. (*A*) MTS assay was performed on C2C12 cells incubated for 8 h with MVs isolated from the serum of control nontumor and LLC tumor-bearing mice. The same assay was performed on primary myoblasts (*B*) incubated for 20 h with MVs derived from serum of patients who were diagnosed with pancreatic adenocarcinoma and who suffered from cancer cachexia. As control, MVs derived from the serum of healthy donors were used. All experiments were performed in triplicate. Results are presented as average ± SEM. $*P < 0.05$; $**P < 0.01$.

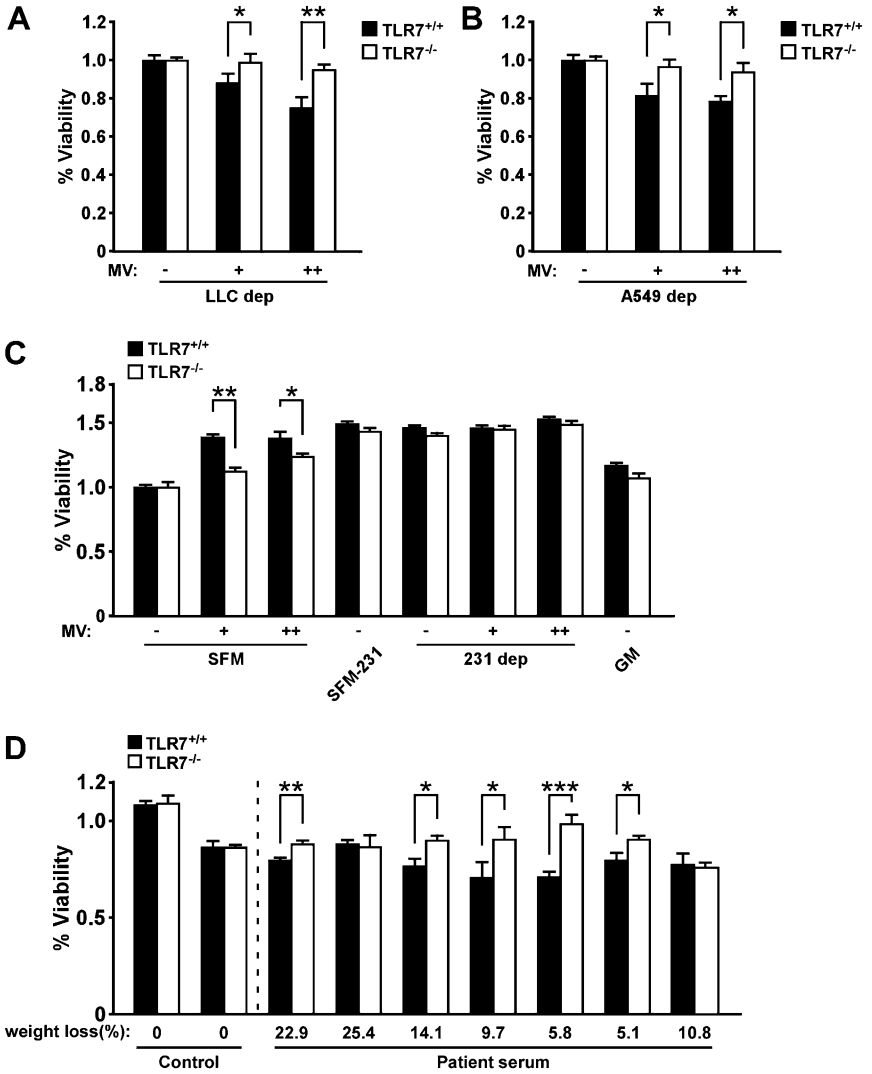

Fig. S3. *TLR7* receptor is required for MV-induced cell death on primary myoblasts. (*A*) Primary myoblasts isolated from $TLR7^{+/+}$ and $TLR7^{-/-}$ mice were incubated for 48 h with LLC-derived MVs diluted in LLC-conditioned medium depleted of MVs (LLC dep), and MTS assay was performed. "+" and "++" indicate a low and high amount of MVs being used for the treatment. (*B*) The same experiment as in *A* was performed using MVs derived from A549 cells. (*C*) Primary myoblasts isolated from $TLR7^{+/+}$ and $TLR7^{-/-}$ mice were incubated for 8 h with MDA-MB-231-derived MVs diluted in either serum-free medium (SFM) or MDA-MB-231-conditioned SFM depleted of MVs (231 dep). Conditioned medium derived from MDA-MB-231 cells (SFM-231) or growth medium (GM) were used to treat primary myoblasts as controls. MTS assay was used to assess cell viability. (*D*) MVs isolated from control serum of healthy donors ($n = 2$) and cachectic sera ($n = 7$) of pancreatic cancer patients who suffered from cachexia were subsequently used to treat primary myoblasts from $TLR7^{+/+}$ and $TLR7^{-/-}$ mice. Twenty hours after incubation, MTS assay was performed to assess cell viability. Results are presented as average ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

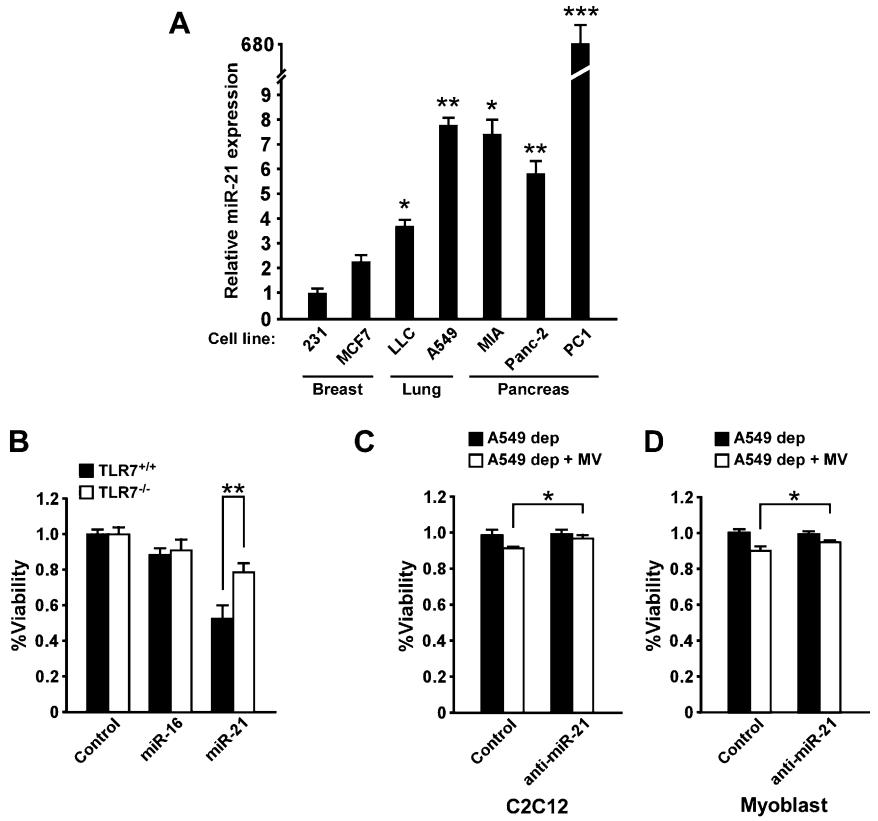

Fig. S4. miR-21 is required for *TLR7*-mediated cell death. (*A*) Quantitative real-time PCR performed on the MVs isolated from indicated cancer cell lines. Data were normalized with respect to exogenous controls ath-miR159a and cel-miR-248. Nanosight analysis was performed to determine the amount of MVs secreted per cell, and the expression level of miR-21 in MVs was further normalized to the MV number secreted per cell. Graph represents the relative expression level of miR-21 in the total MVs secreted per cell, with the lowest number set as 1 from the 231 cell line. Treatments with MVs derived from MDA-MB-231, MIA-PaCa-2, and AsPC-1 are indicated as 231, MIA, and PC1, respectively. (*B*) $TLR7^{+/+}$ and $TLR7^{-/-}$ primary myoblasts were incubated for 48 h with Dotap formulation of miR-16, miR-21, or Dotap alone, and then MTS assay was performed. The same assay was performed on C2C12 cell line (*C*) and primary myoblasts (*D*) incubated with MVs derived from A549 cells previously transfected with LNA-anti-miR-21. Results are presented as average ± SEM. $*P < 0.05$; $**P < 0.01$.

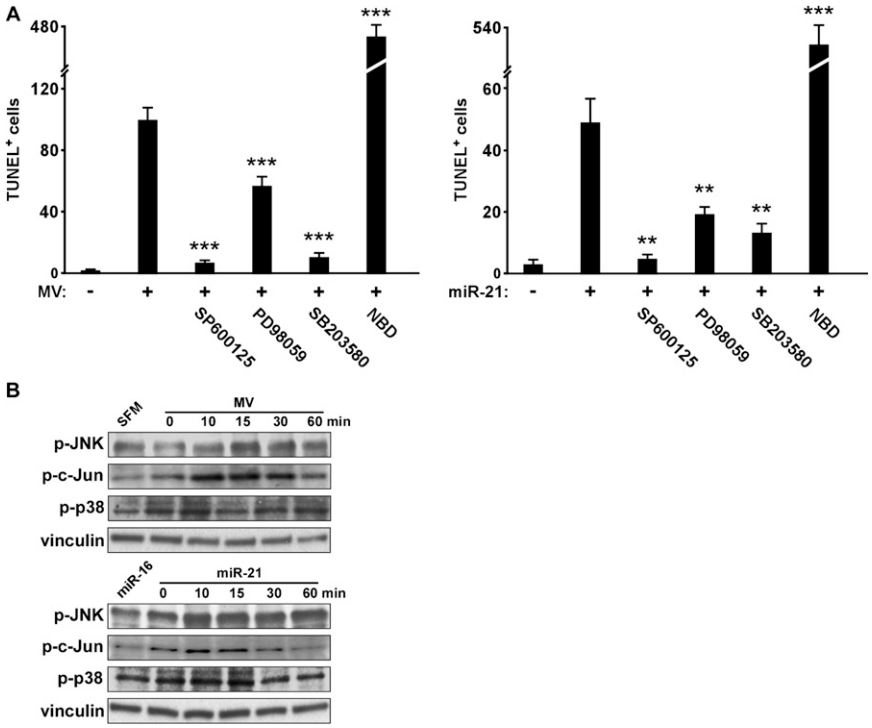

Fig. S5. Inhibition of apoptosis protects myoblasts from MV-induced cell death. (*A*) MVs isolated from A549 cells (*Left*) and synthetic miR-21 (*Right*) were used to treat C2C12 cells that had been pretreated for 1 h with either DMSO (control) or different apoptosis inhibitors (SP600125, phosphor-c-jun inhibitor; PD98059, MEK inhibitor; SB203580, p38 inhibitor) or NF-κB inhibitor (NBD). After 8 h of treatment, apoptosis of C2C12 cells was assessed by TUNEL staining. "+" indicates MVs or synthetic miR-21 being added. (*B*) C2C12 cells were treated with either LLC-derived MVs (*Upper*) or synthetic miR-21 (*Lower*, using miR-16 as control). Phospho-JNK, phospho-c-jun, and phospho-p38 were probed by Western blot at different times, as indicated. Results are presented as average ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

Table S1. Quantitation of secreted MV number

Cancer cell lines	MV number per cell
AsPC-1	9.8×10^7
MIA-PaCa	1.6×10^7
LLC	1.46×10^7
Panc-2	5×10^6
A549	2.8×10^6
MEFs	1.3×10^6
MDA-MB-231	9.6×10^5
MCF7	7.44×10^5

Cancer cell lines secrete different amount of MVs. MVs isolated from cancer cell lines were subjected to Nanosight analysis. Values reflect average number of MVs secreted per cell for each indicated cell line.