Supporting Information Methods

Cell lines

The human Ad5 E1-expressing retinoblastoma (911) or colorectal adenocarcinoma (CaCo-2, HTB-37, ATCC, Manassas, VA) cells were used.

RNA extraction and real-time reverse-transcription PCR assay

Total RNA was isolated from the cell pellets using an RNeasy mini kit (Qiagen). Contaminating DNA was removed by RNase-free DNase (Qiagen). One and a half micrograms of total RNA was reverse transcribed using Omniscript RT kit (Qiagen) with random hexamers (Applied Biosystems, Carlsbad, CA). Msln and GAPDH mRNAs were analyzed by RT-qPCR using the QuantiTect SYBR green PCR kit (Qiagen) in an ABI sequence detection system (ABI PRISM 7400, Applied Biosystems) and each experiment was conducted in triplicates. The relative mRNA levels were normalized to GAPDH mRNA. Supporting Information Table 2 shows the primers for Msln and GAPDH. PCR-cloned Msln and GAPDH were used as copy number standards. Template-negative samples served as a negative control for RT-qPCR.

Western blot

Cell pellets were lysed with sample buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 5% □-mercaptoethanol, 10% glycerol) plus protease inhibitor (Complete protease inhibitor cocktail EDTA-free mini tablet, Roche Applied Science, Indianapolis, IN) for 30 min on ice with gentle vortexing. After homogenization on ice by multiple passages through a 26-gauge needle, cellular debris was removed by centrifugation (14000 rpm, 10 min) at

4°C. Subsequent to denature for 5 min at 95°C (except for the detection of Msln protein), lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad). Membranes were blocked for 1 h at 4°C with 5% nonfat dry milk (NFDM) in 0.05% Tween 20-PBS, and probed with primary antibody (mouse monoclonal anti-Ova antibody, 1:100 dilution, 3G2E1D9; Santa Cruz Biotechnology, Inc.: goat polyclonal anti-Msln antibody, 1:100, D-16; Santa Cruz Biotechnology, Inc.: mouse monoclonal anti-beta-actin antibody, 1:10000, AC-15, Sigma-Aldrich, St. Louis, MO), diluted in 0.05% Tween20-PBS (with 5% NFDM for the detection of Msln protein) overnight at 4°C. Membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse antibody (GE Healthcare, Piscataway, NJ) or donkey anti-goat antibody (Santa Cruz Biotechnology, Inc.) diluted in 0.05% Tween20-PBS (with 5% NFDM for the detection of Msln protein) for 1 h at 4°C, developed by chemiluminescence (ECL plus, GE Healthcare), and exposed to KODAK BioMax MR Film (Carestream Health, Inc., Rochester, NY). Beta-actin was used as an internal control to adjust for differences in the amount of protein loaded in each lane.

Evaluation of Pan02-derived cell lines

Ova expression in Pan02-Ova was confirmed by Western blot. Luc expression in Pan02-LuciGFP was assessed by luciferase activity assay using Luciferase assay system (Promega). The results were read with FLUOstar Omega (BMG LABTECH, Cary, NC). GFP expression in Pan02-LuciGFP was observed by FCM and IF. Msln protein expression in Pan02-Msln was evaluated by FCM, IF, and Western blot.

Detailed construction methods of replication-defective recombinant Ad40 vectors

The rAd40pΔE1-Luc and rAd40pΔE1-CMV-Luc, Ova or Msln were generated by the homologous recombination between a *ClaI*-linearized plasmid coding Ad40wt and a rescue plasmid with homologous regions of Ad40-left and right-ends for recombination (left, 441bp between 1 and 441 of GenBank accession no. L19443; right, 2809bp between 1693 and 4501) as well as Luc, CMV-promoter-driven Luc, Ova or Msln cassette in *E. coli* BJ5183. The resultant plasmid was amplified with DH5alpha (Invitrogen).

Viruses were generated by tranfecting to 911 cells with the vector coding plasmids after linearization with *AsiSI*, and subsequently amplified and purified. All transfections and infections were performed in a dedicated Ad40 work incubator isolated from other viruses. Seven days after transfection, crude viral lysates (CVLs) were prepared by three freeze-thaw cycles and centrifugation (3000 rpm, 10 min), and a series of 1:3 dilution of viral lysates were infected to fresh 911 cells.

In order to prepare vectors suitable for *in vivo* studies, the same amplification steps were repeated using 1:3 dilutions of CVL, which was prepared by three cycles of freeze-thawing of 1.7 ml suspension, and centrifugation (3000 rpm, 10 min), until an apparent cytopathic effect (CPE, grape-like clusters and >90% cell detachment at the 2^{nd} passage) was observed in a 60-mm dish. After observing an obvious CPE, the vectors were then amplified in 911 cells (4×10^4 cells/cm^2) in a 100-mm dish, a 75-cm^2 flask, or 1, 2, 4, 8, and 16 175-cm^2 flasks for subsequent CsCl centrifugation (10^{th} passage).

Vectors were purified with double CsCl centrifugation and titrated with optical density at 260 nm (OD260; virus particle, VP). We performed plaque forming unit (PFU, 20) and tissue culture infectious dose 50 ($TCID_{50}$) assays (Supporting Information Table S1),

and tested for contamination by PCR (QIAGEN fast cycling PCR kit, Qiagen) as previously reported (20). The PCR was performed on a 2720 thermal cycler (Applied Biosystems) with specific primers for Ad40 E1, IX, IVa2 E3, fibers and E4orf2, Luc, and Ad5 E2 (Supporting Information Table S2) for 35 cycles. Subsequently, PCR fragments were cloned and sequenced (20).

Confirmation of the genetic integrity of rAd40pΔE1-Luc produced by our method was first done by shotgun sequencing. More than 80% of rAd40pΔE1-Luc sequence (88.8%, 30791 of 34685 bp) was covered by shotgun sequencing and was found to be identical to the expected sequence. The remaining portion of rAd genome was sequenced by PCR with primers based on the known sequence. Restriction analysis of rAd40pΔE1-Luc with *ClaI*, *HindIII*, and *SwaI* agreed with the expected sequence.

In vivo bioluminescence in tumor models

Mice were anaesthetized with isofluorane (Hospira, Lake Forest, IL) injected intraperitoneally with 15 mg/ml D-luciferin (Molecular Imaging Products, Bend, OR) and imaged 10 min later with a charge coupled device camera (IVIS 100, Xenogen, Alamenda, CA). After acquiring a bright-field image (grey-scale), a bioluminescent image was obtained using 20 cm field of view (binning factor of 8, 1/f stop and open filter, 30-second exposure). Regions of interest (ROIs) were defined manually, signal intensities were calculated using the Living Image software (Xenogen), and were expressed as photons per second. Background photon flux was defined from a ROI drawn over a control mouse where no Pan02-LuciGFP had been administered (abdominal bioluminescent total flux $<1 \times 10^6$ photons/sec).

Supporting Information Table 1. Titers of Ad vectors.

	rAd40pΔE1-Luc	rAd40pΔE1-CMV-Luc	rAd5-CMV-Luc	rAd40pΔE1-CMV-Ova	rAd5-CMV-Ova	rAd40pΔE1-CMV-Msln
VP/ml	**7.18×10^{11}**	**3.86×10^{11}**	**5.20×10^{11}**	**2.34×10^{11}**	**7.64×10^{11}**	**1.13×10^{12}**
PFU/ml[a]	**1.02×10^{8}[b]**	**1.60×10^{6}**	**1.25×10^{9}**	**0.95×10^{6}**	**3.60×10^{9}**	**7.40×10^{6}**
	(0.96-1.08)	**(1.45-1.75)**	**(0.42-1.67)**	**(0.83-1.07)**	**(2.60-4.60)**	**(7.10-7.70)**
$TCID_{50}$/ml[a]	**1.25×10^{8}**	**1.99×10^{6}**	**1.99×10^{9}**	**1.25×10^{6}**	**1.99×10^{10}**	**1.25×10^{8}**
	(1.15-1.34)	**(1.39-2.58)**	**(1.18-2.80)**	**(1.03-1.47)**	**(1.83-2.14)**	**(1.15-1.34)**

[a]Titer on 911 cells gives the titer in plaque-forming units (PFU) per ml and in tissue culture infectious dose 50 ($TCID_{50}$) per ml.

[b]The median (interquartile range) of three independent experiments are shown.

VP, viral particle.

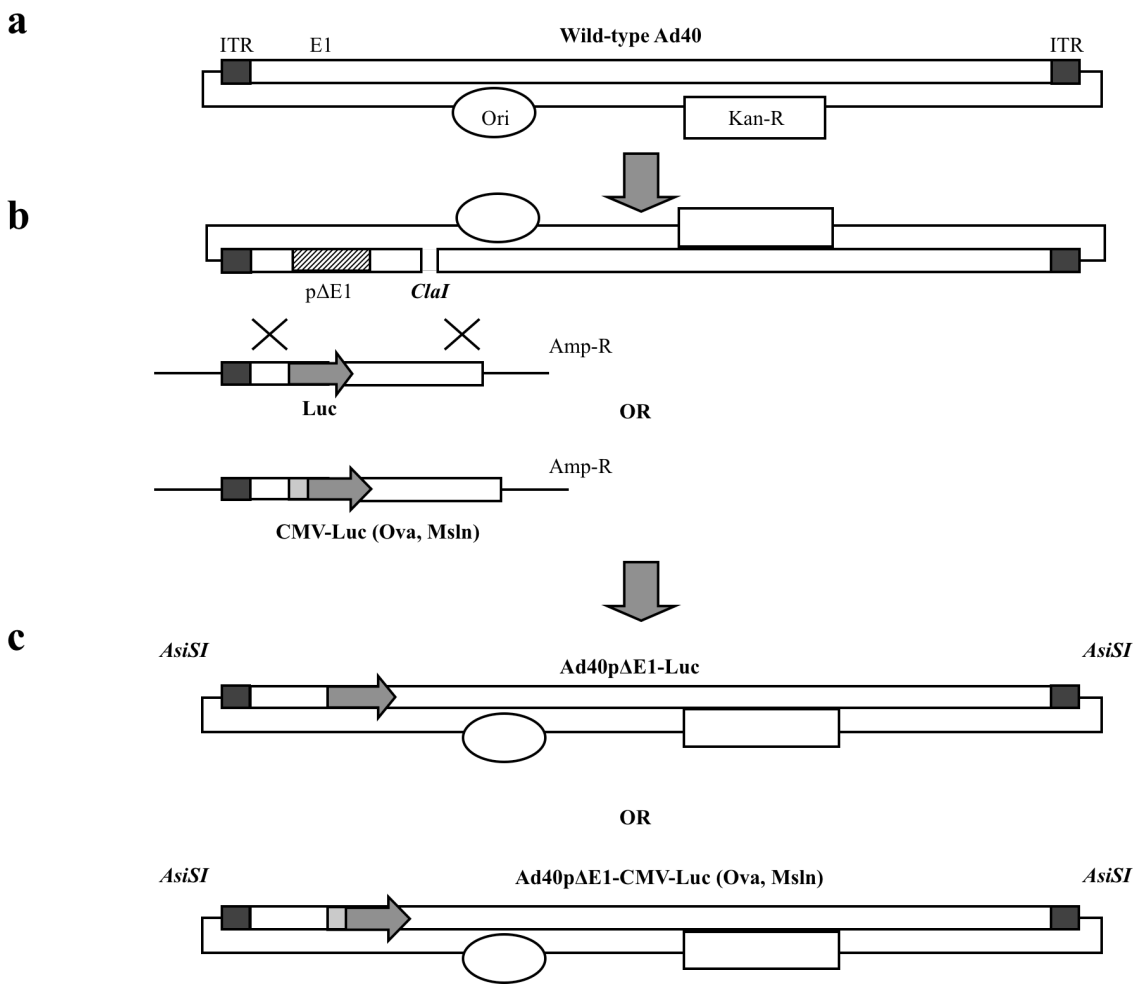
The VP titers of rAd expressing Luc (Ova, Msln) were all in the same order of magnitude. However, rAd5-CMV-Luc (Ova) titers measured by biological titrations (PFU and $TCID_{50}$) were 10 and 1000 times higher than those of rAd40pΔE1-Luc and rAd40pΔE1-CMV-Luc (Ova), respectively. These data show that PFU and $TCID_{50}$ assays did not accurately represent the biological titration of rAd40, probably because newly developed rAd40 has not been adopted for replication in widely used Ad producer cells.

Supporting Information Table 2. Sequences of primers used in this study.

Assay	Primer		Location[a]	Sequence (5' to 3')
PCR	Ad40 E1	F	418-437	ACTCTTGAGTGCGAGCGAGT
		R	3149-3129	TTAATCCTCATCGCTGGATTC
PCR	Ad40 E1/IX/IVa2	F	1719-1736	ATGGAGCGCCCAAACTCA
		R	4501-4482	GTCATTGGGGTCATTTACGG
PCR	Ad40 E3/short fiber	F	27035-27054	TTCCTGCGCTAACGTAACCT
		R	29179-29198	TAAAGCCTAACGCTCCGGTA
PCR	Ad40 long fiber	F	29977-29996	TGACTTCAACCCCGTCTACC
		R	30488-30469	GGGGGCTAGAAAACAAAACC
qPCR	Ad40 fiber	F	30372-30394	AACTTTCTCTCTTAATAGACGCC
	for qPCR	R	30489-30471	AGGGGGCTAGAAAACAAAA
PCR	Ad40 E4orf2	F	33412-33431	CGTGTCACCTGCAGTTCATT
		R	33740-33721	CCTGAGCCCCTGATGTTTTA
PCR	Ad5 E2	F	22527-22546	AAACTCAGGCACAACCATCC

		R	22867-22848	ACCTTTTGATGCCACTACGG
PCR, qPCR	Luciferase reporter gene	F	1395-1419	CCGCCTGAAGTCTCTGATTAAGTAC
		R	1466-1446	TGGAGCAAGATGGATTCCAAT
qPCR	Mouse GAPDH	F	649-668	CCAGAACATCATCCCTGCAT
		R	715-695	GTTCAGCTCTGGGATGACCTT
RT-qPCR	Mouse mesothelin mRNA	F	778-798	GCAGTCAGGGAGGTTCTGAGG
		R	846-826	GGTGGAGACTGACCACTTCGA
RT-qPCR	Mouse GAPDH mRNA	F	311-335	GGTGCTGAGTATGTCGTGGAGTCTA
		R	407-388	CGGAGATGATGACCCGTTTG

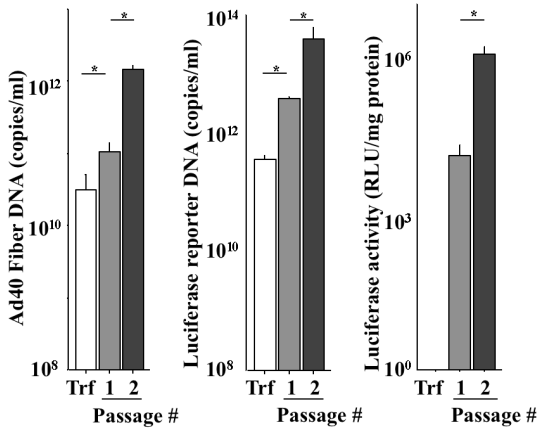
[a]The nucleotide positions of Ad40, Ad5, luciferase reporter gene, mouse GAPDH, mouse mesothelin mRNA, and mouse GAPDH mRNA, respectively, are based on GenBank (NCBI) accession sequence L19443, AY339865, U47295, M32599, NM018857, and M32599. F, Forward; R, Reverse; qPCR, real-time PCR; RT-qPCR, real-time reverse-transcription PCR.



Supporting Information Figure 1. Derivation of replication-defective recombinant Ad40 vectors. A schematic representation of the strategy to engineer rAd40pΔE1-Luc and rAd40pΔE1-CMV-Luc, Ova or Msln. (a) The plasmid encoding wild-type Ad40 (pAd40wt). (b) The pAd40wt linearized by a unique *ClaI* restriction enzyme site. prAd40pΔE1-Luc or prAd40pΔE1-CMV-Luc (Ova, Msln) generated by the homologous recombination between a *ClaI*-digested pAd40wt and the rescue plasmid. (c) The prAd40pΔE1-Luc or prAd40pΔE1-CMV-Luc (Ova, Msln) linearized by *AsiSI* for

transfection of 911 cells. ITR, internal tandem repeat; Ori, *E. coli* origin of replication; Kan-R, kanamycin-resistance gene; Amp-R, ampicillin-resistance gene.

a



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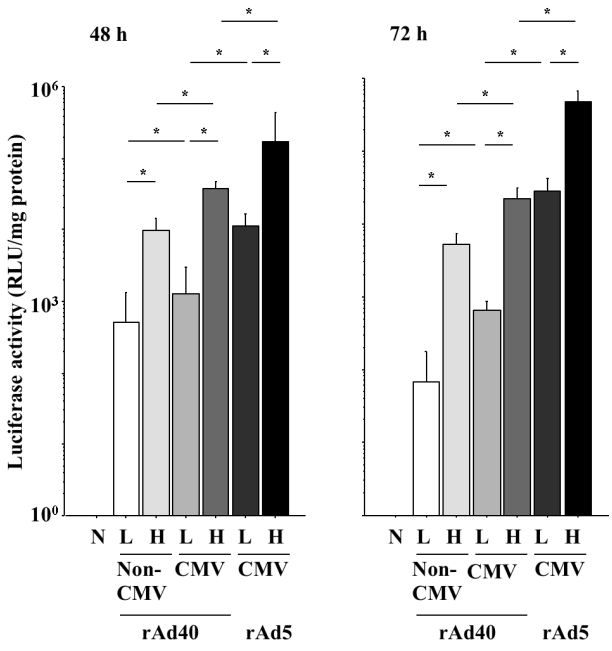
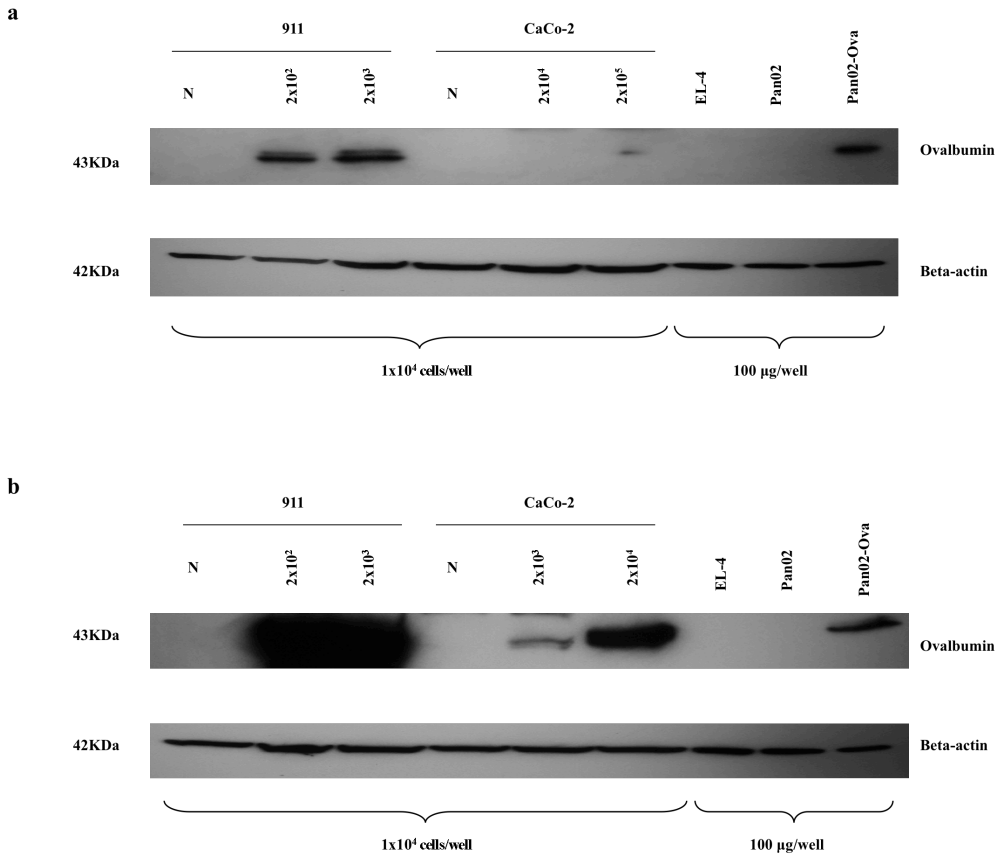


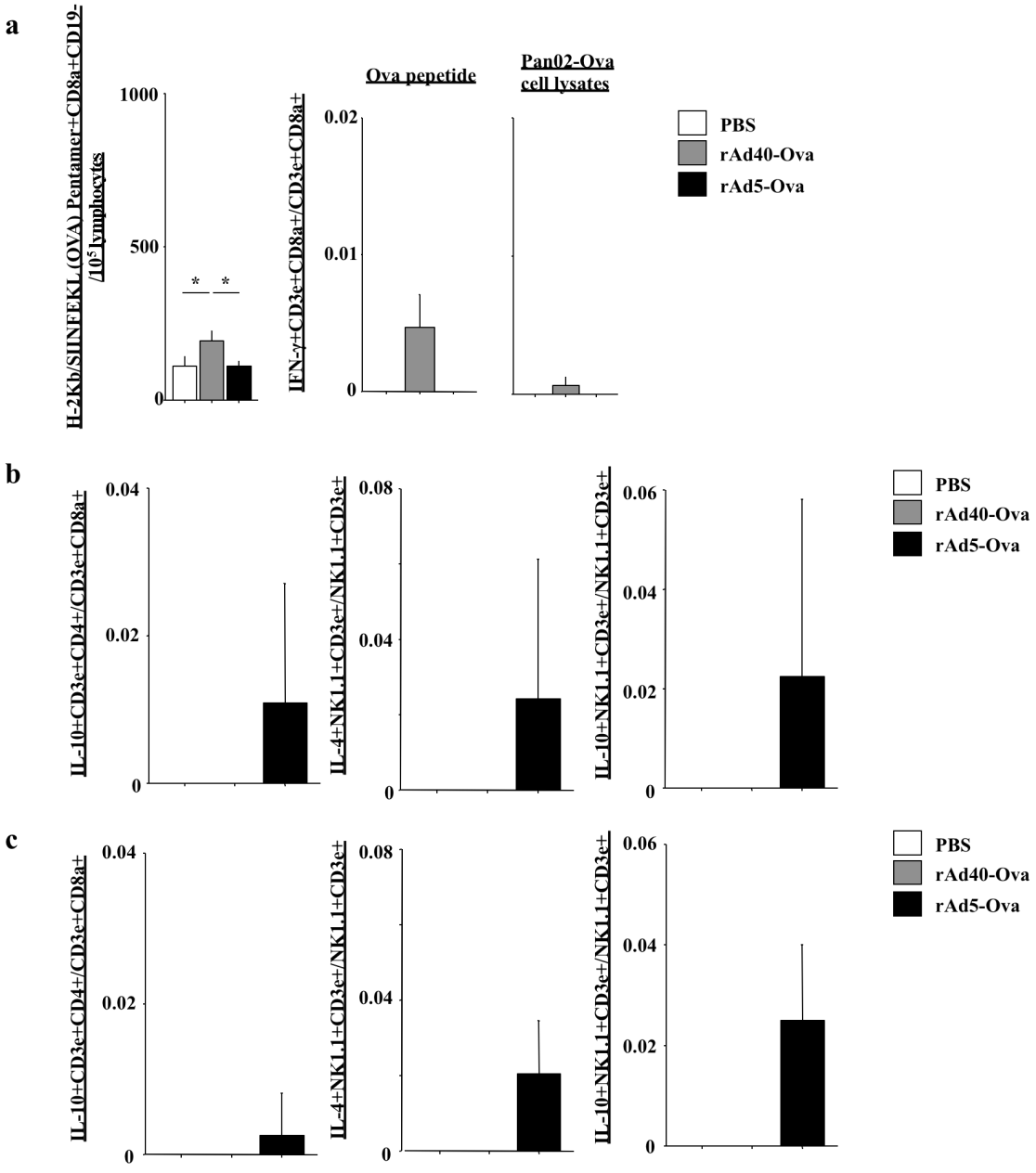
Supporting Information Figure 2. The replication-defective recombinant Ad40 vectors; replication and luciferase expression *in vitro*. (a) Replication of rAd40pΔE1-Luc in 911 cells. The replication of rAd40p□E1-Luc in 911 cells was evaluated by

determining viral copy number in CVL with real-time PCR for Ad40 fiber and Luc, and by cellular luciferase activity. At every round of infection, viral copy number and luciferase activity increased exponentially by about one order of magnitude. Trf, Transfection; RLU, relative luminescence units. (b) The luciferase activities. The luciferase activities in CaCo-2 cells infected with rAd40pΔE1-Luc (Non-CMV-Luc), rAd40pΔE1-CMV-Luc (CMV-Luc) and rAd5-CMV-Luc increased in a dose dependent manner. At both 48 and 72 h after infection, the activities of rAd5-CMV-Luc were the highest, and the activities of rAd40pΔE1-CMV-Luc were higher than those of rAd40pΔE1-Luc. N, no virus: L, low; 200 VP/cell: H, high; 2000 VP/cell: RLU, relative luminescence units.



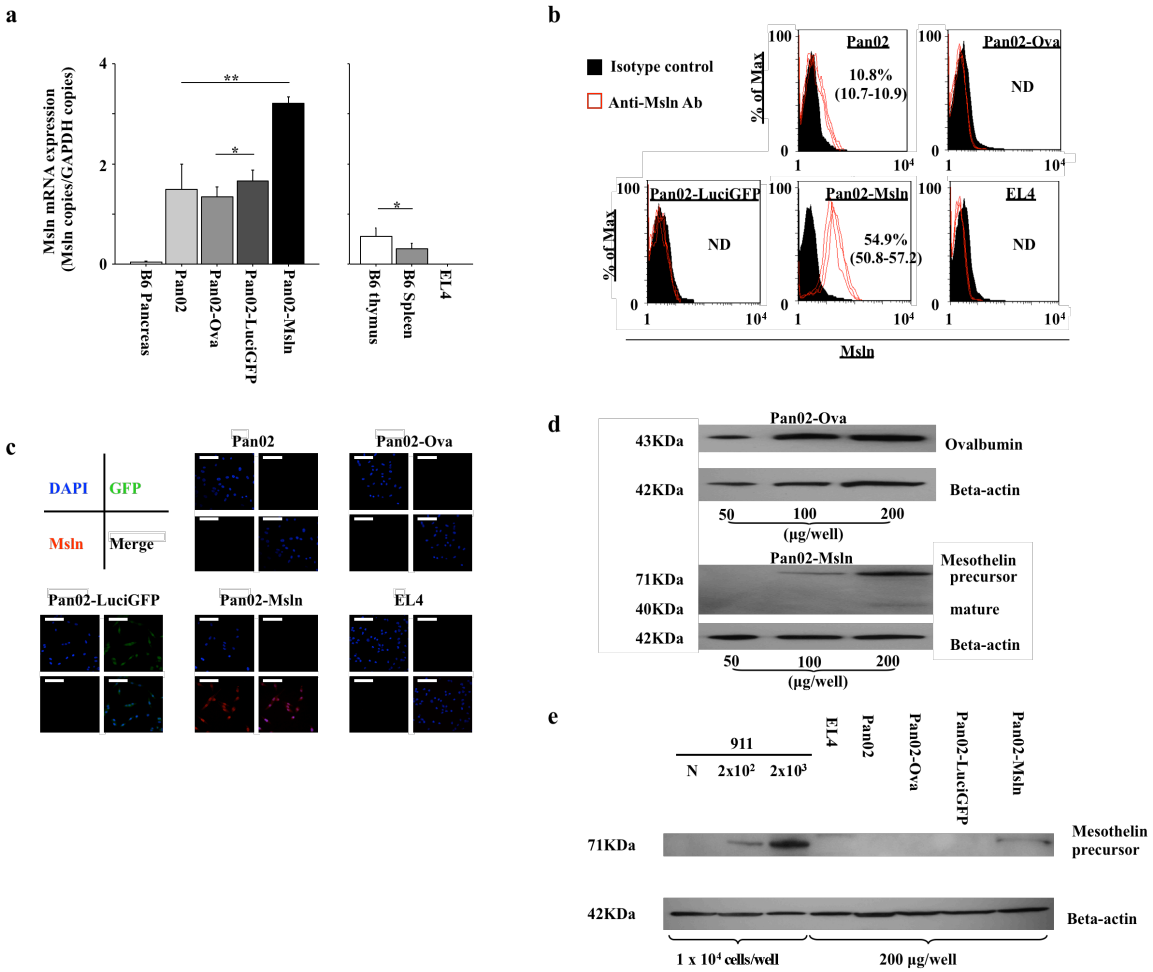
Supporting Information Figure 3. The ovalbumin protein is expressed at lower levels in rAd40pΔE1-CMV-Ova compared with rAd5-CMV-Ova. Expression of ovalbumin (Ova) in rAd infected cell lines. 911 (lanes 1-3) and CaCo-2 cells (lanes 4–6) were non-infected (N, lanes 1 and 4), or infected for 2 h with 2 x 10^2-10^5 VP/cell either rAd40pΔE1-CMV-Ova (a) or rAd5-CMV-Ova (b). Protein (48 h post infection) was extracted from infected cells and 1 x 10^4 cells were used for Western blot detection of Ova protein expression. For comparison, lane 7-9 shows the expression of Ova in EL-4,

Pan02, and Pan02-Ova cells with a stably transfection of an Ova expression plasmid. Beta-actin was used as an internal control to adjust the differences in the amount of protein loaded in each lane. Western blot analysis of total cell lysates in 911 and CaCo-2 cells showed lower Ova expression levels by rAd40pΔE1-CMV-Ova than those by rAd5-CMV-Ova. Thus, the *in vitro* level of protein expression was lower with rAd40 compared with rAd5.



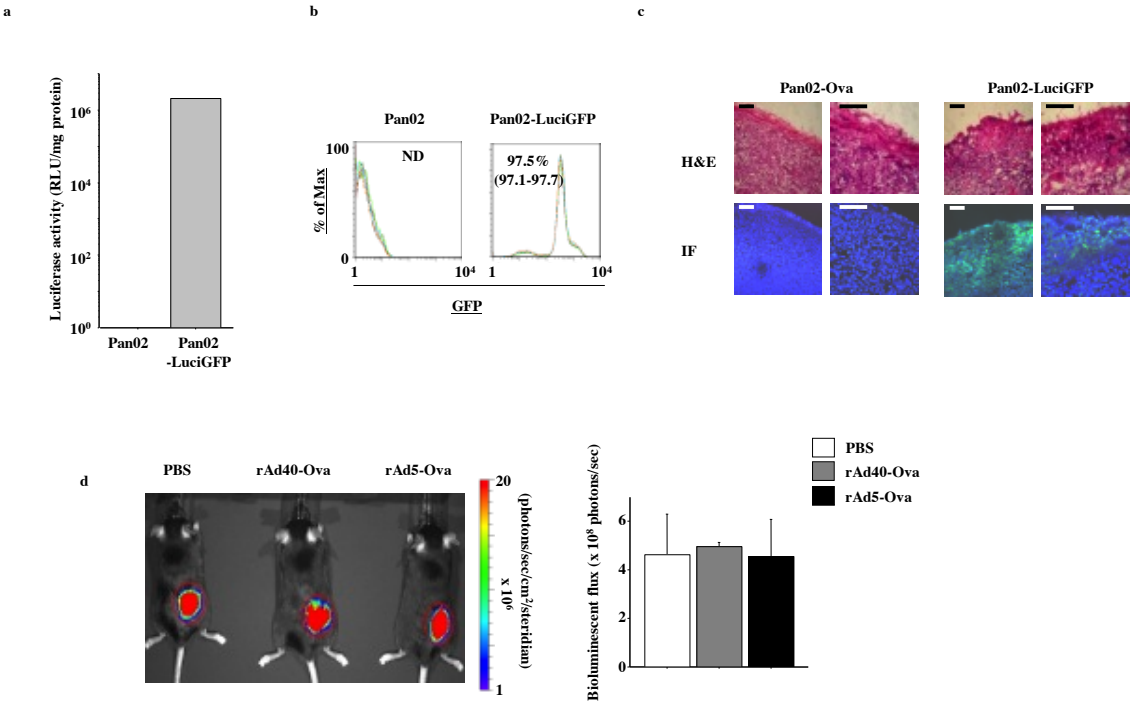
Supporting Information Figure 4. Splenic ovalbumin-specific T cells in subcutaneous Pan02-LuciGFP tumor inoculation after a single intravenous rAd administration.

Splenocytes were extracted for FCM at 30 days after Pan02-LuciGFP tumor inoculation in PBS-, rAd40-Ova-, or rAd5-Ova-pretreated mice. (a) The number of H-2K^{b}-SIINFEKL (Ova)-specific CD8a+CD19- cells in the spleen of PBS-, rAd40-Ova-, or rAd5-Ova-pretreated mice and the ratio of Ova peptide- or Pan02-Ova cell lysates-specific IFN-□-secreting CD8+ T cells to CD8+ T cells in the spleen were measured by FCM. Functional Ova-specific CTLs were detected only in rAd40-Ova-pretreated mice. (b) The ratio of Pan02-Ova cell lysates-specific IL-10-secreting CD4+ T cells to CD4+ T cells, and IL-4- or IL-10-secreting NKT cells to NKT cells. Pan02-LuciGFP-specific IL-10-secreting CD4+ T cells were detected by rAd5-Ova-pretreatment in Pan02-LuciGFP models. (c) The ratio of Pan02-LuciGFP cell lysates-specific IL-10-secreting CD4+ T cells to CD4+ T cells, and IL-4- or IL-10-secreting NKT cells to NKT cells. Pan02-LuciGFP-specific IL-4- and IL-10-secreting NKT cells were detected by rAd5-Ova-pretreatment after Pan02-LuciGFP inoculation. These data indicated that systemic tolerance was induced by liver stimulation and tumor inoculation.



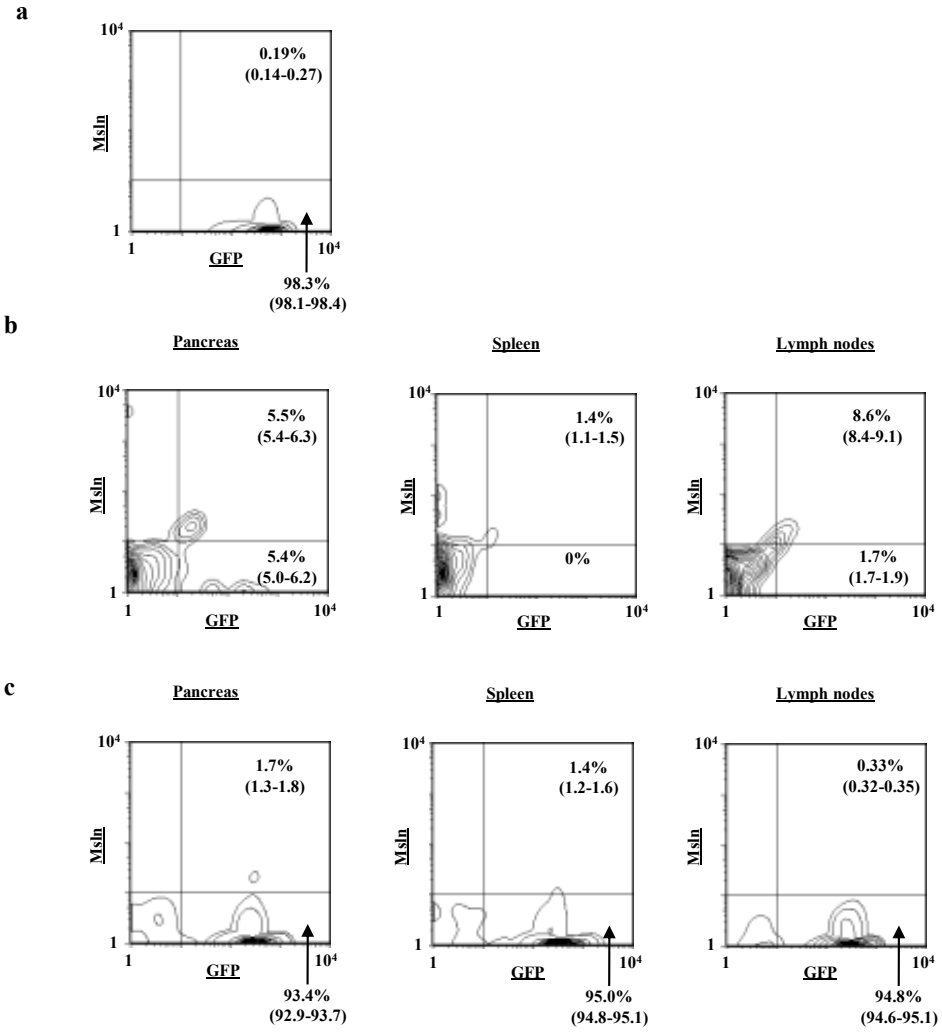
Supporting Information Figure 5. Mouse mesothelin expression in Pan02 and rAd40-Msln infected cells. (a) Mouse mesothelin (Msln) mRNA was expressed in Pan02 cells. Msln mRNA expression (RT-qPCR) in cells and tissues of B6 mice normalized to GAPDH mRNA. Msln mRNA copy numbers of the four Pan02-derived cells (Pan02, Pan02-Ova, Pan02-LuciGFP, and Pan02-Msln) were significantly higher than those of B6 pancreatic tissues. Msln mRNA expression was not detected in EL4 cells. (b) Msln protein expression was detected in Pan02 and Pan02-Msln by FCM. The isotype control

was used to distinguish between fluorescent positive and negative cell populations. ND, not detected. (C) Msln protein was expressed in Pan02-Msln by IF. Merge contains the combined image of DAPI (blue), GFP (green), and Msln (red). Bar = 100 μm. (d) Western blots of Pan02-Ova for Ova expression and Pan02-Msln for Msln protein expression. Beta-actin was used as an internal control to adjust for differences in the amount of protein loaded in each lane. In analyzing Msln protein expression, 200 □g/well was required for the constant detection. (e) Msln protein was detected in rAd40-Msln by western blot. 911 cells were non-infected (N, lane 1) or infected for 2 hours with 2 x 10^2 or 10^3 VP/cell rAd40-Msln. Protein (48 hours post infection) was extracted from infected cells and 1 x 10^4 cells were used for detection of mesothelin precursor protein expression. For comparison, lane 4-8 shows the expression of mesothelin precursor in EL4, Pan02, Pan02-Ova, Pan02-LuciGFP, and Pan02-Msln.



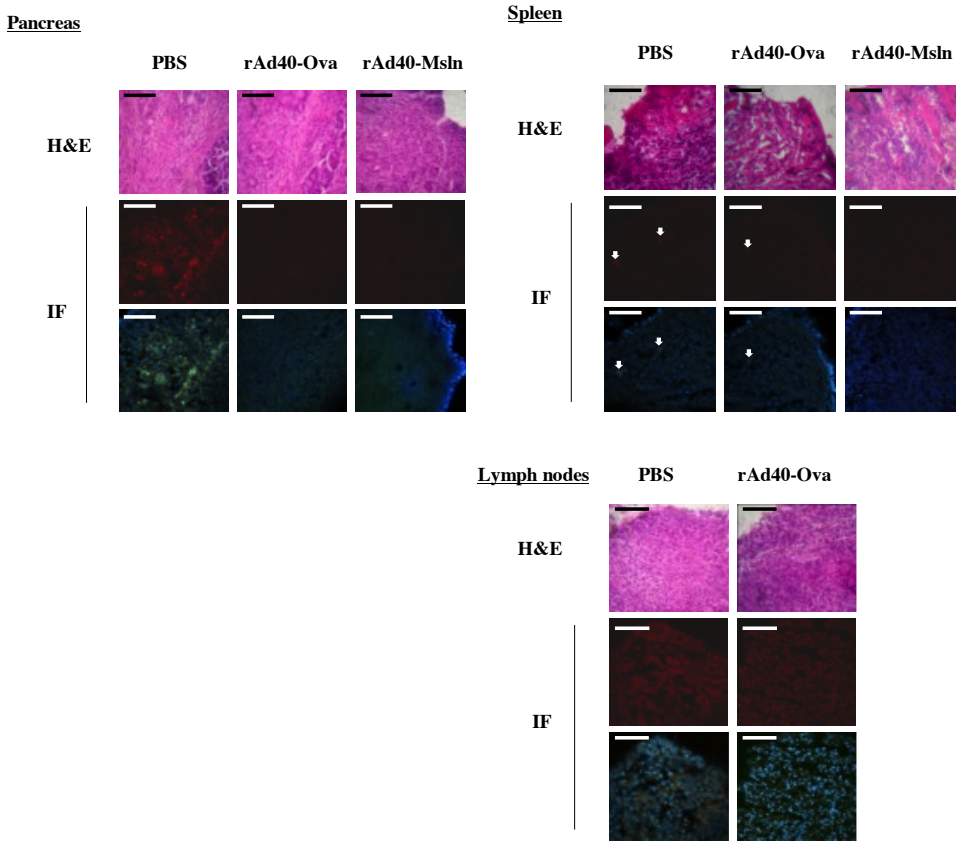
Supporting Information Figure 6. Luciferase activity, and GFP and mouse mesothelin expression in Pan02-LuciGFP cells. (a) Luciferase activity in Pan02 and Pan02-LuciGFP. (b) GFP expression in Pan02 and Pan02-LuciGFP cells evaluated by FCM. ND, not detected. Subcutaneous mouse models with Pan02-Ova and Pan02-LuciGFP. (c) The top and bottom panels show H & E staining and IF for merge, which contains the

combined image of DAPI (blue), GFP (green), and Msln (red). Msln protein was not expressed in subcutaneous mouse models with both Pan02-Ova and Pan02-LuciGFP. Bar = 100 μm. (d) Subcutaneous transplanted Pan02-LuciGFP tumors at 30 days after tumor inoculation in PBS-, rAd40-Ova-, or rAd5-Ova-pretreated mice. The growth of Pan02-LuciGFP tumors was detected by live animal imaging using bioluminescence imaging (left panel). The right graph shows quantitation of luminescence signals generated by the Pan02-LuciGFP tumors in the left panel. Regions of interest (ROIs) are marked with red circles in the left panel. There was no significant difference in bioluminescence among PBS-, rAd40-Ova-, and rAd5-Ova-pretreated mice.

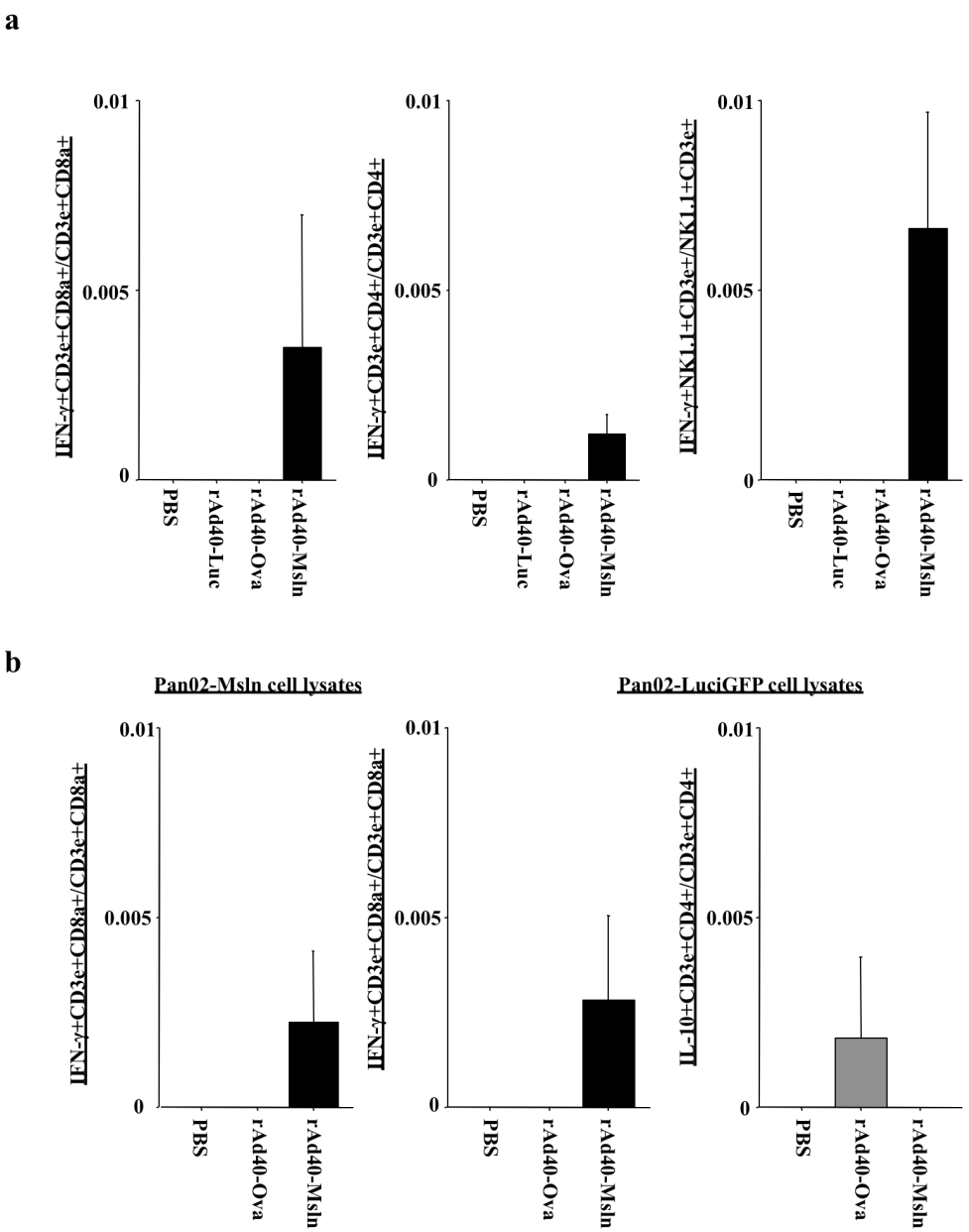


Supporting Information Figure 7. Reducible mouse mesothelin protein expression in Pan02-LuciGFP cells of orthotopic pancreatic cancer mouse models. Msln and GFP double-positive cells were observed in both pancreatic and metastatic tumors (i.e., spleen and lymph nodes) by FCM. Gates were set for detecting tumor cells in FSC/SSC dot-

plots as the 7-AAD-negative population. The panel shows GFP/Msln plots of the FSC/SSC gating. (a) Msln and GFP double-positive cells in Pan02-LuciGFP *in vitro*. (b) Msln and GFP double-positive cells in orthotopic mouse tumors at 28 days after tumor inoculation of 2.5 x 10^5 Pan02-LuciGFP into the pancreas. (c) Msln and GFP double-positive cells in orthotopically transplanted Pan02-LuciGFP tumors after *ex vivo* 14 days culture in puromycin-containing medium. After *ex vivo* culture, almost all Pan02-LuciGFP expressed only GFP, but not Msln protein.



Supporting Information Figure 8. Inhibition of mesothelin protein expression induced by a single intravenous administration with rAd40 expressing mouse mesothelin in orthotopic pancreatic cancer mouse models. Msln protein expression (red, middle panel) in tumor at 29 days after tumor inoculation detected by IF stained for nuclei (DAPI, blue) and observed for GFP (green). Msln protein expression was observed in pancreas of PBS-pretreated mice, and in metastatic diseases (i.e., spleen, lymph nodes) of PBS- and rAd40-Ova-treated mice. White arrows indicate Msln+/DAPI+/GFP+ triple-positive cells. Lower panel shows merging of the Msln, DAPI, and GFP panels. Bar = 100 μm.



Supporting Information Figure 9. Systemic immune responses induced by a single intravenous administration with rAd40 expressing mouse mesothelin. (a) The ratio of Pan02-Msln cell lysates-specific IFN-□-secreting CD8+ T cells to CD8+ T cell, IFN-□-secreting CD4+ T cells to CD4+ T cell or IFN-□-secreting NK1.1+ T cells to NK1.1+ T cells in the spleen at 14 days after intravenous administration with PBS, rAd40-Luc, rAd40-Ova, or rAd40-Msln. rAd40-Msln induced Pan02-Msln-specific systemic

immune responses along the CTL and Th1-cell dependent pathway. (b) Mice at 14 days after intravenous administration with PBS, rAd40-Ova, or rAd40-Msln were implanted with 2.5 x 10^5 Pan02-LuciGFP into the pancreas. At 29 days after tumor inoculation, mice were sacrificed and splenocytes were extracted for FCM. The ratio of Pan02-Msln cell lysates-specific IFN-□-secreting CD8+ T cells to CD8+ T cell, or Pan02-LuciGFP cell lysates-specific IFN-□-secreting CD8+ T cells to CD8+ T cell or IL-10-secreting CD4+ T cells to CD4+ T cells in the spleen was measured by FCM. Pan02-Msln- and Pan02-LuciGFP-specific IFN-□-secreting CD8+ T cells were found only in rAd40-Msln-pretreated mice. In contrast, Pan02-LuciGFP-specific IL-10-secreting CD4+ T cells were detected in rAd40-Ova-pretreated mice, indicating systemic tolerance induced by tumor-specific stimulation following tumor inoculation.